CANCER-ASSOCIATED DEFECTS IN THE DNA DAMAGE RESPONSE: DRIVERS FOR MALIGNANT TRANSFORMATION AND POTENTIAL THERAPEUTIC TARGETS

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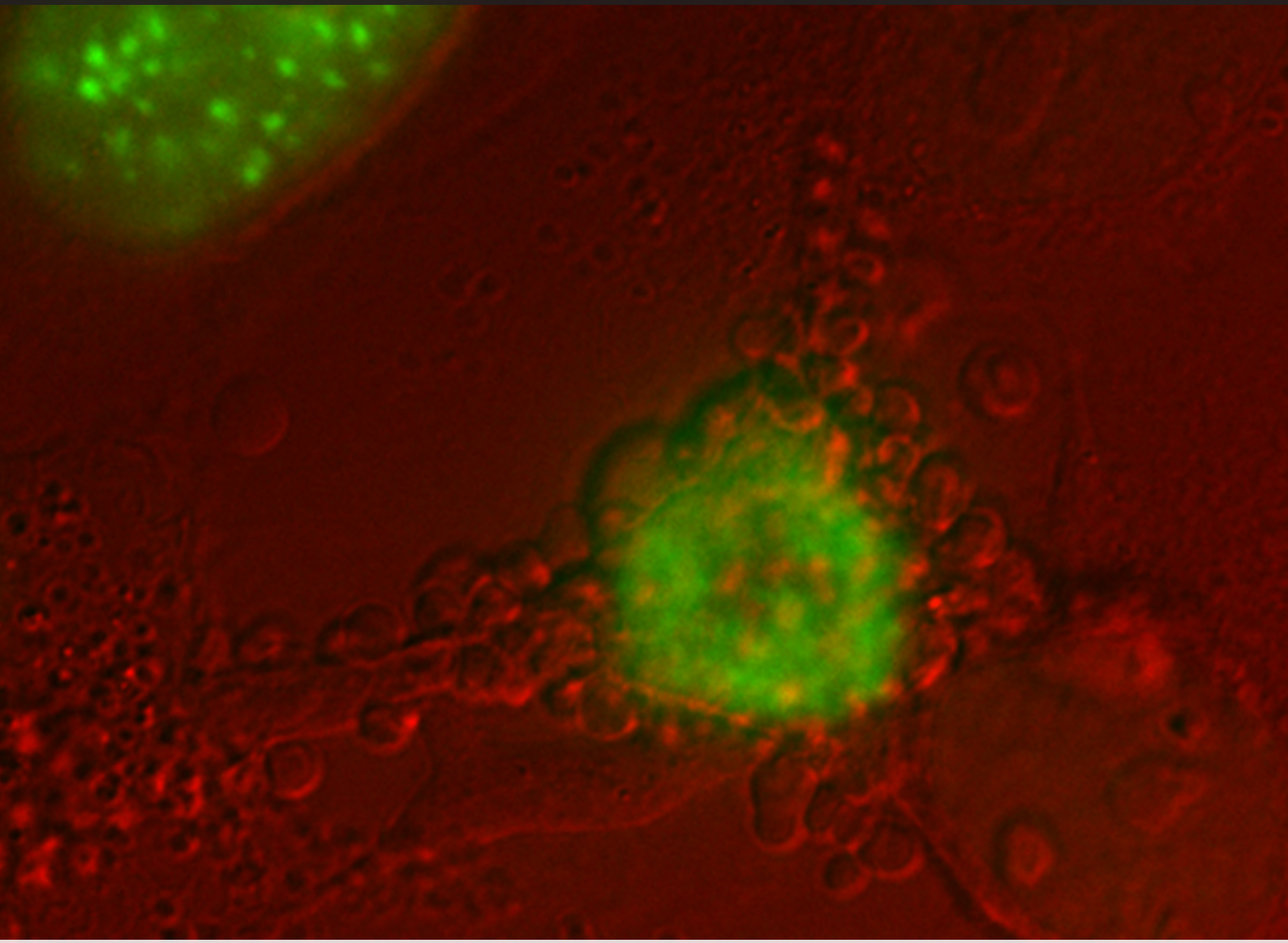

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CANCER-ASSOCIATED DEFECTS IN THE DNA DAMAGE RESPONSE: DRIVERS FOR MALIGNANT TRANSFORMATION AND POTENTIAL THERAPEUTIC TARGETS

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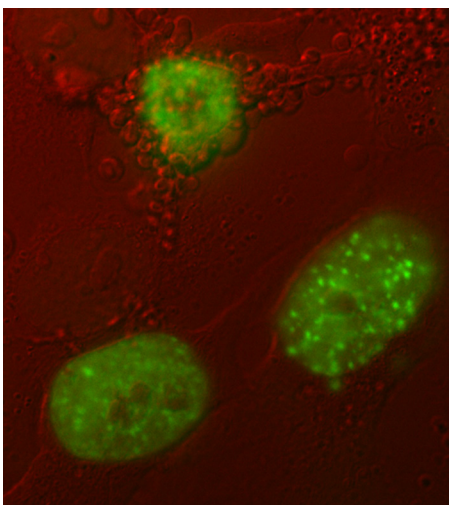

HCC38 human breast cancer cell line, stable expressing GFP-Mdc1, treated with cisplatin (courtesy of Anne Margriet Heijink and Marcel van Vugt).

For this eBook, and the associated Research Topic in Frontiers in Genetics, entitled: 'Cancer-associated defects in the DNA damage response: drivers for malignant transformation and potential therapeutic targets' we have selected 10 papers that each discusses important, yet distinct aspects of the response to DNA damage in normal cells and cancer cells.

Using an evolutionary conserved signaling network called the 'DNA damage response (DDR)' cells maintain the integrity of their genome, and thus safeguard cellular functioning and the ability to create viably progeny. Initially, the DDR appeared to consist of few linear kinase-driven pathways. However, research over the past decades in model organisms, as well as in the human system has revealed that the DDR is a complex signaling network, wired by multiple parallel pathways and displaying extensive crosstalk. Besides phosphorylation, multiple other post-translational modifications, including ubiquitination and sumoylation, are involved to achieve chromatin remodeling and initiation of DNA repair. Also, rather than being a cell-intrinsic phenomenon, we increasingly appreciate that cell-cell communication is involved.

The recognition and repair of DNA damage is essential to maintain normal physiology. Multiple pathological conditions have been attributed to defective DNA repair, most notably accelerated aging, neurodegeneration and cancer. In the context of cancer, through repair of DNA damage or elimination of irreparably damaged cells, the DDR clearly has a tumor-suppressive role. Indeed, many tumor cells show partially inactivated DDR signaling, which allows proliferation

in the context of DNA damage-inducing oncogenes. Simultaneously, loss of specific DDR signaling nodes creates a specific dependence of tumor cells on their remaining DDR components, and thus creates therapeutic opportunities. Especially in the context of cancer treatment, numerous targeted agents are under investigation, either to potentiate the cytotoxic effects of chemo-radiotherapy, or to induce synthetic lethality with cancer-specific alterations, with the treatment of BRCA1/2 mutant cancers with PARP1 inhibitors as a prototype example.

We have selected four review articles that provide insight into the key components and the wiring of the DDR and DNA repair. Torgovnick and Schumacher review the involvement of DNA repair in the initiation and treatment of cancer, Brinkmann et al., describe the involvement of ubiquitination in DNA damage signaling and Jaiswal and Lindqvist discuss how cell-extrinsic signaling participates in communication of DNA damage to neighboring cells. In addition, Shatneyeva and colleagues review the connection between the cellular response to DNA damage and escape from immune surveillance. Concerning the therapeutic application of targeting the DDR and DNA repair, three articles were included. Krajewska and van Vugt review the wiring of homologous recombination and how this offers therapeutic opportunities. Additionally, Knittel and colleagues describe how genetic loss of the central DDR component ATM in chronic lymphocytic leukemia can be exploited therapeutically by targeting certain parallel DNA repair pathways. Syljuasen and colleagues report on how targeting of the DDR can be used as a therapeutic strategy in lung cancer. Finally, three chapters describe newly identified regulators of the cellular response to DNA damage. Von Morgen et al. describe the R2TP complex, Lezzi and Fanciluuli review the involvement of Che-1/AATF in the DDR, and Ohms and co-authors describe how retrotransposons are at the basis of increased genomic instability.

Altogether, these articles describe how defective responses to DNA damage underlie disease - and especially in the context of cancer -can be exploited to better treat disease.

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EDITORIAL
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Editorial: Cancer-Associated Defects in the DNA Damage Response: Drivers for Malignant Transformation and Potential Therapeutic Targets

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Keywords: checkpoint blockade, cell cycle, DNA repair, synthetic lethality, DNA damage response (DDR), rewiring

The Editorial on the research topic

Cancer-Associated Defects in the DNA Damage Response: Drivers for Malignant Transformation and Potential Therapeutic Targets

Transformation of normal cells into cancer cells almost invariably goes along with increased levels of DNA damage. An important source of DNA damage is the enhanced activity of growth-promoting transcription factors, such as *MYC*. Oncogenic activation of these transcription factors aberrantly stimulates DNA replication, which leads to replication stress and ensuing DNA breaks. Cells respond to this type of stress by activation of the DNA damage response (DDR). The DDR is a complex signaling network, displaying multiple levels of cross-talk and feed-back control. Its kinase-driven signaling axes ensure rapid responses to DNA lesions, which is complemented by its transcriptional axis that warrants maintained signaling. Ultimately, activation of the DDR prevents further proliferation and thus provides time to repair genotoxic lesions, and in case of excessive levels of DNA damage promotes permanent cell cycle exit (senescence) or programmed cell death (apoptosis). Activation of the DDR thus prevents the outgrowth of incipient tumor cells, early during tumorigenesis.

In line with the ability to eliminate damaged cells from the proliferative compartment, the DDR clearly has a tumor-suppressive role. Indeed, many tumor cells have inactivated parts of the DDR, which allows proliferation in the context of DNA damage-inducing oncogenes. Not only does partial inactivation of the DDR allow growth of transformed cells, it also provides opportunities for therapeutic intervention. Loss of specific DDR components leaves tumor cells more dependent on their remaining DDR components, especially under conditions of elevated levels of DNA damage induced by chemo/radiotherapy. Identification of such synthetic vulnerabilities may lead to more targeted therapies, in which therapeutic inactivation of the DNA damage response in cancer cells will create more potent anti-cancer strategies.

DNA damage and the repair thereof are increasingly recognized as key pathways in normal physiology, as well as being pathways defective in multiple pathological conditions, including accelerated aging, neurodegeneration and cancer. In addition, the fundamental research into the molecular underpinning of DNA repair that was initiated more than 50 years ago has now translated into drugs that inactivate key components of the DDR with high levels of specificity. Noteworthy, PARP inhibitors, the first molecularly targeted anti-cancer drugs that exploit the DNA repair defect present in *BRCA1*- or *BRCA2*-mutant cancers were FDA-approved at the end of 2014. The increasing importance of this field is illustrated by the 2015 Albert Lasker Awards for

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Biomedical Research being awarded to Stephen Elledge and Evelyn Witkin, pioneers in uncovering the cellular response to DNA damage. In addition, the 2015 Nobel prize for chemistry was awarded to Thomas Lindahl, Paul Modrich, and Aziz Sancar for their seminal work on DNA repair.

In this "Research Topic" entitled: "Cancer-associated defects in the DNA damage response: drivers for malignant transformation and potential therapeutic targets," 10 papers have been published, focusing on various aspects of DNA damage signaling, its effects on cellular viability and its use in cancer therapy.

Increasingly, we realize that the DDR is complex. Rather than being a cell-intrinsic kinase-driven linear pathway, we understand that cell–cell communication is involved, and that it encompasses multiple different post-translational protein marks. Brinkmann et al., describe how ubiquitin signaling plays a central role in the DNA damage response, whereas Jaiswal and Lindqvist describe how extracellular signaling is used to communicate the presence of damaged DNA to neighboring cells. Von Morgen et al. and Lezzi and Fanciluuli, describe the R2TP complex and Che-1/AATF, respectively, as novel components of the cellular response to DNA damage. Ohms et al. describe how retrotransposons are at the basis of increased genomic instability and Shatneyeva et al. portray the interplay between the DNA damage response and escape from immune surveillance.

Torgovnick and Schumacher describe how defects in DNA repair contribute to cancer initiation, and conversely, create opportunities for targeted treatment of those cancers. As an example of these therapeutic consequences, Knittel et al., report how *ATM* loss in chronic lymphocytic leukemia creates synthetic lethal interactions with inactivation of certain DNA repair pathways. Additionally, Syljuasen et al., describe how therapeutic inactivation of DNA damage checkpoint kinases could be exploited in the treatment of lung cancer, whereas Krajewska and van Vugt review how modulation of DNA repair through homologous recombination can be utilized as a therapeutic strategy.

Combined, the papers in this Research Topic underscore the complexity of the cellular response to DNA damage, and highlight how mechanistic insight into the (re)wiring of DDR signaling in cancer cells can be exploited to develop novel cancer therapeutics.

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REVIEW
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DNA repair mechanisms in cancer development and therapy

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DNA damage has been long recognized as causal factor for cancer development. When erroneous DNA repair leads to mutations or chromosomal aberrations affecting oncogenes and tumor suppressor genes, cells undergo malignant transformation resulting in cancerous growth. Genetic defects can predispose to cancer: mutations in distinct DNA repair systems elevate the susceptibility to various cancer types. However, DNA damage not only comprises a root cause for cancer development but also continues to provide an important avenue for chemo- and radiotherapy. Since the beginning of cancer therapy, genotoxic agents that trigger DNA damage checkpoints have been applied to halt the growth and trigger the apoptotic demise of cancer cells. We provide an overview about the involvement of DNA repair systems in cancer prevention and the classes of genotoxins that are commonly used for the treatment of cancer. A better understanding of the roles and interactions of the highly complex DNA repair machineries will lead to important improvements in cancer therapy.

Keywords: DNA repair, cancer therapy, aging, genome instability, progeroid syndromes, xeroderma pigmentosum, ataxia telangiectasia, Fanconi anemia

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Introduction

Living organisms have the crucial task to preserve their genome and faithfully transmit it across generations. Transmission of genetic information is constantly in a selective balance between the maintenance of genetic stability versus elimination of mutational change and loss of evolutionary potential. The DNA molecule is under the continuous attack of a multitude of endogenous and exogenous genotoxic insults and it has been estimated that every cell experiences up to 10^5 spontaneous or induced DNA lesions per day (De Bont and van Larebeke, 2004).

Endogenous damage can result from DNA base lesions like hydrolysis (deamination, depurination, and depyrimidination) and alkylation (6-*O*-Methylguanine) or oxidation (8-oxoG) by intracellular free radical oxygen species (ROS) that can occur as by-products of mitochondrial respiration (Lindahl and Barnes, 2000). Mutations can also arise during normal cellular metabolism for instance by erroneous incorporation of deoxyribonucleotides (dNTPs) during replication.

Environmental sources of damage can be physical [e.g., ultraviolet (UV) light, ionizing radiations (IRs), and thermal disruption] or chemical (e.g., chemotherapeutic drugs, industrial chemicals, and cigarette smoke) and their effects varies from the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidone photoproducts (6-4PPs) following UV exposure, to the introduction of single and double DNA strand breaks upon IR treatment, or to inter- and intrastrand DNA crosslinks, which result from various chemotherapeutic drugs (**Table 1**; Ciccia and Elledge, 2010).

TABLE 1 | Distinct DNA repair systems are specialized to repair the various types of DNA lesions.

Repair mechanism	Lesion feature	Genotoxic source (examples)
Base excision repair (BER)	Oxidative lesions	Reactive oxygen species (ROS)
Nucleotide excision repair (NER)	Helix-distorting lesions	UV radiation
Translesion synthesis	Various lesions	Various sources
Mismatch repair (MMR)	Replication errors	Replication
Single stand break repair (SSBR)	Single strand breaks	Ionizing radiation, ROS
Homologous recombination (HR)	Double-strand breaks	Ionizing radiation, ROS
Non-homologous end joining (NHEJ)	Double-strand breaks	Ionizing radiation, ROS
DNA interstrand crosslink repair pathway	Interstrand crosslinks	Chemotherapy

DNA lesions can alter the primary structure of the double helix thereby affecting transcription and replication. Erroneous repair of lesions can lead to mutations in the genome that can be inherited to daughter cells with deleterious consequences for individual's health. As a consequence, eukaryotic cells have evolved a complex signaling network of repair processes known as the DNA damage response (DDR). The importance of DNA repair mechanisms is highlighted by the existence of many devastating human syndromes that are caused by defects in DDR genes. Notably, many of these mutations generally display increased sensitivity to DNA damaging agents and predispose to the development of specific cancer types (Curtin, 2012). Already Theodor Boveri recognized cancer as a disease of the genome. Indeed mutations and chromosomal aberrations can lead to alterations in the gene function. Uncontrolled tumorous cell growth occurs when oncogenes are activated or tumor suppressor genes inactivated (**Figure 1**). The underlying role of DNA damage in cancer development has become particularly evident when genetic defects in DNA repair systems lead to increased cancer susceptibility.

DNA Repair Defects Lead to Tumor Development

Xeroderma Pigmentosum

Xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) are rare autosomal recessive diseases caused by defects in the nucleotide excision repair (NER) pathway that protects the DNA molecule from the damage inflicted by UV irradiation (Cleaver, 2005). Indeed, XP was initially described by the dermatologists Hebra and Kaposi (1874) and was the first syndrome associated with a defect in a DNA processing pathway (Cleaver, 1968).

The NER-associated diseases share an increased sun-sensitivity and freckling in the skin areas exposed to the sun but while XP is a skin cancer-prone (>1000-fold increase) disease (basal cell cancer, squamous cell cancer, and malignant melanoma; Kraemer et al., 1987), CS and TTD are not.

Bypass of unrepaired DNA lesions during replication in dividing cells of XP patients can lead to mutations. Mutations can alter the sequence and consequently the function of tumor suppressors and oncogenes. Consequently, XP patients bear not only a highly elevated risk for developing skin cancer but also a >10- to 20-fold increase of internal malignancies like leukemias, brain and lungs tumors before the age of 20 (Bootsma et al., 2001).

XP patients present differences in sunburn reaction that inversely correlate with cancer risk: 60% of the cases have an extreme UV light sensitivity directly after birth while the remaining 40% only show visible signs from the age of 2 years where a freckle-like pigmentation becomes more evident on the face. Paradoxically, the latter ones have higher risk to develop cancer. XP patients can also present, in about 20–30% of cases, neurological abnormalities (Diderich et al., 2011).

Complementation studies from fibroblasts derived from XP patients have shed light on the fundamental players involved in this pathway: mutations in seven different NER genes [from Xeroderma pigmentosum, complementation group A (XPA) to Xeroderma pigmentosum, complementation group G (XPG); De Weerd-Kastelein et al., 1972] plus a variant form, Xeroderma pigmentosum, complementation group V (XPV), defective in the translesion DNA polymerase eta (Lehmann et al., 1975), lead to XP.

Nucleotide excision repair repairs the major lesions caused by UV light, the CPDs and 6-4PPs that distort the DNA double helix. A similar type of damage, as well resolved by NER, is caused by polycyclic aromatic hydrocarbons (tobacco smoke or DNA crosslinking agents like Cisplatin or Benzopyrene; Leibeling et al., 2006) and ROS-generated cyclopurines. NER comprises two subpathways: global genome-NER (GG-NER) that scans the entire genome for helix-distorting lesions and transcription coupled-NER (TC-NER), which is operating only on actively expressed genes and is activated when RNA polymerase II stalls at a lesion. The NER mechanism consists of four main different steps: damage recognition, DNA unwinding around the lesion, cleavage and excision of the damaged strand and synthesis of the new DNA with concomitant final ligation. The only difference between the two NER branches resides in the DNA damage recognition phase and for the fact that TC-NER is faster than GG-NER in damage resolving (Hanawalt and Spivak, 2008).

In the first step of GG-NER, the protein complexes XPC-HHR23B-Centrin2 and XPE-DDB2 sense the damage and initiate the repair process by recruiting other NER factors. The multiprotein complex transcription factor IIH (TFIIH; TFIIH subunits: XPB, GTF2H1 GTF2H2, GTF2H3, GTF2H4, XPD, MNAT1, CDK7, CCNH, GTF2H5) generates a transiently open DNA structure by using the 3′-5′ and 5′-3′ nuclease activity of the two ATP-dependent helicases XPB and XPD (Evans, 1997). The fundamental role of these proteins is underline by the fact that XPB and XPD knockout mice are not viable (Cleaver, 2005). XPD is required not only for its helicase unwinding capacity but also to verify the damage after XPC loading. The Arch and Fe-S cluster domains of XPD form a channel where the damaged DNA is scanned in a 5′-3′ direction. After unwinding of a 27–30 bp

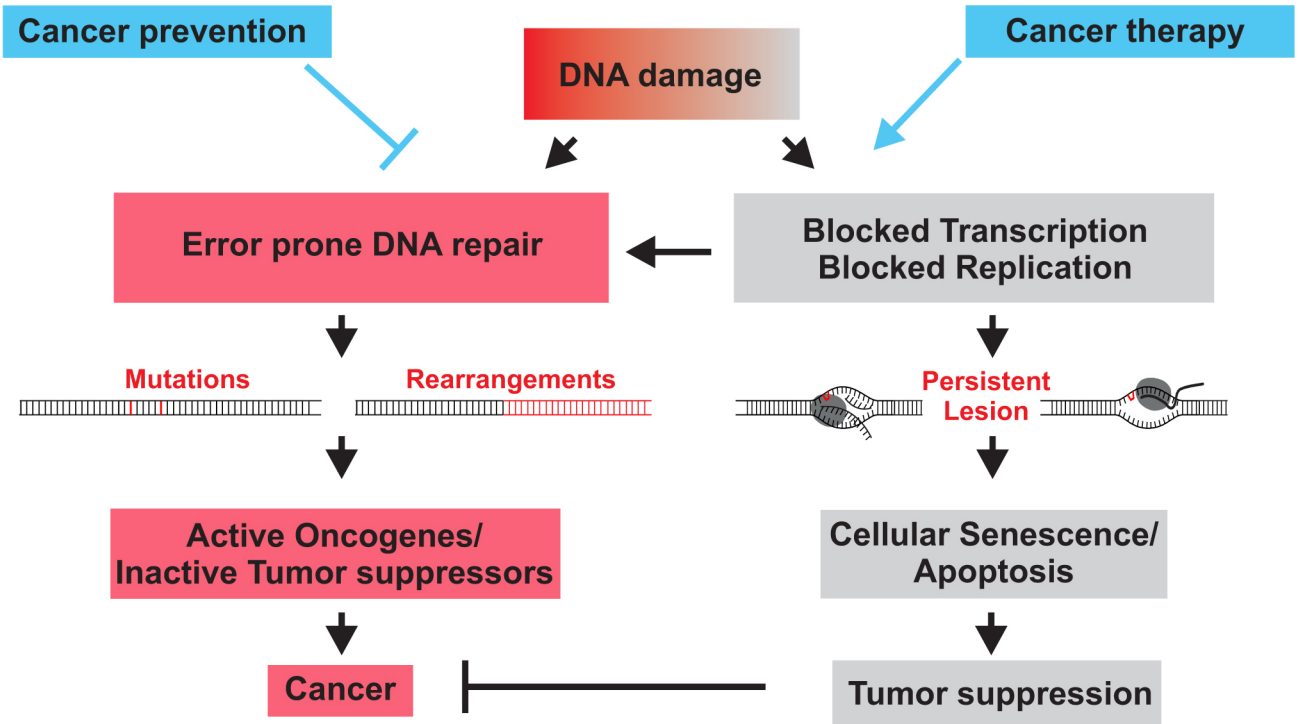

FIGURE 1 | DNA damage causes cancer development when erroneous DNA repair leads to mutations of chromosomal aberration that activate oncogenes or inactivate tumor suppressors genes (red). When DNA damage persists and interferes with replication or transcription, DNA damage checkpoints trigger cellular senescence or apoptosis that inactivate or eliminate damaged cells and thus suppress tumorigenesis (gray). DNA repair mechanisms prevent cancer by preventing mutations. Chemo- and radiotherapy often inflict DNA damage to halt cancer cell proliferation or trigger the apoptotic demise of cancer cells.

DNA tract, the exposed filament is completely covered by the replication protein A (RPA; de Laat et al., 1998). RPA, together with XPA loading on the 5′ side of the lesion (Krasikova et al., 2010), is involved in the correct positioning of the endonucleolytic cleavage mediators. The incision step is carried out by two structure specific endonucleases respectively named XPF-ERCC1 and XPG. The first cut at the 5′-end of the lesion by XPF-ERCC1 is than followed by the action of XPG on the opposite DNA filament (Fagbemi et al., 2011). By using the complementary strand as a template, DNA polymerase δ (in non-replicating cells) and ε (in dividing cells) synthesize the new error-free sequence starting from the 3′-hydroxyl extremity generated by XPF-ERCC1. The remaining 3′-end incision is finally closed by Ligase I or III (Moser et al., 2007).

The TC-NER subpathway initiates when RNA polymerase stalls at a DNA lesion. XPC, which without XPE is incapable of binding to CPDs (Fitch et al., 2003; Sugasawa et al., 2005), is dispensable for TC-NER (Venema et al., 1991). Upon RNAPII stalling the Cockayne syndrome protein B (CSB) recruits Cockayne syndrome protein A (CSA), whereupon the same NER core machinery is activated as following GG-NER-mediated damage recognition. In 80% of the cases CS patients have mutations in CSB (Natale, 2011) and show neurodegeneration and cachectic dwarfism. A possible explanation for the lack of tumors observed in CS patients is the high susceptibility of CS-derived cells to undergo cell death after DNA damage (McKay et al., 2001). In addition, it was shown that CS mouse models exhibit reduced levels of circulating growth factors such as IGF-1 (van der Pluijm et al., 2007), suggesting that a reduced endocrine growth environment might prevent cancer development (Schumacher et al., 2008).

Ataxia Telangiectasia

The major regulators of the DDR are the two serine-threonine kinases ATM [ataxia telangiectasia (AT) mutated] and ATR (ATM and RAD3-related) which both belong, together with SMG-1 (suppressor of mutagenesis in genitalia), DNA-PKcs (DNA-dependent protein kinase catalytic subunit) and mTOR (mammalian target of rapamycin), to the phosphonositide 3-kinase (PI3K)-related protein kinases (PIKKs) family. All of them share a conserved C-terminal kinase domain structure flanked by the FAT and FATC domains, two conserved regions, with high sequence similarity, regulating the kinase activity (Cimprich and Cortez, 2008).

The overlapping substrates of ATM and ATR comprise more than 700 different proteins mainly involved in DNA repair, cell cycle arrest, and transcription but also in developmental processes, immunity and intracellular protein traffic (Matsuoka et al., 2007). Among the most important, ATM and ATR respectively target the two serine-threonine protein kinases: checkpoint kinase

2 (CHK2) and CHK1, that function as key signal transducers of the DDR (Bartek and Lukas, 2003). In contrast to ATM and CHK2, the ATR and CHK1 kinases are indispensable for the viability of mammalian cells (Brown and Baltimore, 2000; de Klein et al., 2000).

Humans carrying homozygous mutations (0.5–1%) in the ATM gene (432 mutations have been reported without any hotspots and generally lead to protein instability—Leiden Open Variation database) suffer from the neurodegenerative disease AT, which is characterized by radiation sensitivity, chromosomal instability and predisposition to cancer. Up to 30% of AT patients develop lymphoid tumors since ATM play a critical role in the differentiation of T and B cells (Lumsden, 2004). Carriers of heterozygous missense mutations leading to the expression of inactive but stable variants acting as dominant ATM version against the wild type allele have higher incidence to develop breast, colorectal and stomach cancer (Thompson et al., 2005; Paglia et al., 2009).

Hypomorphic mutations in ATR lead to Seckel syndrome. The main features of this disease are growth retardation, microcephaly and a characteristic "bird-headed" facial appearance (O'Driscoll et al., 2003). While germline ATR mutations have not yet been reported, ATR was recently found to be downregulated in head and neck cancers (Moeller et al., 2011) and mutations within the FAT domain were observed in oropharyngeal-tumor tissue (Tanaka et al., 2012).

Although they share many substrates, ATM and ATR are activated in different ways. ATR is mainly induced upon DNA single strand breaks (SSBs) originated by replication fork stalling or as result of double strand breaks (DSBs) processing and NER activity. On the other hand, ATM primarily responds to DSBs caused by IR or ROS as well as breaks coming from physiological processes like meiosis, telomere maintenance, or immune system maturation (assembly of the T cell receptor and immunoglobulin genes via V(D)J recombination; Shiloh, 2003).

In the ATR activation process: RPA, after coating the single strand DNA, recruits the ATR interacting protein (ATRIP). This complex helps to localize the site of damage (Zou and Elledge, 2003) and to direct the loading of the RAD9-RAD1-HUS1 (9-1-1) clamp through the interaction with the RAD17-replication factor C (RFC). After 9-1-1 is loaded on the 5′ end of the ssDNA, the ATR activator topoisomerase-binding protein-1 (TOPBP1) can be recruited and activates ATR in an ATRIP-dependent manner (Cimprich and Cortez, 2008). Another mediator of ATR activation is Claspin (Smits et al., 2010). Activated ATR phosphorylates CHK1 on Ser317 and Ser345 residues. Additional substrates of ATR phosphorylation include: ATRIP, Rad17, Rad9, TopBP1, Claspin, H2AX, WRN, BLM, BRCA1 (breast cancer susceptibility gene 1), and FANCD2 (Cimprich and Cortez, 2008).

Ataxia telangiectasia mutated is found in the nucleus of undamaged cells in the form of inactive dimers or higher order multimers, configuration that inhibit, by masking with the FAT domain, the kinase domain. Upon DNA damage, ATM undergoes autophosphorylation on residues Ser367, Ser1893, and Ser1981 with the last one located within the FAT domain. These posttranslational modifications result in dimer dissociation and release of active kinase monomers (Bakkenist and Kastan, 2003; Kozlov et al., 2006). Upon formation of a DSB, the sensor complex MRE11-RAD50-NBS1 (MRN), which is composed by the meiotic recombination protein 11 (MRE11), the DNA repair protein RAD50 and the Nijmegen breakage syndrome protein-1 (NBS1), localize to the damaged area together with ATM (Lee, 2004). Recently it was reported that ubiquitination of NBS1 by SCF-Skp2 E3 ligase trigger the recruitment and activation of ATM on DSB formed upon IR treatment (Wu et al., 2012). Activated ATM phosphorylates the histone variant H2AX, which is then bound by the mediator of DNA damage checkpoint protein-1 (MDC1). MDC1 induces the recruitment of other ATM–MRN complexes resulting in the establishment of a positive feedback-loop that leads to further H2AX phosphorylation and amplification of the initial signal (Lavin et al., 2005). The pool of activated ATM within the cell appear to be divided in two fractions: the first one is physically bounded to DSB sites while the other one is free to reach other targets that required to be activated (Shiloh, 2003).

Ataxia telangiectasia mutated exerts its survival function through the induction of cell cycle checkpoints. In the G1-S checkpoint, ATM phosphorylates the tumor suppressor p53 on S15 leading to the disruption of the inhibitory association with MDM2. Activated p53 induces p21, which binds to and inhibits the S-phase-promoting Cdk2-CyclinE complex (Sancar et al., 2004).

During the G2-M checkpoint, ATM phosphorylates monomers of CHK2 on Thr68 allowing the formation of CHK2 dimers that have as a main target the cell division cycle 25 homolog A (CDC25A). Phosphorylated CDC25A can finally be degraded by the proteasome and prevents cyclin-dependent kinase 2 (CDK2) and CDK1 dephosphorylation, which is required for progression through the cell cycle (Cimprich and Cortez, 2008).

Fanconi Anemia

DNA inter- and intrastrand crosslinks represent a dangerous form of damage blocking vital cellular processes like transcription and replication. The Fanconi anemia (FA) pathway is responsible to repair these aberrations arising in the DNA structure as a result of chemotherapeutics drugs treatment, like cisplatin or mitomycin C (van der Heijden et al., 2004), or naturally evolved due to the interaction with lipid peroxidation products such as malondialdehyde (Stone et al., 2008). FA is an autosomal recessive disease that affects 1 every 100,000 births (Rosenberg et al., 2011) and it is characterized by growth retardation, infertility, bone marrow failure and susceptibility to acute myeloid leukemia. Solid tumors like head and neck, kidney, liver, medulloblastoma, gynecological, oesophageal, and skin cancers are also common between FA patients (Cerbinskaite et al., 2012).

Fanconi Anemia is a heterogeneous genetic disease, 16 different genes are involved in the establishment of the disorder and they can be divided in three major groups: the FA core complex, the I-D2 complex and downstream FA proteins. Eight proteins form the core complex, respectively named FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM while the I-D2 complex is constituted by FANCD2 and FANCI (Walden and Deans, 2014). In the initial phase of the process, FANCM, which forms and heterodimer with FAAP24 (FA-associated protein 24 kDa), recognizes DNA interstrand cross-links (ICL) lesions and recruits other FA factors to the damaged site, the

stalled replication fork. The association of FANCM with the chromatin is strengthened by histone fold protein 1 (MHF1) and 2 (MHF2; Singh et al., 2010) and it is followed by ATR activation (Schwab et al., 2010). Monoubiquitination of FANCD2 on Lys 561 and FANCI on Lys 523 by the core complex, which essentially constitutes a multisubunit E3 ubiquitin ligase, is the key step in the activation of the FA pathway (Taniguchi et al., 2002; Smogorzewska et al., 2007).

Despite the fact that FANCD2 was shown to have intrinsic nuclease activity (Pace et al., 2010), other nucleases are involved in the FA pathway and which one is responsible to perform the first cut and start unhooking the crosslinked DNA is still unknown. The best candidate to assume this function seems to be SLX4 (FANCP), which is a multidomain scaffold protein directed toward branched DNA and Holliday junction (HJ) structures and able to interact with three distinct nucleases: SLX1, XPF-ERCC1, and MUS81-EME1. The interaction between SLX4 is with the NER endonuclease XPF-ERCC1 was indeed shown to be crucial for the removal of ICLs (Crossan et al., 2011). FAN1 (FA-associated nuclease 1) is another nuclease recruited to the damaged site by ubiquitinated FANCD2. FAN1 abrogation does not affect ICLs-induced DSBs formation most likely resembling the possibility that FAN1 is required further down in the steps of the repair process (Kratz et al., 2010).

The FA pathway allows resolving the replication fork stalling by inducing the formation of a DSB and by coordinating the action of three critical repair mechanisms: translesion synthesis (TLS) bypasses the lesion and, after toxic adducts removal by NER, the gap is closed by homologous recombination (HR). The ID complex is finally able to leave the previously damaged area thanks to deubiquitination mediated by USP1 (ubiquitin specific peptidase 1) and UAF1 (USP1-associated factor 1; Nijman et al., 2005).

Although further work is required to fully understand each steps of the FA pathway, some of the downstream players involved are: FANCJ (BRIP1), DNA-dependent ATPase and 5′-3′ DNA helicase able to interact with BRCA1; FANCD1 (BRCA2), able to bind ssDNA and dsDNA and to stimulate RAD51 action; FANCN (partner and localizer of BRCA2, PALB2), required for FANCD1 stabilization and for the recruitment of BRCA2 and RAD51; and FANCO (RAD51C) involved in HJ resolution (Kottemann and Smogorzewska, 2013).

The tumorigenesis of FA is difficult to interpret due to the overlapping functions of all the aforementioned proteins working also in homology-directed repair. Of note, the FA pathway is also active in physiological conditions by preserving the replication fork stability during S-phase (the I-D2 complex was found to be ubiquitinated in undamaged cells; Schlacher et al., 2012) and acts as a barrier against error-prone repair processes such as non-homologous end joining (NHEJ). Accordingly, genomic instability, a typical feature of FA patients, was rescued in *C. elegans*, DT40 chicken and mammalian cells by inhibiting NHEJ components (Adamo et al., 2010; Pace et al., 2010).

Breast Cancer Susceptibility Gene 1 and 2 (BRCA1 and 2)

Double strand breaks are the most threatening forms of DNA damage, if left unrepaired they can lead to chromosomal rearrangements or to cell death. To counteract DSBs, cells have evolved two different repair mechanisms: HR and NHEJ. HR is an error-free way to repair DSBs which takes place during S and G2 phases of the cell cycle where a sister chromatid is used as a homologous template (Roy et al., 2011). *Vice versa*, NHEJ, which fuses two broken chromosomal ends, can be mutagenic and can act independently of the cell cycle status (Caestecker and Van de Walle, 2013).

Two different ways of HR repair coexist: the classic model and the alternative synthesis-dependent strand-annealing (SDSA) model. In the first one, also known as Double HJ model, the 5′ and 3′ ends of a DSB are resected by nucleases (endonuclease Sae2, exonuclease Exo1, helicases Sgs1, and Dna2) and the 3′ ssDNA filament invades the intact sister chromatid, which is used as a template to repair the lesion. The displacement of the second strand results in the formation of a D-loop. The extension of the 3′ invading strand transforms the D-loop to a cross-shape structure known as HJ. The second 3′ overhang, not involved in the initial strand invasion, also produces a HJ with the homologous chromosome. This way of repair may result in the formation of chromosomal crossovers and principally takes place during meiosis (Helleday et al., 2007). To avoid the production of crossover in somatic cells, event that will end up in loss of heterozygosity (LOH), the double HJ can be dissolved by bloom helicase (BML) and Topoisomerase III (Wu and Hickson, 2003).

The SDSA process shares all the steps of the classic HR repair model except for the absence of the D-loop structure formation (Sung and Klein, 2006). SDSA always leads to non-crossover products and is supposed to be the most used way of HR in mitosis.

Both BRCA1 and BRCA2 are involved in the HR pathway. *BRCA1* and *BRCA2* mutations are found in approximately 5–7% of all hereditary breast cancers (Roy et al., 2011). In mice, homozygous BRCA1 and BRCA2 knockouts die at day 8–9 of development (Hakem et al., 1996; Suzuki et al., 1997).

Breast cancer susceptibility gene 1 plays major roles in different DNA repair mechanisms. It acts in HR, NHEJ and single-strand annealing (SSA) through its different interaction domains. Located at the N-terminus, the RING domain is the site for the interaction with BARD1 (BRCA-associated RING domain 1), a structurally-related protein responsible for BRCA1 stabilization and activity (Wu et al., 1996). The BRCA1/BARD1 heterodimer possesses E3 ubiquitin ligase activity and, upon DNA damage, mediates downstream signaling events through ubiquitination of other DDR targets including CtIP, H2AX, RNAPolII, and CstF (Caestecker and Van de Walle, 2013). At the C-terminus, BRCA1 has a domain shared between many DDR proteins: the BRCT1 domain (BRCA1 C-terminal), required for binding phosphorylated proteins during the DDR (Koonin et al., 1996) and essential for transcriptional regulation and chromatin unfolding (Monteiro, 2000; Ye et al., 2001). In the central part of the protein we find the DNA binding domain (DBD), the nuclear localization and exporting sequences and, most importantly, the serine-glutamine (SQ) and threonine-glutamine (TQ) motifs which are indispensable for BRCA1 activation through ATM/ATR phosphorylation (Caestecker and Van de Walle, 2013). Most of the

cancer-associated BRCA1 mutations are found in the RING and BRCT domains (Roy et al., 2011).

Breast cancer susceptibility gene 1 is a component of three different multiprotein complexes involved in all cell cycle checkpoints: the BRCA1A complex (composed of Abraxas, BARD1, RAP80, BRCC36, BRCC45, and MERIT40), responsible to recruit BRCA1 to damaged sites; the BRCA1B complex (formed with BRIP1 and TOPBP1), mainly associated with replication-coupled DNA repair and the BRCA1C complex (formed together with CtIP and the MRN complex), which promotes HR despite NHEJ (Huen et al., 2010).

Interestingly, *BRCA1/BARD* mutations cannot only fuel genome instability due to impaired HR activity, but also promote genome stability as recently shown in *C. elegans* mutants of the *smc-5/6* complex that leads to replicative impediments and DSB formation at stalled replication forks (Wolters et al., 2014). The genome instability in *smc-5/6* mutants could be revered upon inactivation of the *BRCA1/BARD* complex. It is tempting to speculate that mutations in *BRCA1* might be sustained in the human genome as under certain conditions of replication fork breakdown prevention of HR could benefit genome stability.

The BRCA2 protein was recently purified and functionally validated by three independent research groups (Jensen et al., 2010; Liu et al., 2010; Thorslund et al., 2010). In contrast to the multiple functions of BRCA1, BRCA2 main role is to mediate the recruitment of RAD51 to DSBs during HR. BRCA2 carries, in the central part of the protein, a DBD able to bind both single and double stranded DNA and eight BRC repeats indispensable for the interaction with RAD51. Cancer-associated BRCA2 point mutations are found between these repeats (Venkitaraman, 2009).

Breast cancer susceptibility gene 2 prevents RAD51 binding to dsDNA and specifically direct it to ssDNA where it displace RPA (Thorslund et al., 2010). The PALB2, also known as FANCN, is the connection between BRCA1 and BRCA2. PALB2 is required for the colocalization of BRCA1, BRCA2, and RAD51 to the damaged sites and its dysfunction leads to severe HR defects (Zhang et al., 2009a).

Mismatch Repair

The critical role of mismatch repair (MMR) in tumorigenesis is highlighted by the fact that loss of expression of MMR proteins predispose to colorectal, gastric, endometrial and ovarian cancers and inherited defects in the MMR genes are associated with the most prevalent cancer syndrome in humans, the Lynch syndrome (LS), previously known as hereditary nonpolyposis colorectal cancer (HNPCC; Guillotin and Martin, 2014). Moreover, MMR deficiency is present in 15% of all primary cancers (Furgason and Bahassi el, 2013).

The MMR pathway recognizes base–base mismatches and insertion-deletion loops (IDLs; Jiricny, 2006) originating from base misincorporation, tautomeric shifts, slippage of DNA polymerases, damage that acts as mismatch, and recombination duplex. The sequential events in MMR repair comprise damage recognition, excision, and resynthesis steps (Hsieh and Yamane, 2008). The MutSα and MutSβ complexes are the MMR lesion detectors. The first complex is composed by MSH2 and MSH6 and recognizes single base-base mismatches and 1–2 bp IDLs while the second one, formed by the MSH2 and MSH3 proteins, principally find and repair 2–12 bp IDLs (Iyama and Wilson, 2013).

Upon DNA binding, one of the three different heterodimeric complexes MutLα (MLH1-PMS2), MutLβ (MLH1-MLH3), and MutLγ (MLH1-PMS1) can be recruited to form, with MutS, a ternary structure. The complex formed with MutLα is the most important in the MMR pathway, is able to translocate in both directions along the damaged area and to recruit proliferating cell nuclear antigen (PCNA), RFC, and EXO1 to perform the excision step (Guillotin and Martin, 2014). MutLβ function is currently unknown whereas MutLγ is involved in meiotic recombination (Zhang et al., 2005). After damage resection, resynthesis is carried out by DNA polymerase δ and sealing of the nick by DNA ligase I (Larrea et al., 2010).

Being part of the replication fork, the MMR machinery operates mostly in dividing cells (Wagner and Meselson, 1976), nonetheless few publications report an active presence of MMR in the brain (Brooks et al., 1996).

Mismatch repair dysfunction accounts for the mutator phenotype in which base substitution and frameshift mutations are highly increased due to microsatellite instability (MSI). Microsatellites are short tandem repeated DNA sequences of 1–4 base nucleotides spread all over the genome. Replication of these repeats has high error risk and when they are present in tumor suppressor genes, a defective repair may have detrimental consequences (MSI; Guillotin and Martin, 2014).

DNA Damaging Agents in Tumor Therapy

Cancer therapy was jumpstarted at the end of the Second World War by serendipity resulting from some of the darkest chapters of chemical warfare that brought so much suffering during the First World War. Already in the trenches of the First World War bone marrow suppression and lymphoid aplasia were reported upon exposure to the chemical warfare sulfur mustard. The critical link to its therapeutic potential became evident a few decades later when the secret load of the American vessel *S.S. John Harvey* was unleashed in the Italian harbor of Bari during a German air raid. Physicians detected reduced white blood counts in autopsies following the incidence. It turned out that the vessel's load of nitrogen mustard had attacked the white blood cells suggesting that leukemias could be targeted by nitrogen mustard therapy. Already a few years later the first alkylating agents were introduced to cancer therapy. Strikingly, it was found that effective chemotaxis such as nitrogen mustard and cisplatin evoke damage in nuclear DNA that then results in cell death. Therefore, DNA damage not only causes tumor development but could also battle cancers by impairing cancer growth and ultimately triggering the death of malignant cells (**Figure 1**).

Cisplatin

Also known as Peyrone's chloride, cisplatin (*cis*-diamminedichloroplatinum) is one of the most widely used chemotherapeutic drugs. Its antitumor potential was discovered in the sixties by Rosenberg et al. (1965) when he accidentally found out that this metal salt was able to inhibit *Escherichia coli* cell division. Cisplatin soon drew interest in the scientific

community and, after its efficacy was proven in mouse models (Rosenberg et al., 1969), it entered clinical trials and was finally approved by FDA in 1978 as a chemotherapeutic drug for the treatment of testicular and bladder cancers (Kelland, 2007b). The therapeutics properties of cisplatin were then extended to many other types of cancer including small and non-small cell lung, head and neck, ovarian, cervical, and colorectal (Lebwohl and Canetta, 1998; Galanski, 2006).

Once in the cytoplasm, cisplatin gets activated upon reaction with water, which can substitutes one or both the two *cis*-chloro groups of the molecule. The mono aquated form of cisplatin is the most reactive one, it can react with many cytoplasmic nucleophiles substrates including reduced glutathione (GSH), methionine and metallothioneins (MT) but its cytotoxic effect comes from the capacity to target DNA (Galluzzi et al., 2011). Inside the nucleus, cisplatin attacks the N7 nucleophilic site of purine bases leading to the formation of monofunctional adducts. Such adducts are able to form intra-strand crosslinking structures [90% 1,2 d(GpG) and 10% 1,2 d(ApG)] which represent the major type of DNA damage exerted by this chemotherapeutic drug (Dasari and Tchounwou, 2014). Cisplatin-mediated damage arrests cells in the G2 phase of the cell cycle and concomitantly triggers the activation of DNA repair pathways. If the damage is too severe, programmed cell death will be induced through the ATM/ATR/TP53 pathway (Damia et al., 2000; Pabla et al., 2008). Although cisplatin is a really potent apoptotic inducer, intrinsic or acquired resistance can represent an obstacle for its use in tumor therapy. Moreover, cisplatin resistance can either take place before or after DNA binding.

The copper transporter 1 (CTR1) regulates cisplatin cellular uptake. Cisplatin treatment of $Ctr1^{-/-}$ mouse embryonic fibroblasts (MEFs) is associated with a reduced intracellular accumulation respect to wild type MEFs (Holzer et al., 2006) and CTR1 downregulation is found in cisplatin-resistant lung cancer cell lines (Song et al., 2004). Copper pretreatment of cochlear derived HEI-OC1 cells reduced cisplatin cytotoxicity (More et al., 2010). In addition to copper transporters, also organic cation transporters (OCTs) were recently discovered to be involved in cisplatin intake. Even if the uptake is the main cause of altered intracellular cisplatin level, the efflux process must be considered as well. The ABC ATPases-like multidrug resistance proteins (MRP) MRP1, MRP2, MRP3, MRP5, and the copper ATPases ATP7A and ATP7B mediate cisplatin export and were found to have altered expression in cisplatin resistant tumors (Burger et al., 2011).

Cisplatin resistance can also be established through the interaction with intracellular thiol-containing molecules such as GSH and MT. They can both sequester cisplatin within the cytoplasmic compartment and correlations between their expression level and cisplatin resistance were found in ovarian, cervical, lung, and bladder cancer cell lines (Köberle et al., 2010).

Cisplatin-induced DNA damage is primarily repaired by the FA pathway (Deans and West, 2011), as well as by NER and MMR. Enhanced activity of these repair mechanisms can promote cisplatin resistance. Indeed, higher and lower expression levels of the NER endonucleases ERCC1 and XPF were respectively found to be associated with resistance and sensitivity in ovarian and testis cancer cell lines (Köberle et al., 1999; Ferry et al., 2000; Welsh et al., 2004). Moreover, siRNA mediated downregulation of the ERCC1-XPF complex renders lung, ovarian and breast cancer cells more prone to death after cisplatin treatment (Arora et al., 2010).

Like NER, also MMR deficiency compromises cisplatin-induced apoptotic signaling (Topping et al., 2009) and it was observed to be always associated with an increased translesion synthesis (TLS) activity (Jung and Lippard, 2007). The specialized TLS polymerases are therefore another critical target to overcome resistance in patients carrying MMR mutations.

While cisplatin has a strong anti-cancer activity, it also exerts negative side effects like nephro- and neurotoxicity (Kelland, 2007a). The negative aspects and the concomitant possibility to acquire resistance after a certain period of treatment have pushed researchers, during the last 40 years, to design new platinum based drugs.

Approved by FDA in 1989, Carboplatin has, instead of the two *cis*-chloro groups, a bidentate dicarboxylate ligand, which slow down reactivity and unfavorable side effects. Carboplatin is actually used in the treatment of ovarian, head and neck, and lung tumors (Dasari and Tchounwou, 2014). The adducts formed by this molecule are the same ones introduced by cisplatin (Harrap, 1985) and thrombocytopenia is its main negative side effect.

The last platinum drug approved by FDA in 2002 is oxaliplatin. The large 1,2-diaminocyclohexane ligand plus the oxalate leaving group confers to oxaliplatin completely new characteristics: it is less dependent on the CTR1 transporter (Holzer et al., 2006) and forms DNA adducts which are not recognized by MMR (Fink et al., 1996). Apart from being effective in the treatment of cisplatin and carboplatin-resistant tumors (Raymond et al., 2002), oxaliplatin, in combination with 5-fluorouracil, is successfully employed in colorectal cancer treatment (FOLFOX therapy; Kelland, 2007b).

Between the recently developed platinum based drugs, phenanthriplatin is one of the most promising. This new compound kills cancer cells more efficiently than cisplatin and oxaliplatin and appear to be immune to acquired resistance mechanisms (Park et al., 2012).

Nucleoside Analogs

Nucleoside analogs are anticancer metabolites that were developed based on modifications of physiological purine (adenosine, guanosine, inosine) and pyrimidine (cytidine, thymidine, uridine) nucleosides, the fundamental precursors of ATP, DNA, and RNA. This class of drugs is widely used in hematological malignancies and solid tumors and, as well, for the treatment of viral infections (Galmarini et al., 2002).

Nucleoside analogs exert their cytotoxic activities after being incorporated into DNA and RNA molecules leading respectively to replication and transcription inhibition, or by directly interfering with critical enzymes such as polymerases, kinases, ribonucleotide reductases, methyltransferases, nucleoside phosphorylases, and thymidylate synthases (Jordheim et al., 2013). The cellular uptake of these hydrophilic antimetabolites is mediated by two major families of nucleoside transporter (NT) proteins: the equilibrative NTs (ENTs) and the concentrative NTs (CNTs; Zhang et al., 2007). Within the cell, the same enzymes [deoxycytidine kinase (dCK), deoxyguanosine kinase (dGK), thymidine

kinase 1 (TK1) and 2 (TK2)] that are responsible for providing dNTPs for DNA synthesis in resting cells sequentially phosphorylate nucleoside analogs to mono, di- and tri-phosphate variants. Triphosphates represent the active cytotoxic form of nucleoside analogs (Jordheim and Dumontet, 2007).

Targeting every proliferating cell, the lack of specificity of nucleoside analogs leads to negative side effects ranging from bone marrow suppression with immune system depletion to neurotoxicity. Concomitantly, targeted cells can also develop resistance to nucleoside analogs due to decreased activity of the dCK/dGK activating enzymes or by loss of expression of the NTs.

Cytarabine or ara-c was the first nucleoside analog developed starting from modification of 2-deoxycytidine and approved by FDA in 1969 for acute myeloid leukemia (AML) treatment (Johnson, 2001). Ara-c carries a hydroxyl group inserted at the 2′ position of the sugar and, once inside the cell, becomes phosphorylated by dCK. The triphosphate form, ara-CTP, can be inserted into the DNA in active synthesis instead of deoxycytidine triphosphate (dCTP). Since the 3′–5′ proofreading activity of DNA polymerases is slower than ara-CTP incorporation, the modified newly inserted nucleoside, which is not a good 3′ substrate for DNA polymerases, will lead to the stalling of the replication fork (Ross et al., 1990). Gemcitabine is also a 2-deoxycytidine analog with two fluorine introduced in the 2′ position of the sugar. Like cytarabine, the antitumor activity of this molecule is due to the incorporation of the triphosphate form into DNA and concomitant competition with dCTP (Hertel et al., 1990). Gemcitabine has the capacity to inhibit ribonucleotide reductase and therefore decreasing the deoxynucleotide pools (Wang et al., 2007). This nucleoside analog is active in solid tumors such as pancreatic, breast, ovarian and non-small cell lung cancers (Ewald et al., 2008). Gemcitabine was shown to have a better cellular uptake, a longer retention time (Plunkett et al., 1995) and to enhance the antiproliferative capacities of cisplatin in combination regimen treatment (van Moorsel et al., 2000).

While the stereochemical form of natural nucleosides is the β-D-configuration, Troxacitabine is a different kind of pyrimidine analog forming the opposite conformation, the β-l. Its uptake is not mediated by ENTs or CNTs and it is phosphorylated by a different type of kinase, the 3-phosphoglycerate kinase. Troxacitabine's antiproliferative activity was demonstrated in clinical trials for both solid and hematological malignancies (Swords and Giles, 2007). CNDAC is a cytosine analog with a completely different way of action. In contrast to ara-c, gemcitabine and troxacitabine-mediated cytotoxicity that is achieved through replication fork stalling with concomitant S-phase arrest, CNDAC antiproliferative effects are derived from the capacity to induce G2 arrest and to induce DNA DSBs (Wang et al., 2008). Fludarabine and Cladribine, which are used for the treatment of blood malignancies, represent examples of purine analogs based on modifications of 2′-deoxyadenosine. Fludarabine has a fluorine atom at the 2′ position of adenosine plus a phosphate group at the 5′ carbon of the arabinose ring while the only modification of cladribine is, instead of the fluorine, a chlorine atom in the 2′ site of the sugar. Like their pyrimidine analogs, also these molecules are internalized by the NTs, they undergo the same activation steps and they ultimately kill cells by activating the DDR upon DNA incorporation (Huang et al., 1990). Fludarabine and Cladribine were reported to also interfere with the activity of ribonucleotide reductase, DNA ligase, DNA primase (Clarke et al., 2001) and to induce apoptosis through APAF-1 (Genini et al., 2000). Of note, both drugs result cytotoxic also for non-dividing cells (Galmarini et al., 2002).

Clofarabine is another purine analog that was developed in order to ameliorate the two aforementioned predecessors and it was brought into use in 2006 for the treatment of pediatric acute lymphoblastic leukemia (ALL; Bonate et al., 2006). It carries a fluorine atom at the 2′ site of the purine which increases the stability of the molecule and, like gemcitabine and fludarabine, the triphosphate form of clofarabine blocks DNA synthesis, inhibits ribonucleotide reductase and triggers apoptosis by directly affecting the release of cytochrome c from the mitochondria (Ewald et al., 2008). In addition, clofarabine showed *in vitro* cytotoxicity also in non-small cell lung, colon, central nervous system, ovarian, renal, prostate, and breast cancer cell lines (Bonate et al., 2006).

Alkylating Agents

Alkylating agents are one of the oldest antineoplastic drugs. The first glimpse of a therapeutic potential of this class of compounds appeared during the first world war when it was noticed that people exposed to sulfur mustard, a chemical warfare, were developing bone marrow suppression and lymphoid aplasia (Krumbhaar and Krumbhaar, 1919). In 1949, Chlormethine, sold under the name of Mustargen, was the first alkylating agent to be approved by FDA for the treatment of leukemia and lymphomas. Alkylating drugs function during all phases of the cell cycle via formation of reactive intermediates, which attack nucleophilic groups on DNA bases with high negative potential. Of consequence, the primary targets of alkylating agents are purines with N7- and O6-methyl guanine being the most stable *in vitro* methylation adducts (Kondo et al., 2010). Base alkylation can also occurs on adenines on positions N1, N3, N6, N7. Pyrimidines can as well be alkylated: cytosines on positions N3 and O2 and thymidines on O2, N3, and O4 sites (Puyo et al., 2014). Alkylation of oxygen atoms can be highly mutagenic, while *N*-akylations are more cytotoxic. RNA, proteins, and lipids can also be targets of alkylation. Alkylating agents can be either mono- or bifunctional depending on the number of active sites they have and the possibility to react with one or two DNA strands. Monoalkylating agents transfer one alkyl group to their targets resulting in a single base modification and, if not promptly repaired, lead to relative base mispairing (alkylated guanines can wrongly pair with thymines) or to strand breakage due to the formation of an apurinic/apyrimidinic (AP) site. On the other side, the two electrophilic sites of bifunctional agents can attack two different bases on the same or on opposite DNA filaments to form intra- or interstrand crosslinks, respectively, which potentially inhibit strand separation during replication or transcription.

DNA crosslinks can also be introduced as a result of the interaction between two adjacent bases previously modified by monofunctional agents (Fu et al., 2012). Alkylating agents used in chemotherapy are divided in six groups: nitrogen mustards, alkyl sulfonates, ethylenimines, triazines, and nitrosoureas.

Nitrogen mustards represent the oldest group of bifunctional alkylating agents initially used to treat cancer patients. Due to the short half-life and high toxicity, the use of chloremethine, the progenitor of this class of compounds, is actually restricted to veterinary medicine but many of its derivatives were developed and are actually applied in the treatment of different neoplasias. Chlorambucil and Bendamustine are used for treating chronic lymphocytic leukemia (CLL). Melphalan, apart from being implied in breast and ovarian cancers, Hodgkin's disease and neuroblastoma, is the standard treatment, in combination with prednisone, for multiple myeloma (Alexanian et al., 1967).

Cyclophosphamide, the most used drug of this class of agents, possesses the broadest spectrum of anticancer activity. In addition to its beneficial role in hematological malignancies, it is also effective in the treatment of solid tumors like bladder, brain, breast, cervix, endometrium, lung, ovary, and testis (Emadi et al., 2009). Ifosfamide is structurally similar to cyclophosphamide and it is as well utilized in solid tumors such as cervix, testes, head and neck, breast, ovary, and lung tumors. Cyclophosphamide and ifosfamide are prodrugs that require activation in the liver by cytochromes p450.

Busulfan belongs to the class of alkyl sulfonates and is one of the most important bifunctional agent for the cure of chronic myelogenous leukemia (CML; Haut et al., 1961), lymphomas and myeloproliferative disorders.

Thiotepa and altretamine are examples of another class of bifunctional alkylating agents, ethyleneimines. The first one is used for ovarian, breast, and bladder cancer (van Maanen et al., 2000), while the second one has shown positive effects for recurrent ovarian cancer following cisplatin therapy (Chan et al., 2003).

Triazines and nitrosoureas represent two classes of monofunctional alkylating agents with the main difference in their donor alkyl group: a methyl for triazines and chloroethyl for nitrosoureas. Examples of triazines are dacarbazine, an hepatic activable agent included in the treatment of melanoma (Hersh et al., 2011) and temozolomide which is used for primary brain tumors thanks to its high bioavailability in the nervous system (Stupp et al., 2005). Nitrosoureas reduce the *in vitro* proliferation of different cancer cell lines (Gnewuch and Sosnovsky, 1997) and possess activity against solid and non-solid tumors. Carmustine, lomustine, nimustine, and fotemustine are examples of nitrosoureas derivatives that need to be considered for the treatment of brain tumors and skin cancer.

The classic negative side effects of alkylating agents are nausea and fatigue as well as myelo- and immunosuppression and cardiac dysfunction. In addition, most of these chemotherapy agents have mutagenic and carcinogenic potential.

The products of mono *N*-alkylation are repaired by base excision repair (BER) or direct reversal. BER is initiated by DNA glycosylases, which recognize and remove the DNA lesion with the concomitant formation of an abasic (AP) site. The AP site is then processed by specific endonucleases and the missing nucleotide is inserted by DNA polymerase-β. Sealing of the nick is performed by DNA ligase, which finally restores the DNA integrity (Kim and Wilson, 2011). The BER pathway specifically repairs N7MeG, N3MeA, and N3MeG and downregulation of BER components [APE1 endonuclease, polymerase-β, poly (ADP-ribose) polymerase (PARP)] was shown to sensitize tumors to alkylating agents (Liu and Gerson, 2004).

The human AlkB homologs ABH2 and ABH3 are demethylases that catalyze the direct reversal of the following lesions: N1MeA, N3MeC, N3MeT, and N1MeG (Aas et al., 2003). Like for BER deficiency, inhibition of AlkB proteins enhances the chemotherapeutic effects of alkylating drugs (Ralhan and Kaur, 2007). Alkylations of the oxygen atoms, on the other hand, are targets of the repair protein methylguanine DNA methyltransferase (MGMT), which is able to transfer the inserted alkyl groups into its own active site in an auto-inactivating reaction (Pegg et al., 1994). MGMT importance is underlined by the notion that *mgmt* deficient cells are more sensitive than wild type to methylating agents (Day et al., 1980) while MGMT overexpression correlates with resistance to temozolomide (Kaina et al., 2007). MGMT is an optimal candidate to be taken in consideration to sensitize alkylating agent-resistant cancers. In this regard, inhibitors like O6-benzyl guanine (O6-BG), a pseudosubstrate of MGMT, have been proved to enhance the response to temozolomide in cells with high level of MGMT (Zhang et al., 2009b).

O-alkylations can also be repaired by NER or MMR. In contrast to MGMT, MMR presence is indispensable for the antiproliferative activity of alkylating agents: in MMR deficient cells, the damage accumulates but is not translated in the apoptotic signal. Abrogation of MMR rescues the sensitivity of *mgmt* $^{-/-}$ mice to *N*-methyl-*N*-Nitrosourea (Klapacz et al., 2009).

All the aforementioned repair systems act together with HR, FA, and TLS pathways to solve the more complex lesions caused by the action of bifunctional alkylating agents. The interstrand DNA crosslinks introduced by the latter are usually repaired previous transformation in DSBs (Kondo et al., 2010). Targeting key proteins involved in these processes could represent an attractive strategy to enhance the tumor response to this class of chemotherapeutic drugs.

PARP1 Inhibitors

Personalized medicine uses targeted therapies on specific patients cohorts and PARP1 inhibitors represent a new promising class of chemotherapeutic drugs adopted to exclusively disrupt PARP1 function in HR-defective cancers.

PARP1 belongs to a family of 17 ADP-ribosyltransferases which utilize nicotinamide adenine dinucleotide (NAD^+) molecules as a substrate to form polymers of ADP ribose units (PAR) on target proteins. This post-translational modification, known as PARylation (Chambon et al., 1964), is a reversible fundamental process of the DDR necessary for recruiting to the damaged site PAR-binding factors involved in chromatin architecture and DNA repair. PARP1 is the most expressed member of the family, it has nuclear localization and it plays a major role in BER by associating with SSBs and recruiting crucial repair proteins like X-ray repair cross-complementing protein 1 (XRCC1; Rouleau et al., 2010). In addition, PARP1 is part of the HR and NHEJ machineries thanks to the interactions respectively with MRE11, RPA, RAD51 (Bryant et al., 2009), and ligase IV (Li et al., 2013).

Synthetic lethality is the phenomenon by which combinations of mutations in two or more genes is lethal whereas single

mutation of only one is compatible with viability (Reinhardt et al., 2009).

PARP1 inhibition was found to be effective in the treatment of tumors carrying mutations in BRCA1 or BRCA2 genes. In these tumors, the accumulation of SSBs, upon treatment with PARP1 inhibitors, leads to stalling of replication forks and to the formation of DSBs, which cannot be repaired in the absence of functional BRCA1 and/or BRCA2 proteins finally resulting in high level of genomic instability and eventually cell death. Thus, by exploiting the concept of synthetic lethality, PARP1 inhibitors selectively kill malignant cells that are HR deficient (Rouleau et al., 2010). Since PARP1 dissociation from DNA is mediated by auto-PARylation, PARP1 inhibitors exert their cytotoxic effects also by causing a permanent bound of PARP1 to SSBs thereby inhibiting the accessibility of other PARP proteins to the DNA lesion (Elvers et al., 2011).

Besides BRCA1 and BRCA2, sensitivity to PARP1 inhibitors was also observed *in vitro* for the deficiency of other HR genes including RAD51, RAD54, DSS1, RPA1, NBS1, ATR, ATM, CHK1, CHK2, FANCD2, FANCA, and FANCC (McCabe et al., 2006). This finding support the notion that BRCA associated cancers respond to PARP1 inhibitors due to abnormal HR and indicate this therapy as a possible treatment for all of the tumors displaying features of "BRCAness." Olaparib was the first PARP1 inhibitor to be approved by the US FDA for the treatment of ovarian cancers with BRCA mutations but many others (e.g., Iniparib, Rucaparib, Niraparib, Veliparib, and BMN-673) are currently assessed in clinical trials, alone or in combination with either chemo or radiotherapy, for several "non-BRCA" tumors (Tangutoori et al., 2015).

Radiotherapy

Together with surgery and chemotherapy, radiotherapy represents a common treatment option for 50% of cancer patients (Delaney et al., 2005). By releasing large amounts of energy that can be adsorbed by atoms or molecules, IR can directly damage the chemical structure of genetic material and it is consequently used to block cancer cells proliferation and inducing cell death (Jackson and Bartek, 2009). Radiotherapy is given alone or in combination with chemotherapy (chemoradiotherapy) or before (neoadjuvant treatment), during (concurrent treatment) and after surgery (adjuvant treatment) and it can be delivered on patients either with external devices or, internally, with sealed radioactive sources placed inside the body near the tumor area (brachytherapy; Baskar et al., 2012). Unsealed radiation sources (such as iodine, phosphorus, strontium, or samarium), sometimes bound to an antibody directed to the malignant cells, represent the last method to deliver IR in tumor therapy. This class of radiopharmaceuticals drugs are present in liquid forms and usually administered orally or by vein injection (Wallner, 2006).

Apart from being used for curing, radiotherapy can also be adopted with palliative intent to release the pain associated with specific types of cancer.

Photons (X-rays and gamma rays) and charged particles are the main forms of IR utilized in cancer therapy. X- and gamma rays represent widely used photon beams with low radiation charge generated respectively from electrons exciting devices and from the decay of radioactive substances like caesium, cobalt or radium. Once they enter the body, electromagnetic waves of photons do not stop on their targets but they keep going and affecting the surrounding healthy tissues by interacting with the electrons of other molecules. Moreover, the radiation dose decreases as the depth of penetration in the body increase (Hall and Giaccia, 2011).

In photon therapy, most of the DNA damage is inflicted indirectly by the reaction with free radicals species formed upon ionization of water components. Of consequence, the availability of oxygen becomes one of the major limitations in treating solid tumors that are known to be hypoxic. To overcome this problem, chemical radiosensitizer that can react with free radicals in a similar way to oxygen have been developed. Nimorazole and Sanazole represent the best examples of oxygen mimicking drugs actually adopted in the clinic (Lomax et al., 2013).

Charged particles radiation therapy use cyclotron and synchrotron to accelerate electrons, protons or heavy ions like carbon causing direct DNA damage due to the higher linear energy transfer (LET) capacity. The large mass of protons and other charged particles, and their unique absorption profile (Bragg's peak: maximum release of energy when the particles stop traveling through the body) minimize the lateral side scatter and inflict a more precise damage to the target (Allen et al., 2011). Although radiotherapy is one of the most effective ways to kill a cancer cell, it causes both early (acute) and late (chronic) side effects due to killing of normal cells and triggering inflammatory responses. Fatigue and sore skin are the most common acute side effects while the chronic ones largely depends on which part of the body is treated with the possibility to develop secondary cancers. Technological advances, like the use of image-guided (IGRT) or intensity-modulated radiotherapy (IMRT), have made a great progress in precisely delivering IR to patients without affecting healthy tissues but the effectiveness of the treatment does not rely only on this aspect. Other factors, such as the genetic background of the patient, have to be considered to maximize the benefit of radiotherapy (Thoms and Bristow, 2010). As mentioned before, IR attacks directly or indirectly the DNA molecule inflicting lesions that range from abasic site to the more cytotoxic SSBs and DSBs (Wallace, 2002). DNA damage sensing and repair mechanisms, and their status within a specific tumor subtype, are therefore of great importance in the establishment of cancer cell sensitivity to radiotherapy and for assessing how their modulation can be exploited in chemoradiotherapy. Inhibition of cell cycle checkpoints mediated by CHK1 and CHK2 or proteins involved in BER, such as APE or POLQ, or in DSBs repair, like ATM or DNA-PK, have indeed been shown to sensitize cancer cells to radiotherapy (Begg et al., 2011).

Concluding Remarks

DNA damage occurs on a daily basis by endogenous and exogenous sources. Distinct DNA repair systems recognize and remove the lesions. When the damage remains unrepaired DNA damage checkpoints can halt the cell cycle or induce cellular senescence or apoptosis. Erroneous repair or replicative bypass of lesions can result in mutations and chromosomal aberrations. When mutations affect tumor suppressor genes or oncogenes, cell might

transform into cancer cells. Therefore, DNA repair is essential for preventing tumor development. However, once a cancer has developed, DNA damage can be exploited to reduce cancerous growth and evoke apoptotic demise of cancer cells. Thus, chemo- and radiotherapies are still today, over 60 years after having been first introduced into tumor therapy, important strategies to fight cancer. Given the central role of genome instability in triggering and treating cancer, it is likely that genotoxic treatments will remain an important avenue of cancer therapy. Also the better understanding of DNA repair systems will allow therapies that specifically target selected repair pathways. It will be of particular importance to gain a deeper understanding how the various DNA repair systems interact with each other in the context of cellular homeostasis and DNA metabolism in order to optimize targeted approaches to cancer therapy.

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REVIEW
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Regulators of homologous recombination repair as novel targets for cancer treatment

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To cope with DNA damage, cells possess a complex signaling network called the 'DNA damage response', which coordinates cell cycle control with DNA repair. The importance of this network is underscored by the cancer predisposition that frequently goes along with hereditary mutations in DNA repair genes. One especially important DNA repair pathway in this respect is homologous recombination (HR) repair. Defects in HR repair are observed in various cancers, including hereditary breast, and ovarian cancer. Intriguingly, tumor cells with defective HR repair show increased sensitivity to chemotherapeutic reagents, including platinum-containing agents. These observations suggest that HR-proficient tumor cells might be sensitized to chemotherapeutics if HR repair could be therapeutically inactivated. HR repair is an extensively regulated process, which depends strongly on the activity of various other pathways, including cell cycle pathways, protein-control pathways, and growth factor-activated receptor signaling pathways. In this review, we discuss how the mechanistic wiring of HR is controlled by cell-intrinsic or extracellular pathways. Furthermore, we have performed a meta-analysis on available genome-wide RNA interference studies to identify additional pathways that control HR repair. Finally, we discuss how these HR-regulatory pathways may provide therapeutic targets in the context of radio/chemosensitization.

Keywords: recombination, Cell Cycle, genomic instability, DNA Repair, PARP inhibitors

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Introduction

The DNA in each single cell is constantly exposed to a variety of endogenous and exogenous factors that cause DNA lesions, such as UV light and genotoxic chemicals. In addition, normal physiological processes also significantly contribute to generating DNA damage, including cellular metabolism, which produces reactive oxygen species (ROS) as side-products, and DNA replication, which is not an error-free process. To cope with this constant assault on genomic integrity, cells have evolved a complex signaling network called the 'DNA damage response' (DDR). The DDR detects DNA lesions, initiates checkpoints that arrest the ongoing cell cycle and in parallel activates dedicated DNA repair pathways (Jackson and Bartek, 2009). Additionally, when the amount of DNA damage exceeds the repair capacity, DDR signaling will clear damaged cells from the proliferative population through senescence or apoptosis.

Defects in DNA repair are frequently observed in cancer and influence the responsiveness of such cancer cells to therapeutic regimens. Particularly, defects in homologous recombination (HR)-mediated repair of DNA breaks caused by hereditary *BRCA1* and *BRCA2* mutations result in increased sensitivity to DNA damaging agents, particularly platinum-based chemotherapeutics (Tan et al., 2008; Alsop et al., 2012). These observations suggest that modulation of HR repair in HR-proficient tumor cells might constitute an effective manner to sensitize cancers for chemotherapy.

Important in this context is the emerging recognition that DNA break repair is under control of many signaling pathways. Also various HR repair-regulatory pathways have been described and a better understanding of how these pathways control HR may provide insight into how HR repair can be inhibited therapeutically to induce chemosensitization. Therefore, we here present an overview of cell-intrinsic or extracellular pathways that control HR repair. Additionally, we performed a meta-analysis on genome-wide siRNA studies to uncover novel HR regulators. Finally, we will elaborate on the potential therapeutic targets within these pathways.

Repair of DNA Breaks

Among the various types of DNA lesions, single strand breaks (SSBs) are very prevalent. SSBs can be efficiently repaired through base replacement via base excision repair (BER) or alternatively through removing whole nucleotides via nucleotide excision repair (NER; Caldecott, 2008). Unrepaired SSBs or SSBs that occur during replication can be converted into DNA double strand breaks (DSBs), which are far more toxic. If left unrepaired, only a very limited amount of DNA DSBs is required to cause cell death. Proper repair of these DSBs is therefore crucial for cellular survival. Cells are equipped with two fundamentally different pathways to repair DSBs; non-homologous end-joining (NHEJ) and HR (**Figure 1A**). Non-homologous end-joining can be performed throughout the cell cycle and directly ligates DNA-ends in a non-conservative fashion. Since broken DNA-ends may need cleaning up prior to ligation, NHEJ repair can be mutagenic (a detailed review of NHEJ can be found in Lieber, 2010).

In stark contrast, HR repair utilizes a DNA template for repair with significant sequence homology, and this type of repair is conservative in nature and non-mutagenic (Wyman et al., 2004). Most frequently, sister chromatids are employed for HR, which restricts this type of repair to late S phase and G_2 phases of the cell cycle, after DNA replication has occurred (Johnson and Jasin, 2000; Krejci et al., 2012). During the highly regulated process of HR, three main phases can be distinguished. Firstly, 3′-single-stranded DNA (ssDNA) ends are generated by nucleolytic degradation of the 5′-strands. This first step is catalyzed by endonucleases, including the MRN complex (consisting of Mre11, Rad50, and Nbs1). In a second step, the ssDNA-ends are coated by replication protein A (RPA) filaments. In a third step, RPA is replaced by Rad51 in a BRCA1- and BRCA2-dependent process, to ultimately perform the recombinase reaction using a homologous DNA template (**Figure 1B**). More detailed descriptions of HR repair can be found elsewhere (Li and Heyer, 2008; San Filippo et al., 2008). Importantly, HR is not only employed to repair DNA lesions induced by DNA damaging agents, but is also essential for proper chromosome segregation during meiosis. The relevance of HR in these physiological processes is illustrated by its strict requirement during development. Mice lacking key HR genes, such as *Brca1, Brca2,* or *Rad51,* display extensive genetic alterations which lead to early embryonic lethality (Gowen et al., 1996; Hakem et al., 1996; Lim and Hasty, 1996; Ludwig et al., 1997; Sharan et al., 1997; Suzuki et al., 1997). Whereas homozygous inactivation of HR genes is usually embryonic lethal, heterozygous inactivation of for instance *BRCA1* and *BRCA2* does not interfere with cellular viability and rather predisposes to cancer, including breast and ovarian cancer (Futreal et al., 1994; Miki et al., 1994; Wooster et al., 1994; Lancaster et al., 1996). The tumors that develop in individuals with heterozygous *BRCA1/2* mutations invariably lose their second *BRCA1/2* allele, indicating that in certain cancers, the absence of BRCA1/2 is compatible with cellular proliferation. How exactly such tumors cope with their HR defect is currently not fully understood (Elledge and Amon, 2002). What is clear, however, is that these HR-deficient cancers are hypersensitive to various DNA damaging agents, including specific chemotherapeutics (Tan et al., 2008; Alsop et al., 2012). Recent studies have indicated that HR-defective tumors are also exquisitely sensitive to novel agents, such as inhibitors of poly-(ADP-ribose) polymerase (PARP; Bryant et al., 2005; Farmer et al., 2005; Tutt et al., 2010). These insights have prompted the search for cancer-associated mutations in HR genes, to be used for patient stratification for PARP1 inhibitors or other drugs that differentially affect HR-deficient cancers. Additionally, novel components and regulators of the DNA repair machinery are being searched for, to uncover the mechanistic wiring of DNA repair and to uncover potential therapeutic targets for treating cancer.

Control of HR by the DNA Damage Response

A predominant pathway that controls HR activity is the DDR, which consists of multiple kinase and ubiquitin ligases working in parallel signaling axes to coordinate a cell cycle arrest with DNA repair and induction of apoptosis (Ciccia and Elledge, 2010). Components of the DDR can be functionally classified as (1) sensors of DNA damage, (2) signal transducers, and (3) effectors. Various 'DNA damage sensor' complexes exist in order to detect different types of DNA lesions. In the context of DNA breaks, the Mre11/Rad50/Nbs1 (MRN) complex acts as a sensor for DNA DSBs. The MRN complex recruits and activates the upstream DDR kinase ataxia telangiectasia mutated (ATM) (Lee and Paull, 2007). Subsequently, ATM recruits and phosphorylates all MRN members (Gatei et al., 2000; Lim et al., 2000; Zhao et al., 2000; Yuan et al., 2002; Trenz et al., 2006; Linding et al., 2007; Matsuoka et al., 2007). ATM-mediated phosphorylation of these

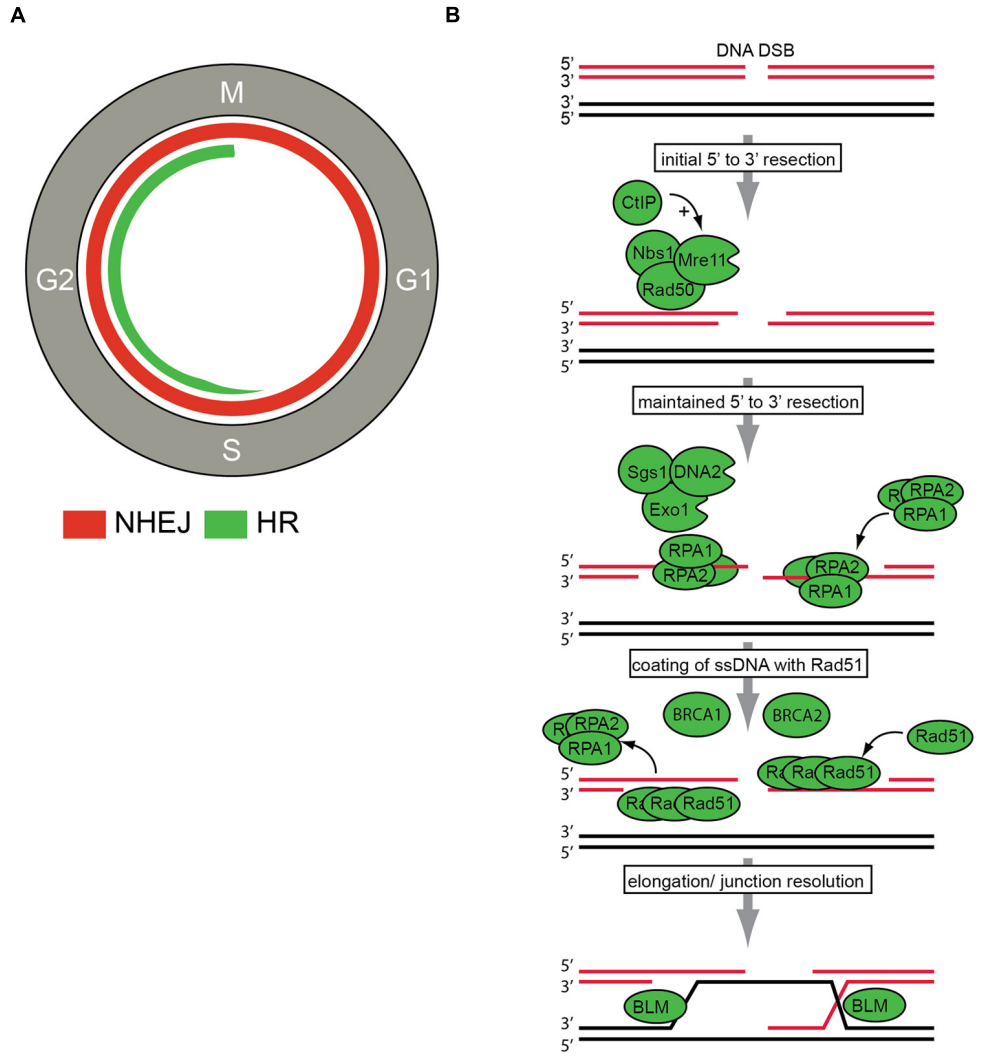

FIGURE 1 | DNA double strand break (DSBs) repair. (A) DNA DSBs repair pathways in the context of cell cycle regulation. Non-homologous end joining (NHEJ) can be performed throughout the cell cycle and is indicated with the red line. Homologous recombination (HR) can only be employed in S/G2 phases of the cell cycle and is indicated in green. **(B)** The key steps in HR repair pathway are indicated. After DSB recognition, 5′–3′ end resection is initiated by the MRN (Mre11, Rad50, Nbs1) complex and CtIP. Subsequently, further resection by the Exo1, DNA2, and Sgs1 proteins is conducted to ensure 'maintained' resection. Then, resected DNA-ends are bound by replication protein A (RPA). The actual recombination step within HR repair, termed strand exchange, is executed by the recombinase Rad51. Rad51 replaces RPA to eventually assemble helical nucleoprotein filaments called '*presynaptic filaments.*' This process is facilitated by other HR components, including BRCA1 and BRCA2. Final step of junction resolution is executed by helicases including Bloom syndrome, RecQ helicase-like (BLM) helicase.

HR components is relevant, as mutational inactivation of these ATM phosphorylation sites prevents the formation of the MRN complex at the sites of damage induced by ionizing radiation (Lim et al., 2000; Zhao et al., 2000), and precludes subsequent cell cycle checkpoint activation and DNA repair (Gatei et al., 2011). MRN/ATM activation consequently leads to the recruitment of additional MRN complexes to the DSB site (Kozlov et al., 2011) and goes along with phosphorylation of other, HR components by ATM, including Brca1 (Cortez et al., 1999; Li et al., 2000) and CtIP (Wang et al., 2013).

Although ATM phosphorylates multiple HR components, it remains unclear to what extent ATM is required for HR. Genetic inactivation of *ATM* in chicken DT40 cells disrupted the formation of irradiation-induced Rad51 and Rad54 foci pointing at impaired HR repair (Morrison et al., 2000; Köcher et al., 2012). However, complete loss of the *Atm* gene in mice did not affect HR capacity in mouse somatic cells in another study (Kass et al., 2013). In contrast, chemical inhibition consistently abrogates HR repair, and points at dominant-negative effects of chemically inhibited ATM (Choi et al., 2010; Kass et al., 2013).

Besides ATM, also ataxia telangiectasia and Rad3 related (ATR) was shown to play a role during HR. In parallel to ATM activation upon DSB formation. The ATR kinase is activated in response to ssDNA, which predominantly occurs

at stalled replication forks (Zou and Elledge, 2003). However, ssDNA is also an intermediate product during HR as a result of DSB processing, and leads to ATR activation in response to DSBs (Jazayeri et al., 2006). Later studies showed that ATR activation not merely is a side-product of DNA-end processing, but is actively involved in the process of HR. Specifically, ATR-dependent hyperphosphorylation of CtIP in response to DSBs is required for CtIP accumulation on the chromatin and extension of DNA-end-resection (Peterson et al., 2013). Combined, it appears that ATM is required for an early resection, whereas ATR is responsible for extensive resection and full checkpoint activation. Although the exact roles of ATM and ATR in the regulation of DNA-end-resection during HR are not yet fully understood, the observation that ATR inhibitors block HR repair warrants further investigation of DDR kinases as therapeutic targets to block DNA repair (Prevo et al., 2012).

Besides regulating the recruitment of HR factors to sites of DNA DSBs, also the actual recombination phase of HR repair is regulated by DDR members. ATM, together with c-Abl, regulates the post-translational modification and assembly of Rad51 filaments (Chen et al., 1999). Furthermore, the downstream checkpoint kinase Chk1 was shown to play a role during recombination. Specifically, Chk1 phosphorylates Rad51 at Thr-309 (Sørensen et al., 2005), which consequently facilitates the assembly of Rad51 nucleofilaments by promoting the displacement of RPA with Rad51 and Rad52 (Sleeth et al., 2007). Importantly, Chk1-depletion resulted in abrogation of Rad51 nuclear foci formation in cells exposed to hydroxyurea, illustrating the functional importance of this interaction (Sørensen et al., 2005). In addition, also Chk2 is involved in regulating HR repair, and Chk2-mediated phosphorylation of Brca1 at Ser-988 was shown to be essential for proper recombination repair (Zhang et al., 2004).

Also negative regulators of DNA-end resection, including 53BP1 and Rif1, are phosphorylated by ATM and are recruited to sites of DNA damage in an ATM-dependent fashion (Escribano-Díaz et al., 2013). Specifically, 53BP1 is phosphorylated by ATM at multiple residues and removal of these sites prevents efficient recruitment of 53BP1 to sites of DNA breaks. In turn, Rif1, which is also phosphorylated by ATM, binds 53BP1 in a phospho-dependent manner and is required to block HR to promote NHEJ repair.(Callen et al., 2013; Escribano-Díaz et al., 2013). How exactly DDR signaling can simultaneously promote pro-HR and anti-HR factors is unclear. Very likely, integration of other signaling pathways, including cell cycle kinases, may be important in fine-tuning this response.

Although DDR kinases are clearly important for HR repair, it remains difficult to separate the DNA repair functions from the checkpoint functions of these DDR kinases. For instance, mutation of the multiple ATM phosphorylation sites on Brca1 not only blocks HR repair, but also results in defective intra-S and G_2/M checkpoint function (Cortez et al., 1999; Xu et al., 1999). Concluding, HR repair appears to be tightly controlled by DDR signaling. However, intense crosstalk and the plethora of proteins that function both in DDR checkpoint signaling as well as in DNA repair, makes it difficult to pinpoint the exact HR regulatory steps in these pathways.

Cell Cycle Regulation

Homologous recombination repair is tightly coordinated with cell cycle progression, which is in large part governed by cyclin-dependent kinases (CDKs). Yeast studies provided the first notion that HR repair is limited to S and G_2 phases of the cell cycle and that it is sensitive to chemical CDK inhibition (Aylon et al., 2004). Subsequently, many HR components were shown to be under control of CDKs and that cell cycle kinases, including non-CDKs, control several steps within HR (Aylon et al., 2004; Branzei and Foiani, 2008).

DNA-end resection constitutes the critical decision point to utilize HR or NHEJ for repair of DSBs, and this switch is under prominent control of CDKs. Importantly, if DNA-end resection at sites of DNA breaks has been initiated there is no point of return, because ssDNA cannot be used as a substrate for NHEJ DNA repair (Symington and Gautier, 2011). Clear evidence that break-induced DNA-end resection requires CDK1 was provided in budding yeast (Ira et al., 2004). An important CDK substrate in this process appeared to be Sae2 (in humans called CtIP, encoded by the *RBBP8* gene), which is phosphorylated on Ser-267 in a CDK-dependent fashion (Huertas et al., 2008; Huertas and Jackson, 2009). CDK-mediated phosphorylation of CtIP appeared essential for MRN-mediated DNA-end resection (Limbo et al., 2007; Sartori et al., 2007). In addition to CtIP, also Nbs1 is a CDK target, phosphorylation of which stimulates MRN-dependent end-resection, further underscoring the control of end resection by CDKs (Falck et al., 2012).

Whereas lower eukaryotes have limited numbers of CDKs, mammalian cells have multiple CDKs that can partner with several cyclins (Morgan, 1997), which complicates the analysis of DDR-cell cycle interactions. Nevertheless, initial studies showed that Cdk2 phosphorylation of CtIP stimulates the multimeric interaction between CtIP, Brca1, and the MRN complex (Yu and Chen, 2004; Chen et al., 2008). Specifically, Mre11 is thought to bring Cdk2 and CtIP in close proximity to subsequently promote Cdk2-mediated CtIP phosphorylation (Buis et al., 2012). This interaction has been shown functional, since loss or inhibition of Cdk2 diminishes HR capacity and also results in increased sensitivity to DNA damaging agents (Buis et al., 2012). However, more recent data show that also Cdk1 inactivation decreases HR repair activity (Johnson et al., 2011). These findings may illustrate that different cell types have different CDK activity profiles, and corresponding CDK requirements. Indeed, studies in murine CDK knockout strains illustrated that not one individual CDK but the overall CDKs level highly influences DDR activation in mammalian cells (Murga et al., 2011).

Cyclin-dependent kinases requirements in HR are not restricted to the initiation of DNA-end resection. Even after DNA break resection has been initiated, CDK activity seems to influence HR. Specifically, the stabilization of ssDNA tails is cell

cycle-dependent through CDK-mediated phosphorylation of RPA (Anantha et al., 2007). Phosphorylation of the RPA subunit RPA2 at Ser-13 by Cdk1-cyclin B was observed in response to treatment with the chemotherapeutic drug camptothecin. Mutation of these CDK sites in RPA resulted in increased numbers and longer retention of gamma-H2AX and altered cell cycle distribution, and reduced recruitment of other DNA repair factor to sites of DNA damage (Anantha et al., 2007).

Interestingly, recent studies have revealed that not only CDKs but also their bindings partners can influence HR. Two germline specific Cdk2 cyclins (A1 and A2) where shown to potentiate HR repair (Müller-Tidow et al., 2004). Although activity of both cyclin A1 and A2 was reported to be required for HR, only cyclin A1 expression was induced by γ-irradiation in a p53-dependent fashion. Additionally, cyclin D1 emerged as a regulator of HR repair (Jirawatnotai et al., 2011). Upon irradiation, Brca2 recruits cyclin D1 to sites of DNA damage, where it directly interacts with Rad51. Moreover, cyclin D1 appears to be essential for Rad51 function, because decreased levels of cyclin D1 severely affected Rad51 recruitment, and consequently resulted in impaired HR. This requirement appeared independent of the canonical cyclin D-binding partners Cdk4 or Cdk6 (Jirawatnotai et al., 2011).

Cyclin-dependent kinases have also been implicated in the regulation of late-stage processes of HR. After recombination has occurred, sister-chromatids can be connected through so-called Holiday junctions, which are resolved by, among others, the Bloom syndrome, RecQ helicase-like (BLM) helicase. Resolution of Holiday junctions, surprisingly, appears to be negatively regulated by CDKs. Notably, Cdk1-dependent phosphorylation of the BLM helicase during mitosis results in dissociation of BLM from the nuclear matrix (Dutertre et al., 2002). However, the functional consequences of this regulation for HR fidelity still remain unclear. Also Brca2 was shown to be negatively regulated by CDKs. Phosphorylation of Brca2 at Ser-3291 within its C-terminal domain prevents the Brca2–Rad51 interaction and thus impairs Rad51-mediated foci formation (Esashi et al., 2005). The phosphorylation of Brca2 at Ser-3291 appears to depend on Cdk1, since chemical inhibition of Cdk1 activity diminishes Brca2-Ser-3291 phosphorylation (Krajewska et al., 2013). In line with Cdk1 activity being most prevalent during mitosis, this mechanism may functionally restrict HR to those phases of the cell cycle when sister chromatids are available for HR repair. Notably, mitotic inactivation of HR can be exploited using Wee1 inhibitors that can aberrantly activate Cdk1. This results in a block in HR repair, underscoring that CDKs not only activate HR during S-phase, but also block HR during mitosis.

Beyond the CDK-mediated regulation of HR, also other cell cycle kinases were shown to influence HR fidelity. Polo-like kinase 1 (Plk1), for instance, which is required for mitotic entry and mitotic progression (van Vugt and Medema, 2005) was shown to regulate HR. In concert with the cell cycle kinase Casein kinase-2 (CK2), Plk1 phosphorylates Rad51 at Ser-14, which is required for the Rad51 filament formation (Yata et al., 2012). Subsequently, CK2 phosphorylates Rad51 at Thr-13 to enhance the interaction between Rad51 and Nbs1 and to facilitate Rad51 recruitment to sites of DNA damage (Yata et al., 2012). In addition to a direct regulation of Rad51, Plk1 binds, and phosphorylates Brca2 (Lin et al., 2003). This interaction appears to be abrogated after DNA damage induction, suggesting that Plk1 may also negatively influence HR repair. Additionally, Plk3 was implicated in the regulation of DNA break repair through modification of CtIP (Barton et al., 2014). In addition, cells lacking Plk3 were shown to be sensitive to PARP inhibitors, which suggests a role for Plk3 in the HR repair (Turner et al., 2008). However, the exact role for Plk3 within the HR machinery needs to be elucidated. Combined, these data imply that cell cycle kinases other than CDKs are required to properly activate HR repair as well as control its silencing when appropriate (**Figure 2**).

Protein Stability Control

As for all cellular pathways, correct protein folding is essential for proper execution of DNA repair. Protein folding is mediated by so-called 'protein-stability control' pathways, controlled by heat-shock protein (HSP) family members (Lindquist and Craig, 1988) Among their many client proteins, several cell cycle control, and DNA repair components appear to be under control of these molecular chaperons. Specifically, Hsp90 appears to control the stabilization, folding, and activation of key HR repair signaling proteins. Most prominently, inhibition of Hsp90 using 17-AAG resulted in Brca2 destabilization (Noguchi et al., 2006; Dungey et al., 2009; **Figure 2**). In line with blocking Brca2 function, Hsp90 inhibition delayed Rad51 filament formation (Noguchi et al., 2006) and resulted in radiosensitization, which was enhanced by the addition of PARP inhibitors (Dungey et al., 2009). Later studies using the more potent Hsp90 inhibitor NVP-AUY922 confirmed these HR defects and described potent radiosensitiziting effects *in vivo* (Zaidi et al., 2012).

The observations that Brca2 depends heavily on protein-stability chaperons have initiated investigations to see whether Brca2 could be destabilized by mild hyperthermia. Indeed, Brca2 is efficiently but transiently destabilized by a short-term cellular hyperthermia (41–42,5°C; Krawczyk et al., 2011). As a consequence, hyperthermia blocked the recruitment of Rad51 to sites of DNA damage and led to impaired HR (Krawczyk et al., 2011). These HR defects coincided with radiosensitization and increased sensitivity for PARP inhibitors *in vitro* and *in vivo* (Krawczyk et al., 2011). Clearly, these findings offer clinical opportunities, since it allows local induction of HR deficiency, which could be used to sensitize tumors for concomitant radiotherapy and PARP inhibitor treatment (Eppink et al., 2012). In addition, the observed Brca2 destabilization offers an appealing explanation for the earlier observed radio- and chemosensitizing effects of hyperthermia, both pre-clinically and clinically (Overgaard et al., 1995; Vernon et al., 1996; Sneed et al., 1998; van der Zee et al., 2000). However, it should be noted that defective HR through Brca2 inactivation does not explain the entire radiosensitizing effect of hyperthermia. Using isogenic cell lines with defects in various repair pathways, HR only partially contributed (Kampinga et al., 2004). In addition,

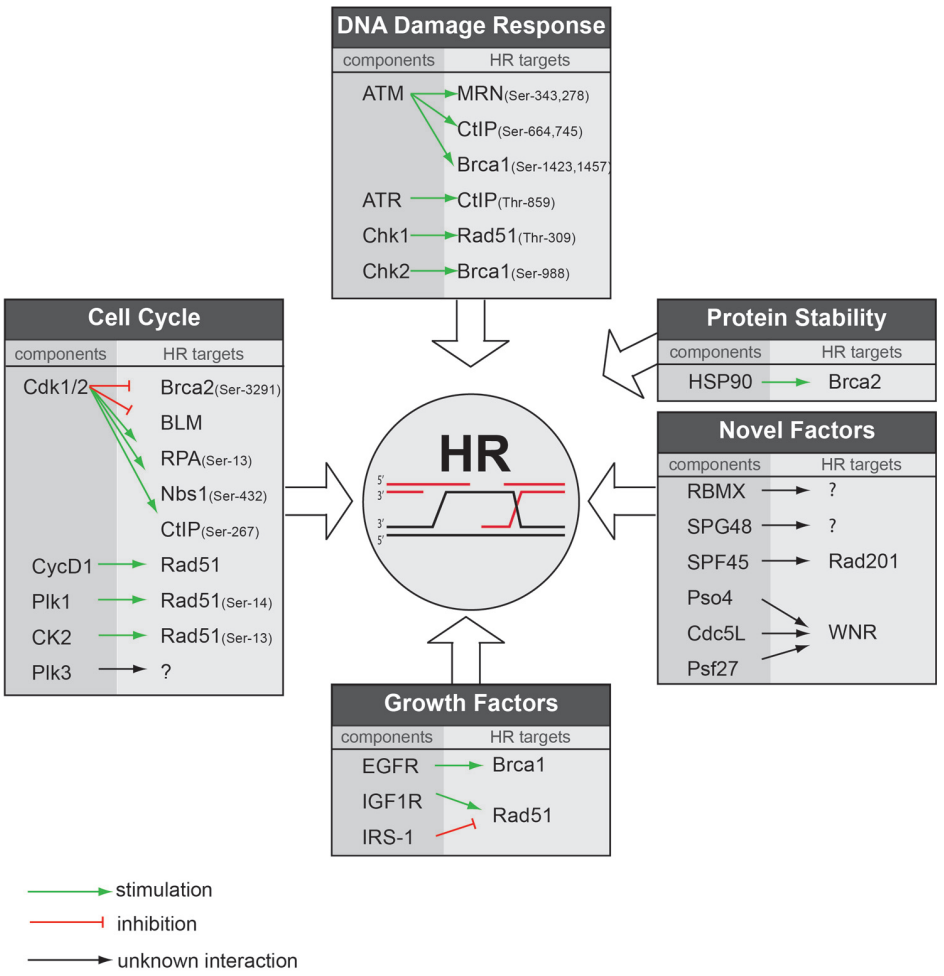

FIGURE 2 | Regulators of HR repair as potential therapeutic targets. Based on literature and the GSEA performed in this report, an overview is provided on the various cellular pathways that regulate HR repair. For each pathway, the responsible component is highlighted along with its substrate within HR. Green arrows indicate stimulatory interactions, red bars indicate inhibitory interactions, and black arrows indicate interactions with unclear effect.

effects of hyperthermia were observed in all cycle phases, which does not support the cell cycle-restricted action of HR repair, suggesting additional targets for hyperthermia in DNA repair (Dewey et al., 1978; Kim et al., 1978). Summarizing, current data support the protein-stability machinery as a feasible therapeutic target to decrease HR capacity, either using Hsp90 inhibitors or through mild hyperthermia. Further, these results warrant clinical studies to combine these approaches with genotoxic therapies that are especially effective in HR-deficient cancers, including PARP inhibitors and platinum-containing chemotherapeutics.

In addition to control of HR DNA repair by HSPs, multiple other enzymes have been to control the stability of DNA repair components. Classically, modification of proteins with ubiquitin has been linked to protein-stability control (Hershko and Ciechanover, 1998). Within the DDR, however, ubiquitilation (as well as SUMOylation) have been shown primarily with activation and protein complex formation (Jackson and Durocher, 2013). However, recently the key HR component CtIP was shown to be ubiquitilated by the APC/C-Cdh1 in a cell cycle and DNA damage-dependent fashion (Lafranchi et al., 2014). Whether the APC/C-Cdh1 controls other DDR proteins upon DNA damage, and whether this affects DNA repair needs further investigation.

Regulation of HR Repair by Growth Factor Receptor Signaling

Growth hormone receptor pathways encompass multiple signaling cascades, controlling many cellular processes including proliferation, cellular survival, and migration. These pathways, including the epidermal growth factor receptor (EGFR) pathway, are frequently hyperactivated in cancers through mutation or amplification and constitute so-called 'oncogenic drivers' (Sharma et al., 2007). However, part of their oncogenic potential may also be explained by promoting DNA repair. Indeed,

growth hormone receptor signaling contributes to increased resistance to radio- or chemotherapy, which likely is related to modulation of DNA repair (Mukherjee et al., 2009). With growth factor receptors being oncogenic drivers, multiple therapeutics have been clinically developed to target growth factor receptors (including antibodies and small molecule inhibitors targeting the EGFR, HER2, and IGF1R). When tested in combination with chemo-radiotherapy, these agents appear to improve responses to radio- and chemotherapy in several cancer types (Huang and Harari, 2000; Bonner et al., 2006). Furthermore, small molecule tyrosine inhibitors that ablate kinase activity of oncogenic variants of these receptors (including erlotinib and gefitinib) have clinical benefit in combination with chemotherapeutics in multiple pre-clinical and clinical studies (Moyer et al., 1997; Knight et al., 2004; Mellinghoff et al., 2005; Quatrale et al., 2011).

Since growth factor receptors control various downstream pathways related to growth and survival, it has remained difficult to pinpoint the influence of growth factor receptor signaling on DNA repair. In addition, it is not completely clear through which mechanism(s) growth factor receptor signaling influences DNA repair, and which DNA repair subtypes are actually modulated by such pathways. In the context of DNA DSB repair, both NHEJ as well as HR were shown to be under control of growth hormone receptor-mediated signaling. Treatment of cells with EGF was shown to increase levels of NHEJ as well as HR (Golding et al., 2009; Myllynen et al., 2011). In the context of promoting NHEJ, the EGFR was reported to associate with the catalytic subunit of DNA-PK, an essential NHEJ component (Liccardi et al., 2011). Additionally, nuclear localization of the EGFR required for DNA repair stimulation, occurs through its interaction with DNA-PK (Liccardi et al., 2011). Additionally, stimulation of the EGFR or the insulin-like growth factor receptor 1 (IGF1R) also elevates levels of HR repair (Golding et al., 2009; Myllynen et al., 2011). Concerning the role of the EGFR in HR repair, it was shown that EGFR activity is required for Brca1 localization to the nucleus (Li et al., 2008). Consequently, blocking the EGFR using erlotinib prevents nuclear Brca1 localization, interferes with Rad51 recruitment to sites of DNA damage and attenuates HR repair (Li et al., 2008). Surprisingly, the role of the IGF1R in HR repair appeared to be mechanistically distinct. IGF1R signaling promotes cellular trafficking of Rad51 through a direct interaction between the insulin receptor substrate-1 (IRS-1), which is recruited to sites of DNA lesions in response to DNA damage (Trojanek et al., 2003). In line with this observation, blocking IGF1R function through deletion of the *Igf1r* gene in mice, or IGF1R depletion ablates IRS-1 phosphorylation, precludes Rad51 translocation to the nucleus and eventually impairs HR repair (Trojanek et al., 2003). Also estrogen-mediated phosphorylation of IRS-1 by the estrogen receptor beta (ERβ) affects HR repair (Urbanska et al., 2009). In contrast to insulin receptor signaling and EGFR signaling, surprisingly, ER signaling negatively impacts the Rad51 function, and inhibition of ERβ-mediated IRS-1 translocation to the nucleus significantly improved DNA repair fidelity and prevented genomic instability (Urbanska et al., 2009). Collectively, multiple growth factor or hormone receptors impact on DNA repair through direct or indirect interactions with DNA repair proteins, albeit that different receptors may have opposite effects in regulating DNA repair.

Since part of the synergistic effects of combined radio/chemotherapy with targeting growth-factor-activated receptors may be explained by interfering with DNA repair, a synthetic lethal context with agents such as PARP inhibitors may be created. Early preclinical evidence indeed underscores this notion, since EGFR inhibition with lapatinib sensitized breast cancer cells to the PARP inhibitor ABT-888 *in vitro* (Nowsheen et al., 2012). In addition, therapeutic targeting of the PI3 kinase, which operates downstream of the EGFR and IGF1R efficiency blocked HR repair through down regulation of both Brca1 and Brca2 and sensitized cells for PARP inhibition (Ibrahim et al., 2012). Concluding, HR DNA repair is not just a cell-intrinsic repair mechanism. Many pathways, including growth factor-activated pathways, were shown to regulate HR, providing a rationale for combined inhibition of growth factor activated pathways with DNA damaging agents.

Novel Regulators of Homologous Recombination

The development of fluorescence-based reporter systems to read out HR efficiency (Pierce et al., 1999) has enabled high-throughput microscopy studies to uncover novel regulators of HR in mammalian cells. Two important studies have taken a genome-wide approach to identify genes that are required for HR repair (Słabicki et al., 2010; Adamson et al., 2012). Many of the identified genes from these studies are highly conserved and also appear to be essential for HR in single-cell organisms such as yeast, including *BRCA1*, *BRCA2,* and *RAD51* (McKinney et al., 2013). In addition to these well-known HR components, novel HR regulators were identified. Notably, genes that control post-transcriptional processing of RNA, including mRNA splicing, where found to control HR repair (Adamson et al., 2012). For instance, depletion of the RNA binding protein RBMX led to diminished Brca2 levels and a consequent failure to recruit Rad51 to sites of DNA damage. Additionally, a putative helicase SPG48 is required for HR repair, although it remains mechanistically unclear which step of HR it controls (Słabicki et al., 2010). The availability of two independent genome-wide siRNA screens for genes that regulate HR allowed us to compare these data sets and identify common pathways and genes. To this end, we applied Gene-Set Enrichment Analysis (GSEA; Subramanian et al., 2005) to uncover additional pathways that modify HR repair, of which targeting could have therapeutic potential (**Figures 3A,B**). Four well-known pathway databases were used for enrichment analysis, KEGG, Biocarta, Reactome, and GeneOntology (**Figure 3C**).

The most striking enrichment was identified in genes that participate in proteasome or RNA biology. These findings match earlier reports demonstrating that proteasome inhibition sensitizes tumor cells to DNA damaging agents, including crosslinking agents, and radiation (Pajonk et al., 2000). Initially,

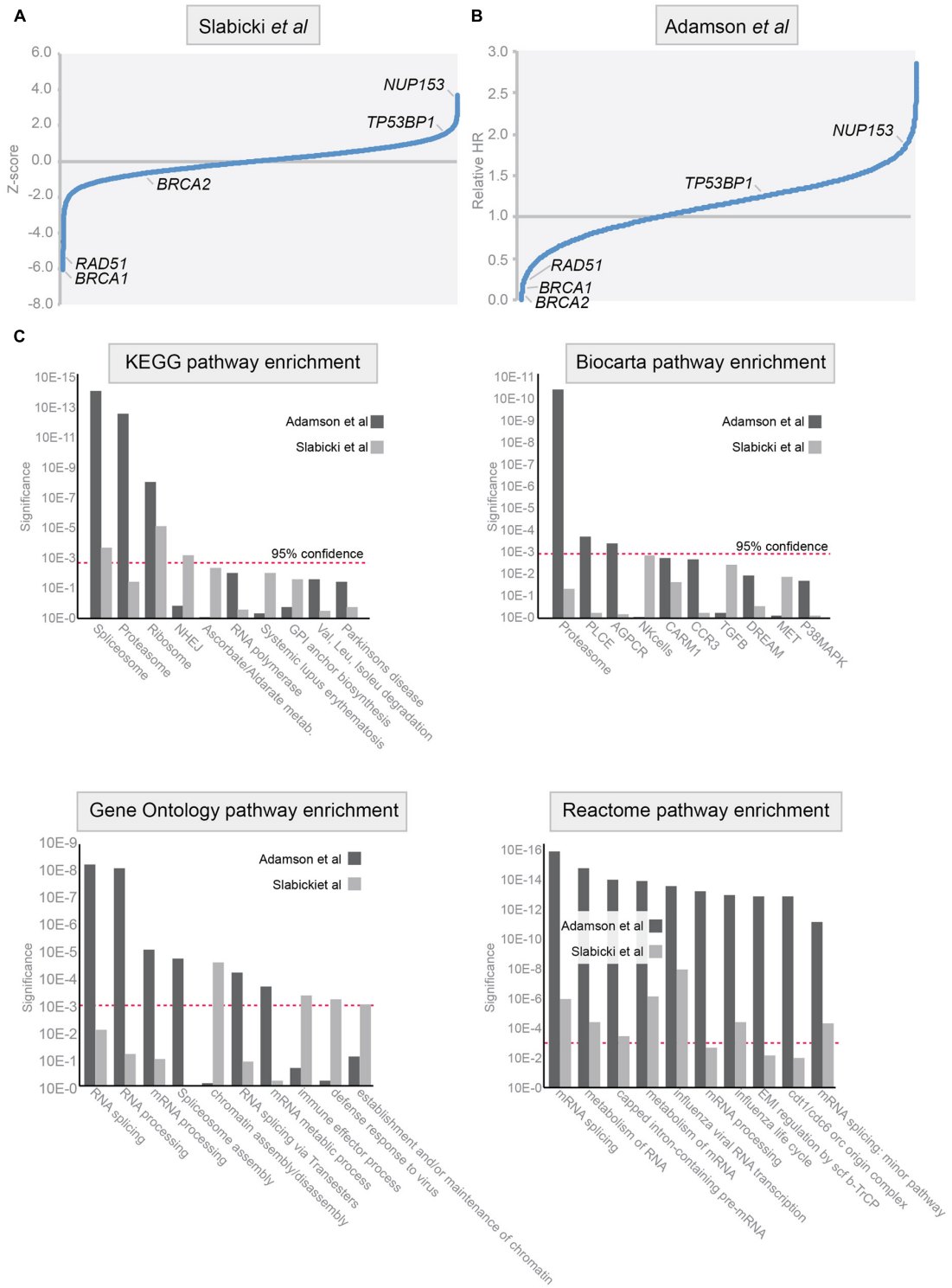

FIGURE 3 | Pathways involved in homologous recombination repair of DSBs. (A,B) Analysis of the data from genome-wide siRNA screens. Data were adapted from published studies (Słabicki et al., 2010, **A**) and (Adamson et al., 2012, **B**). In these studies HR efficiency was assessed using DR-GFP assay in HeLa cells (left) and in DR-U2OS cells (right). Relative HR scores in *Z*-score (left panel) and 'Relative HR score' (right panel) are indicated for genes with an established role in HR: *BRCA1, BRCA2, TP53BP1, NUP153,* and *RAD51*. **(C)** Gene-Set Enrichment Analysis (GSEA) was performed on the data presented in **(A,B)**. Four pathway databases were used for enrichment analysis: KEGG, Biocarta, Reactome, and GeneOntology. Results of the GSEA on the dataset by Słabicki et al. (2010) are represented in light gray, and results on the dataset by Adamson et al. (2012) are represented in dark gray. Red dashed line indicates 95% confidence interval.

these effects were explained by proteasome-mediated control of pro-apoptotic p53 signaling (Vaziri et al., 2005). Thereafter, a more direct role for the proteasome in controlling DNA repair was identified. Inhibition of the proteasome using MG132 or bortezomib, or genetic inactivation of proteasome components blocked the recruitment of DNA repair components FancD2, Brca1, and Rad51, whereas upstream the DDR signaling components H2AX and Mdc1 appeared unaffected (Jacquemont and Taniguchi, 2007; Cron et al., 2013). Due to defective recruitment of these HR factors, inhibitors of the proteasome suppress homologous DNA recombination in mammalian cells (Murakawa et al., 2007). In line with this notion, proteasome inhibition using bortezomib was shown to prevent repair of PARP-inhibitor induced DNA breaks (Neri et al., 2011). Importantly, combined treatment with bortezomib and the PARP inhibitor ABT-888 resulted in sustained levels of H2AX, with defective recruitment of HR repair components leading to enhanced killing of tumor cells. Combined, these results show that the proteasome is involved in HR repair and that therapeutic targeting of the proteasome, using for instance bortezomib, can be used to induce 'BRCAness' in tumor cells. Also, the fact that genome-wide *in vitro* assays identified the proteasome as a system that controls HR repair argues that other HR-controlling pathways may also be uncovered using these approaches.

Among the enriched pathways involved in HR repair, RNA processing was highly abundant (**Figure 3C**), as is matched by the recent identification of RNA-modifying enzymes as regulators of HR (Adamson et al., 2012). In mammalian cells, not much data exist that mechanistically link RNA to HR repair. However, some data from model organisms have provided evidence that RNA processing is involved in DNA repair. Using genetic analysis in *Drosophila*, the RNA splice factor SPF45 was shown to combine a function in RNA splicing and protection against DNA damage caused by MMS exposure (Chaouki and Salz, 2006). Notably, mutations that abolish the RNA splicing function also fail to protect against DNA damage-induced toxicity. Mechanistically, SPF45 appears to interact with Rad201, a member of the RecQ/Rad51 family (Chaouki and Salz, 2006). An unbiased proteomic screening in budding yeast underscored the link between RNA metabolism and DNA repair, for instance by identifying the splicing factor PRP19, as being involved in the DDR (Smolka et al., 2007). In human cells, a mRNA splicing complex consisting of Pso4, Cdc5L, and Psf27 was found to interact with the WNR DNA helicase and was identified to be required for interstrand cross-link repair (Zhang et al., 2005; **Figure 2**). Additionally, Dicer and Drosha RNA products produced from sites of DNA damage where shown to be required for proper DDR signaling (Francia et al., 2012). Again, large-scale proteomic analysis confirmed this notion, and identified multiple factors involved in RNA metabolism, illustrating the intricate connection between RNA splicing and DNA repair (Matsuoka et al., 2007). Part of this relationship can be explained by the observation that uncontrolled mRNA maturation disturbs the DNA-RNA interaction and have deleterious effects on genomic stability (Montecucco and Biamonti, 2013).

Targeting HR-Deficient Tumors Clinically

In oncology, many radio- and/or chemotherapeutic regimens are used in daily practice, which induce high levels of DNA damage directly or indirectly. These therapeutic regimens often induce interstrand DNA crosslinks and DNA DSBs, of which accumulation is very cytotoxic and requires HR for faithful repair. Consequently, tumors in which HR repair is compromised due to mutations or epigenetic silencing of HR repair genes are generally more sensitive to specific DNA damage-inducing factors. Extensive *in vitro* and *in vivo* preclinical studies provided compelling evidence that HR defects are causally related to the vulnerability of such cancer cells to certain DNA damaging chemotherapeutics and radiotherapy. Studies comparing various neo-adjuvant chemotherapeutic regimens in *BRCA1* mutant breast cancers, found that highest response rates were observed with neo-adjuvant cisplatin chemotherapy (Byrski et al., 2010), which increased progression free survival (Byrski et al., 2008). In analogy, responses of ovarian cancer lines to cisplatin were also influenced by their *BRCA1/2* mutation status as well as the status of related HR genes (Taniguchi et al., 2003). These data indicate that inactivation of the HR pathway, either through germ-line or somatic *BRCA1/2* inactivation is linked to increased sensitivity to DNA damaging therapeutics, notably platinum-based agents.

The fact that DNA repair defects through cancer-associated mutations lead to specific vulnerabilities is exploited in synthetic lethal approaches: mutation or inhibition of two separate pathways leads to cell death, whereas loss of function in either one of these pathways does not affect viability. The prototypical synthetic lethal interaction was described for the *BRCA1* and *BRCA2* mutations, which are synthetic lethal with loss of PARP-1 (Bryant et al., 2005; Farmer et al., 2005). Mechanistically, inhibition of PARP1 blocks BER and leads to accumulation of SSBs, which are converted to DSBs in replicating cells (Bryant et al., 2005; Farmer et al., 2005). These DSBs cannot be properly repaired due to the HR defect in *BRCA1/2* mutant cells, which results in cytotoxicity Recent studies provided evidence that PARP is additionally required for restarting of stalled replication forks (Bryant et al., 2009; Petermann et al., 2010). PARP1 accumulates at stalled replication forks and recruits Mre11 to catalyze DNA-end processing for replication restart and recombination. As a consequence, PARP inhibition also results in replication forks collapse, which again leads to accumulation of toxic DNA structures in HR-deficient cells (Schlacher et al., 2011). The finding that *BRCA1/2* mutant cells are selectively sensitive to PARP inhibition constituted the starting point for several clinical trials and the clinical development of various PARP inhibitors. In 2005, the first phase I clinical study investigating a PARP inhibitor (Olaparib, AZD2281) demonstrated that more than 90% of PARP enzymatic activity could be inhibited, which was well tolerated and did not increase toxicity in the *BRCA1/2* mutation carriers group (Fong et al., 2009). More importantly, this study showed clinical benefit of PARP inhibition in patients with *BRCA1/2* mutations (Fong et al., 2009). This early success prompted the clinical development of various PARP inhibitors as single agents or

as part of combined treatment with DNA damaging agents in phase II clinical trials (Audeh et al., 2010; Tutt et al., 2010). In addition the reported beneficial effect of PARP inhibition in breast and ovarian patients with *BRCA1/2* mutations boosted the interest in this type of therapies for other tumors types, including colon cancers, prostate cancer, and gastric cancer (Barreto-Andrade et al., 2011; Sebastian de Bono et al., 2011; Davidson et al., 2013). Results of preformed or ongoing clinical trials with PARP inhibitors are therefore eagerly awaited.

The preclinical studies and early clinical studies raised high potential for therapeutic use of PARP1 inhibitors, but unfortunately PARP inhibitors have not yet delivered the clinical success that preclinical studies promised. Several findings can be attributed to these discrepancies.

Firstly, it appears difficult to effectively select patients for PARP1 inhibitor treatment. The most straightforward strategy to select patients is to obtain the mutation status of *BRCA1/2* in cancer specimens. However, *BRCA1* or *BRCA2* are not only inactivated through gene mutation, also DNA hypermethylation of the genes is frequently reported for several cancers (Esteller et al., 2000; Rice et al., 2000; Cancer Genome Atlas Research Network, 2011). Patients with hypermethylated *BRCA1/2* genes may benefit from PARP1 inhibitors, but may be missed when only *BRCA1/2* mutations status is analyzed. In contrast, when selection criteria are not sufficiently strict, effects of PARP inhibitors may be missed. For example, some studies included all TNBCs, whereas only a subset of these patients may harbor HR defects, and may therefore have clinical benefit from PARP inhibitors (Gelmon et al., 2011). A straightforward, but labor-intense solution to successfully implement PARP inhibitors is to functionally assess HR efficiency in fresh tumor biopsies (Willers et al., 2009). Alternatively, measuring consequences of defective HR, such as genome-wide copy number variation analysis using aCGH may identify tumors with defective HR (Vollebergh et al., 2011). Besides selecting *BRCA1/2* mutant tumors, also mutation of other HR genes results in cancers with similar characteristics, including *PALB2* (Rahman et al., 2007) or *RAD51C* (Clague et al., 2011). Mutation of these genes appeared to result in PARP1 inhibitor sensitivity (Loveday et al., 2012; Min et al., 2013). Also novel regulators of HR may be important in this respect, such as the negative regulator of Brca1 called ID4 (Turner et al., 2007). Whether ID4 mutations or mutations in other novel HR components are frequently found in cancers needs to be studies. Clearly, these observations indicate that there is a strong need to reliably identify cancers with defective HR repair, in order to stratify patient for therapies that target HR-deficient cancers, including PARP1 inhibitors.

Secondly, not all PARP inhibitors appear to be very efficient PARP inhibitors *in vivo*. Moreover, some PARP inhibitors have additional effects. For instance, iniparib (BSI-201) also inhibits other enzymes, including GAPDH (Bauer et al., 2002). It is therefore difficult to verify whether observed clinical benefit in phase II study was achieved exclusively due to PARP inhibition.

A third complicating factor in developing PARP inhibitors for HR-deficient cancers, is the recent observation that secondary mutations may dramatically alter the HR defect of *BRCA1/2* mutation cancers. These secondary mutations can be sub-classified into two categories. The first category consists of intragenic secondary mutations in affected *BRCA1* or *BRCA2* alleles that have been described to restore their reading frame (Sakai et al., 2008; Swisher et al., 2008) and result in resistance to cisplatin. The second category involves secondary mutations in other genes, which have been shown to reverse a HR defect. Mutation of *TP53BP1* for instance, reverses the HR defect in *BRCA1* mutant cancers and render these cancers resistant to PARP1 inhibition. More recently, Rif1 was shown to counteract Brca1 function, and *Rif1* mutations could rescue the genomic instability of mouse *Brca1*$^{-/-}$ cells (Bouwman et al., 2010; Chapman et al., 2012; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Feng et al., 2013; Zimmermann et al., 2013). Whether *Rif1* mutations also account for therapy resistance in *BRCA1* mutant cancers remains to be tested. What is clear is that *BRCA1/2* mutations do not per se reflect a defect in HR, and that functional testing of HR capacity may be required to reliably classify the DNA repair defect in cancers.

Future Perspectives

If HR function could be locally inhibited in cancer cells, this would allow exploitation of the enhanced sensitivity for platinum-containing chemotherapeutics, radiotherapy, or PARP inhibition. The most straightforward approach would be to directly target HR components. The identification of druggable HR genes is therefore actively being pursued. One approach is to chemically inhibit Rad51, the most downstream HR component. Recent studies making use of high-throughput screens have identified chemical Rad51 inhibitors, which increased the sensitivity of glioblastoma cells to alkylating agents (Quiros et al., 2011), and were shown to sensitize various human cancer cells to DNA crosslinking agents, including mitomycin C (Budke et al., 2012).

In addition to directly targeting HR components, the reports described in this review show that modulation of regulatory pathways controlling HR components may be useful as well to achieve an HR-deficient phenotype and thereby sensitize tumor cells to DNA damaging agents (an overview of cellular pathways that regulate HR repair is presented in **Figure 2**). This has been elegantly shown in pre-clinical studies using cell cycle modulators, hyperthermia, DDR inhibitors and Hsp90 inhibitors. Novel approaches, including genome-wide siRNA screens and proteomic interaction maps, may add novel regulators to this growing list of potential therapeutic targets that control HR and warrant translation of these novel targets to uncover their therapeutic potential.

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Regulation of the DNA damage response by ubiquitin conjugation

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In response to DNA damage, cells activate a highly conserved and complex kinase-based signaling network, commonly referred to as the DNA damage response (DDR), to safeguard genomic integrity. The DDR consists of a set of tightly regulated events, including detection of DNA damage, accumulation of DNA repair factors at the site of damage, and finally physical repair of the lesion. Upon overwhelming damage the DDR provokes detrimental cellular actions by involving the apoptotic machinery and inducing a coordinated demise of the damaged cells (DNA damage-induced apoptosis, DDIA). These diverse actions involve transcriptional activation of several genes that govern the DDR. Moreover, recent observations highlighted the role of ubiquitylation in orchestrating the DDR, providing a dynamic cellular regulatory circuit helping to guarantee genomic stability and cellular homeostasis (Popovic et al., 2014). One of the hallmarks of human cancer is genomic instability (Hanahan and Weinberg, 2011). Not surprisingly, deregulation of the DDR can lead to human diseases, including cancer, and can induce resistance to genotoxic anti-cancer therapy (Lord and Ashworth, 2012). Here, we summarize the role of ubiquitin-signaling in the DDR with special emphasis on its role in cancer and highlight the therapeutic value of the ubiquitin-conjugation machinery as a target in anti-cancer treatment strategy.

Keywords: DNA damage, apoptosis, ubiquitylation, genotoxic anti-cancer therapy, p53, Bcl-2

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Ubiquitin—Small Molecule Generating a Broad Range of Cellular Actions

Ubiquitin (Ub) is an essential, highly conserved, 76 residue protein that is ubiquitously expressed in cells. It can be found either in a free form or covalently attached to a target protein (Schlesinger et al., 1975; Hershko et al., 1983; Ciechanover et al., 1985; Hershko and Ciechanover, 1998). Ub acts as a versatile cellular signal that controls a wide range of biological processes, including protein degradation, DNA repair, endocytosis, autophagy, transcription, immunity and inflammation. Ub, E1-, E2-, and E3-enzymes are successively required to target a certain substrate for degradation. Ub is attached to specific substrates in a three-step mechanism, with distinct enzymes catalyzing each step (**Figure 1**). In a first activating step, Ub becomes covalently conjugated to the side chain of an E1-cysteine via its carboxy-terminal (C-terminal) glycine in an ATP-dependent reaction. Activated Ub is then transferred to an E2-enzyme (ubiquitin-conjugating enzyme) via a thioester-bond between the C-terminal glycine residue of Ub and an E2 internal cysteine. Finally, Ub-bound E2 interacts with an E3 Ub ligase that catalyzes Ub transfer from E2 to a specific target protein (Ciechanover et al.,

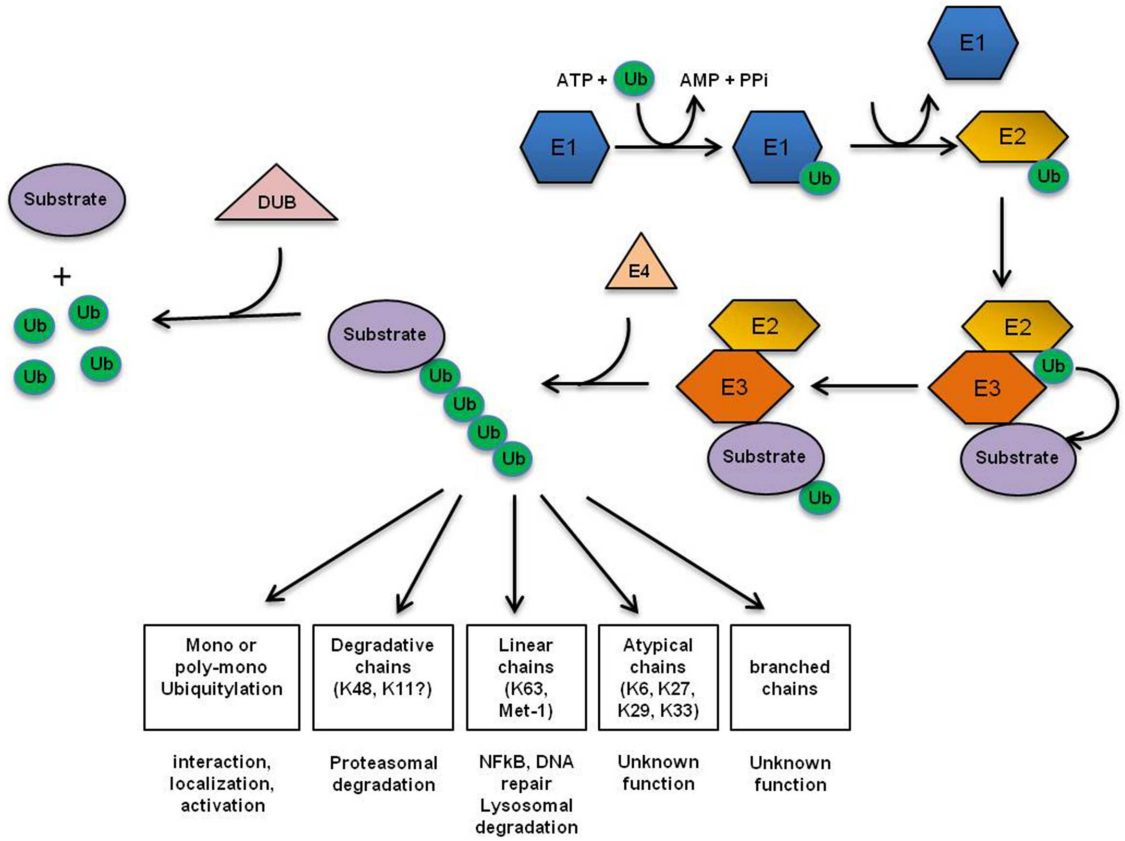

FIGURE 1 | Ubiquitin conjugation machinery. Ub is attached to specific substrates in a three-step mechanism, with distinct enzymes catalyzing each step. First, Ub gets activated by the Ub-activating enzyme (E1). Next, activated Ub is transferred by one of several dozens of Ub-conjugating enzymes (E2) to one of approximately 500 substrate-specific Ub-ligases (E3s) that finally attaches Ub to the substrate (Pickart, 2001). In some cases, the extension of short ubiquitin chains requires additional elongation factors, termed E4 enzymes. About 100 substrate-specific deubiquitylating enzymes (DUBs) counteract the activity of UB-conjugating enzymes (Nijman et al., 2005). The first Ub is either transferred to a ε-NH2 group of a lysine residue (K) of the target protein to generate an isopeptide bond, or in a linear manner to the N-terminal residue of the substrate (Breitschopf et al., 1998; Pickart, 2001). Subsequent Ub addition can occur through isopeptide linkage on all of ubiquitin's seven lysine residues as well as its N-terminal primary amino group, thereby generating a diverse range of chain topologies (Met1-linked, K6, K11, K27, K29, K33, K48, K63 or mixed) that can drive a variety of different protein fates.

1984; Scheffner et al., 1995; Hershko and Ciechanover, 1998). This cascade of sequential interactions results in the formation of an isopeptide bond between the C-terminus of Ub and the ε-amino group of a lysine residue in the target protein (Hershko and Ciechanover, 1998). In some cases, the extension of short Ub chains requires additional elongation factors, called E4 enzymes. *Saccharomyces cerevisiae* ubiquitin fusion degradation 2 (*Ufd2)* is the first discovered E4 enzyme (Koegl et al., 1999; Hoppe, 2005). About 100 substrate-specific deubiquitylating enzymes (DUBs) counteract the activity of Ub-conjugating enzymes (Nijman et al., 2005). The specificity of Ub signaling is achieved by alternative conjugation signals (monoubiquitylation and more complex Ub chains) on alternative substrate sites (Haglund and Dikic, 2005). Diverse chain topologies can specify a variety of different protein fates by providing a platform for the interaction with specific binding partners. These interacting partners depend on Ub binding domains (UBD) or Ub interacting motifs (UIM) to either associate with Ub or to decode ubiquitylated target signals into biochemical cascades (Peng et al., 2003; Komander and Rape, 2012). For instance, monoubiquitylation plays a role in recognizing DNA double strand breaks (DSBs), K63-linked Ub chains are involved in the generation of signaling platforms during DNA repair (Chen et al., 2005a) and polyubiquitin chains covalently connected via K48 linkages mainly target proteins for degradation by the proteasome (Ciechanover et al., 1984; Thrower et al., 2000) (**Figure 1**). The ubiquitin/proteasome system (UPS) is one of the main regulators of protein stability and—among multiple cellular pathways—plays an important role in the execution of the DDR. Multiple studies using proteasome-inhibitors validated the UPS as a valuable therapeutic target in cancer (Voges et al., 1999; Orlowski and Kuhn, 2008); however, targeting one of the major cellular pathways governing protein turnover may cause broad and unspecific off-target cellular responses. Accordingly, ongoing efforts aim to identify the specific targets within the UPS system to selectively target the relevant Ub-conjugation process. Hence, novel Ub ligases or DUBs are frequently evaluated as potential specific targets for anti-cancer therapy.

Recognition of DNA Damage Sites

Massive Ub accumulation around sites of DNA damage can be detected as soon as 15 s following the damage event (Feng and Chen, 2012). Ubiquitylation of the H2A, H2B, and H2AX histone subunits is one of the initial events promoting the destabilization of the nucleosome (Li et al., 1993; Biswas et al., 2011). CHFR (*checkpoint with Forkhead-associated (FHA) and RING finger domain protein*), which is recruited to DSBs by PAR (*poly (ADP) ribose*), regulates the first wave of histone ubiquitylation (Wu et al., 2011a). CHFR ubiquitylates PARP1 (*PAR polymerase 1*) via K48- (site K88, E2: UbcH5C) and K63-linked (E2: Ubc13) Ub chains, and this ubiquitylation is thought to promote the dissociation of PARP1 from damage sites (Liu et al., 2013). Epigenetic inactivation of *CHFR* has been described in several types of cancer, including breast cancer (Erson and Petty, 2004), nasopharyngeal carcinoma (Cheung et al., 2005), colorectal cancer (Toyota et al., 2003), head and neck cancer (Toyota et al., 2003), gastric cancer (Satoh et al., 2003), lung cancer (Mizuno et al., 2002), esophageal cancer (Shibata et al., 2002), hepatocellular cancer (Sakai et al., 2005) and T-cell lymphoma (van Doorn et al., 2005). Furthermore, increasing evidence indicate the regulatory impact of Ub on cancerogenesis. Monoubiquitylation of H2A by RNF2-BM1, a member of the Polycomb repressive complex 1 (PRC1), is thought to be important for the transcriptional repression by inhibiting of RNA-PolII-elongation (Zhou et al., 2008). Interestingly, around 15% of H2A has been described to be constitutively ubiquitylated (Levinger and Varshavsky, 1980). RNF2-BM1 is also involved in monoubiquitylation of H2AX at K119 and K120 (E2: UbcH5C), which in turn initiates the recruitment of the apical PI3K-related kinase *ataxia telangiectasia mutated* (ATM) (Pan et al., 2011; Wu et al., 2011a). ATM is a protein kinase that phosphorylates several key proteins involved in the DDR. So far, no role for this initial histone ubiquitylation in the recruitment of the functionally related apical kinases ATR (*ATM/Rad3-related kinase*) or DNA-PK (*DNA-dependent protein kinase*) has been demonstrated. ATM and ATR transduce the most upstream DDR signal by phosphorylating the checkpoint kinases CHK1/CHK2 and the tumor suppressor protein p53, resulting in cell cycle arrest to allow time for DNA repair, or DDIA after prolonged checkpoint activation, respectively (Shiloh, 2003). Even though the main function of DNA-PK appears to be the induction of cell cycle arrest and DNA repair, specifically the non-homologous end-joining (NHEJ) repair pathway, DNA-PK has also been reported to phosphorylate p53, thus cooperating with ATM/ATR to induce p53-mediated cell cycle arrest and apoptosis (Kim et al., 1999). Notably, the ubiquitin-selective segregase Cdc48/p97/VCP, which is a central regulator of the UPS, influences the DDR by participating in ubiquitylation and proteasomal degradation of the catalytic subunit of DNA-PK(cs) in eukaryotes (Acs et al., 2011; Meerang et al., 2011; Dantuma and Hoppe, 2012; Jiang et al., 2013). Consequently, the interplay of ubiquitylation and phosphorylation events regulates the association of several DDR proteins, most prominently p53, with regulatory E3 ligases or DUBs.

p53—Signal Transducer from DNA Damage to Cellular Actions

Activated p53 translocates into the nucleus where it induces the transcription of several target genes involved in cell cycle regulation, DNA repair, and apoptosis, including the pro-apoptotic molecule BAX (Miyashita and Reed, 1995) and the BH3-only proteins PUMA (Nakano and Vousden, 2001) and NOXA (Oda et al., 2000), which are central in initiating DDIA (**Figure 2**). Loss of p53 function is described in over 50% of human cancers and is frequently associated with a poor patient prognosis (Hollstein et al., 1994). The mechanisms by which p53 differentially triggers cell cycle arrest, senescence, and apoptosis are far from being completely understood; however, different post-translational modifications of p53 (e.g., phosphorylation) have been described that either alter its DNA binding capacity directly or that control its association with different binding partners, including transcriptional activators and repressors, thereby affecting the p53-induced transcriptome in response to DNA damage (Aylon and Oren, 2007). Moreover, it appears that p53 has different affinities toward different p53-responsive elements and different levels of p53 protein might fine-tune its promoter choice, thus determining cell fate. Indeed, low p53 levels tend to favor growth arrest, whereas higher levels trigger apoptosis (Laptenko and Prives, 2006). p53 protein stability is efficiently regulated by the UPS and several E3-ligases (**Table 1**) and DUBs (**Table 2**) have been reported as its direct regulators. Not surprisingly, most E3s or DUBs that regulate p53 stability are also implicated in cancer and further represent promising targets for anti-cancer therapy.

Regulation of p53 by Ubiquitin Ligases

The E3 ligase MDM2 has been shown to directly target p53 for proteasomal degradation while ATM/ATR-mediated phosphorylation of p53 hampers this interaction (Momand et al., 1992); however, MDM2 only mediates monoubiquitylation of p53, but not its polyubiquitylation, arguing for the involvement of additional Ub ligases (Lai et al., 2001). Interestingly, MDM2 is a transcriptional target of p53 and thus acts in a negative feedback loop. Furthermore, MDM2 itself is also a target of ATM and ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage (Khosravi et al., 1999). ATM-mediated phosphorylation of MDM2 at S395 induces MDM2 protein destabilization. One major molecule that has been further implicated in regulating MDM2-mediated p53 proteolysis is MDMX (MDM4). MDMX activity seems to be essential for MDM2-mediated p53 proteolysis by converting MDM2 into an active conformation (Di Conza et al., 2012) and further stimulating MDM2 ligase activity (Linke et al., 2008; Wang et al., 2011; Wade et al., 2012). ATM-mediated MDM2/MDMX phosphorylation disrupts MDM2 oligomerization, thereby inactivating MDM2, leading to p53 stabilization and activation (Cheng and Chen, 2010); however, MDM2 additionally promotes ubiquitylation and degradation of MDMX in response to DNA damage, again acting in a negative feedback loop (de Graaf et al., 2003; Kawai et al., 2003; Pan and Chen, 2003; Pereg et al., 2005; Xia et al., 2008). Additional studies showed that

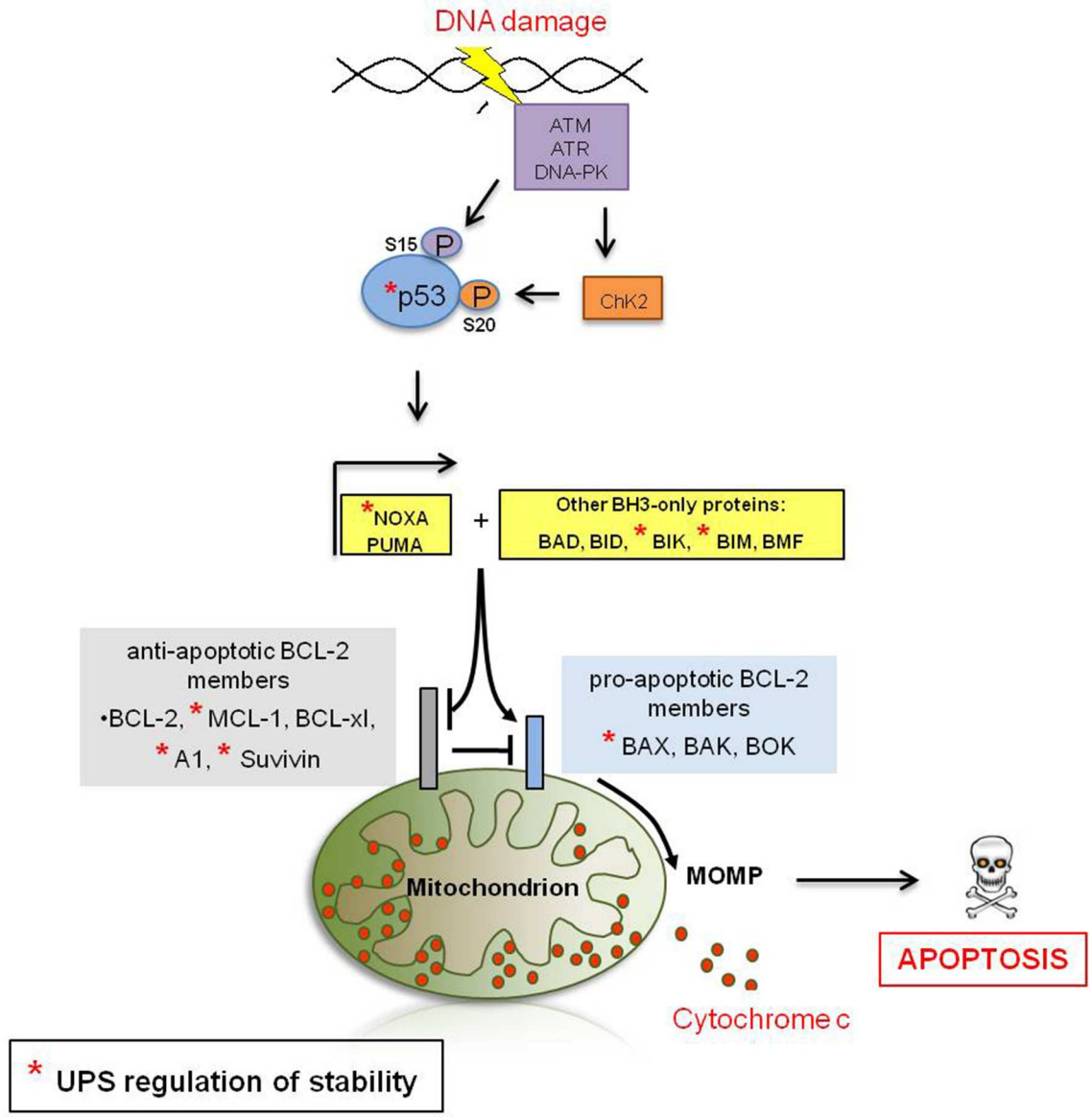

FIGURE 2 | DNA damage-induced apoptotic signaling. The recruitment of ATM, ATR or DNA-PK to the site of DNA damage is a central event during DDR signaling. ATM and ATR transduce the DDR signal by phosphorylation of the checkpoint kinases CHK1/CHK2, which results in cell cycle arrest and either DNA repair or DDIA (Shiloh, 2003). Moreover, ATM and ATR are directly responsible for the post-translational stabilization and thus accumulation of the tumor supressor p53, a key player in transducing the DDR signal (see below in this figure). ATM directly phosphorylates p53 at residue S15 (Banin et al., 1998) and indirectly through the induction of the CHK2 kinase at residue S20 (Shiloh and Ziv, 2013). Phosphorylation of p53 is believed to be critical for the stabilization of p53. Activated p53 translocates into the nucleus where it induces the transcription of several targets involved in cell cycle regulation, DNA repair or apoptosis, including the pro-apoptotic molecule BAX (Miyashita and Reed, 1995) and the BH3-only proteins PUMA (Nakano and Vousden, 2001) and NOXA (Oda et al., 2000) which in turn induce MOMP either directly or in cooperation with other BH3-only proteins. Anti-apoptotic Bcl-2-family members inhibit apoptosis by antagonizing the induction of MOMP. Upon MOMP, multiple pro-apoptotic molecules are released from the mitochondrial intermembrane space (IMS) to activate aspartate proteases, called caspases, which ultimately coordinate most of the hallmarks of apoptosis and cellular self-destruction.

MDM2 generates non-degradative polyubiquitin chains indicating an additional function of MDM2/MDMX-ubiquitylation other than the previously demonstrated degradative target ubiquitylation (Badciong and Haas, 2002). An additional factor in the negative feedback loop regulating p53 is the phosphatase Wip1. Wip1 acts as a gatekeeper in this regulatory loop by dephosphorylating and thus stabilizing MDM2, promoting MDM2-mediated p53 proteolysis (Lu et al., 2007). Elevated expression of negative regulators of p53-stability is reported for numerous tumors and is strongly associated with a poor patient prognosis.

TABLE 1 | E3 ligases involved in DDIA.

E3 ligase	Target
MDM2	MDMX (de Graaf et al., 2003; Kawai et al., 2003; Pan and Chen, 2003; Pereg et al., 2005; Xia et al., 2008), p53 (Momand et al., 1992)
COP1	p53 (Dornan et al., 2004a)
ARF-BP1/Mule	p53 (Chen et al., 2005b; Zhong et al., 2005; Parsons et al., 2009), ARF-BP1/Mule (Chen et al., 2005b), MCL-1 (Zhong et al., 2005)
PIRH2	p53 (Sheng et al., 2008), CHK2 (Bohgaki et al., 2013), PIRH2 (Logan et al., 2004; Abou Zeinab et al., 2013), p73 (Jung et al., 2011; Wu et al., 2011c)
Cul4B	p53 (Nag et al., 2004; Thirunavukarasou et al., 2014)
E6-AP	p53 (Scheffner et al., 1993)
Cul4A-DDB1	p53 (Nag et al., 2004), p73 (Malatesta et al., 2013)
ITCH	p63 (Rossi et al., 2006), p73 (Rossi et al., 2005), tBID (Azakir et al., 2010)
SCF^{Fbw7}	MCL-1 (Inuzuka et al., 2011; Wertz et al., 2011)
$SCF^{\beta TrCP}$	MCL-1 (Ding et al., 2007), BIM (Dehan et al., 2009)
APC/Cdc20	MCL-1 (Harley et al., 2010)
TRIM17	MCL-1 (Magiera et al., 2013)
SAG/RBX2	BIM (Li et al., 2014)
TRIM2	BIM (Thompson et al., 2011)
Culin/ElonginB-CIS	BIM (Ambrosini et al., 2009)
RNF186	BNip1 (Wang et al., 2013)

TABLE 2 | DUBs involved in DDIA.

DUB	Target
USP7	p53 (Li et al., 2002), ARF-BP-1/Mule (Khoronenkova and Dianov, 2013)
USP4	ARF-BP-1/Mule (Zhang et al., 2011)
USP2a	MDM2 (Stevenson et al., 2007), MDMX (Allende-Vega et al., 2010)
USP10	p53 (Yuan et al., 2010)
USP42	p53 (Hock et al., 2011)
USP29	p53 (Liu et al., 2011)
UCH-L1	*p53 (Li et al., 2010; Xiang et al., 2012), NOXA (Brinkmann et al., 2013)
Otubain 1	*p53 (Sun et al., 2012)
USP9X	MCL-1 (Schwickart et al., 2010)
USP18	*BIM (Santin et al., 2012)

**Indirect stabilization, no direct deubiquitylation reported.*

For instance, elevated expression of MDM2 has been identified in breast cancer (Bueso-Ramos et al., 1996), leukemias (Bueso-Ramos et al., 1993), and in lung cancer (Dworakowska et al., 2004). Gene amplifications of MDM2 have been described in 7% of tumors of diverse origin, with the highest frequency observed in soft tissue tumors, osteosarcomas and esophageal carcinomas (Momand et al., 1998). MDM2 is also a substrate for alternative splicing and the production of aberrantly spliced MDM2 RNA is associated with a shortened overall survival of cancer patients (Bartel et al., 2002). Remarkably, a functional interaction of p53/MDM2 is dispensable for embryonic development, whereas it is essential for DDIA, thus emphasizing the potential of the p53/MDM2-interaction as a target in anti-cancer therapy (Zhao et al., 2013).

The E3 ligase COP1 regulates p53 stability in an ATM-dependent manner (Dornan et al., 2004a). Upon DNA damage, activated ATM phosphorylates COP1 on S387 which in turn stimulates a rapid autodegradation mechanism of COP1 resulting in p53 stabilization (Dornan et al., 2006). COP1 itself is a transcriptional target of p53, thus representing yet another feedback loop for controlling p53 stability (Dornan et al., 2004a). Overexpression of *COP1* has been observed in breast cancer (Dornan et al., 2004b), ovarian adenocarcinoma (Dornan et al., 2004b), gastric cancer (Li et al., 2012; Sawada et al., 2013), and in hepatocellular carcinoma (Lee et al., 2010) and this high expression is mostly associated with a poor prognosis.

p53 stability is also negatively controlled by ARF-BP1/Mule encoded by the *Huwe1* gene, which is a binding partner of the alternative binding frame (ARF) tumor suppressor (Chen et al., 2005b; Zhong et al., 2005; Parsons et al., 2009). ARF-BP-1/Mule activity is limited by self-ubiquitylation and subsequent proteasomal turnover (Chen et al., 2005b). Increased ARF-BP1/Mule degradation causes p53 stabilization, which is antagonized by the DUBs USP7 (Khoronenkova and Dianov, 2013) and USP4 (Zhang et al., 2011). ARF-BP1/Mule was found to be expressed at high levels in lymphoma cell lines (Qi et al., 2012) and in colorectal and breast cancer cells (Xie et al., 2002) and it promotes Myc-driven tumorigenesis (Qi et al., 2012), whereas it suppresses Ras-driven tumorigenesis (Inoue et al., 2013).

Another specific E3 ligase for p53 is PIRH2, which was initially named p27(Kip1) and implicated in cell cycle regulation (Leng et al., 2003). Remarkably, PIRH2 preferentially ubiquitylates the transcriptional active form of p53 (Sheng et al., 2008). Moreover, PIRH2 also regulates the stability of the effector kinase CHK2 (Bohgaki et al., 2013) and phosphorylation of PIRH2 by calmodulin-dependend kinase 2 impairs its ability to ubiquitylate p53 (Duan et al., 2007). Again, PIRH2 levels are regulated by self-ubiquitylation following proteasomal turnover (Logan et al., 2004; Abou Zeinab et al., 2013). *PIRH2* is overexpressed in a variety of tumor cells including hepatocellular carcinoma (Wang

et al., 2009; Hu et al., 2012), head and neck cancers (Shimada et al., 2009), clear renal cell carcinoma (Wu et al., 2013), lung cancer (Duan et al., 2004), and prostate cancer (Logan et al., 2006).

A number of additional E3 ligases are reported to regulate p53 degradation, including Cul4B (Thirunavukarasou et al., 2014), E6-AP (Scheffner et al., 1993), and Cul4A-DDB1 (Nag et al., 2004; Thirunavukarasou et al., 2014). Strikingly, regulation of p53 protein is also influenced by the activities of the E4 ligases UBE4B/UFD2a/Ufd2 (Wu et al., 2011b) and CBP (CREB-binding protein)/p300 (E1A binding protein p300) (Shi et al., 2009).

E4 ligases mediate the polyubiquitylation of specific monoubiquitylated substrate proteins, including p53. Recently, CBP and p300 were identified to possess E4 activity and can elongate monoubiquitylated p53 into the cytosolic polyubiquitylated form (Shi et al., 2009). In addition, the E4 ligase UBE4B interacts physically with p53 and MDM2 to polyubiquitylate p53 (Wu et al., 2011b). Consequently, elevated levels of UBE4B are linked to brain tumors and medulloblastoma cell lines. It was further observed that the gene locus of *UBE4B* (1p36.22) is a susceptible candidate locus for hepatitis B virus (HBV) related hepatocellular carcinoma (HCC), forming a possible link between UBE4B/UFD2 and cancer development and tumor suppression (Zhang et al., 2010; Wu and Leng, 2011; Wu et al., 2011b).

Regulation of p53 by DUBs

So far, several DUBs are known to regulate p53 stability, either directly by deubiquitylation and stabilization of p53 itself, or by regulating its key regulators or binding partners. The ubiquitin-specific protease USP7 (HAUSP—herpes virus associated USP) was initially found to be a specific DUB of p53 and its activity stabilizes p53 protein (Li et al., 2002). However, whereas decreased USP7 expression levels had the expected effect of destabilizing p53, ablation of USP7 expression was found to have the opposite effect, resulting in p53 stabilization (Sheng et al., 2006). This p53 stabilization seems to result from increased ubiquitylation and destabilization of MDM2, the E3 ligase largely responsible for p53 ubiquitylation (Cummins and Vogelstein, 2004; Li et al., 2004; Meulmeester et al., 2005). USP2a has been described as a specific DUB of MDM2 (Stevenson et al., 2007) and MDMX (Allende-Vega et al., 2010) and thereby acts as a negative regulator of p53 stability. USP10 is a cytosolic DUB that specifically deubiquitylates p53, while ATM-mediated phosphorylation results in USP10 stabilization as well as nuclear translocation, resulting in p53 stabilization (Yuan et al., 2010). USP42 and USP29 are DUBs for p53 and improve p53 stability under stress conditions (Hock et al., 2011; Liu et al., 2011). Similarly, positive regulation of p53 stability has also been described for OTUB1 (Otubain1), which stabilizes p53 indirectly and independently of its catalytic activity by binding the E3 ligase MDM2. This interaction inhibits the cooperation between MDM2 and UbcH5s, the E2 enzyme important for MDM2-mediated p53 ubiquitylation (Sun et al., 2012). UCH-L1 has also been reported to regulate p53 protein stability (Li et al., 2010; Xiang et al., 2012); however, the molecular details are yet not clear.

Examples for DUBs that might antagonize E4 dependent polyubiquitylation are USP47, a regulator of Base Excision Repair (BER) that controls DNA polymerase β and OTUB1, which mediates DNA damage-dependent deubiquitylation of p53/MDM2 in the cytoplasm (Parsons et al., 2011; Sun et al., 2012).

Regulation of the p53 Homologs p63 and p73 by Ubiquitin Ligases

Interestingly, p53 is required for the DDR in certain but not all cell types (Clarke et al., 1993; Lowe et al., 1993; Strasser et al., 1994). Even though the primary role is exerted by p53 itself, the p53 homologs p63 and p73 can substitute for the downstream activities of p53. p63 and p73 share 60% similarity with the p53 DNA binding domain, allowing them to transactivate some of the same target genes. Like p53, p73 proteasomal turnover is regulated by the E3 ligase PIRH2 (Jung et al., 2011; Wu et al., 2011c) and also by the E4 ligase UFD2a (Hosoda et al., 2005). Furthermore, p63 and p73 protein stability are directly regulated by the ubiquitin ligase ITCH (Rossi et al., 2005, 2006). p73 is a substrate of the Cul4A-DDB1 Ub ligase complex which monoubiquitylates p73 thereby reducing its transcriptional activity without affecting its turnover (Malatesta et al., 2013). MDM2 also binds p73 without supporting its degradation (Balint et al., 1999). Likewise, MDMX, but not MDM2, has been shown to regulate p63 transactivation potential by inhibiting p63 nuclear localization (Kadakia et al., 2001).

DNA Repair Mechanisms

DSB repair is mediated by two extensively studied major repair pathways that have evolved in eukaryotic cells (Chapman et al., 2012). The error prone NHEJ pathway reunites free DNA ends at DSBs with little or no sequence homology and is responsible for most of the repair events in eukaryotes (Lemmens and Tijsterman, 2010). Repair via NHEJ can be rather inexact because the rejoining of non-complementary DNA ends is subject to end-processing by the nuclease activity of Artemis and DNA-PK(cs), which remove damaged or mismatched nucleotides (Bunting and Nussenzweig, 2013). Accurate ligation depends on the presence of loose complementary cohesive DNA ends and is mediated by the NHEJ repair proteins Ku70/80 and XRCC4-Ligase IV (Dahm-Daphi et al., 2005; Moynahan and Jasin, 2010). A second repair pathway is homologous recombination (HR), which dominates in highly proliferative somatic cells in S- and G2-phase. HR is a high fidelity repair pathway that relies on recombination between undamaged sister chromatids or homologous chromosomes (Clejan et al., 2006). Ubiquitylation of substrate proteins plays an important role in specifying the use of a specific DNA repair pathway, as differential ubiquitylation leads to orchestrated recruitment of specific repair factors such as p53-binding protein 1 (53BP1) or Breast Cancer Susceptibility Gene 1 (BRCA1) (Jackson and Durocher, 2013). 53BP1 accumulation promotes NHEJ activation and HR inhibition, whereas BRCA1 recruitment triggers HR (Yun and Hiom, 2009). Their recruitment to chromatin surrounding DSB sites is controlled by the action of the RING-finger protein RNF8, which acts as a central E3 ligase in DDR

and exhibits two distinct roles: it catalyzes the ubiquitylation of substrate proteins either via a protein-recruiting K63- or via a destabilizing K48 specific linkage (Lok et al., 2012). Upon DNA damage RNF8 detects motifs in mediator of DNA damage checkpoint protein 1 (MDC1) previously phosphorylated by ATM and performs K63-linked monoubiquitylation of histones H2A and H2AX. Histone monoubiquitylation promotes RNF8-dependent recruitment of a second E3 ligase, RNF168, to the damage site, which can identify ubiquitylated RNF8 substrates via its Nterminal ubiquitin-binding domains (Mailand et al., 2007; Doil et al., 2009). Subsequently, polyubiquitylation of H2AX further promotes the recruitment of RNF168 to the damage site, amplifying RNF8-dependent histone ubiquitylation by ubiquitylating other substrate proteins via K63 (Doil et al., 2009; Ramadan and Meerang, 2011). The outcome of RNF8/RNF168-dependent K63-linked ubiquitylation is the generation of a molecular landing platform for the accumulation of checkpoint and DNA repair proteins like BRCA1 or 53BP1; however, 53BP1 itself cannot directly bind to K63 polyubiquitin chains since it lacks any relevant binding site (Al-Hakim et al., 2010). Therefore, other mechanisms for 53BP1 recruitment are necessary. For example, 53BP1 accumulation is promoted by p97 segregase activity that removes the polycomb protein L3MBTL1 from DNA DSBs. p97 binds to ubiquitylated L3MBTL1 and extracts it from chromatin. The displacement of L3MBTL1 unmasks 53BP1 binding sites that can now be occupied (Acs et al., 2011).

In addition, RNF8 also ubiquitylates K48-dependent substrates such as the lysine demethylase JMJD2A (Mallette et al., 2012), the NHEJ repair protein Ku80 (Feng and Chen, 2012), and the DNA polymerase sliding clamp proliferating cell nuclear antigen (PCNA), which is involved in DNA synthesis and repair (Zhang et al., 2008). Consequently, these proteins are removed from chromatin for proteasomal degradation.

In accordance with the postulated molecular switch model of PCNA, E4-mediated polyubiquitylation might alter ubiquitin-dependent signaling fates upon damage induction, possibly in a cell type specific manner (Hoppe, 2005). This regulatory mechanism thereby provides another layer of regulation to fine-tune the highly dynamic cascade of ubiquitylation events during the DDR, which can also be reversed by DUB activity.

Besides K48-linked ubiquitylation, PCNA undergoes a switch mechanism from a mono- to a polyubiquitylated form at position K164, regulating its activity in DNA repair (Hoege et al., 2002). This modification triggers translesion synthesis (TLS), i.e., DNA synthesis across lesions. In addition, other factors are needed to extend the modification by a K63-linked polyubiquitin chain leading to an error-free pathway of damage avoidance (Hoege et al., 2002; Daigaku et al., 2010).

A different ubiquitin chain linkage was reported for the E3 ubiquitin ligase BRCA1, which exhibits tumor-suppressor activities and is crucial for maintaining genomic integrity. As a heterodimer with its binding partner BARD1 it specifically catalyzes the formation of K6-linked polyubiquitin chains on substrates, such as RNA Polymerase II and γ-Tubulin (Wu-Baer et al., 2003; Irminger-Finger and Jefford, 2006).

DDIA

In addition to the activation of DNA repair, multicellular organisms acquired a dynamic safe-guard system involving the apoptotic response to dispose of damaged cells when the extent of damage is beyond the cellular repair capacity (Levine et al., 1997). The decision whether a cell survives or dies upon DNA damage is not yet completely understood, however, as mentioned above, the level of p53 abundance is a key factor in the cellular decision of life or death in response to DNA damage. Similarly, the quality of p53 downstream death signaling—the induction of intrinsic/mitochondrial apoptosis—plays a crucial role in the coordinated cellular death upon DNA damage. Specifically, the expression level of pro- and anti-apoptotic proteins, in particular, members of the Bcl-2-family (see below), is decisive for the outcome of the DDR signaling. Furthermore, the nature of the DNA damage, the physiologic status and the origin of the damaged cell may impact on cellular responses to DNA damage. For instance, thymocytes are highly primed to undergo DDIA, whereas primary fibroblasts appear to resist DDIA (Norbury and Zhivotovsky, 2004). Indeed, the capability of the apoptotic machinery in immune cells is central during the cellular differentiation of this tissue. For instance, almost 90% of pre-T- and B-cells undergo apoptosis during maturation. Further, apoptosis triggers the shutdown of the immune response when infection has been overcome (Brinkmann and Kashkar, 2014). In conclusion, several cell types are primed for a rapid induction of apoptosis which is achieved by a "close-to-death" composition of pro- and anti-apoptotic proteins, in particular, Bcl-2-family members (Letai et al., 2002).

Nevertheless, a tight regulation of the response to DNA damage is obligatory in germ cells and somatic cells. In germ cells, mechanisms for limiting genome alterations are required for faithful propagation of the species, whereas in somatic cells, responses to DNA damage prevent the accumulation of mutations that might lead to altered cellular homeostasis.

Bcl-2 Protein Family—Regulators of Mitochondrial Apoptosis

Mitochondria represent a central regulatory node in the apoptotic machinery through the mitochondrial outer membrane permeabilization (MOMP) as the decisive event. Upon MOMP, multiple pro-apoptotic molecules, including cytochrome C are released from the mitochondrial intermembrane space (IMS) to activate aspartate proteases, called caspases, which ultimately coordinate most of the hallmarks of apoptosis and cellular self-destruction. Specifically, cytosolic cytochrome C forms a complex, the apoptosome, with ATP, APAF1, and pro-caspase 9 (pro-casp9), resulting in the activation of caspase 9 (casp9). Casp9 activates the downstream executioner caspase 3 (casp3) which ultimately lead to apoptosis.

Inefficient MOMP has been suggested to be one of the key determinants of therapeutic success of a number of anti-cancer regimens in cancer patients (Adams and Cory, 2007) and members of the Bcl-2 protein family are the key-regulators of this process. The Bcl-2 protein family comprises three classes of member. The first group consists of the anti-apoptotic Bcl-2 protein family

members, including BCL-2, BCL-xl, BCL-w, A1, and MCL-1, that efficiently inhibit MOMP and block apoptosis. The second group consists of pro-apoptotic members such as BAK, BAX, and BOK, trigger apoptosis by directly promoting MOMP. A third divergent class of BH3-only proteins including BIM, BID, PUMA, BAD, and NOXA regulates the activity of pro-and anti-apoptotic Bcl-2 proteins (Adams and Cory, 2007) (**Figure 2**).

Members of the Bcl-2 protein family share at least one conserved Bcl-2 homology domain (BH domain), which is characterized by several α-helical segments. The BH domain does not possess enzymatic activity but it allows pro- and anti-apoptotic members to bind to and to inhibit each other (Adams and Cory, 1998; Cory and Adams, 2002). Binding affinity assays using BH3-only peptides revealed that not all pro- and anti-apoptotic Bcl-2 proteins can antagonize each other, but the affinity differs within the family. The BH3-only proteins BIM, BID, PUMA, and BMF can bind and antagonize all anti-apoptotic Bcl-2 proteins. In contrast, BAD can only bind BCL-2, BCL-xl and BCL-w, and NOXA is restricted in binding to MCL-1 and A1. To date, there are two proposed models that explain how the Bcl-2 protein family regulates MOMP: (i) the indirect activator model and (ii) the direct activator-derepressor model. Both models result in the activation of BAX and BAK and the permeabilization of the outer mitochondrial membrane. The indirect activator model postulates that BAX and BAK are bound in a constitutively active state to anti-apoptotic Bcl-2 proteins. Competitive interactions with pro-apoptotic BH3-only proteins and anti-apoptotic Bcl-2 proteins are sufficient to release active BAX and BAK and induce MOMP. In the direct activator-derepressor model (also called neutralization model), BAX and BAK are activated by the interaction with a subset of BH3-only proteins, such as BID and BIM, called direct activators. In this model, anti-apoptotic Bcl-2 proteins either inhibit MOMP by antagonizing BAX or BAK directly or by sequestering the direct activator BH3-only proteins, thus preventing them from activating BAX or BAK. A second subset of BH3-only proteins, called sensitizers, such as NOXA or BAD, cannot directly activate BAX or BAK but antagonize anti-apoptotic Bcl-2 proteins and thereby release BAX and BAK for the activation by direct activator BH3-only proteins (Tait and Green, 2010).

In response to DNA damage activated p53 translocates into the nucleus where it induces transcription of several pro-apoptotic Bcl-2 proteins, including BAX (Miyashita and Reed, 1995), PUMA (Nakano and Vousden, 2001), and NOXA (Oda et al., 2000), which in turn induce MOMP. The transcriptional upregulation of these pro-apoptotic members in response to DNA damage however may not suffice the required pro-apoptotic trigger toward MOMP as this process is tightly regulated by a number of other Bcl-2 members and only the ultimate pro-apoptotic composition of these proteins can efficiently induce cell death (Ni Chonghaile and Letai, 2008). Accordingly, the genes of some BH3-only proteins appear to be constitutively transcribed in cancer cells as reported for BIK or NOXA (Hur et al., 2004; Brinkmann et al., 2013; Dengler et al., 2014). The majority of these cells however resist apoptosis suggesting that the imbalance in Bcl-2 protein family members (e.g., upregulation of anti-apoptotic members or downregulation of BAX/BAK) efficiently counter the pro-apoptotic action of these factors. More strikingly, non-transcriptional regulation of Bcl-2 protein family members turn-over was repeatedly shown to control the apoptotic process under physiological or pathological condition. This enables cells to rapidly respond to stress cues by regulating protein abundance without employing protein *de novo* synthesis.

The clinical successes of proteasome inhibitors for the treatment of cancer have highlighted the therapeutic potential of targeting cellular process governing protein turn-over. Strikingly, the expression levels of a number of Bcl-2 protein family members including NOXA (Qin et al., 2005; Brinkmann et al., 2013), MCL-1 (Adams and Cory, 2007), A1 (Kucharczak et al., 2005), BCL-2 (Dimmeler et al., 1999), BAK (Qin et al., 2005), BIK (Marshansky et al., 2001; Hur et al., 2004), BIM (Nikrad et al., 2005) was altered when the proteasome was inhibited indicating an essential role of the UPS in regulating Bcl-2-family protein abundance. However, a direct regulation of Bcl-2-protein level via the UPS has only been reported for BAX (Chang et al., 1998; Li and Dou, 2000), BIM (Akiyama et al., 2003), BCL-2 (Dornan et al., 2004b), NOXA (Brinkmann et al., 2013), MCL-1 (Zhong et al., 2005), A1 (Kucharczak et al., 2005), and BCL-B (van de Kooij et al., 2013), while the identities of the responsible E3 ligases and DUBs are largely unknown with some exceptions (**Tables 1**, **2**).

Previous data showed that the stability of the anti-apoptotic BCL-2 protein is regulated through ubiquitylation which is in turn controlled by its phosphorylation (Breitschopf et al., 2000a; Basu and Haldar, 2002). Specifically, MAP kinase-mediated BCL-2 phosphorylation was shown to block BCL-2 ubiquitylation and proteasomal degradation (Dimmeler et al., 1999). Furthermore, BCL-2 turn-over is inhibited by its direct S-nitrosylation (Azad et al., 2006; Chanvorachote et al., 2006). These data showed that BCL-2 undergoes S-nitrosylation by endogenous nitric oxide (NO) in response to multiple apoptotic stimuli. S-nitrosylation of BCL-2 in turn inhibits its proteasomal degradation.

The level of MCL-1 protein is regulated by the action of at least five distinct E3-ligases, namely ARF-BP1/Mule (Zhong et al., 2005), SCF^{Fbw7} (Inuzuka et al., 2011; Wertz et al., 2011), $SCF^{\beta TrCP}$ (Ding et al., 2007), APC/Cdc20 (Harley et al., 2010), Trim17 (Magiera et al., 2013), and the DUB USP9X (Schwickart et al., 2010). Whether different E3-ligases are engaged in different cellular action and in response to different stimuli is not determined. Independently, ubiquitylation of MCL-1 has been mainly considered as a regulatory circuit controlling its abundance. Not surprisingly, dysregulation of MCL-1 ubiquitylation and turn-over have been repeatedly associated with cancer and cancer chemoresistance (Schwickart et al., 2010; Wertz et al., 2011). Mule-dependent MCL-1 ubiquitylation is enhanced by NOXA, which targets Mule to MCL-1 and competes with USP9X in MCL-1 binding (Gomez-Bougie et al., 2011). These data suggest that NOXA, in addition to the functional antagonization of MCL-1, controls MCL-1 turn-over by regulating the physical interaction of MCL-1 with ubiquitin conjugation/deconjugation machinery.

Independent of its own inherent pro-apoptotic activity, the critical role of NOXA in regulating MCL-1 is a unique property of this protein among other BH3-only protein family members. NOXA was initially identified as a primary p53-responsive gene,

providing the first evidence for the transcriptional regulation of NOXA in response to genotoxic stress (Oda et al., 2000). In addition to transcriptional regulation, NOXA stability is controlled by post-translational mechanisms. In particular, ubiquitylation of NOXA has recently been shown to be involved in the regulation of NOXA protein turn-over and thereby influences cellular stress responses (Baou et al., 2010; Dengler et al., 2014). Interfering with this process, dysregulation of NOXA ubiquitylation has been shown to be an efficient strategy of some tumor cells in order to resist the genotoxic chemotherapy (Brinkmann et al., 2013). Specifically, these data showed that NOXA was strongly ubiquitylated in some tumor samples. The elevated NOXA ubiquitylation and reduced stability was a result of epigenetic silencing of NOXA-specific DUB, UCH-L1, which directly deubiquitylates and stabilizes NOXA (Brinkmann et al., 2013). Furthermore, NOXA can be degraded by an ubiquitin-independent mechanism suggesting that the disruption of 26S proteasome function by various mechanisms triggers the rapid accumulation of NOXA based on the capability of NOXA to act as a sensor of 26S proteasome integrity (Craxton et al., 2012).

Ubiquitin-dependent degradation of BIM is regulated by the E3 ligases SAG/RBX2 (Li et al., 2014), TRIM2 (Thompson et al., 2011), Cullin/ElonginB-CIS (Ambrosini et al., 2009), and $SCF^{\beta TrCP}$ (Dehan et al., 2009) while phosphorylation through different kinases including ERK1/2, MAPK and the cell cycle kinase Aurora A precedes its turnover (Ley et al., 2003; Ramesh et al., 2008; Dehan et al., 2009; Wiggins et al., 2010). USP18 has also been shown to be involved in regulating the stability of BIM upon cytokine-induced cell death. Specifically, USP18 inhibition in INS-1E cells enhanced BIM expression level in untreated and IFNγ-treated conditions (Santin et al., 2012).

Analysis of BAX stability in human prostate adenocarcinoma showed that BAX is highly instable and the reduced BAX protein levels was associated with increased Gleason scores of prostate cancer (Chang et al., 1998; Li and Dou, 2000). These results identified the UPS-mediated BAX degradation as a novel survival mechanism in tumor cells and suggested that a selective targeting of this pathway should provide a unique approach for treatment of human cancers, especially those overexpressing BCL-2 (Chang et al., 1998; Li and Dou, 2000).

The stability of the BH3-only protein BNip1 is regulated via the action of the E3 ligase RNF186. BNip1 co-localizes with RNF186 at the ER and is poly-ubiquitylated by RNF186 through K29 and K63 linkage *in vivo*. This modification promotes BNip1 transportation to mitochondria but has no influence on its protein level (Wang et al., 2013).

Extrinsic apoptotic cascade results in the proteolytic activation of BID by caspase-8 (Luo et al., 1998). The COOH-terminal cleavage fragment of BID (truncated BID, tBID) becomes localized to mitochondrial membranes and triggers the release of cytochrome c. Truncated BID was shown to be ubiquitylated and subsequently degraded by the 26 s proteasome which is believed to control the extent of apoptosis in living cells (Breitschopf et al., 2000b). Further analyses identified the ubiquitin ligase ITCH, as a specific ubiquitin ligase of tBID which was not able to use intact BID as a substrate and initiate its proteasomal degradation (Azakir et al., 2010). The N-terminal cleavage product of BID has also been shown to be a substrate of unconventional ubiquitylation and degradation as the acceptor site are neither lysines nor N-terminus (Tait et al., 2007). Acceptor sites reside predominantly but not exclusively in helix 1, which is required for ubiquitylation and degradation of tBID-N. Rescue of tBID-N from degradation blocked BID's ability to induce mitochondrial outer membrane permeability but not mitochondrial translocation of the cleaved complex.

The increasing number of ubiquitin-conjugation events, regulating the abundance or function of Bcl-2 protein family members, is a strong indication of the central role of Ub in DDIA and provides at the same time a promising therapeutic target for cancer treatment.

Exploiting Ubiquitin-Signaling in DDR as a Therapeutic Target in Cancer

The ultimate central goal of conventional cancer therapy is the effective elimination of tumors by invoking DDIA. Since the balance of protein abundance and functionality are decisive for DDR outcomes, it is not surprising that deregulation of ubiquitin-signaling pathways is intimately associated with tumorigenesis and therapy resistance. Accumulating recent evidence conclusively identified ubiquitin-signaling as a valuable target in DDR and cancer chemoresistance. The majority of these efforts focused on the regulation of p53 as one of the central determinants of DDR outcomes. Accordingly, an increasing number of specific regulators of p53 have been identified and evaluated as therapeutic targets. RITA (reactivation of p53 and induction of tumor cell apoptosis) is a small molecule that blocks p53/MDM2 interaction (Issaeva et al., 2004); however, it appeared to be rather unspecific since its pro-apoptotic capacity was described to be p53-independent in several tumors, including myelomas (Surget et al., 2014) and additional data indicated that RITA cannot inhibit this interaction *in vitro* (Krajewski et al., 2005). Nutlins are also described to block the interaction of p53 and MDM2 (Vassilev, 2004). These molecules activate the p53 pathway and suppress tumor growth *in vitro* and *in vivo* in tumor xenograft models of solid and hematologic tumors (Vassilev et al., 2004; Tovar et al., 2006; Sarek and Ojala, 2007). MI-63 and MI-219 are small molecules also designed to block the interaction between p53/MDM2 and early preclinical evaluations demonstrated p53-mediated cell cycle arrest or apoptosis in tumor xenograft models upon treatment with Mi-219 (Shangary et al., 2008). P28 is a peptide fragment derived from azurin, a redox protein secreted from *Pseudomonas aeruginosa*, which stabilizes p53 by blocking its interaction with COP1 (Yamada et al., 2013a,b). The first preclinical trials demonstrated inhibition of tumor growth in xenograft models of p53 positive solid tumors (Jia et al., 2011).

Conclusion

Tumor cell resistance to genotoxic chemotherapy poses a significant challenge in the treatment of cancer patients. As already discussed, protein ubiquitylation is central to the orchestration of the DDR and impacts on susceptibility to conventional

genotoxic chemotherapy. Recent studies using proteasome-inhibitors validated the UPS as a therapeutic target in cancer and provided an impetus to promote the development of effective novel drugs that more specifically interfere with the ubiquitin-conjugating machinery. Thus, a better understanding of the specific link between the DDR and the ubiquitin-conjugating machinery will undoubtedly identify novel targets involved in cancer and will promote the development of new therapeutic strategies to overcome cancer chemoresistance. In line with this notion, based on its ability to inhibit apoptosis, the Bcl-2 protein family has garnered the most attention as a promising therapeutic target in cancer. Accordingly, efforts have lately been focused on the development of drugs targeting Bcl-2 proteins with considerable therapeutic success (Brinkmann and Kashkar, 2014). In view of the fact that the acquired imbalance of Bcl-2 proteins is involved in cancer together with our increasing knowledge about the central role of ubiquitin-conjugation governing Bcl-2 abundance and function support the idea that cancer-treatment may strongly benefit from novel therapeutic protocols targeting ubiquitin-regulation of Bcl-2 family.

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frontiers in Genetics

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Targeting ATM-deficient CLL through interference with DNA repair pathways

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Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in the Western world and accounts for approximately 30% of adult leukemias and 25% of non-Hodgkin lymphomas. The median age at diagnosis is 72 years. During recent years numerous genetic aberrations have been identified that are associated with an aggressive course of the disease and resistance against genotoxic chemotherapies. The DNA damage-responsive proapoptotic ATM-CHK2-p53 signaling pathway is frequently mutationally inactivated in CLL either through large deletions on chromosome *11q* (*ATM*) or *17p* (*TP53*), or through protein-damaging mutations. Here, we focus on the role of ATM signaling for the immediate DNA damage response, DNA repair and leukemogenesis. We further discuss novel therapeutic concepts for the targeted treatment of ATM-defective CLLs. We specifically highlight the potential use of PARP1 and DNA-PKcs inhibitors for the treatment of *ATM*-mutant CLL clones. Lastly, we briefly discuss the current state of genetically engineered mouse models of the disease and emphasize the use of these preclinical tools as a common platform for the development and validation of novel therapeutic agents.

Keywords: chronic lymphocytic leukemia, DNA damage response, PARP inhibitor, DNA-PKcs inhibitor, precision medicine

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Background

Genome maintenance is a major challenge for all life on earth. In mammals, genomic integrity is preserved through mechanisms that ensure the faithful transmission of fully replicated and undamaged DNA during each cell division (Hoeijmakers, 2001, 2009). For this purpose, eukaryotic organisms evolved a complex DNA surveillance program: Prior to mitosis, cells progress through G_1/S-, intra-S and G_2/M cell cycle checkpoints (Bartek and Lukas, 2007; Reinhardt and Yaffe, 2013). These checkpoints are activated in response to incomplete DNA replication (e.g., due to stalled replication forks), as well as genotoxic damage induced by internal and external sources, such as UV radiation, reactive oxygen species, ionizing radiation (IR) or DNA-damaging chemotherapeutic agents (Weinert, 1998; Zhou and Elledge, 2000; Abraham, 2001; Kastan and Bartek, 2004; Lukas et al., 2004; Bartek and Lukas, 2007). Active checkpoints halt cell cycle progression and thus provide the time necessary to resolve genomic damage (Reinhardt and Yaffe, 2013). If the genotoxic insult exceeds repair capacity, additional signaling cascades, leading to programmed cell death, are activated (Reinhardt and Yaffe, 2013). Thus, DNA damage checkpoints serve as an effective mechanism to provide and maintain genomic stability (Zhou and Elledge, 2000; Kastan and Bartek, 2004; Reinhardt and Yaffe, 2013).

Coherent with a prominent role of the DNA damage response (DDR) in genome maintenance, many DDR-associated genes have been found to be altered in the germline of patients suffering from cancer-prone inherited syndromes, such as *Li-Fraumeni* (*TP53*), *Ataxia telangiectasia* (*ATM*), Seckel syndrome (*ATR*), *Nijmegen breakage syndrome* (*NBS1*), *A-T-like disease* (*MRE11*), *Xeroderma pigmentosum* (XP complementation groups) or familial breast and ovarian cancer (*BRCA1*, *BRCA2*, *RAD51C*) (Frebourg and Friend, 1992; Lavin and Shiloh, 1997; Lehmann, 2003; O'Driscoll et al., 2003; Shiloh, 2003; Taylor et al., 2004; Nevanlinna and Bartek, 2006; Fackenthal and Olopade, 2007; Meindl et al., 2010). Disabling mutations within DDR genes have been proposed to result in a so-called "*mutator phenotype*," which is thought to drive the runaway proliferation of incipient cancer cells through the accumulation of additional cancer-driving or resistance-causing genomic aberrations (Loeb et al., 2003, 2008; Jiricny, 2006; Jackson and Bartek, 2009; Lord and Ashworth, 2012). While defects in DDR genes appear to facilitate malignant transformation, exploiting these genome-destabilizing alterations for targeted anti-cancer therapy offers a promising therapeutic avenue. In this review, we will focus on cancer-associated defects in ATM-mediated DNA double-strand (DSB) repair and their potential targeting. We will further pinpoint the lack of suitable genetically engineered mouse models of CLL as a critical bottleneck for the rapid preclinical evaluation of novel targeted therapies.

DNA Double Strand Break Repair

DSBs can be inflicted by different agents, such as IR and topoisomerase II inhibitors (e.g., etoposide) (Reinhardt and Yaffe, 2009). Mammalian cells use two major DSB repair mechanisms (**Figures 1A,B**). The error-prone non-homologous end joining (NHEJ) pathway, which does not depend on an intact DNA replication product as a template for repair, can be employed throughout all cell cycle phases (**Figure 1B**) (Dietlein and Reinhardt, 2014; Dietlein et al., 2014a). NHEJ is primarily used throughout G_1-phase, when no intact sister chromatid is available as a template for repair. NHEJ-mediated DSB repair relies on the catalytic activity of the protein kinase DNA-PKcs, which is recruited to the break site through physical interactions with the non-catalytic subunits Ku70 and Ku80 (Lees-Miller and Meek, 2003). DNA-PKcs activity mediates the assembly of additional NHEJ factors, such as XRCC4- and Lig4, which facilitate re-ligation of the DSB ends during NHEJ (Lees-Miller and Meek, 2003). Homologous recombination (HR)-mediated DSB repair is the second DSB repair pathway employed by mammalian cells (**Figure 1A**). HR is an error-free DSB repair mechanism that requires the presence of an intact DNA replication product, which is used as a template. This template dependence leads to a restriction of HR use to late S- and G_2-phase (Chapman et al., 2012; Dietlein and Reinhardt, 2014; Dietlein et al., 2014a). One of the earliest steps of the HR process is resection of the DSB to create a single-stranded 3′-DNA overhang, which is engaged and coated by the single-stranded DNA (ssDNA)-binding protein RPA (Cimprich and Cortez, 2008; Lyndaker and Alani, 2009). RPA is subsequently replaced by RAD51 in an ATM/CHK2/BRCA1/BRCA2/PALB2-dependent process (Sung and Klein, 2006; San Filippo et al., 2008; Heyer et al., 2010; Krejci et al., 2012). This ssDNA overhang then serves to invade the intact sister chromatid as an intact copy for DNA repair (Sung and Klein, 2006; San Filippo et al., 2008; Krejci et al., 2012). During the HR process, RAD51 fulfills a key role by mediating homology search, strand exchange, and Holliday junction formation (Chapman et al., 2012).

ATM Signaling and the DNA Damage Response

The proximal DDR kinase ATM, which is mutated in the human cancer-prone disorder *Ataxia telangiectasia* (A-T), is a key regulator of the cellular DDR and essentially controls three different functional outcomes of DDR signaling: cell cycle checkpoints, DNA repair and apoptosis (Reinhardt and Yaffe, 2009; Shiloh and Ziv, 2013). Immediately following the occurrence of a DSB, the trimeric MRN complex, consisting of *M*RE11, *R*AD50 and *N*BS1, is recruited to the site of the lesion (Chapman and Jackson, 2008; Reinhardt and Yaffe, 2013). In parallel, ATM is activated and tethered to the site of the DSB via a physical interaction with the C-terminus of NBS1 (Falck et al., 2005). ATM subsequently phosphorylates histone H2AX on Ser-139. The resulting phospho-H2AX is commonly referred to as γ-H2AX (Rogakou et al., 1998, 1999; Bartek and Lukas, 2007; Reinhardt and Yaffe, 2009). The phosphorylated Ser-139 residue in the C-terminal region of γ-H2AX subsequently binds with high affinity to the phosphopeptide-recognizing BRCT domains of the mediator protein MDC1 (Lee et al., 2005; Stucki et al., 2005; Lou et al., 2006), which in turn is phosphorylated by ATM at multiple residues (Matsuoka et al., 2007). In addition, MDC1 is phosphorylated by the constitutively active Ser/Thr kinase CK2 (Spycher et al., 2008). The resulting phospho-motif is recognized through the phosphopeptide-binding FHA and/or BRCT domains of NBS1 (Chapman and Jackson, 2008; Spycher et al., 2008; Lloyd et al., 2009). This CK2-dependent NBS1 recruitment retains the MRN complex and NBS1-bound ATM at the DSB site (Melander et al., 2008; Spycher et al., 2008). Thus, MDC1, through ATM- and CK2-directed phosphorylation, tethers both the MRN complex and active ATM at the break site, essentially forming an ATM auto-amplification loop.

Coherent with its role in checkpoint signaling and genome maintenance, *ATM* is frequently mutated in various human cancer entities, ranging from solid tumors to lymphomas and leukemias (Haidar et al., 2000; Ripolles et al., 2006; Ding et al., 2008; Waddell et al., 2015). Moreover, bi-allelic loss of *ATM* was shown to be associated with resistance against genotoxic chemotherapy and reduced patient survival (Ripolles et al., 2006; Austen et al., 2007; Skowronska et al., 2012). Recent *in vitro* experiments suggest that ATM is required for the execution of chemotherapy-induced p53-mediated apoptosis (Jiang et al., 2009). Together these data might rationalize why disabling *ATM* alterations

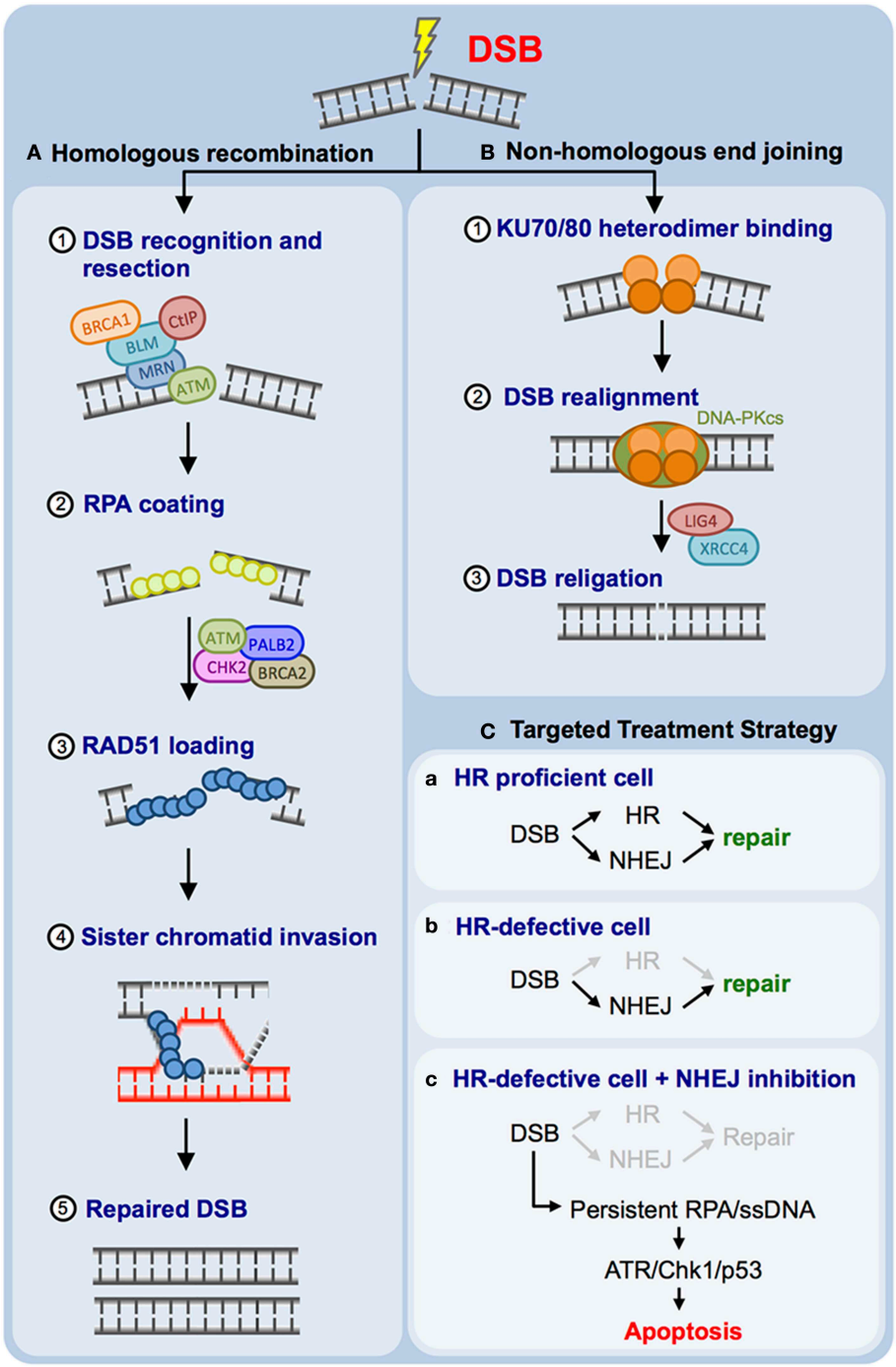

FIGURE 1 | Mammalian cells employ two principal DNA double-strand break (DSB) repair pathways. (A) Schematic representation of the error-free homologous recombination (HR) pathway. DSB resection (1), RPA coating (2), RAD51 coating (3), strand invasion (4), and DSB repair are illustrated (5). **(B)** Schematic representation of non-homologous end joining (NHEJ). Ku70/Ku80 binding (1), DNA-PK holo-enzyme assembly and recruitment of additional NHEJ factors, such as LIG4 and XRCC4 (2), as well as DSB religation (3) are illustrated. **(C)** Proposed targeting of HR-defective human cancer through DNA-PKcs inhibition is outlined (for details please refer to the main text).

are a selected genomic aberration in human neoplastic disease.

Intriguingly, ATM is not only a critical mediator of DNA damage-induced apoptosis, but has also been shown to play a major role in DNA repair, specifically HR-mediated DSB repair, with a less well-characterized role in NHEJ (Luo et al., 1996; Dar et al., 1997; Chen et al., 1999; Morrison et al., 2000; Yuan et al., 2003; Kuhne et al., 2004; Riballo et al., 2004; Xie et al., 2004;

Bredemeyer et al., 2006; Shrivastav et al., 2009). Experiments performed with *ATM*-deficient DT40 cells, as well as *A-T* cells derived from patients have shown that these cells display a mild, but distinct HR defect as the result of impaired assembly and functioning of RAD51-associated protein complexes (Morrison et al., 2000; Shiloh, 2003; Yuan et al., 2003). Specifically, a decreased and delayed formation of RAD51 foci was observed in *A-T* cells following IR (Shiloh, 2003; Morrison et al., 2000; Yuan et al., 2003). As detailed above, RAD51 recruitment requires an RPA-coated 3′-single-stranded overhang and thus prior DSB resection. This DSB resection process was shown to be ATM-dependent (Adams et al., 2006; Jazayeri et al., 2006; Myers and Cortez, 2006). Further investigation revealed that ATM is specifically involved in HR-mediated DSB repair during the G_2-phase of the cell cycle. For instance, it was recently shown that IR-induced sister chromatid exchanges in G_2 are ATM-dependent (Beucher et al., 2009; Conrad et al., 2011; Jeggo et al., 2011). Furthermore, CtBP-interacting protein (CtIP), which promotes efficient DSB resection during the HR process, recently emerged as an ATM substrate (Shibata et al., 2011). The rather mild DNA repair defect that is observed in *ATM*-deficient cells might be explained by the recent observation that ATM appears to control HR-mediated DSB repair specifically in heterochromatin (HC) regions of the genome (Goodarzi et al., 2008, 2010; Jeggo et al., 2011). These experiments revealed that approximately 85% of IR-induced DSBs are rapidly repaired through a largely ATM-independent process. Approximately 15% of IR-induced DSBs are repaired via a slow-acting repair process that depends on ATM (Goodarzi et al., 2010). Intriguingly, DSBs that undergo delayed repair are mainly restricted to areas of the genome that consist of HC (Goodarzi et al., 2010). It was further shown that ATM directly phosphorylates the HC-building factor KAP-1. This KAP-1 phosphorylation allows HR-mediated DSB repair within HC regions. Furthermore, KAP-1-depletion was demonstrated to rescue the DSB repair defect induced by *ATM* deficiency (Goodarzi et al., 2008, 2010; Jeggo et al., 2011). Altogether these data strongly suggest that the apoptosis-evading effect of *ATM*-deficiency, which likely stems from insufficient p53 activation, is associated with a potentially druggable HR defect.

Defective ATM-dependent DSB Repair as a Potential Therapeutic Target in CLL

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disorder that accounts for approximately 30% of adult leukemias and 25% of non-Hodgkin lymphomas (NHL) (Hallek and Pflug, 2010). It is the most common form of leukemia in the western world with an incidence rate of 4-5/100.000 (Hallek and Pflug, 2010). CLL is a disease of the elderly with <10% of the patients being <40 years of age and a median age at diagnosis of 72 years (Hallek and Pflug, 2010). CLL is extraordinarily heterogeneous in its clinical manifestation, treatment response and course. Some patients live for decades and do not require any therapeutic intervention, while others suffer from rapidly progressive and refractory disease (Cramer and Hallek, 2011). It is this extraordinary heterogeneity, which makes treatment of CLL especially challenging. To date, no curative therapy exists besides allogeneic stem cell transplantation, for which most patients do not qualify due to age or reduced performance status. However, it is important to note that we are witnessing a paradigm shift in the treatment of CLL with new, targeted agents recently approved (e.g., ibrutinib, idelalisib), or being evaluated in advanced approval trials (ABT-199). These novel agents interfere directly with B cell receptor signaling (ibrutinib—BTK Inhibitor, idelalisib—PI3Kδ Inhibitor), or relieve repression of the pro-apoptotic proteins BAX and BAK through BCL2 blockade (ABT-199) (for an excellent review, please refer to Thompson et al., 2015).

A hallmark feature of CLL cells is an extraordinarily high frequency of genomic aberrations, which can be documented in more than 80% of CLL patients (Dohner et al., 2000; Di Bernardo et al., 2008; Crowther-Swanepoel et al., 2010; Ouillette et al., 2010). Moreover, the failure of all conventional chemotherapies to induce long-lasting remissions strongly suggests that the apoptosis-mediating DDR is crippled in CLL. The genomic instability of CLL cells is reflected by a number of cytogenetic abnormalities that occur recurrently in CLL. For instance, deletions of the short arm of chromosome 17 (*del(17p)*) are found in 5–8% of chemotherapy-naïve patients. These deletions almost always include band *17p13*, where the prominent tumor suppressor gene *TP53* is located. CLL patients carrying a *del(17p)* clone show marked resistance against genotoxic chemotherapies that cannot be overcome by the addition of anti-CD20 antibodies in the context of state of the art chemo-immunotherapy (Hallek et al., 2010). Among cases with confirmed *del(17p)*, the majority show mutations in the remaining *TP53* allele (>80%) (Seiffert et al., 2012). Disabling *TP53* mutations are enriched in chemotherapy-treated patients, suggesting that an inactivation of the pro-apoptotic ATM-CHK2-p53 signaling cascade is selected for in CLL (Puente et al., 2011; Quesada et al., 2011).

Deletions of the long arm of chromosome 11 (*del(11q)*) can be found in approximately 25% of chemotherapy-naïve patients with advanced disease stages and 10% of patients with early stage disease (Zenz et al., 2010; Puente et al., 2011; Quesada et al., 2011). These deletions frequently encompass band *11q23* harboring the *ATM* gene. A subset of approximately 40% of patients carrying a *del(11q)* clone display inactivating mutations of the second *ATM* allele and these cases show a poor chemotherapy response, reminiscent of what has been described for *TP53*-defective CLLs (Austen et al., 2007). In addition, patients carrying a *del(11q)* clone typically show rapid progression, and reduced overall survival (Seiffert et al., 2012). As for *TP53*, disabling *ATM* mutations are enriched in chemotherapy-treated patients, again suggesting that an inactivation of the pro-apoptotic DDR is selected for in CLL (Puente et al., 2011; Quesada et al., 2011). It remains to be seen whether the novel agents, including ibrutinib, idelalisib, ABT-199, obinotuzumab or lenalidomide might overcome the reduced prognosis of *del(17p)*/*TP53* and *del(11q)*/*ATM* altered cases.

Recently, two novel potential therapeutic approaches to specifically treat ATM-deficient neoplastic disease have emerged from *in vitro* and *in vivo* experiments performed in different laboratories.

As ATM is involved in HR-mediated DSB repair (**Figure 1A**), it was proposed that repression of NHEJ, the second prominent DSB repair pathway employed by mammalian cells, might display selective toxicity against ATM-defective cells while sparing healthy cells (**Figure 1C**) (Gurley and Kemp, 2001; Jiang et al., 2009; Reinhardt et al., 2009; Riabinska et al., 2013; Dietlein and Reinhardt, 2014; Dietlein et al., 2014a,b). Early experiments performed with $ATM^{-/-}$ and $PRKDC^{-/-}$ (encoding DNA-PKcs) mice revealed that double knockout animals undergo early embryonic lethality (E7.5), while single knockout animals were born alive (Xu et al., 1996; Gao et al., 1998; Gurley and Kemp, 2001). These data revealed a robust synthetic lethal interaction between *ATM* and *PRKDC* and suggest that pharmacological interception of DNA-PKcs signaling might be detrimental to *ATM*-defective *del(11q)* CLLs. Consistent with this hypothesis, combined depletion of *Atm* and *Prkdc* in *Myc*-driven transplanted murine lymphomas led to a massive sensitization of these lymphomas against the anthracycline doxorubicine (**Figure 1C**) (Jiang et al., 2009; Reinhardt et al., 2009). Pharmacological DNA-PKcs inhibition has recently been evaluated in preclinical systems (**Figure 1C**). DNA-PKcs repression with the ATP-competitive small molecule inhibitor KU-0060648 resulted in robust induction of apoptosis of *ATM*-defective cells *in vitro* (Riabinska et al., 2013). Furthermore, KU-0060648 displayed substantial cytotoxicity against *Atm*-depleted *Myc*-driven murine lymphomas, while *Atm*-proficient lymphomas were entirely resistant (Riabinska et al., 2013). The authors next extended their observations to freshly isolated CLL cells. While KU-0060648 displayed marked single agent activity against *del(11q)* CLL cells, cytogenetically normal cells did not show any apoptosis following drug exposure (Riabinska et al., 2013). Further analyses revealed that DNA-PKcs inhibition in *ATM*-defective cells prevents effective DSB repair (Riabinska et al., 2013). On a molecular level, the authors showed that KU-0060648-exposed *ATM*-defective cells initiate DSB resection and accumulate RPA-coated ssDNA intermediates. These structures ultimately trigger apoptotic cell death through activation of the RPA/ATRIP/ATR/CHK1/p53/Puma apoptotic signaling cascade (Riabinska et al., 2013). Further experiments showed that not only *ATM*-deficiency, but also other HR-impairing genetic aberrations, such as *BRCA1*-, *BRCA2*-, *FANCD2*- or *RAD50* mutations were associated with DNA-PKcs dependence (Dietlein et al., 2014b). Together these data suggest that DNA-PKcs inhibitors either as single agents or in combination with DSB-inducing chemotherapeutics might be a viable treatment option for *del(11q)* CLLs. Intriguingly, Celgene has developed CC-115, a small molecule compound that is currently being evaluated in phase I/II clinical trials as a combined DNA-PKcs/mTOR inhibitor for the treatment of both solid tumors and hematological malignancies, including CLL (ClinicalTrials.gov identifier: NCT01353625).

A second potential therapeutic approach for *ATM*-defective human neoplastic disease has recently emerged from preclinical model systems. Different groups have shown that PARP1 inhibitors display selective toxicity against *ATM*-defective cells (Williamson et al., 2012; Gilardini Montani et al., 2013; Kubota et al., 2014) (**Figures 2A–C**). PARP1 inhibitors have recently gained the attention of the biomedical community, as they have been demonstrated to selectively eradicate *BRCA1*- or *BRCA2*-deficient cells and tumors (**Figure 2C**) (Bryant et al., 2005; Farmer et al., 2005). PARP1 inhibitor treatment was shown to induce DNA damage in *BRCA1* or *BRCA2*-proficient and -deficient cells (Farmer et al., 2005). However, only *BRCA1* or *BRCA2*-defective cells were sensitive to PARP1 inhibition, while *BRCA1/2* wildtype cells were PARP1 inhibitor-resistant (Farmer et al., 2005). Subsequent experiments revealed that additional DNA repair-disabling cancer-associated mutations in genes such as *RAD51*, *RAD54*, *DSS1*, *RPA1*, *NBS1*, *ATR*, *ATM*, *CHK1*, *CHK2*, *FANCD2*, *FANCA*, or *FANCC* were also associated with PARP1 inhibitor sensitivity (McCabe et al., 2006). These results motivated additional experiments that tested the hypothesis that *ATM* deficiency could be an actionable genetic alteration that might be susceptible to PARP1 inhibition. In this regard, four pieces of data have recently been published. First, RNA interference-mediated *ATM* repression was shown to sensitize MCF-7 and ZR-75-1 breast cancer cells (ER-positive, HER2-negative, *BRCA1/2* wildtype, *TP53* wildtype) to the PARP1 inhibitor olaparib (Gilardini Montani et al., 2013). Second, a focused gastric cancer cell line screen revealed that low ATM protein expression significantly correlated with olaparib sensitivity (Kubota et al., 2014). A further characterization revealed that pharmacological- or RNA-interference-mediated repression of ATM kinase activity enhanced olaparib sensitivity in gastric cancer cell lines with parallel depletion or inactivation of p53 (Kubota et al., 2014). In addition to these solid tumor entities, PARP inhibitors have also been evaluated in hematological malignancies. In mantle cell lymphoma xenograft transplants it was recently shown that animals carrying lymphomas lacking both *ATM* and *TP53* (UPN2) displayed significant olaparib sensitivity. Similarly, in mice transplanted with lymphomas lacking *ATM* and one copy of *TP53*, olaparib induced a significant survival gain. In contrast, mice transplanted with ATM- and p53-proficient lymphomas (JVM-2), or lymphomas with isolated p53 inactivation (HBL-2), did not derive a survival benefit from olaparib (Williamson et al., 2010, 2012). Lastly, proliferating primary *ATM*-deficient CLL cells were shown to display increased olaparib sensitivity, compared to *ATM*-proficient counterparts (Weston et al., 2010). Both genetic and pharmacological experiments validated that this effect was ATM-dependent (Weston et al., 2010). Furthermore, the authors employed a murine xenograft model of an *ATM*-mutant mantle cell lymphoma cell line to demonstrate a significantly reduced lymphoma burden and an increased survival of animals following olaparib treatment *in vivo* (Weston et al., 2010). Altogether, these data suggest that PARP1 inhibition might be a useful strategy for the treatment of refractory *ATM*-defective CLLs (**Figure 2C**).

Perspectives

One of the biggest hurdles in preclinical CLL research and preclinical development of targeted CLL therapeutics is the lack of mouse models that faithfully mimic the genetic events leading

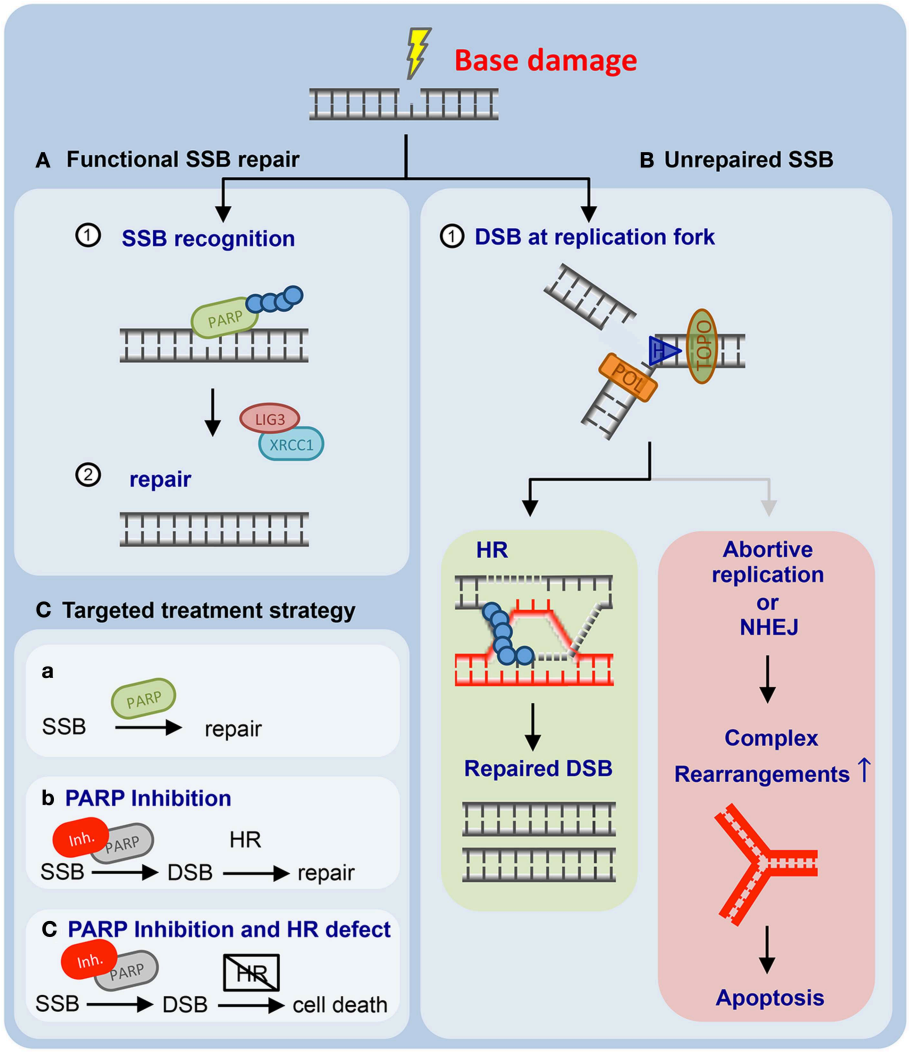

FIGURE 2 | Mammalian cells employ base excision repair to resolve single-strand breaks (SSBs) and non-helix-distorting base modifications. (A) Unperturbed base excision repair (BER) requires PARP1 and LIG3 and XRCC1. **(B)** PARP1 inhibition leads to the accumulation of genotoxic lesions that are subsequently repaired through homologous recombination (HR)-mediated DNA repair (left panel). If HR-mediated DNA repair is unavailable, PARP1 inhibitor-induced genotoxic damage accumulates and ultimately results in apoptotic cells death (right panel). **(C)** Proposed targeting of HR-defective human cancer through PARP1 inhibition is outlined (for details please refer to the main text).

to human CLL development. Although several models exist (for an excellent review, please refer to Simonetti et al., 2014), none of these models truly recapitulates the multistep leukemogenesis typically observed in CLL patients. Specifically the high-risk aberrations, such as *Tp53*- or *Atm* deletion/mutation are thus far not sufficiently recapitulated. Although $Tp53^{-/-}$ mice have been crossed with *Eμ-Tcl1* transgenic animals, the resulting compound-mutant *Eμ-Tcl1*;$Tp53^{-/-}$ mice carried a homozygous germline deletion of *Tp53*, which limits their use as a preclinical model to mirror somatic *del(17p)* or *TP53*-mutation in CLL (Liu et al., 2014). Of note, *Eμ-Tcl1*;$Tp53^{-/-}$ mice develop B-CLL substantially earlier than *Eμ-Tcl1* mice with an early appearance of $CD5^{+}$/IgM^{+} B cells in the spleen (Liu et al., 2014). These animals display an aggressive course of disease development, as well as a drug resistance phenotype reminiscent of human *del(17p)* CLL (Liu et al., 2014). These data suggest that a B cell-specific conditional *Tp53* deletion, for instance through the use of *Cd19-Cre*^{ERT2} deleter mice on the *Eμ-Tcl1* background, might be a useful experimental strategy to faithfully mimic clonal evolution of p53-defective CLL. In addition, B cell-specific conditional *Atm* deletion using the recently published *Atm*^{fl} allele (Zha et al., 2008) should be performed with *Cd19-Cre*^{ERT2} deleter mice in the *Eμ-Tcl1* background. Furthermore, it is desirable to translate recent large scale CLL genome sequencing data into preclinical platforms. For instance, generation of mice carrying a B cell-specific *Myd88*^{L265P} mutation, which has recently been described as a potential early driver lesion in CLL (Landau et al., 2013), should be pursued (**Figure S1**).

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fgene.2015.00207/abstract

Figure S1 | Schematic proposal of the use and early integration of genetically engineered mouse models for the development of novel CLL therapeutics. Recent large-scale chronic lymphocytic leukemia (CLL) genome sequencing efforts have unraveled the identity of numerous potential driver mutations in CLL. With this genomic information in hand, we propose the generation of novel genetically-engineered mouse models of CLL to serve as a preclinical platform for the identification and validation of novel CLL therapeutics.

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frontiers in GENETICS

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Targeting lung cancer through inhibition of checkpoint kinases

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Inhibitors of checkpoint kinases ATR, Chk1, and Wee1 are currently being tested in preclinical and clinical trials. Here, we review the basic principles behind the use of such inhibitors as anticancer agents, and particularly discuss their potential for treatment of lung cancer. As lung cancer is one of the most deadly cancers, new treatment strategies are highly needed. We discuss how checkpoint kinase inhibition in principle can lead to selective killing of lung cancer cells while sparing the surrounding normal tissues. Several features of lung cancer may potentially be exploited for targeting through inhibition of checkpoint kinases, including mutated p53, low ERCC1 levels, amplified Myc, tumor hypoxia and presence of lung cancer stem cells. Synergistic effects have also been reported between inhibitors of ATR/Chk1/Wee1 and conventional lung cancer treatments, such as gemcitabine, cisplatin, or radiation. Altogether, inhibitors of ATR, Chk1, and Wee1 are emerging as new cancer treatment agents, likely to be useful in lung cancer treatment. However, as lung tumors are very diverse, the inhibitors are unlikely to be effective in all patients, and more work is needed to determine how such inhibitors can be utilized in the most optimal ways.

Keywords: checkpoint abrogation, lung cancer, ATR, Chk1, Wee1, replication stress, cancer stem cells, hypoxia

INTRODUCTION

Lung cancer is difficult to treat. Its frequent incidence combined with the low success rate of current treatment strategies, make lung cancer the overall deadliest form of cancer worldwide (Siegel et al., 2012). Although recent progress has demonstrated druggable driver mutations in lung cancer, such as ALK (Anaplastic Lymphoma Kinase) translocations and EGFR (Epidermal Growth Factor Receptor) mutations, these are found only in a small subset of all lung cancer patients, and treatment resistance develops invariable (Chen et al., 2014). Most patients are diagnosed in late stages of the disease and are treated with chemotherapy or radiotherapy, with symptomatic and sometimes life prolonging effect. Overall, 5 years survival is bleak, approaching 18% (National Institutes of Health, 2011; Cancer Registry of Norway, 2012). There is therefore still a strong need for development of new treatment strategies in lung cancer.

In response to DNA damage or replication stress, activation of the checkpoint kinases Chk1 (Checkpoint kinase 1), Wee1 and ATR (Ataxia Telangiectasia and Rad3 related) facilitate S and G2 checkpoint arrest (Sanchez et al., 1997; Liu et al., 2000; Wang et al., 2001; Heffernan et al., 2002; Sørensen et al., 2003; Beck et al., 2012). These kinases may promote survival of tumor cells both in the absence and presence of DNA damaging agents. Inhibitors of these kinases have been developed and are currently in preclinical and clinical testing for cancer treatment (Do et al., 2013; Llona-Minguez et al., 2014; McNeely et al., 2014). For instance, several clinical trials are ongoing with the Wee1 inhibitor MK1775 (AZD1775) for combined treatment with radiation therapy or chemotherapy. These studies are performed in several cancer types, including lung cancer. Trials are also ongoing with the Chk1-inhibitors LY2606368 and SCH 900776 as single agents or in combination with chemotherapeutic drugs (ClinicalTrials.Gov). Of note, non-small cell lung cancer (NSCLC) patients treated with Chk1 inhibitors reportedly showed partial responses in Phase 1 trials (Calvo et al., 2014; Sausville et al., 2014). The first clinical trials with ATR inhibitors were recently initiated, evaluating the safety and biological effects of AZD6738 and VX-970 (ClinicalTrials.Gov).

Here, we briefly review the rationales for using checkpoint kinase inhibitors as anticancer agents, and discuss their potential for treatment of lung cancer. The focus is on how checkpoint kinase inhibition in principle can lead to selective killing of lung cancer cells while sparing the surrounding normal tissue.

GENERAL PRINCIPLES BEHIND THE TUMOR SELECTIVE EFFECTS OF Chk1/ATR/Wee1 INHIBITORS

G2 CHECKPOINT ABROGATION

Following DNA damage, the G2 checkpoint prevents mitotic entry of damaged cells and thereby protects against mitotic catastrophe and cell death (Syljuåsen et al., 2004). The G2 checkpoint is activated mainly through inhibition of the mitosis promoting complex Cyclin B-Cdk1 (Cyclin dependent kinase 1). Wee1

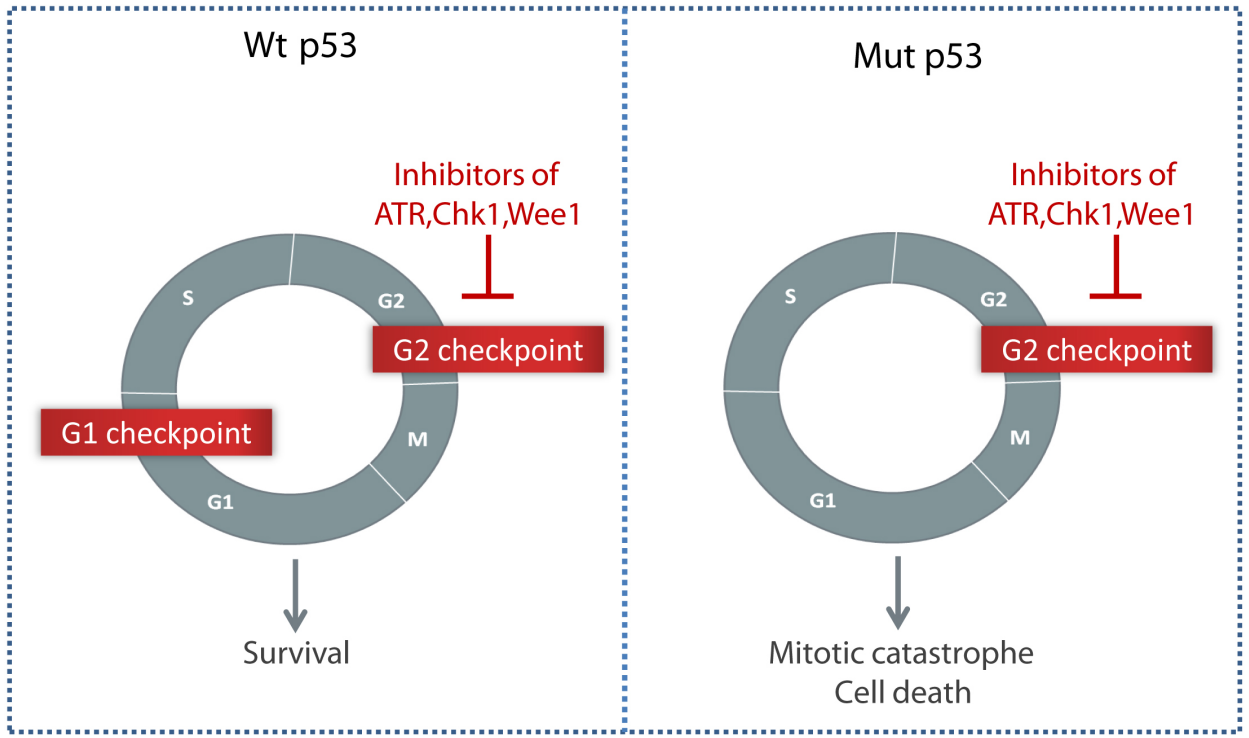

FIGURE 1 | Selective killing of p53 negative tumor cells through G2 checkpoint abrogation by inhibitors of ATR, Chk1, or Wee1. Compared to normal cells with an intact G1 checkpoint, cancer cells lacking the p53-dependent G1 checkpoint may depend more on the G2 checkpoint to survive after DNA damage.

kinase directly phosphorylates Cdk1 on its Tyrosine 15 residue, an inhibitory phosphorylation site negatively regulating Cdk1 activity (Parker and Piwnica-Worms, 1992). Tyrosine 15 phosphorylation is counteracted by the CDC25 (Cell Division Cycle 25) phosphatases, which in turn are negatively regulated by Chk1 (Sanchez et al., 1997). The activity of Chk1 is stimulated by ATR-mediated phosphorylation of Chk1 at the Serine 317 and 345 residues (Zhao and Piwnica-Worms, 2001). Thus, inhibition either of Wee1, Chk1, or ATR leads to decreased inhibitory phosphorylation of Cdk1 and thereby increased Cdk1 activity and G2 checkpoint abrogation.

Importantly, it was hypothesized that cancer cells lacking the G1 checkpoint may depend more on the G2 checkpoint for cell survival (reviewed in Dixon and Norbury, 2002; Ma et al., 2011). The G1 checkpoint is activated through the function of the tumor suppressor p53, and is often absent in cancer cells due to p53 mutations or other defects in the p53 signaling pathway (Nagasawa et al., 1995). Abrogation of the G2 checkpoint by inhibitors of Chk1, Wee1, or ATR may therefore selectively sensitize p53 defective cancer cells to DNA damaging agents, while the surrounding normal cells could be spared (**Figure 1**; Leijen et al., 2010; Ma et al., 2011; Hirokawa et al., 2014).

S PHASE DAMAGE

While Chk1, Wee1, and ATR are widely known as key regulators of the G2 checkpoint, these kinases also regulate CDK activity during S phase, and thereby prevent the induction of DNA damage during normal S phase progression (Syljuåsen et al., 2005; Sørensen and Syljuåsen, 2012). Increased CDK activity in response to checkpoint kinase inhibition promotes unscheduled replication initiation, leading to nucleotide shortage, replication stalling and subsequent activation of endonucleases and DNA breakage (Beck et al., 2012). In addition, shortage of other replication factors such as RPA (Replication Protein A) contributes to replication fork collapse after the unscheduled initiation (Toledo et al., 2013). ATR and Chk1 also play a more direct role in stabilizing stalled replication forks, by mechanisms that are still poorly understood (Brown and Baltimore, 2003; Friedel et al., 2009), but may involve suppression of nucleases (Froget et al., 2008; Forment et al., 2011). Thus, Chk1, ATR, and Wee1 inhibitors do not only cause G2 checkpoint abrogation, but also induce DNA damage in S phase, which may contribute to the cytotoxic effects of these inhibitors (Toledo et al., 2011; Sørensen and Syljuåsen, 2012).

During tumor development, the expression of oncogenes, such as Cyclin E, Myc and Ras, may abnormally increase replication, leading to so-called "replication stress" (Bartkova et al., 2005; Gorgoulis et al., 2005; Halazonetis et al., 2008). Importantly, cancer cells with elevated replication stress activate ATR/Chk1 and may depend more on these kinases for cell survival compared to normal cells (Gilad et al., 2010). In such cells ATR/Chk1 may help restrain the CDK activity and replication to tolerable levels, and these cells also likely depend more on Wee1. When combined with ongoing replication stress caused by oncogenes, checkpoint kinase inhibitors may therefore cause cytotoxic levels of S phase damage in tumor cells, while having minimal effects on normal cells. In addition to G2 checkpoint abrogation in p53 defective

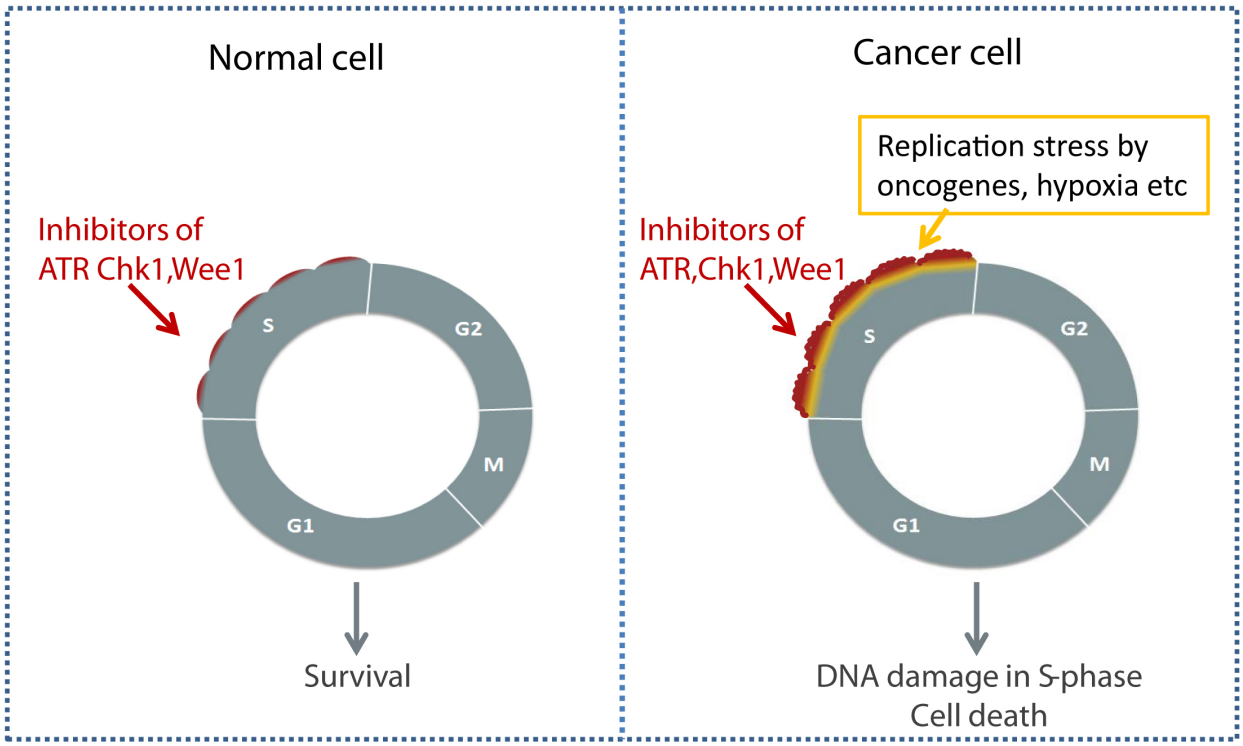

FIGURE 2 | Selective killing of cancer cells through S phase damage induced by checkpoint kinase inhibitors. Elevated replication stress in cancer cells due to oncogenes or hypoxia may lead to increased cytotoxic effects of checkpoint kinase inhibitors in S phase.

cells, increased S phase damage thus represents another reason for tumor-selective effects of Chk1, ATR, and Wee1 inhibitors (**Figure 2**; Sørensen and Syljuåsen, 2012; Do et al., 2013; Lecona and Fernandez-Capetillo, 2014).

INHIBITION OF HOMOLOGOUS RECOMBINATION REPAIR

Another shared function of Chk1, Wee1, and ATR is their role in positive regulation of homologous recombination (HR) repair, a major pathway for repair of DNA double strand breaks. In fact, inhibition of HR repair was suggested as a main mechanism for the radiosensitizing effects of the Chk1-inhibitor AZD7762, besides G2 checkpoint abrogation (Morgan et al., 2010). The Chk1-mediated regulation of HR repair occurs at least partly through direct phosphorylation of the Rad51 recombinase repair protein (Sørensen et al., 2005). A recent study showed that Wee1 inhibition also can inhibit Rad51 function and HR repair (Krajewska et al., 2013). The increased CDK activity after Wee1 inhibition leads to phosphorylation of BRCA2 at the 3291 residue, which in turn inhibits Rad51 loading (Davies and Pellegrini, 2007; Krajewska et al., 2013). The role of ATR in HR repair is less clear. However, ATR may support HR repair through control of the S phase checkpoint allowing time for repair, and through phosphorylation of Chk1 or other factors such as BRCA1 (Brown et al., 2014).

Notably, HR repair is largely restricted to S and G2 phase cells (Jeggo et al., 2011), and inhibition of HR repair will thus not affect non-cycling G0 or G1 phase cells. As tumors typically contain more cycling cells compared to the surrounding normal tissues, inhibition of HR repair in S and G2 phase cells therefore likely contributes to promote tumor selective effects of checkpoint kinase inhibitors.

CANCER-ASSOCIATED CHANGES IN ATR, Chk1, or Wee1 EXPRESSION

ATR, Chk1, and Wee1 are all essential proteins required for embryonic development in mice (Brown and Baltimore, 2000; Liu et al., 2000; Tominaga et al., 2006). Consistent with an essential role, homozygous inactivating mutations of the genes encoding these checkpoint kinases have not been observed in cancer. However, a small subset of human tumors shows heterozygous mutations in ATR or Chk1 (Bertoni et al., 1999; Lewis et al., 2005; Zighelboim et al., 2009), resulting in reduced protein expression. To our knowledge mutations in Wee1 have not been reported. However, Wee1 may be downregulated through other mechanisms such as cancer-associated expression of microRNAs (Butz et al., 2010; Tili et al., 2011). Interestingly, a recent siRNA screen identified ATR itself, and regulators of ATR kinase activity, among the factors protecting cells against the ATR inhibitor VE821 (Mohni et al., 2014). Cancer cells with reduced expression of ATR were thus more sensitive to the ATR inhibitor. This is likely because of more complete ATR inactivation in response to concentrations of VE821 that normally would be sufficient to only partially inactivate the cellular pool of ATR. Hence, it is possible that cancer cells with inherent reduced expression of ATR, Chk1, or Wee1 may respond to low concentrations of checkpoint kinase inhibitors, whereby normal cells could be spared.

On the other hand, ATR, Chk1, and Wee1 are also overexpressed in a subset of human cancers (Iorns et al., 2009; Mir et al., 2010; Cole et al., 2011; Magnussen et al., 2012; Parikh et al., 2014). In some cases, the checkpoint kinases may be upregulated as part of a cellular response to cope with elevated replication stress (Sørensen and Syljuåsen, 2012; Lecona and Fernandez-Capetillo, 2014). For instance, Myc amplification has been linked with elevated Chk1 levels and increased sensitivity to Chk1 inhibitors (Cole et al., 2011; Hoglund et al., 2011). Possibly, such cells will therefore depend on the high levels of ATR, Chk1, or Wee1 to survive. Inhibitors of ATR, Chk1, or Wee1 may thus potentially be more toxic to cancer cells inherently expressing high levels of these kinases. Taken together, this creates a complex picture where either abnormal *low* expression, or *high* expression, of ATR, Chk1, or Wee1 in cancer cells may potentially cause increased sensitivity to inhibitors of these checkpoint kinases.

TUMOR HYPOXIA

Hypoxia is very common in solid tumors and develops due to rapid growth of cancer cells and insufficient growth of new blood vessels, resulting in higher oxygen consumption than supply. Tumors can contain regions of long-term, persistent hypoxia, as well as regions with fluctuations in oxygen leading to cycles of transient hypoxia and reoxygenation (Bertout et al., 2008; Dewhirst, 2009). Hypoxia is a poor prognostic factor and is associated with resistance to conventional cancer therapy (Bristow and Hill, 2008; Horsman et al., 2012; Luoto et al., 2013; Walsh et al., 2014). However, hypoxic tissues also offer the advantage of being distinct from the surrounding normal tissues, and as such may be exploited to obtain selective killing of cancer cells. Importantly, severe hypoxia leads to replication stress and activation of DNA damage checkpoint signaling (Hammond et al., 2002, 2003). Therefore, inhibitors of ATR or Chk1 may in fact represent hypoxic cell cytotoxins (Hammond et al., 2004). Indeed, several studies have demonstrated increased cytotoxic effects of both Chk1 and ATR inhibitors in cancer cells exposed to hypoxia compared to normoxic cells (Hammond et al., 2004; Pires et al., 2012; Cazares-Korner et al., 2013; Hasvold et al., 2013). However, the increased effects of Chk1 inhibitors were observed after reoxygenation following prolonged hypoxic exposure, and not when the Chk1 inhibitors were present only during hypoxia (Hasvold et al., 2013). Chk1-inhibitors may thus be more effective combined with other treatments that cause reoxygenation, such as for instance fractionated radiotherapy. The impact of hypoxia on the effects of Wee1 inhibitors is not clear and largely awaits investigation.

Although more work is needed to elucidate the influence of a hypoxic tumor microenvironment on the responses to checkpoint kinase inhibitors, these studies do indicate that hypoxic tumors may be more sensitive to checkpoint kinase inhibitors compared to the surrounding normoxic tissue.

CANCER STEM CELLS

Intra-tumor heterogeneity may play an important role during cancer treatment. Particularly, small sub-populations of tumor-initiating cells, or cancer stem cells (CSCs), may survive cancer therapy and promote tumor regrowth. Although the characterizing markers (Keysar and Jimeno, 2010) and origin of these cells has been a matter of debate, their existence in human cancers is now mainly accepted (O'Connor et al., 2014). Due to their inherent resistance against conventional cancer treatments and important role in tumor recurrence and metastasis, finding strategies for eradicating these CSCs is a crucial task.

Interestingly, several studies have demonstrated that DNA damage-induced signaling is enhanced in CSCs of various origins (glioblastoma, NSCLC, head and neck, prostate and pancreas), including increased activation of Chk1, and such cells are particularly sensitive to Chk1-inhibitors (Bao et al., 2006; Bartucci et al., 2012; Venkatesha et al., 2012; Wang et al., 2012; Wu et al., 2012; Fang et al., 2013; Bertrand et al., 2014; Signore et al., 2014). Furthermore, inhibition of ATR has been shown to cause depletion of chemoresistant and tumorigenic $CD133^+$ colon cancer cells (Gallmeier et al., 2011), and Wee1 inhibition radio-sensitized glioblastoma stem cells *in vitro* (Mir et al., 2010). The expression of Wee1 was in fact higher in $CD133^+$ compared to $CD133^-$ primary glioblastoma cells (Mir et al., 2010), and Wee1 was among the most downregulated genes upon differentiation of PTEN positive glioblastoma stem cells (Forte et al., 2013), indicating that high levels of Wee1 may be required to maintain a stemlike state. However, another report found no radio-sensitization by the Wee1 inhibitor MK1775 in glioblastoma neural stem cells (Sarcar et al., 2011).

More work is needed to clarify the effects of Chk1 versus ATR and Wee1 inhibition in CSCs, and to understand the mechanisms involved. Reports regarding the repair capacity of CSCs have been conflicting (Bao et al., 2006; McCord et al., 2009; Ropolo et al., 2009), and the effectiveness of Chk1 inhibition in such cells has primarily been coupled to regulation of cell cycle progression and cell death through apoptosis and mitotic catastrophe (Ropolo et al., 2009).

CHALLENGES OF LUNG CANCER TREATMENT

Excellent reviews summarizing and discussing the various therapies and targets of lung cancer in depth have been published elsewhere (Willers et al., 2013; Berge and Doebele, 2014; Chen et al., 2014), and we therefore only briefly summarize some of the main challenges of current lung cancer treatment below. These challenges are relevant with respect to evaluating the potential use of checkpoint kinase inhibitors.

Lung cancer is a common disease, and the number one killer among cancers (Brustugun et al., 2014). However, there exists a huge diversity, both in clinical manifestation and patients. While most patients are or have been daily smokers, some have never smoked. Many patients are old, but some patients get this diagnosis at younger age. Traditionally lung cancers were divided in small cell lung cancer and non-small cell lung cancers. Current treatment algorithms require both histological subtype (adenocarcinoma vs. squamous cell carcinomas) and analyses for specific genetic aberrations. Treatment and follow-up of lung cancer patients vary depending on these specific characteristics (Chen et al., 2014).

Approximately 75% of lung cancer patients are diagnosed with stage four disease, and receive palliative treatment with chemotherapy and/or radiotherapy. The standard therapy is a

platinum (cisplatinum or carboplatinum) combined with a second drug (gemcitabine, pemetrexed, or vinorelbine for instance). The effects are unfortunately not long lasting, and new strategies are needed for a more effective treatment (Bonanno et al., 2014).

A subset of patients is treated with targeted therapy based on genetic aberrations in the tumor. Approximately 10–15% of NSCLCs are mutated in the EGFR gene, more common in Asian populations and among never-smokers. Patients with an EGFR-mutation in their tumor cells are effectively treated with tyrosine kinase inhibitors like gefitinib, erlotinib, or afatinib. These drugs have effects for 8–9 months in median, and second and third line drugs are in development (Melosky, 2014).

A small percentage of the tumors have a translocation involving the ALK-gene. This is present in approximately 2–6% of the adenocarcinomas, and is also effectively treated with targeted therapy (crizotinib, ceritinib; Chia et al., 2014). Unfortunately, resistance develops in all patients. Other genetic alterations are currently being tested in clinical studies. BRAF mutations, ROS1 translocations, PIK3CA mutations, MET amplifications and HER2 aberrations are examples of such alterations, present in only a small percentage of lung cancers and currently being targeted in clinical studies (Chen et al., 2014).

Unfortunately, while lung cancer treatment today can relieve symptoms and prolong life with some months, the disease usually progresses. More knowledge is therefore needed about mechanisms underlying disease progression in order to develop new treatment strategies.

THE POTENTIAL OF ATR/Chk1/Wee1 INHIBITORS FOR TREATMENT OF LUNG CANCER

In light of the general principles behind the tumor selectivity of ATR, Chk1, and Wee1 inhibitors outlined above (summarized in **Figure 3**), there are several specific traits associated with lung cancer that may potentially increase the efficacy of such inhibitors. Below we outline these traits and discuss relevant published experimental work.

p53 MUTATIONS

Firstly, p53 mutations are very common in lung cancer (Takahashi et al., 1989). This is important as loss of p53 is proposed as a major reason behind the tumor specific effects of checkpoint kinase inhibitors (see above). Previous work showed that p53 disruption could sensitize p53 wt lung cancer cells (A549 and LXSN) to the combined effects of radiation and the Chk1-inhibitor UCN-01 (Xiao et al., 2002). Similarly, the Wee1 inhibitor MK1775 radio-sensitized lung cancer cells (A549, H460, H1299) in a p53-dependent manner (Bridges et al., 2011). Furthermore, siRNA mediated depletion of p53 sensitized A549 lung cancer cells to the ATR inhibitor VE821 in combination with cisplatin

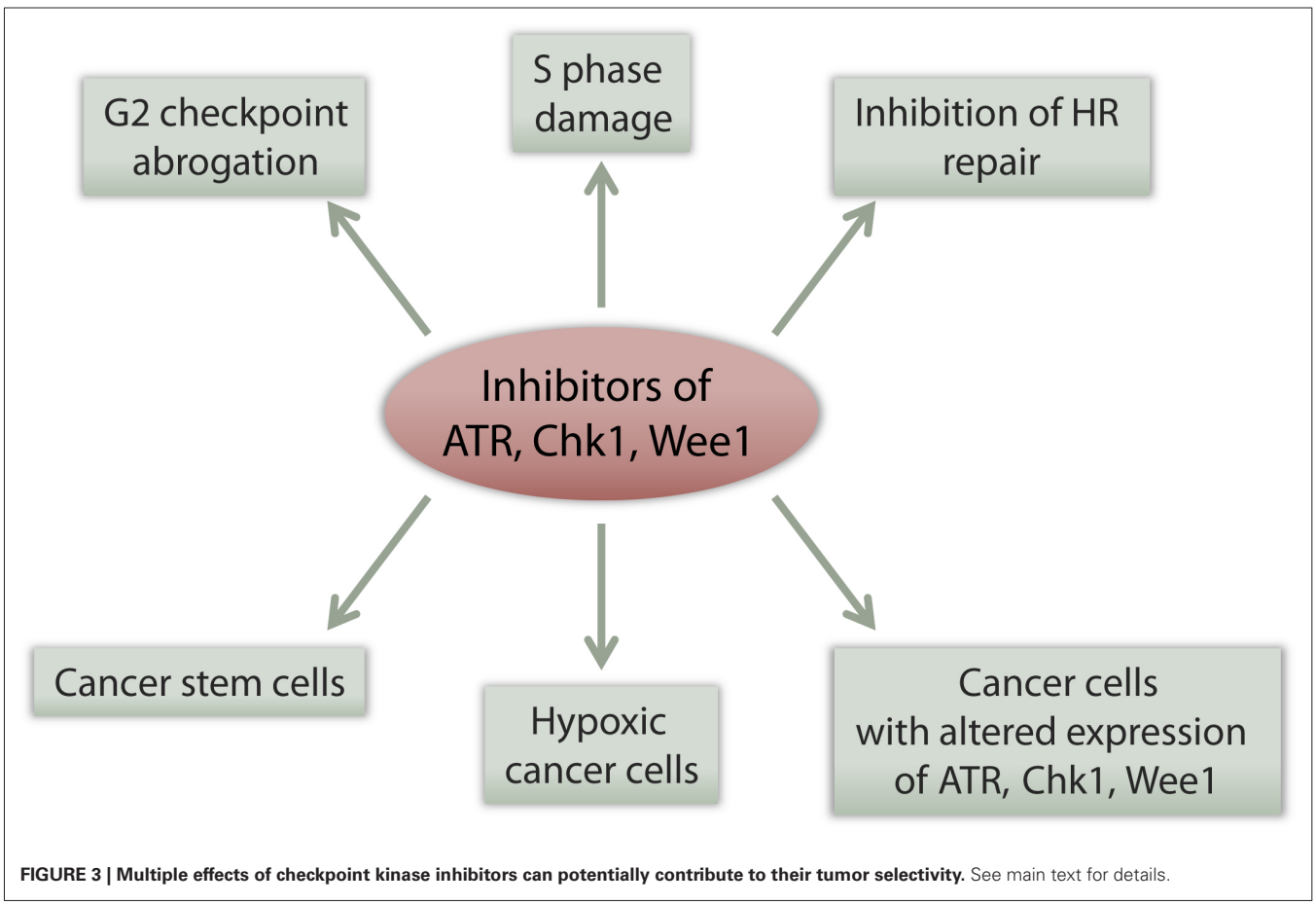

FIGURE 3 | Multiple effects of checkpoint kinase inhibitors can potentially contribute to their tumor selectivity. See main text for details.

(Reaper et al., 2011), and A549 cells depleted of p53 were also sensitized to another ATR inhibitor, VX-970, in combination with various DNA damaging drugs (Hall et al., 2014). These results thus support the hypothesis that inhibitors of ATR, Chk1, or Wee1 can be used to selectively target p53 deficient lung cancer cells. However, although p53 status has proven important for the effects of checkpoint kinase inhibitors in isogenic cell systems, p53 status alone does not seem sufficient to predict responses across large heterogenic cancer cell panels (Petersen et al., 2010; Guertin et al., 2013; Hall et al., 2014). Particularly, the cytotoxic effects of ATR, Chk1, or Wee1 inhibitors given as single agents vary between different cell lines regardless of p53 status (Petersen et al., 2010; Guertin et al., 2013; Hall et al., 2014). It is therefore unlikely that the p53-status alone can fully predict the efficacy of ATR, Chk1, and Wee1 inhibitors in lung cancer patients. However, p53 deficiency is, one among several factors, contributing to increasing the efficacy of these inhibitors.

INCREASED REPLICATION STRESS CAUSED BY GENETIC ALTERATIONS OR HYPOXIA

Secondly, replication stress is a common feature of lung cancer, which could sensitize to checkpoint kinase inhibition by enhancing the S phase damage (see above). For instance, the Myc oncogene is an inducer of replication stress, and some lung cancers are Myc-driven (Little et al., 1983). Exogenous overexpression of Myc caused increased sensitivity to Chk1 inhibitors in various cell types (Cole et al., 2011; Hoglund et al., 2011; Murga et al., 2011). In addition, ATR inhibitors caused increased cell death in Myc overexpressing cells, and partial genetic depletion of ATR prevented growth of Myc-induced tumors in mice (Murga et al., 2011; Schoppy et al., 2012). Thus, Myc overexpression may sensitize to both Chk1 and ATR inhibitors. Furthermore, Ras is mutated in a subset of lung cancers (Vasan et al., 2014). Oncogenic Ras can cause replication stress and increase the efficacy of ATR inhibitors (Gilad et al., 2010; Schoppy et al., 2012), and the Wee1 inhibitor MK1775 was identified in a screen for agents targeting Ras driven malignancies (Weisberg et al., 2014).

In addition, a proportion of NSCLCs reportedly show reduced expression of the repair protein ERCC1 (Postel-Vinay et al., 2012; Wei et al., 2012). Low levels of ERCC1 sensitize cells to platinum-based drugs such as cisplatin, and ERCC1 is currently being tested as a predictive biomarker for cisplatin-based chemotherapy in lung cancer (Postel-Vinay et al., 2012; Wei et al., 2012; Bonanno et al., 2014), although the methods of evaluating the ERCC1 levels have been questioned (Friboulet et al., 2013). Interestingly, a recent siRNA screen for factors protecting against the ATR inhibitor VE821 identified ERCC1 among the strongest hits (Mohni et al., 2014). Cells with low levels of ERCC1 ceased S phase progression and showed increased cell death after ATR and Chk1 inhibition (Mohni et al., 2014). Lung cancer cells with low levels of ERCC1 may therefore be highly sensitive to ATR, as well as Chk1, inhibitors.

Thus, manipulation of Myc, Ras or ERCC1 in various cell systems can cause altered sensitivity to ATR, Chk1, and Wee1 inhibitors. However, it remains to be shown whether Myc, ERCC1 and/or Ras status can predict responses to checkpoint kinase inhibitors across large panels of heterogenic human lung tumors. Potentially, these factors could be valuable as predictive biomarkers for responses to checkpoint kinase inhibitors *in vivo*.

Moreover, hypoxia is common in lung tumors (Bollineni et al., 2012). Hypoxia can induce replication stress (Hammond et al., 2003) and may sensitize to ATR or Chk1 inhibitors (Olcina et al., 2010). Nonetheless, few studies have focused on hypoxia and the effects of checkpoint kinase inhibition in lung cancer. Of note, a recent report demonstrated decreased viability of hypoxic A549, H1299, and H1975 lung cancer cell lines after treatment with a hypoxia-activated Chk1 inhibitor (the CH-01 prodrug; Cazares-Korner et al., 2013), indicating that hypoxic lung tumors may be sensitive to Chk1 inhibitors. In contrast, a single study suggested that hypoxia does not sensitize H1299 lung cancer cells to the Wee1 inhibitor MK1775 (O'Brien et al., 2013).

LUNG CANCER STEM CELLS

Though less studied than CSCs in glioblastoma, several studies have suggested that lung tumors contain sub-populations of such tumor initiating cells (reviewed in Singh and Chellappan, 2014). High expression levels of CSC markers such as CD133 and CD44 have been identified as poor prognostic factors in NSCLC patients (Luo et al., 2014; Wu et al., 2014), and studies with lung cancer cell lines have confirmed the presence of side population (SP) cells and spheroid-forming cells with typical CSC properties, including resistance to chemotherapy agents and radiation (Ho et al., 2007; Salcido et al., 2010; Fang et al., 2013; Lundholm et al., 2013). Furthermore, recent studies have shown that lung cancer cell lines surviving radiation express higher levels of several CSC markers such as CD44 or CD24 (Gomez-Casal et al., 2013).The cell adhesion molecule CD44 in particular was upregulated in cells surviving radiation from two different lung cancer cell lines (Gomez-Casal et al., 2013), suggesting that this marker may be associated with radiation resistance. CD44 positive cells were also found to be resistant to cisplatin in a study of NSCLC cell lines (Leung et al., 2010).

Overcoming such treatment resistance is vital for successful treatment of lung cancer patients, and a few recent studies indicate that Chk1 inhibition might be a promising way to do so. In spheroid-forming cells derived from the NSCLC cell line NCI-H1299, the combination treatment of Chk1 inhibition and gemcitabine enhanced the antiproliferative effect of gemcitabine, though it failed to deplete the CSC population completely (Fang et al., 2013). Even more promising, in a study using cells derived directly from lung cancer patients, activation of Chk1 in response to chemotherapeutic drugs was strongly enhanced in cells grown as spheres (undifferentiated) compared to adherent cells grown in a monolayer (differentiated; Bartucci et al., 2012). These undifferentiated cells, termed NSCLC-SCs, were also resistant to the cytotoxic effects of cisplatin, gemcitabine and paclitaxel, consistent with a strong repair capacity and checkpoint activation. However, inhibition of Chk1 abolished this chemotherapy resistance, and the combination of chemotherapy and Chk1 inhibitors severely decreased the colony-forming ability of these cells, making Chk1 inhibition a promising strategy for the selective targeting of such NSCLC-SCs. The effects of ATR and Wee1 inhibitors in this context are not known.

ALTERED EXPRESSION LEVELS OF CHECKPOINT KINASES IN LUNG CANCER

Only limited information is available regarding the expression levels of ATR, Chk1, and Wee1 in lung cancer. However, ATR and Chk1 may be amplified in a subset of genomic unstable lung cancers (Krajewska et al., 2014). In one report, lung cancer cell lines expressing high levels of Chk1 were hypersensitive to Chk1 inhibitors, suggesting that their growth depended on the high amount of Chkl (Grabauskiene et al., 2013). To our knowledge, ATR and Chk1 are not commonly mutated in lung tumors (http://cancergenome.broadinstitute.org). However, other mechanisms of inactivation, like methylation or microRNA-regulation, might play a role. Loss of Wee1 has been reported in NSCLC (Yoshida et al., 2004), but it is not known whether these cells show altered sensitivity to Wee1 inhibitors.

SYNERGY WITH CONVENTIONAL LUNG CANCER TREATMENTS

While checkpoint kinase inhibitors may show antitumor activity as single agents, they will most likely be used in combination with other treatments. As described above, the current standard treatments of lung cancer include several chemotherapeutic drugs and radiation therapy. Some of these conventional treatments may synergize with checkpoint kinase inhibitors. Multiple studies in different cancer types suggest that ATR and Chk1 inhibitors strongly synergize with gemcitabine and cisplatin (Lecona and Fernandez-Capetillo, 2014; McNeely et al., 2014). This has also been shown in lung cancer. Combination of the Chk1 inhibitor AZD7762 with gemcitabine or cisplatin suppressed growth of lung carcinoma xenografts in mice (Bartucci et al., 2012). H1299 lung cancer cells grown as spheres were resistant to gemcitabine, but could be sensitized by Chk1-inhibition (Fang et al., 2013). In addition, the ATR inhibitor VX-970 sensitized lung cancer cell lines and human lung tumor primary xenografts to cisplatin (Hall et al., 2014). Notably, when comparing the effects of combining the inhibitors with cisplatin or gemcitabine, the ATR inhibitor VX-970 was most effective in combination with cisplatin, and the Chk1 inhibitor AZD7762 in combination with gemcitabine (Hall et al., 2014). Potentiation of the effects of H1299 lung cancer cells to gemcitabine has also been reported with the Wee1 inhibitor MK1775 (Hirai et al., 2009). Furthermore, both Chk1 and Wee1 inhibitors were reported to sensitize lung cancer cells to radiation (Bridges et al., 2011; Yang et al., 2011).

CONCLUDING REMARKS

In conclusion, lung tumors are difficult to treat and inhibitors of checkpoint kinases ATR, Chk1, and Wee1 will potentially be useful in future treatment strategies. Several common traits of lung cancer can contribute to increase the efficacy of checkpoint kinase inhibitors and promote tumor selective toxicity (summarized in **Figure 4**). However, as lung tumors are very diverse, the inhibitors are unlikely to be effective in all patients. The main challenges are to identify which patients that would benefit from such treatment and to utilize the inhibitors in the most optimal ways.

The efficacy of checkpoint kinase inhibitors in lung cancer is determined by multiple genetic factors, including p53, Myc, Ras, ERCC1, and the levels of ATR, Chk1, and Wee1 kinases

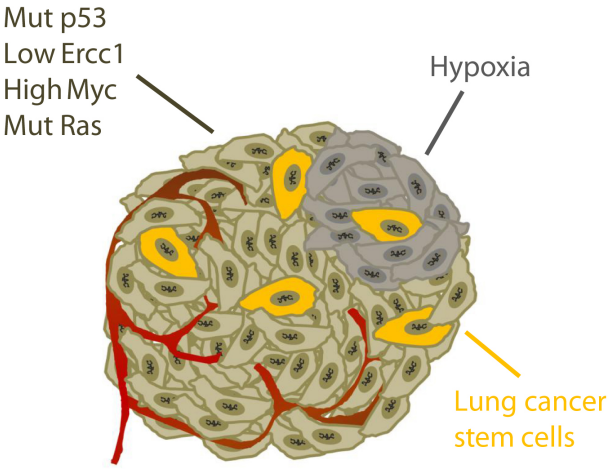

FIGURE 4 | Specific traits of lung cancer potentially causing tumor selective effects of checkpoint kinase inhibitors. See main text for details.

themselves. In addition, the efficacy also depends on other factors, like tumor hypoxia and CSCs. Therefore, it will most likely be difficult to find a single predictive biomarker for responses to checkpoint kinase inhibitors in lung cancer. A combination of several biomarkers may be useful to select patients. In order to identify optimal biomarkers, future studies should aim at understanding mechanisms determining the efficacy of such inhibitors in lung cancer. For instance, the relative contribution of S phase damage versus G2 checkpoint abrogation to the antitumor effects is not well understood. Importantly, the ATR, Chk1, and Wee1 kinases have several distinct functions, which need to be addressed separately. The inhibitors of each of these kinases may therefore be applicable in different situations. Recent preclinical studies have in fact reported synergistic effects when different checkpoint kinase inhibitors were combined, such as for instance Chk1 and Wee1 inhibitors (Carrassa et al., 2012; Russell et al., 2013; Chaudhuri et al., 2014; Chia et al., 2014). The exact mechanism behind this synergy between Chk1 and Wee1 inhibitors is not known, but may likely involve increased S phase damage (Carrassa et al., 2012; Chila et al., 2014). Such combinations should be explored further and be carefully compared to the inhibitors given as single agents at a range of different concentrations.

However, checkpoint kinase inhibitors will most likely be employed in combination with conventional current treatments, such as chemotherapeutic drugs and radiation therapy. Thus, an important issue is how these inhibitors can be utilized in an optimized way together with standard lung cancer treatments. The combined effects of checkpoint kinase inhibitors with chemotherapy and radiation should be further explored in both preclinical as well as clinical lung cancer studies. Particular attention should be given toward potential effects on lung CSCs. As has been shown for other treatment combinations, the sequential treatment timing may also be important (Lund-Andersen et al., 2014). For instance, the optimal time of administrating Chk1 inhibitors in combination with antimetabolites may be after cells have arrested in S phase following the antimetabolite treatment (Grabocka et al., 2014).

Finally, an important issue is whether partial inhibition of checkpoint kinases may increase the risk for the development of genetically unstable normal cells, or potentially lead to more aggressive tumor cells. Few studies have addressed the issue of potential increased genomic instability of cells surviving treatment with checkpoint kinase inhibitors. However, genetic studies from mice suggest that partial, subtle depletion of ATR (by haploinsufficiency) may cause increased genomic instability and accelerate Ras driven carcinogenesis (Gilad et al., 2010). On the other hand, subtle overexpression of Chk1 (by an extra allele of the Chk1 gene) promoted transformation in another report, likely due to increased survival of cells undergoing replication stress (Lopez-Contreras et al., 2012). Low levels of replication stress may therefore allow proliferation of potentially genetic unstable cells, while high levels of replication stress results in cell death. To better evaluate the potential risk associated with checkpoint kinase inhibition, it might be useful to compare the extent of genomic instability in cells surviving after treatment with checkpoint kinase inhibitors with the instability in cells treated with conventional DNA damaging agents.

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Discovering Che-1/AATF: a new attractive target for cancer therapy

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The transcriptional cofactor Che-1/AATF is currently emerging as an important component of the DNA damage response (DDR) machinery, the complex signaling network that maintains genome integrity and prevents tumorigenesis. Moreover this protein is involved in a wide range of cellular pathways, regulating proliferation and survival in both physiological and pathological conditions. Notably, some evidence indicates that dysregulation of Che-1/AATF levels are associated with the transformation process and elevated levels of Che-1/AATF are required for tumor cell survival. It is for these reasons that Che-1/AATF has been regarded as an attractive, still theoretical, therapeutic target for cancer treatments. In this review, we will provide an updated overview of Che-1/AATF activities, from transcriptional regulation to DDR.

Keywords: Che-1/AATF, DNA damage response, transcription, apoptosis, cell cycle regulation, cellular stress, survival

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Che-1/AATF at a Glance

More than 10 years ago human Che-1/AATF was identified by two different groups as both an RNA polymerase II binding protein and a gene downregulated upon TGF β induced differentiation (Fanciulli et al., 2000; Lindfors et al., 2000).

The human *Che-1/AATF* gene is located on chromosome 17, a region of the genome that is very rich in protein-coding genes, segmental duplications, and home to genes implicated in a wide range of human genetic diseases, such as *BRCA1* and *TP53* (Zody et al., 2006). It is highly conserved among eucaryotes and encodes for a protein of 558 aminoacids, whose expression is regulated by a negative feedback mechanism in which Che-1/AATF is present on its own promoter exerting an inhibitory effect (Monaco et al., 2003). At the structural level, the protein is characterized by the presence of an N-terminal acidic domain, a canonical leucine zipper, and three LXXLL motifs for nuclear receptor binding. It also contains two nuclear and two putative nucleolar localization signals (Fanciulli et al., 2000; Lindfors et al., 2000; Scott et al., 2011) and, at the cellular level Che-1/AATF mostly shows a nuclear and nucleolar localization. However, a cytoplasmic localization has also been reported in primary cerebellar granule neurons (Barbato et al., 2003; Di Certo et al., 2007), hippocampal neurons (Guo and Xie, 2004), and mouse embryonic fibroblasts (Höpker et al., 2012).

Che-1/AATF is a Transcriptional Cofactor

Che-1/AATF's ability to bind the RNA polymerase II in addition to the observation that its rat ortholog exhibits transactivation activity (Page et al., 1999), suggested from the beginning that Che-1/AATF could have been a transcriptional cofactor involved in the regulation of gene expression by connecting specific transcription factors to the general transcriptional machinery.

In fact, Che-1/AATF has been shown to interact with nuclear hormone receptors *in vitro* and to enhance transactivation of several steroid hormone receptors, alone or in cooperation with histone acetyltransferase p300 (Leister et al., 2003). In addition, it has also been reported that Che-1/AATF activity on androgen receptor mediated transcription is enhanced by its interaction with the tumor suppressor protein TSG101 (Burgdorf et al., 2004). Up until now, in addition to nuclear hormone receptors, several transcription factors, including the retinoblastoma protein (pRb), p65, and STAT3 (Bruno et al., 2002, 2006; Ishigaki et al., 2010), have been proven to interact with Che-1/AATF, thereby involving it in multiple cellular processes. These interactions are mostly regulated by post-translational modifications, which provide a rapid and reversible manner to modulate Che-1/AATF co-transcriptional activity in response to different stimuli (**Table 1**). In this regard, it is interesting to note that this protein interacts with RNA polymerase II through the C-terminal region of the subunit 11 (hRPB11; Fanciulli et al., 2000). This subunit is encoded by a multigene family which produces, along with the main form hRPB11a, proteins differing in their C-terminal domain, with different binding abilities and differently expressed in several tissues (Grandemange et al., 2001; Benga et al., 2005). Thus, Che-1/AATF action on transcription may depend on its binding of both transcription factors or different forms of hRPB11.

Che-1/AATF in Proliferation and Cell Cycle Control

Che-1/AATF protein is ubiquitously expressed (Fanciulli et al., 2000; Lindfors et al., 2000) and its expression is essential for proliferation and survival since Traube (Che-1/AATF mouse ortholog) knock out mice are embrionically lethal at the preimplantation state (Thomas et al., 2000). Moreover, mutant embryos exhibit a significant reduction in the total number of cells, indicating Che-1/AATF's involvement in cell cycle regulation. Consistent with these data, Bruno et al. (2002) demonstrated that Che-1/AATF promotes cell cycle progression by inhibiting the growth suppression functions of the pRb protein. pRb exerts its anti-proliferative functions by interacting with transcription factors E2F and promoting the assembly of an inhibitory complex containing histone deacetylases (i.e., HDAC1) on the promoters of E2F-responsive genes, whose expression is essential for the transition G1/S (Dick and Rubin, 2013). Che-1/AATF directly binds pRB and removes HDAC1 from the Rb/E2F complex, allowing transcription and progression to the S phase (Bruno et al., 2002). This activity may be modulated by its interaction with IFT88/polaris, a centrosomal protein that negatively regulates G1–S transition and inhibits Che-1/AATF binding to pRb (Robert et al., 2007). Remarkably, Che-1/AATF is hyperphosphorylated and accumulated during the G1/S transition (Bruno et al., 2002), suggesting that post-translational modifications may also regulate Che-1/AATF pro-proliferative functions. In addition, it has been recently shown that Che-1/AATF also participates in the control of mitotic entry by localizing at interphase centrosomes and regulating centrosome duplication and spindle formation (Sorino et al., 2013).

Che-1/AATF is an Anti-Apoptotic Factor

Along with its pro-proliferative role, Che-1/AATF also exhibits strong anti-apoptotic activity. Indeed, the rat AATF protein was originally identified for its ability to interact with and antagonize the activity of Dlk/ZIP (ZIPK), a serine/threonine kinase involved in the induction of apoptosis (Page et al., 1999).

Up until now much of the information regarding the anti-apoptotic function of Che-1/AATF derives from studies performed in the neural tissue, where this protein seems to be involved in the regulation of the apoptotic signaling in both physiological and pathological conditions. Di Certo et al. (2007) showed a direct interaction between Che-1/AATF and neurotrophilin receptor interacting MAGE homolog "NRAGE," an inducer of cell-death during neuronal development. In particular, they demonstrated that Che-1/AATF counteracts NRAGE-induced apoptosis, while NRAGE overexpression induces Che-1/AATF degradation by targeting it to the ubiquitin–proteasome pathways (Di Certo et al., 2007). Similarly, some evidence suggests that Che-1/AATF anti-apoptotic activity is involved in the neurodegeneration process associated with neurodegenerative diseases, such as Alzheimer's. This pathology is associated with extracellular aggregates of the β-amyloid peptide (Aβ) and intraneuronal fibrillar tangles of the microtubule binding protein Tau (Crews and Masliah, 2010). It has been demonstrated that Che-1/AATF can counteract neuronal degeneration induced by Aβ by interacting with prostate apoptosis response-4 (par-4) and blocking the par-4 mediated aberrant production and secretion of the neurotoxic peptide (Xie and Guo, 2004). Moreover, Che-1/AATF interacts with Tau in rat cerebellar granule neurons where this interaction is modulated during neuronal apoptosis (Barbato et al., 2003). A further indication of Che-1/AATF involvement in neurodegeneration is the demontration that it interacts with and is a substrate of cyclin-dependent kinase 5 (Cdk5), a serine/threonine protein kinase, whose activity is deregulated in neurodegenerative diseases (Buontempo et al., 2008).

A protective role of Che-1/AATF has also been reported in human kidney proximal tubule cells, where this protein has been observed to counteract apoptotic cell death following induced-renal injury by preserving mitochondrial function and reducing oxidative damage (Xie and Guo, 2006).

However, a pro-apoptotic role of Che-1/AATF has been recently reported. Ferraris et al. (2012) demonstrated that Che-1/AATF overexpression enhances UV induced apoptosis by promoting phosphorylation and transactivational activity of the pro-apoptotic factor cJun, in a p53 independent manner. Moreover, UV damage induces Che-1/AATF redistribution from nucleolus to nucleoplasm, thus allowing a direct Che-1/AATF-cJun interaction (Ferraris et al., 2012).

TABLE 1 | Che-1/AATF post-translational modifications.

Modification	Residue	Enzyme	Function	Reference
Phosphorylation	S181	ATM	Stabilization upon DNA damage; modulation of protein–protein interactions	Bruno et al. (2006)
	S141 S474 S508	Chk2	Stabilization upon DNA damage; modulation of protein–protein interactions	Bruno et al. (2006)
	T144	HIPK2	Degradation following apoptotic DNA damage	De Nicola et al. (2014)
	S316* S320* S321*		Modulated upon autophagy inhibition	Alayev et al. (2014)
	T366*	MK2	Nuclear translocation	Höpker et al. (2012)
		Cdk5		Buontempo et al. (2008)
Poly(ADP ribosyl)ation		PARP-1	Stabilization upon DNA damage	Bacalini et al. (2011)
Ubiquitination		HDM2	Degradation following apoptotic DNA damage	De Nicola et al. (2007)
Isomerization	P145	Pin1	Prerequisite for ubiquitination and degradation	De Nicola et al. (2007)

*Che-1 and AATF genes were cloned independently by two different groups. They encode for the same protein but in GenBank their sequences differ in the total number of aminoacids. AATF is reported as a protein composed of 560 aa while Che-1 of 558 aa. In order to avoid confusion we reported the position of the modified residue as indicated in the literature and the sequence which is referred to. *indicates AATF sequence.*

Che-1/AATF is Involved in the Cellular Response to Different Kind of Stress

DNA Damage

An increasing number of studies indicate Che-1/AATF as an important component of the DNA damage response (DDR), a complex network of pathways that eucaryotic cells have evolved to maintain genome integrity and prevent tumorigenesis (Jackson and Bartek, 2009; Lord and Ashworth, 2012). DDR coordinates multiple factors that cooperate together to detect genomic lesions, arrest cell cycle in order to allow repair, and promote apoptosis or senescence if damage is too severe (Ciccia and Elledge, 2010).

Upon DNA damage Che-1/AATF is extensively modified by post-translational modifications affecting its localization, half-life and interacting partners. It has been demonstrated that checkpoint kinases MK2, ATM, Chk2 can phosphorylate and activate this protein (Höpker et al., 2012; Bruno et al., 2006). Höpker et al. (2012) have shown that DNA damage promotes Che-1/AATF phosphorylation by checkpoint kinase MK2 at residue T366. This modification induces translocation of Che-1/AATF from the cytoplasm to the nucleus where it inhibits transcription of p53 dependent proapoptotic genes, such as *Puma*, *Bax*, and *Bak* (Höpker et al., 2012). On the other hand, phosphorylation through ATM and Chk2 leads to Che-1/AATF stabilization and accumulation by increasing its resistance to proteasome degradation (Bruno et al., 2006). Moreover, these latter modifications greatly affect Che-1/AATF functions, acting as a molecular switch that moves this protein from the pathways regulating cell cycle progression to the ones involved in cell cycle arrest and survival. In particular, (ATM–Chk2) phosphorylated-Che-1/AATF relocates from E2F1-dependent promoters to the promoters of genes involved in checkpoint activation such as *TP53* and *p21*, thus allowing their transcription and cell cycle arrest at the G2/M checkpoint (Bruno et al., 2006). Interestingly, these modifications also promote a specific interaction between Che-1/AATF and tumor suppressor p53. This binding occurs at the early stage of the DDR and specifically directs p53 toward the transcription of genes involved in cell cycle arrest. Notably, the two proteins detach when DNA damage is not repairable and cells undergo apoptosis (Desantis et al., 2015a). Evidence also shows that upon DNA damage phosphorylated-Che-1/AATF, by ATM and Chk2, promotes the transcription of the anti-apoptotic factor XIAP, an inhibitor of caspase activity. Consistent with this observation, Che-1/AATF overexpression protects cells from apoptosis induced by DNA damaging agents (Bruno et al., 2008).

It is worth remembering that, other than phosphorylation, poly(ADP-ribosyl)ation also participates in regulating Che-1/AATF activities upon genotoxic stress. In fact, it has been demonstrated that poly (ADP-ribose) polymerase 1 (PARP-1) interacts with this protein and promotes its modification, which in turn contributes to Che-1/AATF stabilization upon DNA damage (Bacalini et al., 2011).

A recent study revealed that Che-1/AATF is also part of the spindle assembly checkpoint (SAC), a ubiquitous safety mechanism that ensures the fidelity of chromosome segregation during mitosis and cooperates with the proteins of the DDR network in restricting mitotic progression in response to DNA damage (Zhang et al., 2007; Lara-Gonzalez et al., 2012). In particular, it was shown that DNA damage induces centrosomal accumulation of Che-1/AATF and depletion of this protein is associated with an increase in the number of centrosomes, multipolar spindles, failure to arrest mitosis, and apoptosis in response to genotoxic treatments (Sorino et al., 2013).

In agreement with its anti-apoptotic and prosurvival roles, Che-1/AATF degradation is required to execute the apoptotic program when DNA damage is too severe and cannot be repaired. The complex signaling cascade that leads to Che-1/AATF degradation following apoptotic DNA damage has been recently elucidated in two papers from De Nicola et al. (2007). They showed that upon apoptotic DNA damage the kinase HIPK2 directly interacts with Che-1/AATF and phoshorylates it at residue T144. This phosphorylation allows a conformational change mediated by the prolyl isomerase Pin1, which in turn promotes the interaction with ubiquitin ligase HDM2, thereby promoting Che-1/AATF ubiquitylation and proteasomal degradation. In agreement with these results, Che-1/AATF overexpression interferes with HIPK2 induced apoptosis, while failure in Che-1/AATF degradation upon apoptotic stimuli is associated with reduced cell death (De Nicola et al., 2007, 2014).

As described above, Che-1/AATF strongly affects p53 functions upon DNA damage by activating its transcription, promoting p53 dependent growth arrest and inhibiting p53 dependent apoptosis. Notably, this protein has also a strong impact on the activity of the mutant forms of p53 (mtp53), which are associated with almost 50% of cancer cases (Freed-Pastor and Prives, 2012). Indeed, Che-1/AATF is required for mtp53 transcription and its depletion induces apoptosis, without involving any other stimuli, in several cancer cell lines carrying mtp53. This event is the result of a simultaneous reduction of mtp53 level and activation of pro-apoptotic genes, such as *Puma* and *Noxa,* by tumor suppressor protein p73. In more detail, in the presence of mtp53, Che-1/AATF depletion induces endogenous checkpoint activation that leads to stabilization of the transcription factor E2F1, which in turn, activates p73. Inline with Che-1/AATF's ability to modulate checkpoint activation, Affymetrix microarray experiments have revealed that this protein regulates the expression of genes involved in DNA repair (Bruno et al., 2010).

Cellular Stress

Over the last few years, several pieces of evidence indicate that Che-1/AATF participates in the cellular response to different types of stress, other than DNA damage (**Figure 1**). For example, hyperosmotic stress can activate Che-1/AATF by inducing MK2-mediated phosphorylation (Höpker et al., 2012).

Ishigaki et al. (2010) indicated Che-1/AATF as a component of the unfolded protein response (UPR), an adaptative mechanism activated by endoplasmatic reticulum (ER) stress whose function is to restore ER homeostasis or induce apoptosis if stress cannot be resolved. Che-1/AATF is induced upon ER stress and promotes cell survival by activating transcription on the serine/threonine kinase AKT1, through directly interacting with transcription factor STAT3. Indeed, ectopic expression of Che-1/AATF protects cells from ER stress mediated apoptosis whereas its depletion increases the percentage of apoptotic cells after induction of ER stress (Ishigaki et al., 2010).

More recently, it has been demonstrated that Che-1/AATF protects cells from apoptosis induced by ionizing radiations (IR), hypoxia, or glucose deprivation by inducing autophagy, a self degradative process essential for maintaining cellular homeostasis that allows cells to survive under metabolic stress. In particular, Che-1/AATF inhibits the activity of the kinase mTOR, a central regulator of autophagy, by activating the transcription of its inhibitors Redd1 and Deptor. In agreement with these results, Che-1/AATF depletion decreases autophagy induction after stress, thus leading to apoptosis (Desantis et al., 2015b). Interestingly, it has been recently reported that inhibition of serum deprivation induced autophagy by resveratrol reduces phosphorylation of Che-1/AATF at residues S316, S320, and S321; however, the kinases responsible of these modifications are still unknown (Alayev et al., 2014).

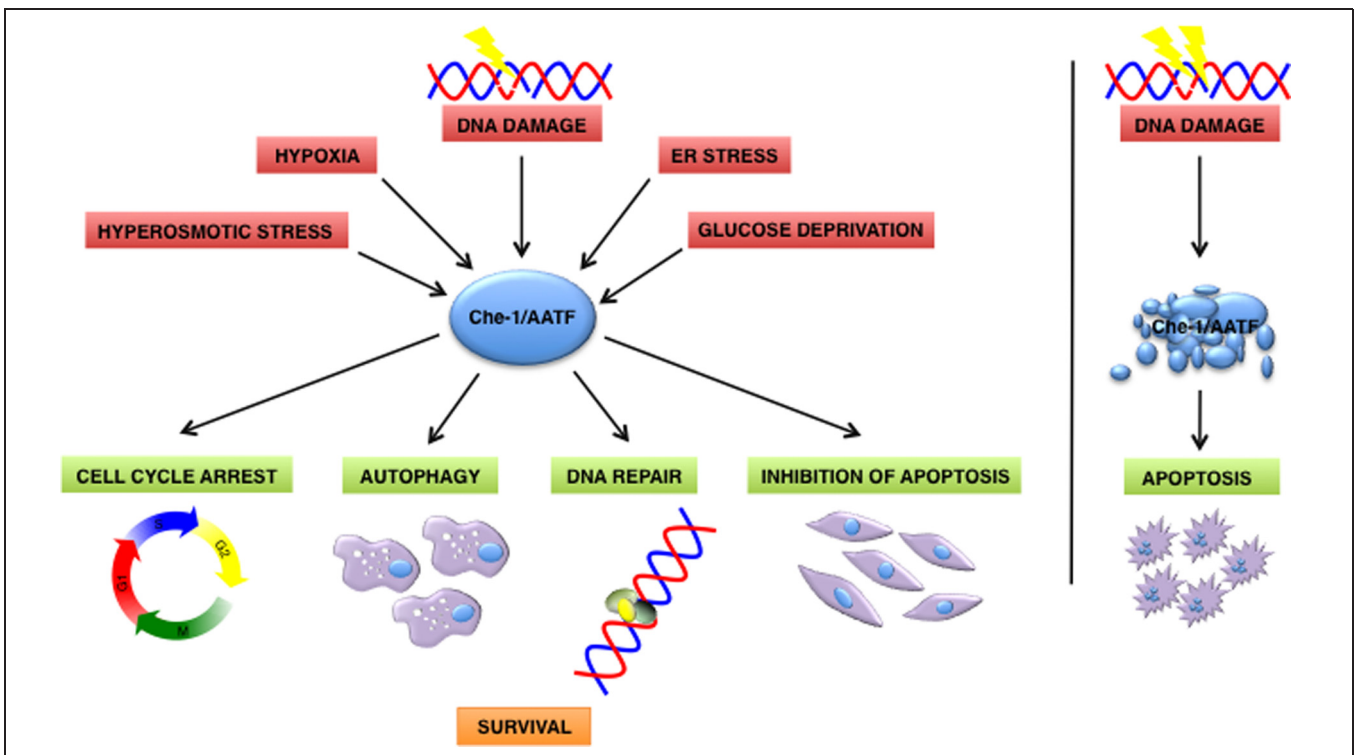

FIGURE 1 | Che-1/AATF is a central mediator of the cellular response to different types of stress. In response to DNA damage and cellular stress Che-1/AATF promotes cell survival by inducing cell cycle arrest, autophagy, DNA repair and inhibition of apoptosis. Otherwise if DNA damage is too severe Che-1/AATF is degradated and cells undergo apoptosis. ER, endoplasmatic reticulum.

Che-1/AATF as a Putative Therapeutic Target in Cancer

Overall, the observations described above strongly indicate that Che-1/AATF plays an important role in many aspects of cancer biology. Indeed, this protein is not only involved in cell cycle progression and in protecting cancer cells from apoptosis induction, but also plays a role in controlling autophagic response and ER stress, appearing to be able to sustain the survival of tumor cells (Desantis et al., 2015b). Moreover, Che-1/AATF deeply affects the activity of p53, by both modulating wild type p53 target specificity and supporting the "gain of function" of the mutated forms of this oncosuppressor (Bruno et al., 2010; Desantis et al., 2015a). However, a screening for Che-1/AATF mutations in 121 breast cancer families has highlighted that no mutations in Che-1/AATF coding sequence can be associated with cancer predisposition (Haanpää et al., 2009). On the other hand, several studies suggest that dysregulation in Che-1/AATF level inside cells could be relevant for the transformation process. In fact, this protein has been found upregulated in several leukemia cell lines and in patients with chronic lymphocytic leukemia (Kaul and Mehrotra, 2007; Bacalini et al., 2012). In addition, *Che-1/AATF* gene was amplified in neuroblastoma patients and increased Che-1/AATF expression levels were associated with poor prognosis and reduced survival (Höpker et al., 2012). Consistent with these observations, Che-1/AATF depletion was shown to enhance the cytotoxic effect of DNA-damaging chemotherapy both *in vitro* and *in vivo* and to induce apoptosis of cancer cells carrying mtp53 (Bruno et al., 2006, 2008, 2010; Höpker et al., 2012). All these findings strengthen the notion that Che-1/AATF could be considered a valid target for novel anticancer therapeutic approaches. Unfortunately, so far no compounds able to inhibit Che-1/AATF activity have been identified. However, future efforts focused on understanding the mechanism of action of Che-1/AATF and the characterization of the pathways implicated in its regulation will provide useful indications towards developing specific inhibitors for this protein.

Concluding Remarks and Open Questions

Much has been learned about Che-1/AATF functions in the years following its identification but a great deal remains to be unveiled.

One question that needs to be addressed is its role in DNA repair. Bruno et al. (2010) have shown that Che-1/AATF expression is necessary for proper repair of damaged DNA, but how this action is exerted is still not entirely understood. The ability of Che-1/AATF to regulate the expression of genes involved in DNA repair is definitely part of this process but other mechanisms may participate. At the structural level, one of the main features of Che-1/AATF is the presence of an extremely acidic domain at its N-terminal region, which in other transcription factors has been associated with chromatin remodeling properties (Hu et al., 1999; Tumbar et al., 1999). Moreover, Che-1/AATF has been found in histone acetyltransferase complexes through its interaction with the transcriptional co-activator ADA3 (Zencir et al., 2013) and it has the ability to induce local histone hyperacetylation by displacing HDAC1 from transcription factors pRb and Sp1 (Bruno et al., 2002; Di Padova et al., 2003). Based on these observations, one could speculate that Che-1/AATF participates in the DNA repair process by regulating the chromatin state and increasing its accessibility. If so, a new scenario for Che-1/AATF functions will be open since chromatin remodeling plays a fundamental role in replicative and transcriptional controls. Indeed, Che-1/AATF could participate in the regulation of gene expression by regulating chromatin structure at specific gene loci where it is recruited by its interaction with both transcription factors and transcriptional machinery.

Furthermore, since Che-1/AATF has already been appointed as a nucleolar stress sensor (Ferraris et al., 2012), it will be interesting to further investigate its involvement in the nucleolar stress response that monitors and maintains ribosome biogenesis and nucleolar integrity. This pathway has a crucial role in maintaining cellular homeostasis and it has been demonstrated that nucleoli disruption leads to activation of p53 in absence of DNA damage (Rubbi and Milner, 2003). Moreover, transformed cells undergo p53 mediated senescence, autophagy, and apoptosis in response to nucleolar perturbation by inhibition of ribosomal RNA synthesis (Woods et al., 2014). Indeed, in the last few years nucleolus and ribosomal gene expression are emerging as new exciting targets for cancer therapy and RNA polymerase I inhibitors are currently entering phase I clinical trials (Quin et al., 2014). In this context, it will be fundamental to explore the possibility that Che-1/AATF plays a role in ribosome biogenesis itself. This idea is supported by the observations that a mouse embryo mutant for *Traube* shows a decrease in the number of ribosomes and *Drosophila* Che-1/AATF mutants arrest the development of the egg chamber at the same stage as the mutants affecting the synthesis of ribosomes, namely when the massive growth starts and cells need to synthesize ribosomes to trigger this growth (Jagut et al., 2013).

Answering these questions will shed further light on additional aspects of Che-1/AATF functions and likely contribute to identifying possible therapeutic approaches involving this protein.

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Substrate recognition and function of the R2TP complex in response to cellular stress

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The R2TP complex is a HSP90 co-chaperone, which consists of four subunits: PIH1D1, RPAP3, RUVBL1, and RUVBL2. It is involved in the assembly of large protein or protein–RNA complexes such as RNA polymerase, small nucleolar ribonucleoproteins (snoRNPs), phosphatidylinositol 3 kinase-related kinases (PIKKs), and their complexes. While RPAP3 has a HSP90 binding domain and the RUVBLs comprise ATPase activities important for R2TP functions, PIH1D1 contains a PIH-N domain that specifically recognizes phosphorylated substrates of the R2TP complex. In this review we provide an overview of the current knowledge of the R2TP complex with the focus on the recently identified structural and mechanistic features of the R2TP complex functions. We also discuss the way R2TP regulates cellular response to stress caused by low levels of nutrients or by DNA damage and its possible exploitation as a target for anti-cancer therapy.

Keywords: R2TP complex, protein folding, DNA damage response, cellular stress, cancer

HSP90 AND ITS CO-CHAPERONES

Chaperones are proteins involved in protein folding and protein-complex assembly or disassembly (Macario and Conway de Macario, 2005). Heat shock protein 90 (HSP90) is an abundantly expressed chaperone implicated in a wide range of cellular processes, including cell signaling, protein degradation, genome maintenance and assembly of transcriptional and translational machineries (Prodromou et al., 1997; Li et al., 2012; Saibil, 2013). HSP90 client proteins are often in near-native state and HSP90 is involved in the late stages of their folding (Jakob et al., 1995). HSP90 acts as a dimer and its substrate specificity and activity are given by its co-chaperones (Ali et al., 2006; Li et al., 2012). Although most of the well-known HSP90 co-chaperones are small single-molecule proteins (CDC37, p23, SGT1, AHA1), recent work has shown that HSP90 co-chaperones can be multi-protein complexes themselves, such as the R2TP complex (Zhao et al., 2005; Li et al., 2012).

R2TP COMPLEX

The R2TP complex was discovered in budding yeasts as an Hsp90-interacting protein-complex (Zhao et al., 2005). It is involved in the assembly of large number of multi-subunit complexes: (a) the small nucleolar ribonucleoproteins (snoRNPs), which are essential for biogenesis of ribosomes, spliceosomes, and tRNAs (Zhao et al., 2008; McKeegan et al., 2009); (b) RNA polymerase II (Boulon et al., 2010) and (c) phosphatidylinositol 3-kinase-related kinases (PIKKs) and their complexes (Horejsi et al., 2010) that are involved in DNA damage signaling (ATM, ATR, DNA-PKcs), transcription regulation (TRRAP), nonsense mediated mRNA decay (SMG1), and nutrient signaling (mTOR; Izumi et al., 2012; **Figure 1**). Although the R2TP complex has become recently focus of many studies, the exact function and the molecular mechanism of its action is still not clear.

COMPONENTS OF THE R2TP COMPLEX

The R2TP complex is highly conserved from yeast to mammals and consists of four subunits: PIH1D1, RPAP3, RUVBL1 and RUVBL2, known under diverse names (**Table 1**; Zhao et al., 2005). The complex also associates with prefoldin and prefoldin-like proteins PFDN2, PFDN6, UXT, WDR92, URI and PDRG1, which form so called prefoldin-like complex, also implied in protein-complex assembly (Cloutier et al., 2009; Cloutier and Coulombe, 2010; **Figure 2**).

RUVBL1 and RUVBL2 belong to AAA+ ATPases family (ATPases associated with a variety of cellular activities) and are essential for viability in all so far examined model organisms (*Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans*; Nano and Houry, 2013). The AAA+ ATPases are characterized by presence of an AAA+ domain containing Walker A and B motifs, sensor domains 1 and 2 and an arginine finger (Patel and Latterich, 1998; Neuwald et al., 1999; Jha and Dutta, 2009). The Walker A motif binds ATP while the Walker B motif is involved in ATP hydrolysis, providing together ATPase activity. The sensor domains detect whether the ATPase is bound to ATP or ADP and the arginine finger either affects ATP hydrolysis or converts ATP hydrolysis into a mechanical output (Guenther

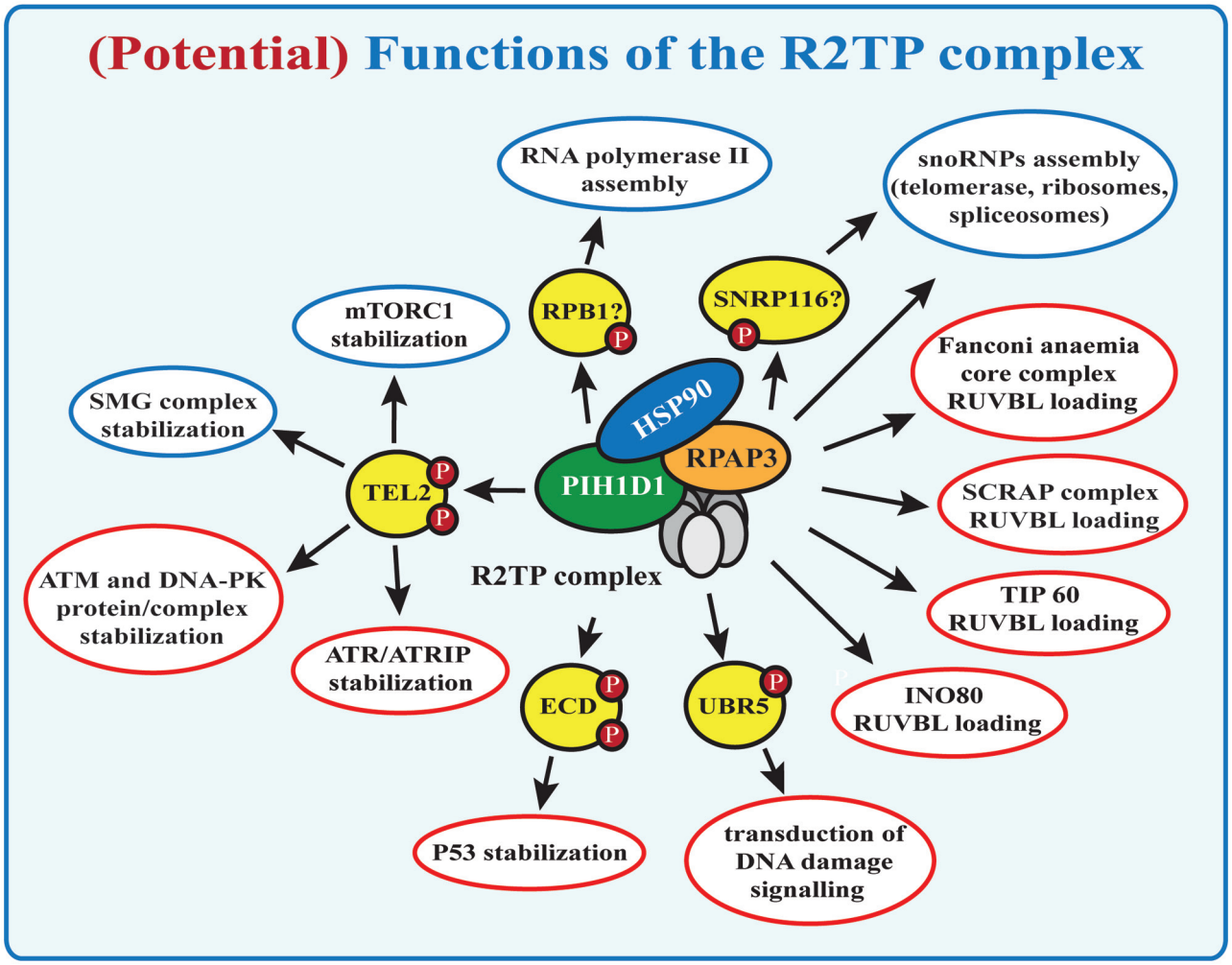

FIGURE 1 | (Potential) functions of the R2TP complex. Known functions of the R2TP complex are indicated in blue, potential R2TP functions are indicated in red. SMG1 complex and mTOR stabilization by the R2TP complex requires binding of PIH1D1 with the PIH-N domain to TEL2. Other potential PIH-N domain binding proteins are indicated, but their role for R2TP complex function remains to be studied.

et al., 1997; Ogura et al., 2004). Although *in vitro* data show no or limited ATPase activity of RUVBL1 and RUVBL2 (Grigoletto et al., 2011), some *in vivo* functions are impaired after mutation of the Walker A or B motifs: for example mutations of the yeast proteins comprising the ATPase activity result in serious growth defects while inactivation of the mammalian RUVBL1/2 activity decreases activation of mTOR and stability of the telomerase component TERC (Jonsson et al., 2004; Venteicher et al., 2008; Kim et al., 2013). It is therefore highly probable that the ATPase activity of RUVBL1/2 is important for at least part of their functions and that they might need other proteins, absent in the *in vitro* assays, that promote the ATPase activity. The purified proteins also exhibit a weak helicase activity (Huen et al., 2010).

The crystal structures of RUVBL1 revealed three structural subdomains – N terminal and C terminal subdomains form the AAA+ domain, while a flexible middle domain (also called the insertion domain), is involved in DNA or RNA binding. The insertion domain is located outside the core of the protein and is specific for RUVBL1/2 but not for other members of the AAA+ ATPases family and its deletion in both RUVBL1/2 increased their ATPase and helicase activity, indicating an auto-inhibitory function of this domain (Matias et al., 2006; Niewiarowski et al., 2010; Petukhov et al., 2012).

AAA+ ATPases often constitute hexamers (Smith et al., 2006) and accordingly, RUVBL1 and RUVBL2 form homo or hetero hexamers and/or double-hexameric structures – dodecamers. The crystal structure of a RUVBL1 monohexamer reveals a strong ADP binding by the AAA+ domain, which could explain its very low ATPase activity and indicating that the monohexamer is possibly not physiologically relevant (Matias et al., 2006). Yeast Rvb1 and Rvb2 incubated together form a hexameric ring, observed by electron microscopy and scanning transmission electron microscopy. Together the proteins have higher ATPase and helicase activity compared with the separate proteins (Gribun et al., 2008). Many studies also show formation of a dodecamer, consisting of both

Table 1 | Alternative names for the components of the R2TP complex.

	Mammals	Yeast
PIH1D1	NOP17	Nop17, Pih1
RPAP3	hSPAGH	Tah1
RUVBL1	Pontin, RVB1, TIP49A, TAP54α, ECP-54, TIH1, p50	Rvb1
RUVBL2	Reptin, RVB2, TIP49B, TAP54β, ECP-51, TIH2, p47	Rvb2

RUVBL1 and RUVBL2 (Puri et al., 2007; Torreira et al., 2008; Niewiarowski et al., 2010; Gorynia et al., 2011). Antibody labeling of Rvb2 in the yeast complex revealed that only one of the two rings contained this protein, arguing for two monomeric hexameres (Torreira et al., 2008). The crystal structure and mass spectrometry analysis of the human RUBVL1/2 complex supports formation of a dodecamer composed from two heterogenic hexameres (Niewiarowski et al., 2010; Gorynia et al., 2011). In the dodecameric complex, ATPase activity of both proteins is required to catalyze the ATP reaction (Puri et al., 2007) and depending on the arrangement of the insertion domain, the RUVBL complex forms a compact or a stretched confirmation (Lopez-Perrote et al., 2012). Interestingly, apart from a 3:3 ratio of RUVBL1/2, hexameres with different stoichiometry of RUVBL1/2 were also detected (Niewiarowski et al., 2010). The different conformations could represent the diverse range of functions these proteins play *in vivo*: one conformation could be important for helicase activity, while another one may be involved in protein-complex assembly (Torreira et al., 2008). Moreover, the independent and sometimes even opposing effects of RUVBL1/2 on transcription suggest that the ATPases can act independently on each other (Gallant, 2007).

RUVBL1 and RUVBL2 take part in many cellular processes: they are components of chromatin remodeling complexes TIP60, SWR/SRCAP, and INO80 and very recently they have been reported to interact with the Fanconi anemia core complex, which is involved in DNA inter-strand crosslink repair (Rosenbaum et al., 2013; Rajendra et al., 2014). RUVBL1/2 also influence transcription, play a role in assembly of the mitotic spindle and telomerase complex and (as a part of the R2TP complex) are involved in RNA polymerase II assembly, PIKK complex formation and snoRNPs biogenesis (Nano and Houry, 2013). The exact role of RUVBL1 and RUVBL2 in these processes remains to be determined. Published data suggest that their main function may be assembly or activation of the complexes in which they are contained. This theory supports the fact, that the protein level of Rvb1 and Rvb2 in yeasts is low compared to the abundance of the complexes in which they are involved and therefore only associate with these complexes transiently (Gallant, 2007; Nano and Houry, 2013).

RPAP3 and PIH1D1 are subunits specific exclusively for the R2TP complex. The yeast RPAP3 homolog Tah1 interacts with Pih1 via its C-terminal part and contains one tetratricopeptide

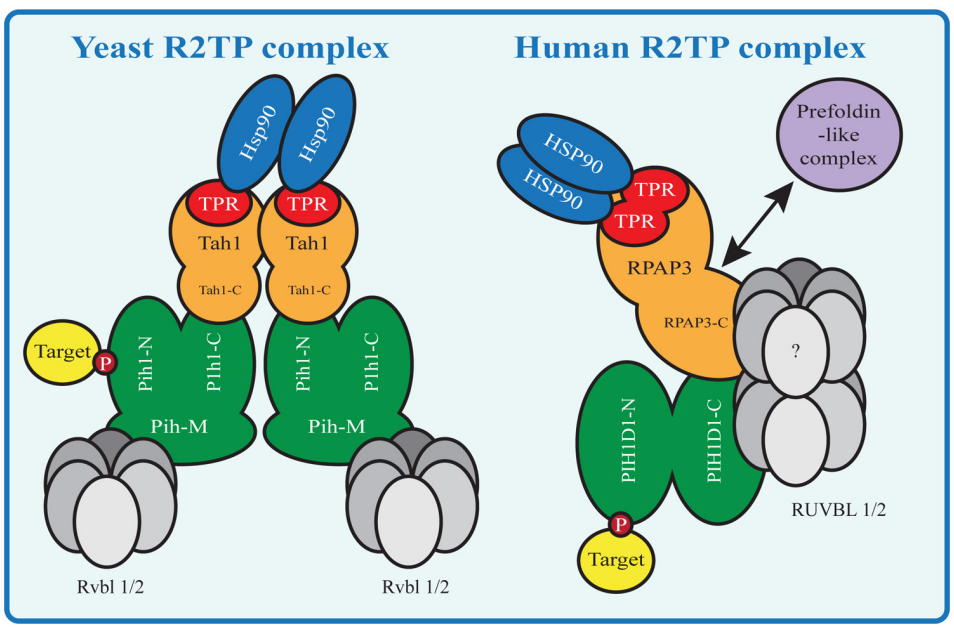

FIGURE 2 | HSP90-R2TP complex in yeast and mammals. Proteins homologous between Yeast and mammals are shown in the same color. N indicates the N-terminal domain, C the C-terminal domain and M the middle domain of a protein. Yeast Tah1 contains one TPR domain and therefore two Tah1 molecules are needed to bind the Hsp90 dimer. Tah1 binds Pih1, which in turn binds R2TP target substrates with its N-terminal domain and the Rvbl proteins with its middle domain. In contrast the mammalian R2TP complex binds the HSP90 dimer with one RPAP3 molecule containing two TPR domains. The RPAP3 C-terminal domain connects directly or indirectly with the other components of the complex, namely PIH1D1 the RUVBLs and the prefoldin-like complex components (absent in yeast R2TP).

(TPR) domain (Zhao et al., 2005; Millson et al., 2008). TPR domains are known to facilitate interactions with HSP70 or HSP90 and are implicated in other protein–protein interactions (Smith, 2004). The Tah1 TPR domain is involved in binding of Hsp90 but not of Hsp70 in stressed stationary cells and is not essential for Hsp90 recruitment to the R2TP complex (Millson et al., 2008; Zhao et al., 2008). While mutation of the Tah1 TPR domain decreased Tah1-Hsp90 interaction, it did not affect Tah1-Pih1 interaction. Tah1 forms a dimer, which binds both Hsp90 proteins present in the Hsp90 dimer, preventing its simultaneous binding to other co-chaperones (Pal et al., 2014). The drosophila RPAP3 homolog Spagh contains one TPR domain that binds both Hsp70 and Hsp90 and stimulates their activities (Benbahouche Nel et al., 2014). The presence of two TPR domains in human RPAP3 and its strong interaction with HSP90 and HSP70 indicate that RPAP3 couples the R2TP complex with HSP90 and possibly with other chaperones (Smith, 2004; Chagot et al., 2014). Crystallographic analysis of RPAP3 shows an interaction between both TPR domains and HSP90 peptides containing the conserved MEEVD sequence. Abolishing HSP90 binding requires mutation of both TPR domains, suggesting that the HSP90 dimer is bound by one RPAP3 molecule (Pal et al., 2014). RPAP3 is expressed in three isoforms: isoform 1 (and possibly isoform 3) interacts with PIH1D1 and is required for its stabilization, isoform 2 lacks an in-frame exon coding for 34 amino acids, present in the other two isoforms, and does not interact with PIH1D1, and therefore it may antagonize the R2TP complex activity. The C-terminal part of RPAP3 binds (independently on the TPR domains) a subunit of the prefoldin-like complex WDR92 and thus may mediate the interaction between R2TP and prefoldin-like complex (Itsuki et al., 2008; Back et al., 2013).

In yeast, Pih1 plays a central role in the R2TP complex by directly binding the other complex components (Kakihara and Houry, 2012). It has been proposed that Hsp90 and Tah1 stabilize the otherwise unstable Pih1 (Paci et al., 2012). Similarly to Tah1, Pih1 has Hsp90 binding properties, although the interaction is relatively weak, and the C-terminal domain of Pih1 binds the C-terminal domain of Tah1 (Zhao et al., 2008; Eckert et al., 2010; Paci et al., 2012; Back et al., 2013; Pal et al., 2014). In addition, Pih1 interacts with Rvb1 and Rvb2 by its middle domain. In mammals, RPAP3 and HSP90 also act together to stabilize PIH1D1 and PIH1D1 possibly connects the components of the R2TP complex (Zhao et al., 2008; Paci et al., 2012). While the middle domain is not present in mammalian PIH1D1, the C-terminal part ranging from amino acid 250 to 290 mediates the interaction with the rest of the R2TP complex, although it is not clear which of the R2TP complex components binds to the C-terminal part directly (Horejsi et al., 2014).

Two recently published papers identified a novel PIH1D1 phospho-peptide binding domain (PIH-N domain), distinct from any so far known phospho-binding-domains. The domain is capable of binding PIH1D1 interaction partner TEL2 independently of the rest of the complex. The PIH-N domain structures of the mouse and the human proteins are very similar. Both studies identified a basic patch within the N-terminal domain of PIH1D1, which is responsible for binding to TEL2 peptide containing a phosphorylated acidic DpSDD sequence. The crystal structure of the human PIH1D1 revealed hydrogen bonds essential for the binding between lysine 57, lysine 64, and arginine 168 of PIH1D1 (lysine 57, 64, and 133 in mouse) and the phosphorylated serine and aspartates of the DpSDD motif. Mutations of these PIH1D1 residues abolished TEL2 peptide binding (Horejsi et al., 2014; Pal et al., 2014). Lysine 57 and lysine 64 are evolutionary conserved and accordingly, mutation of lysine 64 in PIH1D1 abolished interaction with TEL2. Mass spectrometry and pull-down experiments revealed that PIH-N domain interacts directly in a phosphorylation-dependent manner with novel PIH1D1 interacting partners ECD, SNRP116, and UBR5. The mass spectrometry results also suggest that interaction between PIH1D1 and the main subunit of RNA polymerase II RPB1 is phosphorylation-dependent and that lysine 64 is essential for the interaction. Nevertheless, it is not clear whether the interaction with RPB1 is direct or mediated by another factor (Horejsi et al., 2014). These data indicate that PIH-N domain recognizes and recruits specific substrates to the R2TP during assembly of PIKKs, snoRNPs and RNA polymerase II and possibly of other complexes.

A phospho-binding domain similar to the PIH-N is probably also present in PIH1D1 ortholog Kintoun, which is involved in the cytoplasmatic assembly of dyneins, required for cilia motility (Omran et al., 2008). The positively charged lysines important for the PIH-N binding are substituted with positively charged arginines in Kintoun and it is capable of binding the phosphorylated TEL2 peptide with weak affinity (Horejsi et al., 2014; Pal et al., 2014). Since Kintoun interacts with DYX1C1, a TPR domain-containing protein involved in dynein assembly, it could function as a co-chaperone in a similar way as PIH1D1 in the R2TP complex (Tarkar et al., 2013).

ASSEMBLY OF snoRNPs

SnoRNPs are RNA-protein complexes essential for biogenesis of ribosomes, spliceosomes, and telomerase (Smith and Steitz, 1997). The two major groups of snoRNPs are the box C/D snoRNPs (involved in processing of the pre-rRNA by 2′-*O*-methylation) and the H/ACA snoRNPs (involved in pseudouridylation of the pre-rRNA; Kiss-Laszlo et al., 1996; Tycowski et al., 1996). Assembly of snoRNPs is a complicated process, which requires a number of assembly factors.

The box C/D snoRNPs consist of box C/D snoRNAs and four core protiens: Snu13, Nop1, Nop56, and Nop58. Tah1 depletion in yeast led to decreased stability of box C/D snoRNAs and this effect was apparent only in stressed cells in stationary phase, possibly because Tah1 and Hsp90 are required for Pih1 stabilization under these conditions (Zhao et al., 2008). Rvb2 and Pih1 depletion led to a temperature sensitive phenotype and a disturbance in the accumulation and localization of both box C/D and H/ACA snoRNPs (Newman et al., 2000; Boulon et al., 2008). A synthetic genetic array, a method that identifies genes involved in the same pathway or complex, implicated genetic interactions of both Pih1 and Rvbs with Nop58p (Zhao et al., 2008). Pih1 directly binds the box C/D core protein Nop58 via Nop58 C-terminus (Gonzales et al., 2005; Kakihara et al., 2014) and the interaction is stronger in the absence of RNA. Binding of ADP,

ATP, and ATPgS by Rvb1/2 (but not ATP hydrolysis) leads to dissociation of the R2TP complex itself and also releases R2TP from Nop58 C-terminal domain. Since the Walker A and Walker B motifs of Rvb1/2 are essential for yeast C/D snoRNA accumulation, the ATPase activity must play a role at a different step of the snoRNPs assembly (King et al., 2001; McKeegan et al., 2007, 2009; Boulon et al., 2008). Given that the R2TP complex interacts with the earliest stage of the snoRNP complex, which contains only the protein components, it is involved in the early phase of the box C/D snoRNP biogenesis. The mammalian RUVBL1 and RUVBL2 bind box C/D snoRNAs and all components of the R2TP complex are essential for their assembly (Newman et al., 2000; Boulon et al., 2008). Similarly to yeast Pih1, human PIH1D1 binds NOP58 and a snoRNP assembly factor NUFIP. A recent proteomic analysis revealed that RUVBL1 and RUVBL2 are components of various maturation stages of human pre-snoRNPs and are released at the final stage of snoRNP maturation. In accordance with the data from yeast experiments, PIH1D1 and RPAP3 were not detected in any stage of pre-snoRNPs, which raises the possibility that PIH1D1 and RPAP3 may in ATP dependent manner load RUVBL1/2 to the first RNA-free stage of snoRNP formation and do not take part in the latter stages of the assembly (Bizarro et al., 2014).

At least two assembly factors – Shq1 and Naf1 – are important for biogenesis of yeast H/ACA snoRNPs (Machado-Pinilla et al., 2012). Shq1 binds a component of the H/ACA snoRNP complex Nap57 and prevents the binding of Naf1 to the other snoRNP components (Yang et al., 2002; Grozdanov et al., 2009). Accordingly, the release of human SHQ1 from NAP57 is required for H/ACA snoRNP assembly. Cytosolic extracts from Hela cells were able to remove SHQ1 from NAP57 in an ATP independent manner and SHQ1 removal was inhibited by addition of antibodies directed at the R2TP component, but not by addition of HSP90 inhibitors. NAP57 directly binds PIH1D1 *in vitro* and interaction of its unstructured C-terminal part with RUVBL1/2 was essential for its disassociation from SHQ1. These experiments indicate that the R2TP complex takes action at the early stage of the H/ACA snoRNP assembly and is required for removal of inhibitors of H/ACA snoRNPs assembly from the H/ACA snoRNPs precursors. NAP57 lacks the DpSDD PIH-N domain consensus binding-motif, however, it contains phosphorylated acidic sequences that may mediate the interaction with the PIH-N domain. Since the purified R2TP complex was unable to release NAP57 from SHQ1, additional factors may be required for this reaction or for proper assembly of the R2TP complex itself (Machado-Pinilla et al., 2012).

ASSEMBLY OF RNA POLYMERASE II

Eukaryotic cells contain three different RNA polymerases: (a) RNA polymerase I produces ribosomal RNA, (b) RNA polymerase II transcribes small nuclear RNAs and messenger RNAs, and (c) RNA polymerase III produces a range of small RNAs including the transfer RNAs. RNA polymerase II consists of 12 subunits of which Rpb1 and Rpb2 form the active cleft, while the other subunits are located further in the periphery of the complex (Cramer et al., 2008).

The R2TP complex together with the prefoldin-like complex interacts with RNA polymerase II and is involved in its assembly in the cytoplasm and in the transport of the assembled polymerase to the nucleus. Quantitative mass spectrometry analysis revealed that the polymerase is assembled in several steps, which include formation of two RNA polymerase II sub-complexes. The R2TP complex preferentially interacts with unassembled RPB1 and with the sub-complex containing RPB1. Depletion of RPAP3 and inhibition of HSP90 led to destabilization of RPB1 in the cytoplasm. Interestingly, RPAP3 also binds to RNA polymerase II subunit RPB5 independently on RPB1 and is required for its incorporation within the RNA polymerase II complex. RPB5 binds to the component of the human prefoldin-like complex URI, also involved in the assembly of RNA polymerases in the cytoplasm (Miron-Garcia et al., 2013). Thus, the prefoldin-like complex may be also involved in RPB5 assembly in the RNA polymerase II complex. It is highly possible that RPB1 is recruited to the R2TP complex via direct or indirect phosphorylation-dependent interaction with the PIH-N domain, because wild type but not mutated PIH-N domain binds phosphorylated RPB1 (Horejsi et al., 2014). RPB1 does not contain the DSDD motif, recognized by the PIH-N domain, but it contains other phosphorylated acidic Casein Kinse 2 (CK2) consensus sequences, which could bind to PIH-N. Alternatively, the interaction may be mediated by another factor binding to the PIH-N domain and to RPB1.

RNA polymerase I and III are also multi-subunit complexes. Since R2TP complex interacts with several of their subunits, it is highly possible that the R2TP complex is also involved in their assembly (Jeronimo et al., 2004, 2007; Boulon et al., 2012). In addition, PIH1D1 is directly involved in mTORC1-dependent rRNA transcription by RNA polymerase I (Zhai et al., 2012).

ASSEMBLY OF PHOSPHATIDYLINOSITOL 3-KINASE-RELATED KINASES

The PIKK family consists of ATM, ATR, DNA-PKcs, mTOR, SMG1, and TRRAP. ATM, ATR, and DNA-PKcs are essential for DNA damage signaling (Zhou and Elledge, 2000; McKinnon, 2012); SMG1 regulates nonsense-mediated mRNA decay by the mRNA surveillance complex that removes mRNAs with premature stop codons (Yamashita et al., 2005); TRRAP is part of multiple acetyltransferase complexes and facilitates transcription by binding transcription factors like E2F and c-MYC (Grant et al., 1998; McMahon et al., 1998, 2000; Murr et al., 2007); and mTOR is a central player in cell metabolism and regulates processes like cell growth, autophagy, transcription, and actin organization in reaction to growth factor signaling and nutrient availability (Wullschleger et al., 2006). All PIKKs bind to HSP90 co-chaperone TEL2, which forms together with its interacting partners TTI1 and TTI2 so called TTT complex. TEL2 phosphorylated on serine 487 and 491 by CK2 binds to the PIH-N domain present in PIH1D1 and mutation of both serines disrupts its interaction with R2TP complex, but does not affect the binding of PIKKs and HSP90 (Horejsi et al., 2010). Therefore the TTT complex connects PIKKs to both HSP90 and R2TP complex independently of each other. Knock-out of TEL2 and knock-down of TTI1 ad TTI2 lead to depletion of PIKKs from cells (Horejsi et al., 2010;

Hurov et al., 2010). Interestingly, HSP90 inhibition decreases levels of ATM and DNA-PKcs but does not affect levels of mTOR and SMG1 (Takai et al., 2007), while disruption of TEL2-R2TP binding affects stability of SMG1 and mTOR (Horejsi et al., 2010), but also of ATM and DNA-PKcs (Rao et al., 2014). At the same time depletion of RUVBL1 and RUVBL2 leads to decreased PIKKs levels (possibly by affecting both transcription of genes encoding RUVBL1/2 and protein stability) and reduces PIKK signaling (Izumi et al., 2012). It is therefore possible that HSP90 alone is important for proper folding of ATM and DNA-PKcs, while in complex with R2TP it mainly affects assembly of complexes of all PIKKs. As many subunits of large complexes become unstable if the complex formation is disrupted, it is possible that the instability caused by non-functional TEL2 or R2TP complex is not due to improper folding of the kinases themselves, but due to disruption of complexes in which they are involved. This is also supported by the fact that reduced levels of RUVBL1/2 impair formation of the mRNA surveillance complex, which contains SMG1 and mTORC1 complex (Izumi et al., 2010; Kim et al., 2013).

mTOR forms two distinct complexes: (a) mTORC1 is associated with Raptor and regulates protein synthesis, (b) mTORC2 is associated with Rictor and is involved in actin organization (Kim et al., 2002; Sarbassov et al., 2004). Study in mouse and human cells showed that knockdown of TEL2, TTI1, and RUVBL1/2 mediated by siRNA reduced mTOR activity several folds and led to disruption of mTOR dimer and impediment of the mTORC1 assembly (Kim et al., 2002; Sarbassov et al., 2004; Hoffman et al., 2010). The interaction between TEL2 and mTOR was dependent on the ATPase activity of RUVBL1 and RUVBL2 and was destabilized in the absence of ATP or glucose and glutamine starvation conditions (Kim et al., 2013). At the same time, destabilization of TEL2 and mTOR interaction led to decreased mTOR levels. Interestingly, low levels of glucose and glutamine led to decreased stability of other PIKKs as well, suggesting that nutrient signaling regulates also other cellular pathways by affecting the R2TP complex stability. The study in human and mouse cells focused only on members of the TTT complex and RUVBL1/2, but since TEL2 interacts with RUVBL1/2 through direct interaction with PIH1D1, the assembly of mTORC1 is regulated by CK2 (Fernandez-Saiz et al., 2013) and PIH1D1 directly binds to mTORC1, it is highly probable that the whole R2TP complex takes part in the mTORC1 assembly. This is also supported by the fact that the R2TP complex is involved in mTORC1 regulated rRNA transcription (Zhai et al., 2012).

ATR is activated in response to presence of single-stranded DNA, which is generated during repair of wide variety of DNA damage lesions and during replication stress. Upon activation, ATR forms a heterodimer with ATRIP and is recruited to the single-stranded DNA, coated by replication protein A (Nam and Cortez, 2011). Knock-down of TEL2 by siRNA firstly leads to decreased ATR activation and inhibits binding of the ATR/ATRIP heterodimer to DNA damage mediator protein TOPBP1 and later it leads to decreased levels of ATR and ATRIP (Rendtlew Danielsen et al., 2009). It is therefore possible that TEL2 interaction with the R2TP complex is required for formation of the ATR/ATRIP/TOPBP1 complex and that ATR and ATRIP become unstable if they cannot form the active complex.

MOLECULAR MECHANISM OF R2TP FUNCTION AND ITS REGULATION

Although the work on snoRNPs assembly provides some clues about the mechanism of the R2TP complex function, most of it is still largely unknown. It seems that in mammals, PIH1D1 and RPAP3 are required at the early stages of complex formation, probably for loading of RUVBL1/2 to the assembled complex (Bizarro et al., 2014). RUVBL1/2 are required either to disassemble inhibitors of complex formation or are involved in some other steps of assembly and/or activation of the late stages of the assembled complexes (Nano and Houry, 2013). Given that RUVBL1/2 are known to be parts of many diverse cellular complexes, it is intriguing to hypothesize that the R2TP complex is involved in assembly of all these complexes.

The work on assembly of mTORC1 complex revealed that assembly of the R2TP complex itself is regulated by presence of nutrients and the complex becomes disassembled particularly in the absence of glucose and glutamine. The absence or presence of glucose and glutamine also regulates localization of the R2TP complex: in growing yeasts, the R2TP complex is localized in the nucleus and interacts with box C/D snoRNPs, while it relocalizes to the cytoplasm in poorly growing cells (Kakihara et al., 2014). These results show that nutrient signaling affects (via regulation of R2TP complex assembly and localization) various processes in the cells. Although the R2TP complex is also involved in regulation of DNA damage response, it is not known whether the DNA damage signaling affects localization or assembly of the R2TP complex.

R2TP COMPLEX AND THE DNA DAMAGE RESPONSE

In order to protect genome integrity, cells are equipped with an extensive response mechanism that comes into play after DNA damage. Proper function of the DNA damage response pathways is essential for cancer avoidance (Bartkova et al., 2005).

The general mechanism of the DNA damage response consists of sensors, transducers, and effectors. The sensors detect the damaged DNA and activate PIKKs ATM, ATR and DNA-PKcs, which transmit the signal to effector proteins (Zhou and Elledge, 2000). Depending on the amount of the damage, the effector proteins (including transcription factor p53) arrest the cell cycle by activating the checkpoints and either repair the damaged DNA or (in case of too extensive damage) activate pathways leading to cellular senescence or apoptosis (Banin et al., 1998; Canman et al., 1998; Zhou and Elledge, 2000; Vousden and Lu, 2002).

The R2TP complex regulates the DNA damage response by affecting stability of all PIKKs involved in the DNA damage response and by regulation of ATR activity. In addition, mutation of the phospho-binding domain PIH-N caused decreased p53 activation following induction of DNA double strand breaks even in the presence of normal levels of ATM and DNA-PKcs, the two PIKKs responding to this type of DNA damage, suggesting that the R2TP complex regulates activity of ATM and DNA-PKcs or of other components of the DNA damage pathways (Horejsi et al., 2010). One of such components might be ECD, a protein

that directly interacts with p53 and promotes its stabilization by inhibiting its binding to MDM2 (Zhang et al., 2006). ECD contains two DSDD motifs, which are both required for its direct phosphorylation-dependent interaction with the R2TP complex. Hypothetically, the R2TP complex may either guide the interaction between ECD and p53 or may be involved in the MDM2 disassociation from p53. The latter possibility is supported by the fact that HSP90 directly interacts with p53 and regulates its stability (Sasaki et al., 2007).

Another PIH1D1 phospho-binding partner involved in DNA damage is an E3 ubiquitin ligase UBR5, which regulates transduction of the DNA damage signaling to the effectors (Zhou and Elledge, 2000; Gudjonsson et al., 2012). The decreased p53 activity in the presence of mutated PIH1D1 could be due to disruption of the interaction between UBR5 and PIH1D1, although the mechanism is unclear.

The chromatin remodeling complexes TIP60, INO80, and SWI/SCRAP that contain RUVBL1/2 have been all implicated in regulation transcription, replication, recombination, and repair of DNA damage (Murr et al., 2006). The TIP60 complex possesses an acetyl-transferase activity, which is essential for accumulation of repair proteins at the site of the damaged DNA, activation of ATM (100) and removal of H2AX from the chromatin (Sun et al., 2005; Ikura et al., 2007). In addition, acetylation of p53 by TIP60 directs the p53 response toward induction of apoptosis. RUVBL1/2 are required for acetylation of at least some of the TIP60 targets (Ikura et al., 2007; Jha and Dutta, 2009).

INO80 is involved in repair of DNA double strand breaks by sliding nucleosomes along the DNA and their eviction at the site of the damage. Yeast Rvb1/2 bind to Ino80 subunit Arp5 in an ATP but not ATPase dependent manner and their loss leads to disassociation of Arp5 from the complex and to the loss of chromatin remodeling activity of the complex (Shen et al., 2003; Osakabe et al., 2014).

The yeast SWI complex, known as SRCAP complex in mammalian cells, remodels chromatin by catalyzing replacement of histone dimers H2A-H2B in nucleosomes by dimers containing the histone variant Htz1 in yeast or H2AZ in mammals. This

Table 2 | Confirmed and potential targets of the R2TP complex.

	Function		Direct interaction	
R2TP complex target	**Target complex**	**R2TP complex**	**R2TP component**	**Target complex component**
Confirmed				
RNA polymerase II	Transcription	Assembly	?	?
snoRNPs	Biogenesis of ribosomes, spliceosomes, and telomerase			
boxC/D snoRNPs	2′-*O*-methylation of preRNA	Assembly	PIH1D1?	NOP58?
H/ACA snoRNPs	Pseudouridylation of prerRNA	Assembly	PIH1D1?	NAP57?
SMG1	Nonsense mediated mRNA decay	Assembly	PIH1D1	TEL2
mTORC1	Central regulator of cell metabolism	Assembly	PIH1D1	TEL2
ATM/ATR/DNA-PK	Kinases involved in DNA damage signaling	Possibly assembly	PIH1D1	TEL2
Potential				
ECD/p53	Transcription factor involved in DNA damage response	possibly assembly	PIH1D1	ECD
UBR5	E3 ubiquitin ligase involved in DNA damage signaling	?	PIH1D1	UBR5
INO80	Chromatin remodeling complex, involved in repair of DNA double strand breaks	Possibly loading RUVBL1/2	?	?, RUVBL1/2
TIP 60	Chromatin remodeling complex, acetyl-transferase activity important for DNA damage response	Possibly loading RUVBL1/2	?	?, RUVBL1/2
SWI/SCRAP	Chromatin remodeling complex, DNA damage signaling	Possibly loading RUVBL1/2	?	?, RUVBL1/2
Fanconi anemia core complex	Recognition and repair of DNA crosslinks	Possibly loading RUVBL1/2	?	?, RUVBL1/2

process is essential for DNA damage signaling, although the role of RUVBL1/2 in it is not known (Niimi et al., 2012).

Very recently, it has been reported that RUVBL1/2 are associated with Fanconi anemia core complex. This complex is essential for recognition and repair of DNA cross-links. Knockdown of RUVBL1/2 shows similar phenotype as depletion of the Fanconi anemia core complex proteins and conditionally knockout cells depleted from RUVBL1/2 have highly reduced levels of the Fanconi anemia core complex proteins (Rajendra et al., 2014).

The role of RUVBL1/2 in all the mentioned complexes is unknown, but in light of the recent findings about snoRNPs assembly and about the essential role of RUVBL1/2 in incorporation of Arp5 into the Ino80 complex, it is highly probable that RUVBL1/2 are necessary for assembly and activation of these complexes. It would be extremely interesting to know whether PIH1D1 and RPAP3 are involved in the early stages of their assembly by loading RUVBL1/2 onto these complexes.

R2TP COMPLEX IN CANCER

Components of the R2TP complex are over-expressed in cancer: PIH1D1 levels are increased in several breast cancer cell lines (Kamano et al., 2013), RUVBL1/2 are over-expressed in liver and colon cancers (Huber et al., 2008; Jha and Dutta, 2009; Grigoletto et al., 2011) and their higher expression in cancer tissues is positively correlated with the expression of genes involved in metabolic processes activated by mTOR signaling (Kim et al., 2013). HSP90 levels are high in various cancer cell lines (Calderwood et al., 2006) and its activity is essential for variety of processes in cancer cells. It also plays a key role in the conformational maturation of oncogenic signaling proteins such as HER-2/Erb2, Akt, Raf-1, Bcr-Abl, and mutated p53. Multiple drugs that influence the substrates of R2TP or HSP90 chaperone are currently used or tested for the treatment of cancer. HSP90 became an attractive antineoplastic drug target, currently with 17 agents in different stages of clinical trials (Neckers and Workman, 2012) and inhibition of HSP90 reveals significant treatment effects *in vitro* in different types of cancers of unmet need such as the glioblastoma, lung, and pancreas cancer (Mayer et al., 2012; Choi et al., 2014; Pillai and Ramalingam, 2014; Wachsberger et al., 2014). Inhibition or depletion of HSP90 co-chaperones such as p23, Cdc37, and Aha1 further sensitizes cells to HSP90-targeted drugs (Neckers and Workman, 2012). Increased mTOR activity can promote tumor growth and mTOR inhibitors are already used in the clinic (Fruman and Rommel, 2014). CK2 is essential for cellular viability and progression of the cell cycle. It is required for tumorigenesis, but the exact mechanism is not clear (Trembley et al., 2010). Sites phosphorylated by CK2 form protein–protein interaction motifs, critical for regulation of DNA damage response pathways (Iles et al., 2007; Chapman and Jackson, 2008; Horejsi et al., 2010; Yata et al., 2012; Guerra et al., 2014; Horejsi et al., 2014) and for recognition of specific substrate by the R2TP complex. The relevance of CK2 as a molecular target in cancer has led to the development of CK2 inhibitors for clinical use, which are potent, highly specific and

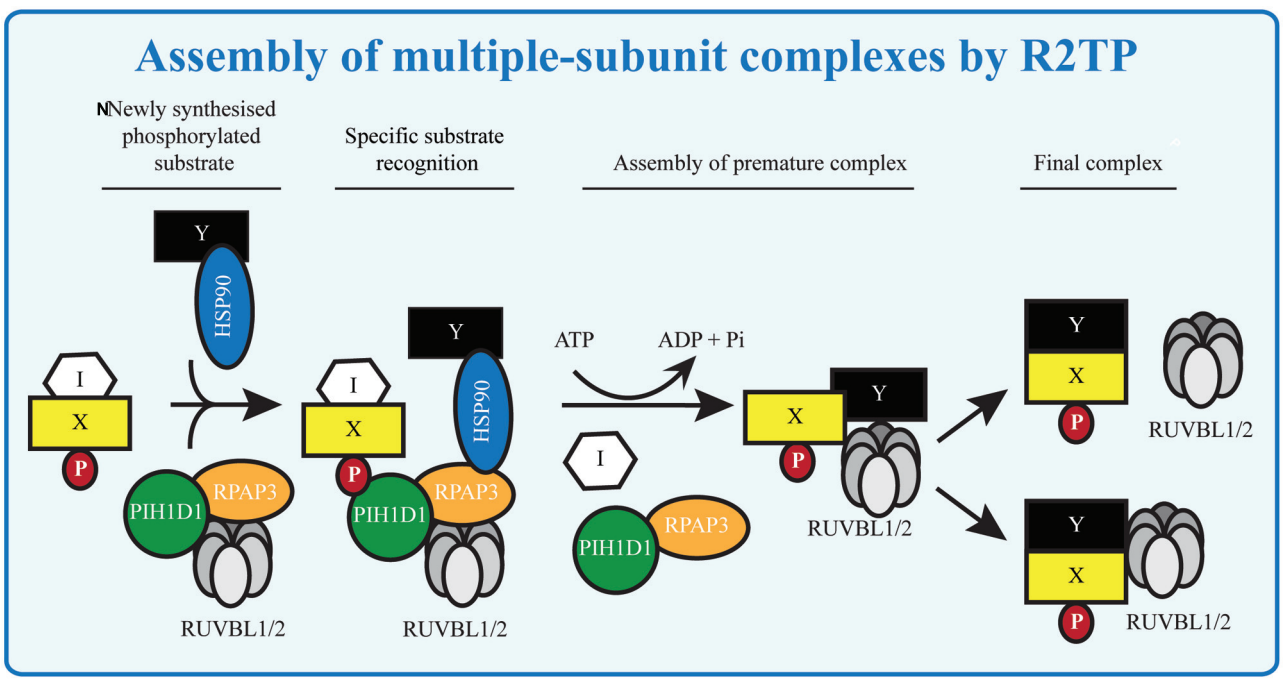

FIGURE 3 | Assembly of multiple-subunit complexes by R2TP. The multiple-subunit complexes in mammalian cells are assembled in several steps: the early phase requires presence of R2TP complex, which may serve to remove assembly inhibitors (I) and/or to load RUVBL1/2 to the premature complexes in ATP dependent manner. RUVBL1/2 helps in the latter stage of assembly forming the mature complexes and either disassociate from or become part of the mature complexes. The substrate (X, Y) specificity is given by presence of phosphorylated sequence (P), recognized by PIH-N domain and possibly by specific interactions with other components of the R2TP complex.

orally available, with known antitumor efficacy in breast, pancreatic and prostate xenograft mouse models (Cozza et al., 2013; Gyenis et al., 2013).

The R2TP complex specifically recognizes sites phosphorylated by CK2 and works in conjunction with HSP90 to assemble multisubunit complexes involved in many cellular processes highly relevant to cancer, which makes it a promising target for new cancer drugs. Potential targets include the ATPase activity of RUVBL1 or RUVBL2 (Elkaim et al., 2012; Grigoletto et al., 2013) and PIH-N domain binding – inhibitors that block the PIH-N phospho-binding by mimicking the DpSDD motif should prevent function of the R2TP. The R2TP complex inhibitors could be of value in a clinical setting either to enhance HSP90, CK2, ATM and ATR inhibitors, to sensitize cells to cancer treatment based on induction of DNA damage (radiotherapy and some types of chemotherapy) or as an alternative to HSP90 inhibitors.

FUTURE DIRECTIONS

The R2TP complex is implicated in the assembly of multiple cellular complexes (**Table 2**). The mechanisms by which the R2TP complex recognizes its substrates and exerts its function are still not completely understood. Strikingly, the experiments with snoRNPs assembly indicate that disruption of protein interactions and loading of RUVBL1/2 onto the premature complexes may be a general R2TP function (**Figure 3**). Indeed, NOP56 and NOP58, components of boxC/D snoRNPs, have homology to the unstructured tail of H/ACA snoRNP component NAP57, important for NAP57 disassociation from SHQ1 (Machado-Pinilla et al., 2012). The identification of the PIH-N domain helps us to understand the principle of recognition of R2TP substrate and could assist in the search for novel R2TP complex substrates. As the interaction between PIH-N and its substrate is phosphorylation-specific and the main kinase involved in the substrate phosphorylation seems to be CK2 (Horejsi et al., 2010, 2014), better understanding of its activation in response to different stimuli and/or identification of other kinases phosphorylating the substrate will lead to better understanding of regulation of R2TP function. Indeed, although CK2 has been regarded as a constitutive kinase not subject to regulation, recently published data show that binding of 5-diphosphoinositol pentakisphosphate (IP7) to CK2 enhances its phosphorylation of TEL2 and increases stability of DNA-PKcs and ATM after DNA damage (Rao et al., 2014).

One of the main functions of the R2TP complex could be regulation of cellular energy balance. The assembly of RNA polymerase II, mTORC1, ribosomes and spliceosomes (through the snoRNPS) is dependent on the R2TP complex. The presence of ATP increases the presence of higher order RUVBL complexes (McKeegan et al., 2009) and stimulates R2TP mediated assembly of mTOR and rRNA transcription (Kim et al., 2013). Therefore R2TP may work as a metabolic switch or master regulator by simultaneous influencing mTOR activity, protein synthesis and other cellular processes such as transcription and response to DNA damage.

Most of R2TPs functional mechanisms still remain elusive: how does R2TP assert its function on its substrates? What is the role of the prefoldin/like complexes associated with the R2TP complex? Are the differences in the structures reported for the RUVBL hexameres relevant for its function? What is the role of HSP90 in the R2TP complex? Is the PIH-N domain always involved in R2TP substrate recognition? Is PIH-N domain involved in regulating assembly processes? Is the R2TP complex generally involved in assembly of complexes containing RUVBL1/2? Answering these questions will allow us to start understanding of the molecular mechanisms of the function of this highly important complex. Also, more studies are required to evaluate the attractive possibility that the R2TP inhibitors are relevant in cancer treatment.

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Bystander communication and cell cycle decisions after DNA damage

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The DNA damage response (DDR) has two main goals, to repair the damaged DNA and to communicate the presence of damaged DNA. This communication allows the adaptation of cellular behavior to minimize the risk associated with DNA damage. In particular, cell cycle progression must be adapted after a DNA-damaging insult, and cells either pause or terminally exit the cell cycle during a DDR. As cells can accumulate mutations after a DDR due to error-prone DNA repair, terminal cell cycle exit may prevent malignant transformation. The tumor suppressor p53 plays a key role in promoting terminal cell cycle exit. Interestingly, p53 has been implicated in communication of a stress response to surrounding cells, known as the bystander response. Recently, surrounding cells have also been shown to affect the damaged cell, suggesting the presence of intercellular feedback loops. How such feedback may affect terminal cell cycle exit remains unclear, but its presence calls for caution in evaluating cellular outcome without controlling the cellular surrounding. In addition, such feedback may contribute to how the cellular environment affects malignant transformation after DNA damage.

Keywords: bystander effect, p53, cell cycle, senescence, DNA damage, cell cycle exit, G2

INTRODUCTION

Changes in the genome can be a potential threat to the cell and to organism survival. However, the genome is continuously exposed to a variety of genotoxic stresses. These are endogenous insults such as the production of reactive oxygen species (ROS) or various metabolite byproducts, or exogenous insults such as UV radiation, heavy metals, air pollutants, bacterial toxins, and inflammatory responses. All of these agents cause structural damage and can hinder or abolish cellular processes as transcription or DNA replication. Of the various DNA lesions, DNA double strand breaks (DSBs) are considered most deleterious, because if unrepaired they can lead to chromosomal aberrations such as deletions, translocations, and amplifications. These chromosomal aberrations may result in deregulation of gene expression and altered cellular function, which may eventually cause cell death or tumor initiation and progression (Lord and Ashworth, 2012).

To minimize the risk to genome integrity, cells have evolved the DNA damage response (DDR)—a highly regulated signaling network that responds to the presence of DNA lesions (Bartek and Lukas, 2007; Jackson and Bartek, 2009). A prime function of the DDR is to ensure that lesions in DNA are recognized and repaired. Simultaneously, the repair needs to be coordinated with other cellular processes, in particular cell cycle progression. Therefore, the DDR can be divided into two major pathways, one that assembles and repairs the lesions and one that amplifies and conveys the signal away from the break site to modify cellular behavior. In all eukaryotes these two processes are initiated by sensor proteins such as the Mre11-Rad50-Nbs1 (MRN) complex or the Ku70/Ku80 dimer, that detect the presence of DSBs. The binding of sensor proteins to damaged DNA recruits the phosphoinositide 3-kinase related kinases ATM, ATR, or DNA-PK leading to activation of these kinases (Falck et al., 2005). Once activated these kinases initiate cascades that enforce local and global rearrangement of chromatin, involving recruitment of multiple proteins and posttranslational modifications as phosphorylation, ubiquitylation, sumoylation, and methylation (Lukas et al., 2011). For example, phosphorylation of histone 2A variant (H2AX) at C-termini near a break site by ATM serves as a platform for the protein MDC1, who in turn can function as a recruitment platform for the ubiquitin ligase RNF8. RNF8-mediated ubiquitylation recruits RNF168, whose ubiquitylation of chromatin proteins attract BRCA1 and 53BP1, proteins that affect how the DNA break will be repaired (Kolas et al., 2007; Bekker-Jensen and Mailand, 2011).

DSB REPAIR IS NOT ALWAYS PERFECT

A majority of DSBs are repaired by three pathways—homologous recombination (HR), non-homologous end joining (NHEJ), and microhomology-mediated end joining (MMEJ). NHEJ is a fast repair process using template independent ligation of two ends of DNA and is functional throughout the cell cycle; in contrast HR is a slow repair process that depends upon the use of a sister chromatid as template and is functional only in late S- and G2 phase of the cell cycle (Chapman et al., 2012). Whereas NHEJ only requires minor modifications of the DNA to allow for ligation, HR requires resection to create stretches of single-stranded DNA that can be used for base-pairing with the sister chromatid (Hartlerode and Scully, 2009). The amount of resection at a

DSB is influenced by proteins as BRCA1 and 53BP1, and is considered as a determinant for which repair mechanism will be utilized (Chapman et al., 2012). Importantly, during NHEJ the DNA ends are frequently modified to allow efficient ligation, resulting in a change in the genetic information (Lieber, 2010). Similarly, MMEJ, an alternative version of end-joining contains even larger modifications of DNA ends, giving rise to deletions (Decottignies, 2013). In contrast, due to the use of a template for sequence information, HR is largely considered as an error-free repair process, although its accuracy is debated. Two different processes are described to support that HR may be an error-prone process—first the unequal sister chromatid exchange (SCE) which has been observed in highly repetitive sequences, and second the involvement of translesion synthesis polymerases in synthesizing the DNA (Guirouilh-Barbat et al., 2014). Thus, cells that have repaired DSBs are likely to contain changes in the genetic information.

CHECKPOINT MAINTENANCE IS NOT ALWAYS PERFECT

The repair of damaged DNA needs to be coordinated with various other cellular processes, in particular cell cycle progression. Therefore, in addition to stimulating repair, the DDR enforces a cell-cycle arrest, referred to as a DNA damage checkpoint. At the heart of the checkpoint are the ATM and ATR kinases, which initiate a signaling cascade by phosphorylating the effector kinases Chk2 and Chk1. Chk1/Chk2 in return phosphorylate cell cycle regulators as Cdc25 phosphatases. Phosphorylation of Cdc25s leads to their functional inactivation and subsequent inhibition of Cdk activity, causing rapid inhibition of cell cycle progression (Peng et al., 1997; Mailand et al., 2000; Karlsson-Rosenthal and Millar, 2006). In addition, inhibition of indirect regulators of Cdk activity as Plk1 and Aurora A support a rapid cell cycle arrest (Smits et al., 2000; Krystyniak et al., 2006). Checkpoint signaling also maintains the arrest by stabilizing p53 that transcriptionally regulates a large number of genes involved in DDR and other stress pathways (Allen et al., 2014). In addition, p38-dependent pathways contribute to regulate protein expression to maintain a checkpoint over time (Reinhardt et al., 2010). However, although the DDR is a very tightly regulated process, evidence of H2AX phosphorylation, chromosomal rearrangements and breakage during the transition from G2 to mitosis suggest that checkpoint signaling is not always stringent (Syljuasen et al., 2006; Deckbar et al., 2007; Lobrich and Jeggo, 2007). Thus, cells that have initiated a DDR and a checkpoint arrest may resume proliferation before all damaged DNA is repaired.

CELL CYCLE EXIT

As an alternative to a temporal cell cycle arrest, cells may permanently leave the cell cycle and become senescent. The duration from infliction of DNA damage to cell cycle exit depends directly on the cell cycle state. Whereas an untransformed G2 cell exits the cell cycle if damage is not repaired within a couple of hours, an S-phase cell first finishes DNA replication and only leaves the cell cycle in G2 (Baus et al., 2003; Krenning et al., 2014; Müllers et al., 2014). Cell cycle exit in G2 phase depends on activation of the ubiquitin ligase APC/C-Cdh1, which efficiently targets a large amount of cell cycle regulators for proteasome-mediated degradation (Wiebusch and Hagemeier, 2010). How APC/C-Cdh1 is activated after DNA damage remains unclear, but the process depends on expression of p53 and its transcriptional target p21, and at least in the case of Cyclin B1, nuclear translocation of the protein to be degraded (Wiebusch and Hagemeier, 2010; Johmura et al., 2014; Krenning et al., 2014; Müllers et al., 2014). Thus, the regulation of p53 is a key determinant for whether cell cycle exit or resumed proliferation occurs after initiation of a DDR.

p53 AND CELL FATE

The level and activity of p53 is upregulated in response to various stresses and has been shown to play a role in different pathways including DDR, hypoxia, apoptosis, metabolism and senescence (Gonfloni et al., 2014; Pflaum et al., 2014). Functioning as a complex signaling node, the p53 protein contains a large amount of post-translational modifications, which together with differential affinity for transcriptional elements and expression of regulatory proteins impact on cell fate decisions (Kruse and Gu, 2009; Carvajal and Manfredi, 2013). Interestingly, although p53 levels are similarly induced, different stimuli can elicit different responses on p53-transcription targets such as p21 (Espinosa et al., 2003; Donner et al., 2007), highlighting that p53 function may be modulated by the integration of a wide variety of signaling pathways (Sullivan et al., 2012). One factor that can affect p53 function is its temporal dynamics in cells. Rather than accumulating at a certain level, cellular p53 can oscillate after induction of DSBs (Lahav et al., 2004). In contrast to sustained p53 induction that stimulates cell cycle exit, the oscillatory pulses of p53 favor eventual resumption of proliferation after damage (Purvis et al., 2012). However, exactly how integration of signals determines p53 behavior remains unclear, in particular in the context of a population of cells.

THE BYSTANDER RESPONSE

During the past few decades the DDR pathway has been studied extensively in cells that have experienced damage directly. However, cells experiencing a DDR can communicate this to surrounding cells (Klammer et al., 2013). The first evidence of propagation of the DDR came from experiments performed in Chinese hamster ovary cell lines, in which 1% of nuclei hit by α-particles resulted in more than 30% of the cell population showing increased incidence of SCE (Nagasawa and Little, 1992). Supported by other observations, this phenomenon was later termed the radiation-induced bystander effect (RIBE), which is defined as physiological changes in unirradiated cells manifested by cells exposed to radiation (Sokolov et al., 2005, 2007; Klammer et al., 2013). Apart from SCE, various biological consequences of RIBE have been observed in different studies such as genomic instability, micronuclei formation, apoptosis, micro RNA (miRNA) regulation, and differentiation (Lorimore et al., 1998; Belyakov et al., 2002; Kovalchuk et al., 2010; Vinnikov et al., 2012). A common feature of RIBE seems to be induction of DNA damage. Indeed, Ku70, Ku80, or DNA-PKcs knockout bystander cells that are repair deficient are sensitive to the induction of mutations and chromosomal aberrations (Little et al., 2003; Nagasawa et al., 2003). However, the number of DSBs generated in directly irradiated and bystander cells differ, and

point mutations are predominant in bystander cells as compared to partial or total gene deletion in directly irradiated cells (Little et al., 1997; Huo et al., 2001; Sedelnikova et al., 2007). Mechanistically, deregulation of redox homeostasis may be a major cause of DNA damage in bystander cells (Azzam et al., 2002; Sokolov et al., 2007). Indeed, addition of Vitamin C or E to cell culture reduces the frequency of micronuclei formation, suggesting that ROS contributes to DNA damage formation (Narayanan et al., 1997; Konopacka and Rzeszowska-Wolny, 2006). The occurrence of DSBs in bystander cells is more frequent during DNA replication or active transcription, indicating that energy-dependent processes may underlie some of the damage (Burdak-Rothkamm et al., 2007; Dickey et al., 2012). In addition, these processes involve opening up double-stranded DNA, suggesting a mechanism for how ROS-induced single-stranded breaks can be transformed to DSBs, and indicating that the bystander effect may be particularly efficient during late cell cycle stages where replication and transcription is high.

The bystander effects appear to be cell and genotype specific and also depend upon the type of radiation (Baskar, 2010). Most of the RIBE studies have been performed in cell and tissue culture models where non-irradiated cells were co-cultured with either irradiated cells or with the conditioned medium from irradiated cells. Using mice models, Koturbash et al. (2008) showed that the bystander effect occurs *in vivo* as cranial irradiation led to DNA damage in protected spleen tissues. The RIBE also led to a profound epigenetic change in different bystander parts of the animal and, interestingly, the bystander response could differ between male and female (Besplug et al., 2005; Koturbash et al., 2006, 2007).

The above observations suggest that paracrine or endocrine signaling molecules from irradiated cells are responsible for the bystander effect. However, in addition to secretion of extracellular factors, transmission through gap junctions has also been implicated in RIBE, suggesting that multiple factors may propagate a bystander effect (Azzam et al., 2001; Hubackova et al., 2012; Klammer et al., 2013; He et al., 2014). Some of the factors implicated in transmitting the bystander response are interleukins, transforming growth factor beta (TGFβ), and nitric oxide (NO) (Iyer et al., 2000; Shao et al., 2002; Dieriks et al., 2010). As a consequence of RIBE, a DNA damage-response pathway is initiated in bystander cells. Apart from the p53 pathway, the DDR also initiates stress signaling through JNK and p38 MAPK signaling cascades including NF-kB, a major regulator of cell survival, inflammation, autophagy, and differentiation (Azzam et al., 1998; Piret et al., 1999). Activation of such a signaling network reprograms a cell to react to external danger and may coordinate a response in a complex tissue environment.

RECIPROCAL BYSTANDER EFFECT

Proper tissue homeostasis is dependent on bidirectional rather than unidirectional communication between cells. It is therefore reasonable to expect that an exchange of signaling molecules between non-irradiated and irradiated cells occurs (Goldberg and Lehnert, 2002; Chen et al., 2011; Widel et al., 2012; He et al., 2014). Indeed, the first observation of bidirectional communication between cells was seen by Mackonis et al. (2007), who reported an increased rate of survival of cells receiving a high radiation dose when their nearby cells received a low radiation dose. This interesting observation was termed a type III effect. Later on Chen et al. (2011) showed that there is a decrease in micronuclei formation and apoptosis in irradiated cells when co-cultured with non-irradiated cells. However, although the mechanisms of a reciprocal bystander effect are not yet clear, recently He et al. (2014) used co-culture of irradiated macrophages and non-irradiated hepatocytes to postulate that cAMP released from bystander hepatocytes could lead to a decreased micronuclei formation in irradiated macrophages. These studies suggest that reciprocal communication is important to react to external damage in an efficient and flexible manner. Interestingly, incorporation of both bystander and reciprocal bystander responses suggests the presence of intercellular feedback loops that may augment responses in both damaged and non-damaged cells.

p53 IN THE BYSTANDER RESPONSE

One of the promising candidates that can function as a connecting link between intrinsic and extrinsic signals is the p53 protein. Apart from cell autonomous responses, such as activation in response to DSBs in bystander cells, p53 also plays a role in transmission of the bystander response (Lorimore et al., 2013). In particular, cytochrome C release from damaged cells has been shown to be involved in RIBE in a p53-dependent manner, suggesting that p53 can both transmit and respond to RIBE (He et al., 2011). The oscillatory behavior of p53 over time has attracted the attention of modeling efforts to predict the potential outcome on cell fate (Lev Bar-Or et al., 2000; Geva-Zatorsky et al., 2006; Wee et al., 2009). A recent study based on mathematical modeling proposed that cytochrome C could couple the p53 oscillatory behavior in damaged and non-damaged cells to enhance the robustness and sustainability of p53 pulses (Kim and Jackson, 2013). Although this model needs further validation in an experimental setup, a reciprocal bystander effect imposed by cytochrome C on p53 pulses may impact on cell fate decisions, as p53 oscillations favor resumed proliferation rather than cell cycle exit (Purvis et al., 2012).

CONCLUSION

As both DNA repair and cell cycle checkpoint maintenance is not perfect, the occurrence of DNA damage to a cell constitutes a risk for establishment and propagation of genomic changes. By forcing a cell to permanently withdraw from the cell cycle, the risk associated with such changes can be reduced. Indeed, a permanent cell cycle exit is suggested to function as a tumor barrier after oncogene-induced DNA damage in S phase (Bartkova et al., 2006; Di Micco et al., 2006), a phase that may be particularly susceptible for RIBE (Burdak-Rothkamm et al., 2007). However, the determinants for when cells exit the cell cycle are not clear. Interestingly, p53, the key regulator of cell cycle exit may both modulate and respond to bystander communication. This opens up for the possibility that feedback within a population impacts on whether cell cycle exit occurs (**Figure 1**).

The original definition of a checkpoint is a mechanism that is checking to see that the prerequisites (for a process as cell cycle progression) have been properly satisfied (Hartwell and Weinert,

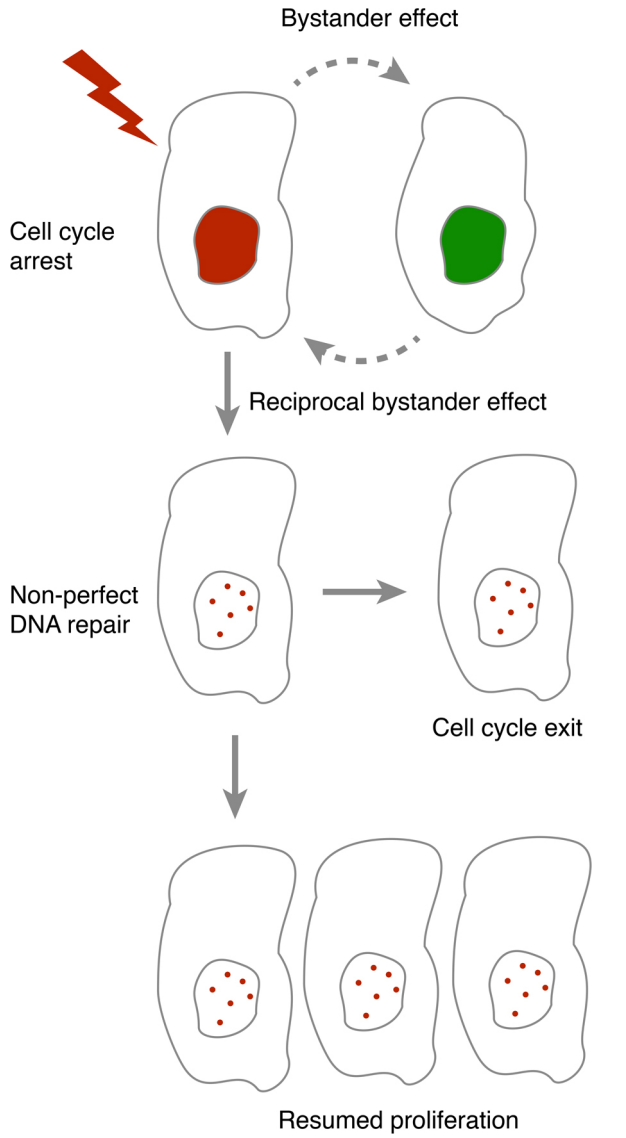

FIGURE 1 | In the presence of DNA damage, cells either pause or terminally exit the cell cycle. As DNA repair and cell cycle checkpoint maintenance are not always accurate, resumption of proliferation after a cell cycle pause may lead to propagation of mutations. Bystander communication forms an intercellular feedback that may contribute to whether resumption of proliferation may occur.

1989). A growing body of evidence suggests that upon damage a cell changes its microenvironment and spreads a signal to neighboring cells to communicate that damage is inflicted. Whether the spread of a signal from a damaged cell is a call for help or a warning is still not clear. The spread is likely to contribute to an effective population response and to assist to eliminate severely damaged cells. However, how cell intrinsic and cell extrinsic pathways interact to determine the fate of a damaged cell remains unclear. Nonetheless, the existence of cell–cell communication affecting DDR pathways calls for caution in evaluating experiments without controlling the local environment, as factors as cell confluence may impact on experimental outcome.

OUTLOOK

The bystander response, as a cause of genome instability, is implicated in induction of mutations leading to secondary cancers (Coates et al., 2008; Lorimore et al., 2008; Mancuso et al., 2008). In contrast to partial or total gene deletion in directly irradiated cells, bystander cells show primarily point mutations (Huo et al., 2001). Thus, surrounding cells may receive a more subtle genomic change that may promote survival. Early tumor development is accompanied by DNA damage also in the absence of treatment, where activation of a DDR can precede p53 mutations and defects in DNA damage signaling (Bartkova et al., 2005; Gorgoulis et al., 2005). Whether a bystander effect may contribute to increase malignant transformation during tumorigenesis remains to be studied. However, it is possible that a group of early tumor cells may not only collectively enhance the amount of DNA damage per cell, but may also impact on whether proliferation will be resumed. Due to the non-perfect DNA repair and checkpoint maintenance, such resumed proliferation may increase the risk for malignant transformation.

p53 and its associated pathways are altered in more than half of all human cancers, likely reflecting the importance of p53 for cellular fate. It is tempting to speculate that alteration in the p53 pathway can give flexibility to a cell to respond to different extrinsic signals and to better adapt to the environment. Understanding how the bystander effect couples to cell fate decision may impact on risk assessment and indicate novel targets to increase the efficiency of chemo- and radiation therapy.

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DNA damage response and evasion from immunosurveillance in CLL: new options for NK cell-based immunotherapies

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Chronic lymphocytic leukemia (CLL) is the most prominent B cell malignancy among adults in the Western world and characterized by a clonal expansion of B cells. The patients suffer from severe immune defects resulting in increased susceptibility to infections and failure to generate an antitumor immune response. Defects in both, DNA damage response (DDR) pathway and crosstalk with the tissue microenvironment have been reported to play a crucial role for the survival of CLL cells, therapy resistance and impaired immune response. To this end, major advances over the past years have highlighted several T cell immune evasion mechanisms in CLL. Here, we discuss the consequences of an impaired DDR pathway for detection and elimination of CLL cells by natural killer (NK) cells. NK cells are considered to be a major component of the immunosurveillance in leukemia but NK cell activity is impaired in CLL. Restoration of NK cell activity using immunoligands and immunoconstructs in combination with the conventional chemotherapy may provide a future perspective for CLL treatment.

Keywords: chronic lymphocytic leukemia, DNA damage response, natural killer cell, immunotherapy, immunoligands, immunoconstructs

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is an indolent lymphoproliferative disorder characterized by the progressive accumulation of monoclonal $CD5^+$ B cells in the peripheral blood, bone marrow and secondary lymphoid tissues (Ghia and Hallek, 2014; Yair et al., 2014). Another typical feature of CLL is extraordinary high frequency of chromosomal aberrations often associated with the DNA damage response (DDR) pathway and dysregulation of the cell cycle (Landau et al., 2013). Recent data suggest that defects of the DDR pathway and interaction with bystander cells of the microenvironment are pivotal factors for CLL progression. Prolonged DNA damage and defective repair results in the release of DNA-HMGB1 complexes from necrotic cells and the induction of inflammatory response that can be hijacked by CLL cells (Jia et al., 2014).

The standard therapy of CLL which includes combined chemotherapy and/or immunotherapy is highly efficient for the depletion of CLL cells from the peripheral blood but not from lymphoid tissue and bone marrow. Moreover, this might result in the selection of resistant clones (Landau et al., 2013). Haploidentical stem cell transplantation (HSCT) is an alternative treatment but restricted to a limited group of patients due to lack of suitable donors and may cause fatal side effects. New successful therapeutic strategies include modulation of the microenvironment and activation of the patient immune system to combat cancer cells (Burger and Gribben, 2014; Yair et al., 2014).

Direct targeting of tumor-associated antigens (TAA) on malignant cells by monoclonal antibodies (mAb) is regarded as a promising approach (Simpson and Caballero, 2014). Thus, antibodies against CD20 (rituximab, Obinutuzumab), CD19 (GBR 401), CD23 (lumiliximab), or CD52 (alemtuzumab) are currently evaluated (Robak, 2013). More recently, immunotherapies with genetically engineered chimeric T-cell receptors (CARs), which detect TAA, were developed (Burger and Gribben, 2014; Yair et al., 2014). So far, T cells with specificity for the common B cell antigens CD19, CD20, and CD23 were generated (Riches and Gribben, 2013). Initial clinical trials revealed feasibility, and increasingly also impressive antitumor effects.

There is emerging evidence that natural killer (NK) cells also play a pivotal role in the immunosurveillance of CLL (Reiners et al., 2013; Huergo-Zapico et al., 2014). Understanding of the molecular mechanisms of evasion from NK cell-mediated immune responses and recovery of their function will help to develop novel treatment strategies. In this review we focus on the role of DDR defects and the immune microenvironment in the evasion from NK cell responses in CLL, as far as on restoration of NK cell function using immunoligands and immunoconstructs.

THE ROLE OF DDR DEFECTS IN CLL DEVELOPMENT: IMPACT ON ESCAPE FROM NK CELL IMMUNE RESPONSE

Recent studies have identified 20 candidate CLL driver genes associated with core signaling pathways, including DNA repair, cell cycle control, Notch signaling, inflammatory pathways, Wnt

signaling, RNA splicing, and RNA processing (Landau et al., 2013), among which mutations associated with DNA repair and cell cycle regulation are most recurrent.

The physiological function of the DDR pathway is to detect DNA damage, to signal its presence and to mediate DNA repair. The proximal DDR constitutes of two major kinase branches, the ATM/Chk2 and the ATR/Chk1 pathways. Activation of ATR, which phosphorylates its effector kinase Chk1, is induced in response to single-strand breaks and bulky DNA lesions. The ATM kinase, signaling through its effector Chk2, is activated primarily in response to DNA double-strand breaks (DSBs), such as those induced by alkylating agents, topoisomerase inhibitors, or ionizing radiation. Chk1 and Chk2 have a protective function providing time to the cell to repair genotoxic lesions. Both kinase pathways result in activation of cell cycle-arresting target genes, DNA repair and apoptosis via p53 activation (Reinhardt and Yaffe, 2009).

Recent sequencing studies identified recurrent somatic gene mutations in CLL patients for proteins involved in DNA damage signaling and DNA repair, including mutations in TP53, ATM, CHEK1, CHEK2, POT1, BRCA1, and CHD2 (Puente et al., 2011; Quesada et al., 2011).

The ATM-Chk2-p53 signaling axis plays an important role in regulation of apoptotic response to DNA damage in CLL, as mutations in ATM and TP53 are enriched in patients with secondary resistance to DNA-damaging chemotherapy (Bartkova et al., 2005; Landau et al., 2013).

ATM gene is frequently inactivated in CLL and is associated with defective apoptosis in response to chemotherapeutic agents (Austen et al., 2007). ATM mutant cells exhibit impaired DNA DSB repair. Poly (ADP-ribose) polymerase (PARP) plays a pivotal role in a direct repair of DSBs and involved in main DNA repair mechanisms: homologous recombination and non-homologous end-joining (Weston et al., 2010). ATM dysfunction is associated with significantly higher PARP activity in CLL patients, which might mediate genomic instability and progression of the disease. *In vivo* studies using xenograft model of an ATM mutant cell line demonstrated significantly reduced tumor load and an increased survival of animals after treatment with the PARP inhibitor Olaparib (Weston et al., 2010). Clinical studies with Olaparib demonstrated sufficient efficacy in patients with ATM deficient, relapsed and refractory CLL (ISRCTN34386131 DOI 10.1186/ISRCTN34386131). Deletions of the short arm of chromosome 17 (del(17p)) where TP53 is located are found in 5–8% of chemotherapy-naïve patients (Dohner et al., 2000). Mutations of TP53 are found in 4–37% of patients with CLL, and have been associated with very poor prognosis (ultra-high risk) in a number of studies (Zenz et al., 2010). Among cases with confirmed del(17p), the majority show mutations in the remaining TP53 allele (>80%). Higher genomic complexity and clinical diversity of CLL are associated with T53 mutations. Impaired DDR promotes a "mutator phenotype," which allows the acquisition of additional genetic lesions driving transformation in CLL (Seiffert et al., 2012).

Mutational inactivation of the DDR is an established hallmark of CLL and associated with high genomic instability (Zenz et al., 2010; Landau et al., 2013).

ATM appears to be a major regulator of the p53 response. They communicate the genotoxic lesion to the apoptotic machinery but they are frequently inactivated in CLL and are associated with poor response to conventional chemotherapy (ten Hacken and Burger, 2014).

The B cell receptor (BCR) pathway inhibitors in CLL have shown high efficacy in the cases with poor chromosomal aberrations such as Del (17p) or p53 mutation, known to acquire resistance to standard chemotherapy. Downstream targets of the BCR such as SYK, Bruton's tyrosine kinase (BTK), or PI3K isoform p110 delta have a promising anti-neoplastic activity in patients with CLL. Responses are typically manifested by rapid regression of enlarged lymph nodes and splenomegaly that is accompanied by transient lymphocytosis (Burger and Gribben, 2014; Yair et al., 2014).

Clinical trials with Idelalisib, PI3K delta isoform inhibitor, have a dramatic and durable response in CLL patients with a markers of poor prognosis, such as mutations in p53, ATM and NOTCH1. Monotherapy with Idelalisib and combination with other therapeutical agents such as Rituximab and Ofatumumab results show good activity in CLL regardless of high-risk prognostic markers (Khan et al., 2014).

Moreover, the DDR is able to alert the immune system toward the stressed cell, mainly through the recruitment of NK cells, which are able to identify and eliminate dangerous cells without prior antigen-mediated stimulation (Raulet, 2006; Bryceson and Ljunggren, 2008). NK cells do not only distinguish between "self" and "non-self," but specifically seek for pathological changes in endogenous cells. One important danger signal is the inducible expression of ligands for cytotoxic NK cell receptors [NKG2D (NK group 2, member D) and NCRs (natural cytotoxicity receptors)] to alarm the innate immune system in response to DNA damage (Gasser et al., 2005; Gasser and Raulet, 2006a,b,c; Gasser, 2007; Soriani et al., 2009; Fine et al., 2010; Norman et al., 2011). Ligands for these NK cell receptors are not expressed on normal cells but are found on cells undergoing cellular stress that causes DNA damage including chemotherapeutics or ionizing radiation (Raulet, 2006).

The expression of NKG2D ligands in response to genotoxic stress and stalled DNA replication forks is induced through canonical DDR in an ATM/ATR-dependent fashion in mouse and human fibroblasts (Gasser et al., 2005). The NKp30 ligand BAG6 is released by stressed cells via the exosomal pathway and has to be associated with these small membrane vesicles to properly activate NK cells (Simhadri et al., 2008). The release of exosomes is known to be regulated by TSAP6 in a p53-dependent manner (Lespagnol et al., 2008). Thus, defects in the DDR such as p53 mutations may directly affect NK cell-dependent recognition and elimination of CLL cells (Reiners et al., 2013). In line, an impaired expression of ligands for two major activating receptors—NKG2D and NKp30—was shown to be associated with CLL probably explaining NK cell anergy in this disease (**Figure 1**; Salih et al., 2008; Nuckel et al., 2010; Costello et al., 2012; Reiners et al., 2013). However, mechanisms of escape from NK response in CLL are not completely clear, but defects in NK cell activity strongly correlate with progression of the disease (Ziegler et al., 1981; Riches and Gribben, 2013).

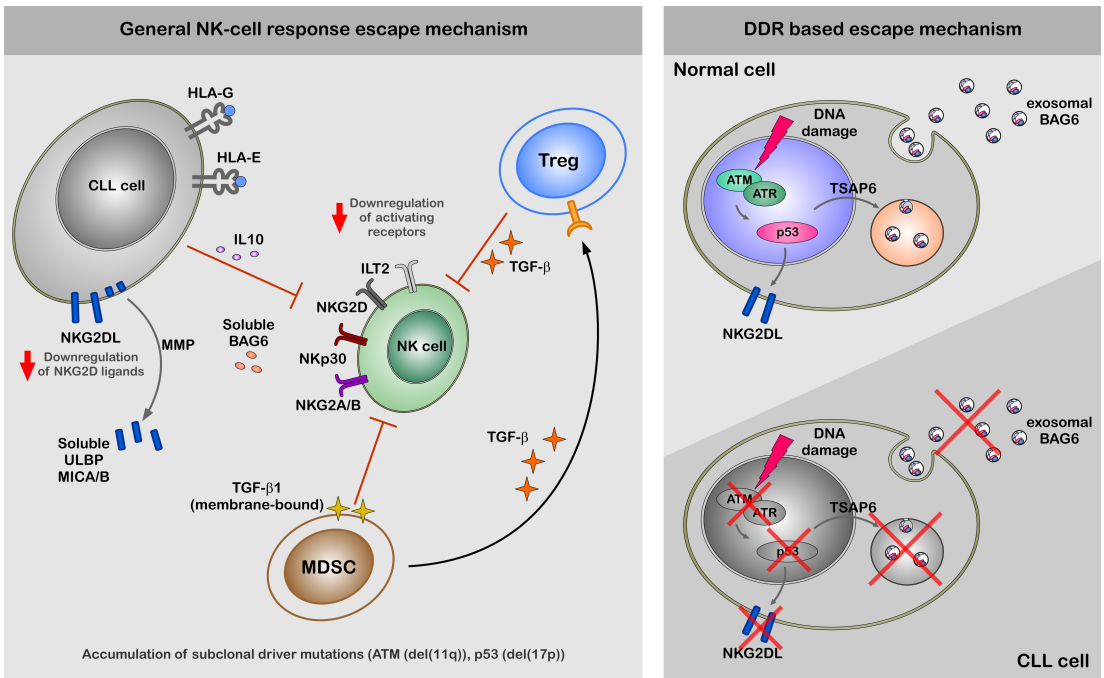

FIGURE 1 | A hypothetical model of CLL escape from NK response. The left panel of the figure demonstrate general mechanism of the escape from NK-cell response by CLL cells. The escape of CLL cells from the NK cell response is regulated at different levels; (1) diminished expression NKG2D ligands on the cell surface, (2) increased levels of ligands for inhibitory receptors ILT2 and NKG2A/B, (3) shedding by MMPs and production of soluble ligands for activating receptors (ULBP2, MICA, and BAG6) NKG2D and NKp30 (Iclozan et al., 2013), which rather suppress than activate NK cells. Indirect suppression of NK cell activity might be regulated by MDSC and Treg cells via production of TGFβ and IL-10, which modulate expression levels of activating receptors on the cell surface of NK cells. The right panel of the figure is focused on the impaired DDR and its role for the escape from NK-cell response. Induction of DDR in healthy cells results in activation ATM-p53 axis. Activation of p53 results in cell surface expression of NKG2D ligands and exosomal release of BAG6 following transcriptional activation of TSAP6. Cell surface expression of NKG2D ligands and exosomal expression of BAG6 is impaired in CLL cells due to defects in DDR.

IMMUNE MICROENVIRONMENT DRIVES CLL PROGRESSION

The tumor microenvironment plays an important role in CLL progression. The functional components of the microenvironment can be divided in three groups. The first group includes bone marrow stromal cells (BMSC), nurse-like cells (NLC), and follicular dendritic cells (FDCs), collectively involved in supporting selection, survival and proliferation of CLL cells. BMSCs send anti-apoptotic signals via VCAM and integrins and protect CLL cells against conventional chemotherapy (Gehrke et al., 2011). NLC attract CLL cells by secreting CXCL12 and CXCL13 and protect from drug induced apoptosis via CXCL12, BAFF, APRIL, CD31, and plexin-B via activation of prosurvival cascades such as NFκB and ERK (Burger and Gribben, 2014). Also NLCs play important role in activation of the BCR signaling cascade. FDCs protect CLL cells from apoptosis by direct contact resulting in upregulation of antiapoptotic protein MCL-1 (Endo et al., 2007).

The second group is represented by regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSCs). They interfere with the complex interaction between the immune system and transformed cells via production of immunosuppressive soluble factors such as TGF-β and IL-10 (D'Arena et al., 2013; Jitschin et al., 2014).

The third group encompasses components of the immune system, including $CD4^+$ T, $CD8^+$ T, and NK cells. Despite an elevated T cell count in the peripheral blood, the T cell compartment is abnormal in CLL, showing profound functional defects and signs of chronic activation [upregulation of CD69, HLA-DR, and CD57 and downregulation of CD28 and CD62L (Pedersen et al., 2002; Burger and Gribben, 2014)]. $CD4^+$ T cells stimulate CLL cells via CD40/CD40L crosstalk to induce the production of CCL17 and CCL22 for attracting Th2 lymphocytes (Nakayama et al., 2004). The NK cell subset in CLL patients shows reduced ability to attack cancer cells partly owing to diminished expression of the activating NK receptor NKp30 on the cell surface (Costello et al., 2012). Also, HLA-G and HLA-E on the CLL cells and high levels of soluble/decoy ligands, for activating NK cell receptors suppress NK cell function (Nuckel et al., 2005).

NOVEL APPROACHES FOR CLL TREATMENT: IMMUNE CONSTRUCTS AS TOOLS TO REDIRECT NK CELLS AGAINST TUMOR CELLS

The improved understanding of the pathology of CLL and the role of the microenvironment resulted in the development of novel less toxic agents (Burger and Gribben, 2014; Yair et al., 2014). These new compounds include inhibitors aimed at BCR signaling pathway (Pallasch and Hallek, 2014), antiapoptotic proteins (Yair et al., 2014), mAbs (Robak, 2013), and immune-modulatory drugs (Burger and Gribben, 2014; Yair et al., 2014).

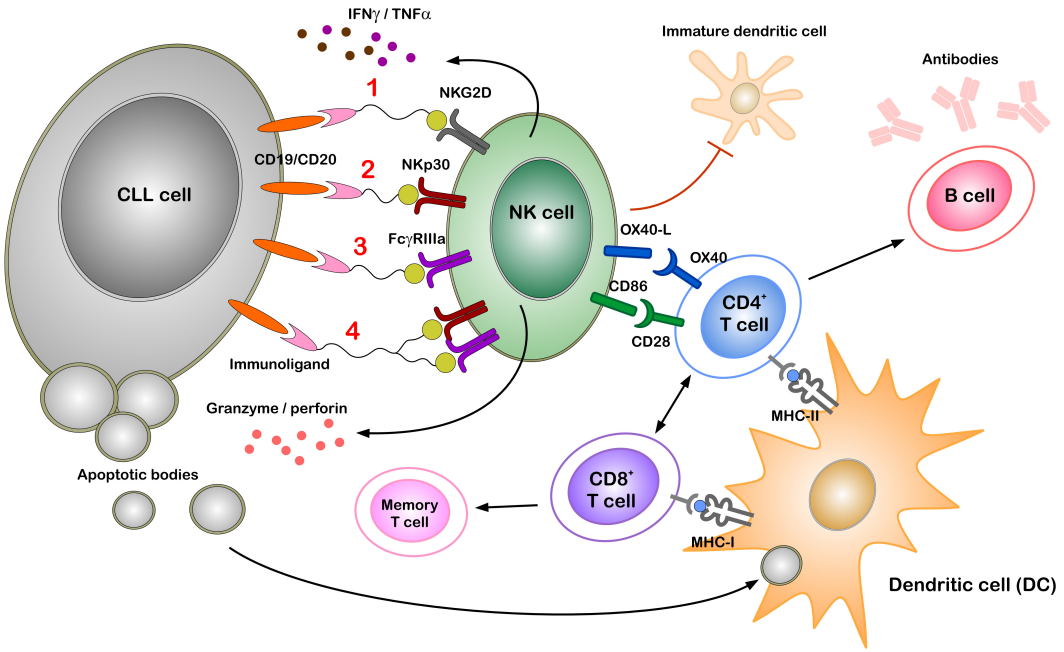

FIGURE 2 | Potential role of immunoligands to redirect NK cells to CLL cells. Immunoligands with specificities for CLL cells (through CD19 or CD20) and for NK cells [through activating receptors on NK cells such as NKG2D, NKp30, FcγRIIIa or simultaneous NKp30 and FcγRIIIa(1–4)] can link and activate respective immune cells even in the presence of active immune suppression. Stimulating of either of the activating receptors on NK cell leads to cytokine secretion (IFNγ, TNFα) and degranulation, thereby killing tumor cells by apoptosis. Professional antigen presenting cells (APCs) such as dendritic cells (DCs) phagocytose components of dying tumor cells and present tumor antigens to both $CD8^+$ and $CD4^+$ T cells, thereby inducing cell-mediated and humoral adaptive immunity and memory response. Direct NK–DC crosstalk in terms of maturation of DCs and killing of immature DCs is mainly attributed to NKG2D and NKp30 activation. Additionally, following activation NK cells express OX40 ligand (OX40L) and CD86 on the cell surface, which can bind to the co-stimulatory receptors OX40 and CD28 expressed by $CD4^+$ T cells. Direct interaction between NK and $CD4^+$ T cells through such co-stimulatory molecules can enhance T cell effector functions.

Current immunotherapy for CLL is mainly intended to stimulate T cells in order to eliminate the tumor (Costello et al., 2012; Burger and Gribben, 2014), whereas NK cell-based therapies are not as advanced. Various recombinant immunoligands and immune constructs to restore impaired NK cell activity have been developed and analyzed pre-clinically. While both represent recombinant constructs, the immunoligands utilize natural ligands for immune receptors fused to tumor-specific single chain variable fragment (scFv) or other antibody-derived fragments whereas the immune constructs utilize antibody-derived components to target both immune and target cells (Vyas et al., 2014). So far, the activating receptors FcγRIIIa, NKG2D, and NKp30 were used as target structures on NK cells.

FcγRIIIa (CD16a) s is one of the main NK cell-activating receptors, which upon stimulation, mediates ADCC through the release of granzyme and perforin (Alderson and Sondel, 2011). The clinical success of many FDA-approved mAbs (e.g., Rituximab) is partially attributed to NK cell-mediated ADCC through FcγRIIIa receptor (Houot et al., 2011). Several bispecific and trispecific immunoconstructs with one arm specific for the human FcγRIIIa receptor have been developed. These constructs target different tumor antigens (CD33, CD123, and CD19) and are currently tested for effectiveness (Singer et al., 2010; Stein et al., 2010).

NKG2D is known as the most important receptor involved in immune evasion of CLL and other tumor cells (Huergo-Zapico et al., 2014). Tumor cells downregulate the expression of the ligands for NKG2D and release soluble ligands upon induced shedding by matrix metalloproteinases (MMPs) that block NKG2D receptor function (Huang et al., 2011; Chitadze et al., 2013). Immunoligands containing human NKG2D ligands have already been generated (Germain et al., 2005; Pogge von Strandmann et al., 2006; Jachimowicz et al., 2011; Kellner et al., 2012b; Rothe et al., 2014). One of them is a recombinant bispecific immunoligand bearing ULBP2 ligand–scFv fusion in a single chain format. The first construct of this kind was ULBP2-BB4, which links NK cells and $CD138^+$ tumor cells through the ULBP2 ligand and the BB4 scFv with specificity for the TAA CD138 (Pogge von Strandmann et al., 2006). ULBP2-BB4 successfully activated and retargeted NK cells against multiple myeloma (MM) tumor cell lines and primary patient tumor cells and showed antitumor activity in a xenograft MM model. Another bispecific protein developed consists of recombinant MICA as NKG2D ligand chemically conjugated to Fab fragments from mAb specific for TAAs such as CD19 and CD20 (Kellner et al., 2012b). Malignant cells, which are otherwise resistant, can be rendered susceptible to NK cell attack by NKG2D ligand MICA in this format (Germain et al., 2005).

Targeting NKp30 to reactivate NK cells in CLL is most promising and feasible as novel activating ligands for NKp30 are identified recently. While BAG6 can activate NK cells via NKp30 to induce target killing of tumor cells and immature DCs when expressed on the surface of exosomes (Pogge von Strandmann et al., 2007; Simhadri et al., 2008), expression of B7-H6 seems to be more tumor-confined as it is also found on the surface of cancer cells (Brandt et al., 2009; Matta et al., 2013). A bispecific immunoligand (B7-H6-7D8) comprising the B7-H6 (NKp30 ligand) ectodomain fused to a 7D8-derived anti-CD20 scFv was generated (Kellner et al., 2012a). The B7-H6-7D8 immunoligand efficiently redirected NKp30-dependent NK cell mediated lysis toward $CD20^+$ lymphoma cells (Kellner et al., 2012a, 2013). Furthermore, this construct enhanced the lysis of target cells when used along with either rituximab (anti-CD20 mAb which activates the FcgRIIIa receptor) or NKG2D activating construct ULBP2-7D8 (Kellner et al., 2012a, 2013). The concept of synergistic activation of NK cells by multiple activating receptors is proven in various systems *in vitro* (Bryceson et al., 2006; Morgado et al., 2011; Deguine et al., 2012) and will be applied in the next generation of immunoligands in a triplebody format (Vyas et al., 2014; **Figure 2**).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In the last decade a better understanding of the role of the immune environment in progression of CLL led to new targeted therapy options. NK cells are the first line to attack the cancer, but their activity is highly impaired in CLL patients. Recent studies show impressive pre-clinical responses to immunoligands and immunoconstructs redirecting NK cells against different cancers. Novel immune construct-mediated restoration of NK cell functionality in CLL patients is an attractive strategy for treatment, which might even pave the way for recovery of patients with high risk cytogenetic aberrations and/or refractory disease.

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LINE-1 retrotransposons and *let-7* miRNA: partners in the pathogenesis of cancer?

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Long interspersed nuclear element-1 (LINE-1 or L1) retrotransposons are insertional mutagens capable of altering the genomic landscape in many ways. Activation of the normally silent LINE-1 retrotransposon is associated with a high level of cancer-associated DNA damage and genomic instability. Studies of LINE-1 have so far focused mainly on changes in gene expression, and our knowledge of its impact on functional non-coding RNAs is in its infancy. However, current evidence suggests that a significant number of human miRNAs originate from retrotransposon sequences. Furthermore, LINE-1 is generally not expressed in normal tissues while its expression is widespread in epithelial cancers. Based on our recent studies, we demonstrate a functional link between aberrant LINE-1 expression and deregulation of *let-7* miRNA expression. Since the expression of *let-7* is modulated by LINE-1 activity, we discuss possible mechanisms for this effect and how the silencing of LINE-1 activation could provide new therapeutic options for cancer treatment. Based on the deep sequencing of small RNAs in parallel with gene expression profiling in breast cancer cells, we have identified potential pathways linking L1 activity to *let-7* processing and maturation and ultimately to the control of stemness in human cancer cells.

Keywords: LINE-1, retrotransposon, *let-7* microRNA, long non-coding RNA, cancer, gene modulation

INTRODUCTION

Retrotransposons, a family of mobile genetic element, are the most common repetitive elements in the human genome. Of these, the long interspersed nuclear element-1 (LINE-1 or L1) and the Alu elements are the most prolific classes of retrotransposon, comprising 28% of the human genomic sequence. L1 is an insertional mutagen capable of copying itself and reinserting into the genome at multiple sites and is thereby capable of wreaking mutational havoc on the genome. The activation of L1 retrotransposons and ensuing L1 retrotransposition is also associated with a high frequency of DNA breaks and genomic instability (Symer et al., 2002) and several studies have shown that there is a direct association between the severity of cancer-associated DNA damage and the activation of L1 expression (Belgnaoui et al., 2006; Wallace et al., 2010). L1 also accelerates the mobilization of Alu elements, certain mRNAs and non-coding RNAs to new sites in the genome (Esnault et al., 2000; Garcia-Perez et al., 2007), further altering cellular function in many ways. Because of these potentially harmful impacts on genomic integrity, normal adult cells have developed a variety of defense mechanisms, including epigenetic silencing, to prevent the expression of L1 elements (Chen et al., 2012b; Rangasamy, 2013). Related to this, studies have shown that hypomethylation of L1 promoters is associated with activation of L1 expression in many types of cancer (Cruickshanks and Tufarelli, 2009). When L1 elements become active, they can rapidly increase in copy number by a "copy-and-paste" mechanism and become a source of genetic mutations. For example, two recent studies have unveiled several tumor-specific *de novo* L1 mutations in lung and liver cancers using the transposable element analyzer (TEA) repeat analysis pipeline and genome-wide mapping (Iskow et al., 2010; Lee et al., 2012).

A recent survey of whole genome sequences from a variety of tumors included in the Cancer Genome Atlas (TCGA) project also identified a number of L1-mediated insertional mutations in colon, prostatic, colorectal, and ovarian cancers, suggesting that L1-induced mutations are common in cancer cells and tissues (Shukla et al., 2013). Despite these findings, questions remain concerning whether the activation of L1 elements is causative of cancer or merely occurs as an epiphenomenon due to the unstable genomic state of cells. Although a clear connection has been established between L1-induced mutations and altered expression of affected genes, it is unclear if these represent cell-type-specific mutations or are sufficiently prevalent to contribute to cancer pathology in general. The activation of L1 retrotransposons occurs mostly in cancers of epithelial origin. In recent studies, we and others have shown that L1 expression occurs in almost all the aggressive forms of human breast cancer characterized by high rates of lymph node metastasis, including estrogen-negative (ER-) tumors, which are characterized by frequent distant metastasis and intrinsic resistance to hormone therapy (Harris et al., 2010; Chen et al., 2012a). In support of these findings, another study has shown that breast carcinomas release retroviral-like particles into the extracellular space that contain high levels of L1-encoded mRNA (Golan et al., 2008). Furthermore, the level of L1 elements is high in the plasma of patients with breast cancer, melanoma, and lymphoma (Balaj et al., 2011), suggesting a link between L1 activity and the recurrent forms of

metastasis. Although the contribution of L1 activity to initiating the expression of certain protein-coding oncogenes such as *c-MET*, typically via alternative promoters, has been recognized (Cruickshanks and Tufarelli, 2009; Wolff et al., 2010), little is known about the regulatory role of L1 elements (if any) for non-coding RNA genes. A recent transcriptome study reports that an L1 transcript driven by a viral HBV promoter, referred to as HBx-LINE-1, does not encode a protein but produces a long non-coding RNA (lncRNA) which induces the β-catenin signaling pathway and facilitates the acquisition of a mesenchymal phenotype and metastatic potential (Lau et al., 2014). Strikingly, HBx-LINE-1 expression has been found to occur in ∼25% of hepatocellular carcinomas examined, and correlates with reduced patient survival. Although this finding suggests a role for L1 elements in the development of liver cancer, it is not clear whether a similar pattern of L1-driven lncRNA expression exists in other types of cancer.

RETROTRANSPOSONS AS THE SOURCE OF NON-CODING RNA

Growing evidence suggests a close association between the presence of retrotransposons in the intergenic regions of the human genome and sources of non-coding RNAs, including miRNAs and lncRNAs. Notably, ∼30% of human lncRNAs originate from retrotransposons, in both sense and antisense orientations. In addition, ∼80% of lncRNAs contain retrotransposon-derived sequences embedded within or nearby their transcription start sites, in which the retrotransposon sequences contribute signals for lncRNA expression, splicing and processing (Kapusta et al., 2013). Genome-wide analyses also reveal that lncRNAs are highly enriched for LTR and HERV elements but are depleted of L1 and Alu elements. Despite the low content of L1-derived sequences, a recent study reported that a point mutation in an L1-containing lncRNA sequence, which is located within an intron of *SLC7A2*, leads to a defect in the expression of the lncRNA and results in a lethal encephalopathy phenotype (Cartault et al., 2012). The presence of this L1 sequence is predicted to contribute to the proper folding of the lncRNA, which is important for its function in the brain.

Alu elements do not encode functional proteins for their mobilization. Instead, Alu rely on the functioning of the L1 machinery. In fact, L1 elements are frequently found overlapping Alu sequences at multiple locations in the genome and in particular, in lncRNA sequences. Several recent studies suggest that Alu elements present in lncRNAs can contribute to the regulatory role of these lncRNAs. One such Alu-mediated lncRNA is *APTR*, which represses *p21* expression by recruiting polycomb repressive proteins to the p21 promoter. The presence of Alu is crucial to the localization of *APTR* to the p21 promoter and thus to regulation of cell growth and proliferation (Negishi et al., 2014). Interestingly, this lncRNA also contains an L1 sequence overlapping with closely spaced pairs of inverted Alu elements. Whether the presence of the L1 sequence has any effects on the functions of the lncRNA remains to be elucidated. The function of Alu elements is also linked to the expression of many disease-related lncRNAs. As a key regulatory element, Alu mediates the expression of an lncRNA, referred to as *ANRIL* (antisense non-coding RNA in the INK4 locus), which binds to polycomb group proteins and interacts with multiple target gene promoters during the process of atherosclerosis (Holdt et al., 2013). The presence of Alu in the lncRNA not only increases the expression of *ANRIL* transcripts but also marks the promoters of target genes for epigenetic silencing. Strikingly, deletion or mutation of the Alu sequence in *ANRIL* normalizes *ANRIL*-regulated gene networks and cellular functions. These findings highlight a new role for retrotransposons in epigenetic *trans*-regulation of gene networks, which might be relevant to other lncRNAs as well.

Another important layer of genetic control that shapes cellular functioning is the expression of microRNAs. Computational studies reveal that miRNA target sites in the 3′-UTRs of genes can be formed from embedded retrotransposon sequences, and also that many miRNAs were initially formed from retrotransposon sequences (Roberts et al., 2014). Most miRNAs are transcribed as long primary transcripts (pri-miRNAs) and processed by Drosha/Dicer to mature miRNAs with lengths of 20–22-nt. miRNAs regulate gene expression post-transcriptionally by binding to one or more mRNAs, ultimately leading to the translational inhibition or degradation of the target genes. Differential expression of miRNAs is observed in many types of cancer with some of these miRNAs playing crucial roles in cancer onset and progression. There is also growing evidence that the seed sequences of miRNAs are derived from retrotransposons (Borchert et al., 2011). For instance, the miRNA-28 family originates from LINE L2B elements. Several computational analyses have reported that some miRNAs share significant sequence homology to retrotransposons (Filshtein et al., 2012). In addition, a substantial number of miRNAs contain hairpin sequences that are related to retrotransposons. In further support of these findings, another recent study has shown that several human miRNAs and miRNA target sites in the 3′-UTRs of genes are, in fact, derived from L1, Alu, and MIR elements (Spengler et al., 2014). Notably, ∼85% of miRNA target sites overlap L1 and Alu elements, indicating that a strong relationship exists between miRNA functionality and the activity of retrotransposons.

Our group has recently shown that L1 elements are not expressed in normal differentiated cells, but that their expression is widespread in all the types of breast tumors and breast cancer cells examined so far, and correlates with poorer patient survival (Chen et al., 2012a). Supplementary Table 1 summarizes the expression of L1 elements determined by different investigators in a variety of cancer cells, tumor tissues, and animal-model studies. To elucidate the molecular functions of L1 elements other than those resulting in insertional mutations, we silenced the expression of endogenous L1 elements in T47D breast cancer cells using an L1-specific endo-siRNA that can specifically silence L1 expression through increased DNA methylation of L1 promoters (Chen et al., 2012b). A genome-wide analysis of miRNA expression using high-throughput deep sequencing showed strong global upregulation of miRNA expression and very marked changes in a number of specific miRNAs secondary to L1 silencing in this cancer cell line (Ohms and Rangasamy, 2014). To our surprise, most of the changes in miRNA expression occur mainly in the *let-7* family of miRNAs (**Figure 1A**). In particular, *let-7a* miRNA

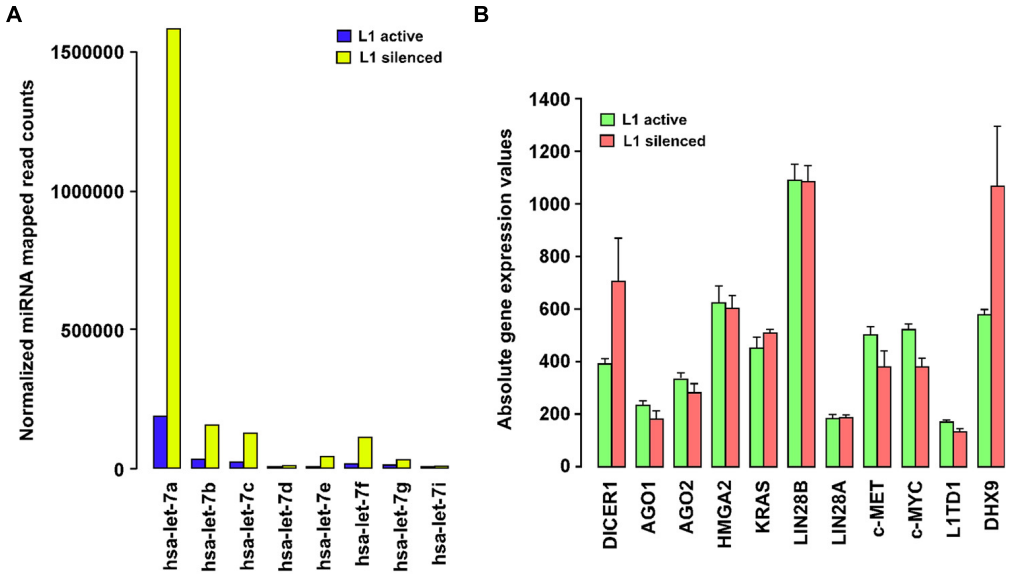

FIGURE 1 | (A) Long interspersed nuclear element-1 (L1) silencing modulates the expression of the *let-7* family of miRNAs. Barplot showing DESeq-normalized absolute read counts for *let-7* family miRNAs from L1-silenced small RNA deep sequencing experiment in T47D breast cancer cells. **(B)** Expression profiling of *let-7a*-target gene expression in T47D breast cancer cells before and after silencing L1. Barplot showing normalized absolute gene expression values for selected genes. Error bars show mean ± SEM ($n = 3$ replicates for each experimental group).

was strongly upregulated from 149,428 normalized mapped reads to 1,855,633 reads in L1 silenced cells, accounting for 40% of the increase in the total normalized read counts in the L1-silenced cells compared to cancer cells in which L1 remained active. This massive increase in *let-7a* expression is intriguingly similar to the differential expression of *let-7a* seen in normal cells and a variety of cancer cells in which the expression of *let-7* is repressed (Boyerinas et al., 2010).

Let-7a has a highly conserved sequence across organisms from *Caenorhabditis elegans* to humans. It regulates the expression of a range of genes through its 5′ seed sequence (5′-UGAGGUA-3′), which binds to corresponding sequences located in the 3′ UTRs of genes. *Let-7a* is also known to target many oncogenes including *c-Myc, HMGA2*, and *Lin28,* and its expression is a hallmark of cell differentiation. Notably, the loss of *let-7a* expression is often considered to have prognostic value since it indicates poor survival in many cancers. Studies performed in lung and renal cell carcinoma reveal that the overexpression of *let-7a* inhibits *in vitro* cancer cell proliferation and *in vivo* tumor regeneration by reducing the expression of *c-Myc* and c-Myc targeted genes (Liu et al., 2012). Another major target of *let-7a* is *c-MET*, which is one of the key genes activated by L1 expression in cancer cells (Wolff et al., 2010). Abnormal expression of *c-MET* induces multiple signal transduction pathways involved in cancer growth and metastasis including the RAS, PI3K, STAT3, and β-catenin pathways. For these reasons, there is growing interest in the therapeutic use of *let-7a* itself, or pharmacological modulators of *let-7a* to treat human cancers in clinical applications.

Expression of *let-7a* is subjected to complex regulation involving positive (p68/p72 helicases) and negative factors (c-Myc, Lin28, hnRNPA1). The p68/p72 RNA helicases, as components of the Drosha microprocessor complex, stimulate the processing of pri-*let-7* miRNAs into mature RNAs by Dicer-mediated processing. The mature *let-7a* also binds to a complementary region in the pri-*let-7* miRNA, recruiting Argonaute and promoting its own downregulation (Zisoulis et al., 2012). Moreover, the expression of *let-7a* is also controlled by c-Myc binding to the *let-7* promoters which decreases its expression. c-Myc also activates *Lin28* expression by binding to the Lin28 promoters and Lin28, in turn, binds selectively to pri-*let-7* miRNAs and blocks Dicer processing of pri-*let-7* miRNAs into mature *let-7a* (Chang et al., 2009). By repressing *let-7a*, *Lin28* often acts as an oncogene in cancer cells (Viswanathan et al., 2009). Strikingly, Lin28 is itself targeted by *let-7a* thus affecting the functioning of Lin28 in a feedback circuit. Currently, however, few studies have addressed the functional role of L1 in the expression of the *let-7* miRNA family. Thus, to clarify the role of L1, we carried out profiling of *let-7a*-target gene expression, in breast cancer cells before and after silencing L1. This study revealed that L1 silencing reduces the expression of some *let-7a*-targeted genes including c-Myc and c-MET, although only to a modest degree (**Figure 1B**). In another study, inhibition of L1 activity by antiretroviral drugs was shown to reduce c-Myc expression in cancer cells (Sciamanna et al., 2005), which may partially explain the ability of L1-silencing to activate *let-7a*. Importantly, our gene expression profiling shows that Dicer is also significantly upregulated in L1 silenced cells, which supports a recent report of Dicer inhibiting L1 activation in human cells (Heras et al., 2013). What is less clear from these studies is how L1 silencing leads to increased expression of *let-7a* miRNA in cancer cells. Thus, the link between the *let-7a* miRNAs and the expression of L1 elements in cancer cells requires further investigation.

DOES L1 EXPRESSION INFLUENCE MIRNA EXPRESSION?

There is little or no direct evidence for a reciprocal relationship between the silencing of L1 and *let-7a* expression in the literature. Also, little is known about the relationship between the expression of L1 elements and other miRNAs. So, how might L1 influence the expression of the *let-7a* miRNA? One possible mechanism is that retrotransposon sequences located in the promoter regions of *let-7* miRNAs might act as functional domains for their regulation. Sequence analysis with the RepeatMasker database reveals the presence of retrotransposon fragments scattered throughout the promoter regions of the *let-7* miRNAs, including L1, Alu, and MIR elements. Given that the methylation status of the *let-7* promoters does not appear to play a significant role in the expression of *let-7* miRNAs (Lu et al., 2007), it is conceivable that these inserted retrotransposon transcripts interact with a variety of host proteins, including RNA binding proteins, chromatin modifiers, and regulators of transcription/translation to form an L1 ribonucleoprotein (RNP) complex. Notably, L1 and Alu sequences have recently been shown to confer binding sites for several chromatin regulatory complexes involving lncRNA expression (Blackwell et al., 2012; Goodier et al., 2013). Regardless of whether the inserted retrotransposons within the promoter regions of *let-7* miRNAs are active or not, there are at least 100 copies of highly active L1 elements present in human cells (Brouha et al., 2003). Several lines of evidence indicate that transcripts from these L1 elements are associated with hnRNPA1, which is an abundant RNA binding protein involved in splicing, processing, and export of several pre-mRNAs to the cytoplasm (Goodier et al., 2007; Sokolowski et al., 2013). Importantly, hnRNPA1 also binds to pri-*let-7a* miRNAs and acts as a repressor of *let-7* biogenesis by antagonizing the docking of KSRP (KH-type splicing regulatory protein), which is a component of Drosha/Dicer complexes and is known to positively regulate processing of miRNAs (Michlewski and Caceres, 2010; **Figure 2**). Strikingly, except for pre-*let-7i*, all the members of the *let-7* family interact with hnRNPA1. Thus, there is evidence of a relationship, at least in the case of *let-7a* miRNAs, between the L1 activity and miRNA expression.

An alternative but not mutually exclusive possibility is that L1 silencing may activate miRNA-inducing proteins such as transcription factors and RNA helicases that activate *let-7* biogenesis. Intriguingly, our gene expression microarray profiling of L1-silenced cells showed significantly upregulated expression of the DEAH box-containing DHX9 protein, which shares high sequence similarity to the *Drosophila* maleless protein (MLE), a protein that regulates dosage compensation (Kuroda et al., 1991). DHX9 is an RNA helicase (also known as RNA helicase A, RHA, or NDHII) involved in the RNA-induced silencing complex (RISC) assembly process (Fu and Yuan, 2013) as an RISC-loading factor and this function is mediated by its dsRNA-binding domains. Recently, DHX9 has been shown to interact with Dicer, AGO2, and TRBP2 in miRNA loading and depletion of DHX9 leads to reduced miRNA processing and RISC assembly. Notably, DHX9 also interacts with the L1 RNP complex, along with other RNA helicases, including MOV10, that can potentially impede RISC function (Goodier et al., 2012). Strikingly, a recent study proposed that Lin28 could antagonize the production of *let-7a* miRNAs by recruiting a DHX9-like RNA helicase to promote its own translation (Kallen et al., 2012). Together, these observations suggest that induction of DHX9 by L1 silencing (either directly or through interaction with other proteins) can activate the expression of *let-7a* miRNAs. However, further research is needed to evaluate the precise function of L1 in the activation of the *let-7* miRNAs. Given that *let-7* is often viewed as a tumor suppressor miRNA, a strategy in which L1 activity is selectively inhibited pharmacologically could provide new therapeutic options for human cancer treatment.

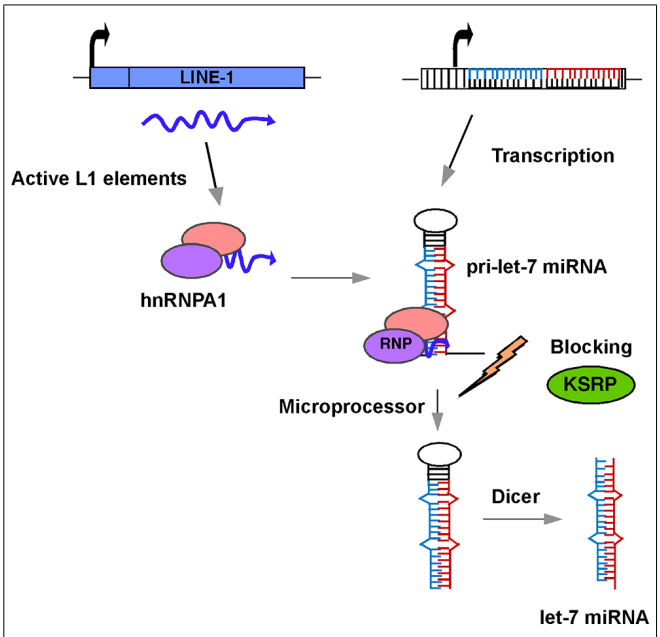

FIGURE 2 | Schematic diagram of a model showing the role of L1 in *let-7* miRNA processing and maturation. L1 transcripts in human cancer cells interact with hnRNPA1 to form an L1-hnRNPA1 complex. This complex binds to pre-*let-7* miRNA and prevents recruitment of KSRP regulatory protein thus blocking the action of the microprocessor and preventing the formation of mature *let-7* miRNAs.

In summary, this study has explored the relationship between the expression of L1 elements and cancer onset and progression. In this study we have shown, first, that L1 activity is widespread in epithelial cancers. Second, the expression of non-coding RNAs including miRNAs and lncRNAs is closely associated with L1 activity. Third, we have demonstrated the interplay between the aberrant expression of L1 elements and miRNAs, and in particular, the tumor suppressor miRNA *let-7a*. As we propose above, there is a clear link between *let-7a* expression and the silencing of L1 elements. Questions remain, however, as to how L1 elements, either directly or in combination with other host proteins contribute to the loss of *let-7* expression in the various types of cancer. Further studies are required to thoroughly test the mechanisms proposed above by which L1 might affect miRNA expression. Regardless of the answers to these questions, the available data suggest that silencing of L1 expression holds great therapeutic potential for the treatment of various epithelial cancers.

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SUPPLEMENTARY MATERIAL

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