THE DNA REPLICATION MACHINERY AS THERAPEUTIC TARGETS

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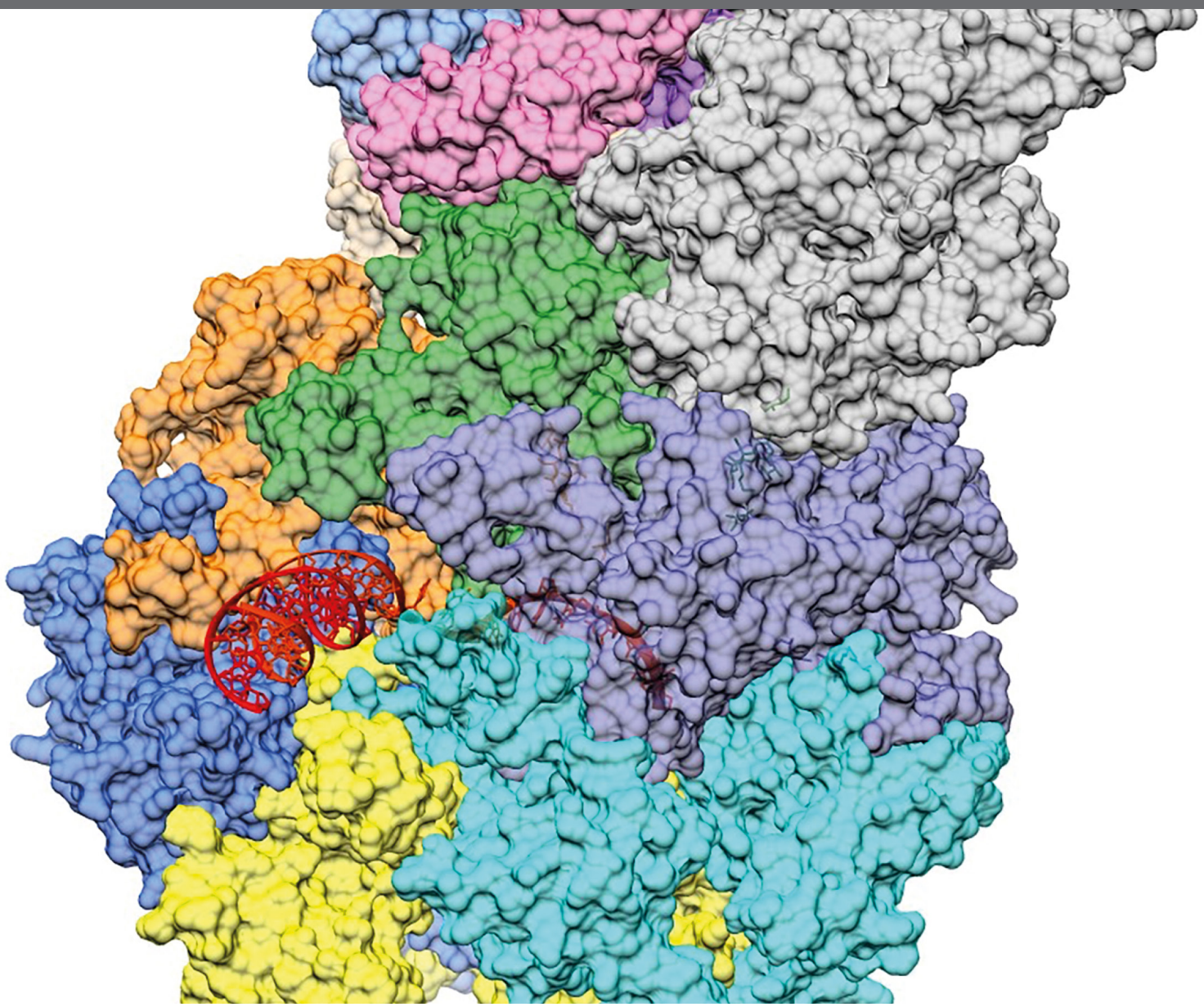

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THE DNA REPLICATION MACHINERY AS THERAPEUTIC TARGETS

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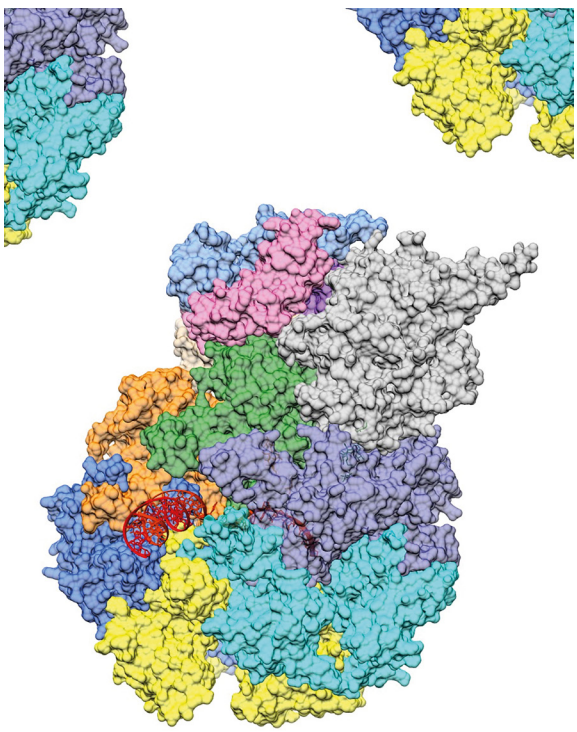

Eukaryotic replicative helicase is a complex of three component. The hexameric mini-chromosome maintenance (MCM) complex, the tetrameric GINS complex and the Cdc45 protein resulting in the eleven proteins CMG (Cdc45, MCM, GINS) complex. The image shows the cryo-EM single particle reconstruction of eukaryotic CMG at a DNA replication fork. DNA is shown in red, MCM2 in lavender, MCM3 in orange, MCM4 in yellow, MCM5 in green, MCM6 in aqua, MCM7 in blue, Cdc45 in gray, GINS PSF2 protein in pink, and GINS SLD5 in pale blue. The two other components of GINS (PSF1 and PSF3) are present, but are in the back of the structure and therefore out of view. Image was created using UCSF Chimera from PDBID: 5U8S.
Image: Amanda Altieri.

In all organisms, the DNA replication machinery is responsible for accurate and efficient duplication of the chromosome. Inhibitors of replication proteins are commonly used in anti-cancer and anti-viral therapies. This eBook on "The DNA Replication Machinery as Therapeutic Targets" examines the normal functions of replication proteins as well as strategies to target each step during the replication process including DNA unwinding, DNA synthesis, and DNA damage bypass and repair. Articles discuss current strategies to develop drugs targeting DNA replication proteins as well as future outlooks and needs.

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EDITORIAL
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Editorial: The DNA Replication Machinery as Therapeutic Targets

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Editorial on the Research Topic

The DNA Replication Machinery as Therapeutic Targets

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Chromosomal DNA replication is a process conserved through all domains of life. For accurate and efficient duplication of the genetic information, DNA replication components must work together in a highly regulated and coordinated fashion. Due to a central role in cell proliferation, the DNA replication machinery is an attractive therapeutic target for treating bacterial and viral infections, autoimmune disorders, and cancer. This eBook, entitled "The DNA Replication Machinery as Therapeutic Targets" explores how DNA replication factors serve as targets for new generations of therapies.

In all organisms, the chromosomal replication process starts at a specific chromosomal region called an origin of replication, at which origin-binding proteins (OBP) bind and locally unwind the duplex DNA. Additional proteins interact with the OBP-DNA complex and are responsible for the assembly of the DNA helicase around the DNA. Once assembled, the helicase unwinds the duplex in an ATP-dependent manner and forms the initial replication bubble. The exposed short regions of single-stranded DNA (ssDNA) at the replication bubble are coated with ssDNA-binding protein (SSB). DNA primase, DNA polymerases, and the rest of the replication machinery are recruited to the SSB-ssDNA complex to initiate bidirectional DNA synthesis. Due to the antiparallel nature of duplex DNA, and the unidirectionality of DNA polymerases, one strand of the chromosome is synthesized continuously (leading strand) while the other is copied discontinuously (lagging strand) as a series of Okazaki fragments (O'Donnell et al., 2013; Kelman and Kelman, 2014; Kunkel and Burgers, 2017). Although these processes are fundamentally conserved in the three domains: archaea, bacteria and eukarya; as well as viruses and bacteriophages, the proteins, and complexes involved differ (Makarova and Koonin, 2013).

Because the replication machinery is composed of a variety of core proteins and regulatory factors, disruption of any of the proteins involved will inhibit the replication process and/or its efficiency, and lead to replication stress. Replication stress is induced by endogenous factors such as dNTP depletion, DNA secondary structures or crosslinks, or by exogenous chemotherapies that damage DNA, such as cisplatin (Kitao et al., 2018). In human cells, replication stress occurs when DNA polymerases uncouples from the replisome and lags behind helicase unwinding (Zeman and Cimprich, 2014). As a result, long stretches of ssDNA are exposed at the replication fork. Replication protein A (RPA, the eukaryotic SSB) binds to the extended stretches of ssDNA, depleting free RPA in the cell and causing replication fork collapse (which may lead to DNA breakage and cell death). Therefore, inducing replication stress by disrupting the replication machinery or its regulation are an attractive strategies for drug design (O'Connor, 2015; Forment and O'Connor, 2018).

Each chapter in this eBook highlights a different therapeutic strategy to effectively target DNA replication. One approach to halt replication is to inhibit DNA polymerases via binding of small molecules to the active site. The three replicative DNA polymerases responsible for the duplication of chromosomal DNA in eukarya, DNA polymerases α, δ and ε (Pol α, Pol δ, and Pol ε), all belong to family B DNA polymerases. In order to develop nucleotide analogs as drugs, one needs to understand how DNA polymerases incorporate natural and modified nucleotides into DNA. The contribution by Daimon et al. adds to our understanding of the structure and function relationships of family B polymerases by solving the structure of two members of this family from *Aeropyrum pernix* (Daimon et al.). The incorporation of various classes of modified nucleotides and nucleotide terminators by another family B DNA polymerase from archaea is described by a review contributed by Gardner et al. These archaeal polymerases share sequence similarity with the eukaryotic enzymes and thus can serve as good model systems for the more complex eukaryotic replication (Makarova et al., 2014).

In addition, the use of nucleotide analogs to treat human autoimmune disorders and cancer is summarized in a contribution by Berdis. Young explores mitochondrial replication and how incorporation of certain nucleoside chain terminator inhibitors by Pol γ (the mitochondrial-specific polymerase) can lead to unintended toxicity by shutting down mitochondrial genome replication (Young). In addition, certain classes of compounds target Pol γ in cancer cells to inhibit mitochondrial replication with the potential to induce tumor cell death (Young).

Despite a central role in copying the chromosome, the inherent processivity of DNA polymerases is low, and only a few nucleotides are incorporated at a time. However, in the replication complex, high processivity DNA synthesis is conferred by a ring-shaped protein, referred to as the DNA polymerase sliding clamp, that encircles DNA and tethers the polymerase catalytic unit to the DNA for processive DNA synthesis (Indiani and O'Donnell, 2006). The sliding clamps cannot assemble themselves around the DNA and require an additional clamp loader complex that assembles the clamp around duplex DNA in an ATP-dependent manner. In addition to interacting with the polymerase, the sliding clamps of bacteria and eukarya also interact with dozens of other proteins involved in DNA replication, repair, and cell cycle progression (Vivona and Kelman, 2003). Therefore, inhibitors of the bacterial and eukaryal sliding clamps are being developed as anti-cancer and anti-bacterial drugs (Georgescu et al., 2008). The current knowledge on the development of sliding clamps inhibitors and their possible use as therapeutic agents is summarized in a review contribution by Altieri and Kelman.

Another key enzyme for cellular replication is the DNA helicase, the enzyme responsible for unwinding double-stranded DNA ahead of the replisome (Sakakibara et al., 2009). The contribution by Datta and Brosh describes the current state of the art in designing helicase inhibitors as anti-cancer drugs, and the issues surrounding the use of helicase inhibitors (Datta and Brosh). In eukarya, the replicative helicase is a complex of three components, the heterohexameric minichromosome maintenance (MCM), the tetrameric GINS complex and the Cdc45 protein. These form the CMG (Cdc45, MCM, GINS) complex (Onesti and MacNeill, 2013; O'Donnell and Li, 2018). Due to the essential role of CMG in chromosome replication, it is a prime target for anti-cancer drugs. The current efforts in the development of CMG inhibitors as anti-cancer drugs are summarized in a contribution by Seo and Kang. Instead of directly inhibiting an enzyme activity (such as DNA polymerase), another strategy is to deplete activity by downregulating gene expression or deregulating protein activity via the ubiquitination pathway (Jang et al.).

In addition, while some drugs are effective on their own, in other cases multiple replication factors can be targeted simultaneously to disrupt multiple pathways and lead to more efficient and effective treatment strategies. *Mycobacterium tuberculosis* is a pathogenic bacterium that is the etiological agent of tuberculosis (TB), which kills more than a million people a year (Bañuls et al., 2015). Reiche and coauthors summarize the current state of the development of drugs against the *M. tuberculosis* replication machinery, including drugs targeting the polymerase (Pol III), the sliding clamp, clamp loader, and other replication proteins (Reiche et al.). In addition, Reiche et al. demonstrate the increased effectiveness of a combination *M. tuberculosis* antibiotic strategy that depletes dNTP pools while inhibiting DNA polymerase activity (Reiche et al.). Another example of a combination strategy is the inhibition of DNA polymerase synthesis with nucleotide inhibitors in combination with DNA damaging agents to create DNA lesion that stall synthesis (Berdis).

REMAINING CHALLENGES AND FUTURE OPPORTUNITIES

Despite effective inhibitors of DNA replication proteins, eventual resistance to these inhibitors leads to tumor recurrence and remains a challenge for long-term therapeutic efficacy. Therefore, it will be important to continue to study molecular mechanisms of tumor resistance to DNA replication inhibitors. For example, DNA polymerase mutants that effectively remove nucleotide chain terminators can lead to drug resistance, as can upregulation of lesion bypass DNA polymerases (Berdis).

We anticipate that knowledge of DNA replication protein expression, regulation, and biochemical properties will continue to address these challenges and accelerate the development of novel strategies for effective treatment. To reach potential as therapeutic targets, more high-resolution structural information is needed for all replisome proteins and complexes to understand important replisome active site architectures and protein interactions. High resolution replisome structures will enable models for docking small molecules to inhibit enzyme activities and disrupt essential replisome interactions.

Finally, new molecular tools will accelerate identification of new DNA replication drugs targets. CRISPR-Cas9 genome engineering tools have revolutionized many scientific disciplines and offer a powerful method to alter genes by

either modifying gene sequence or introducing insertions or deletions to knock out gene function (Doudna and Charpentier, 2014). Genome-wide CRISPR-Cas9 screens aim to disrupt all or a subset of genes in an organism to identify important genes in a pathway (Sánchez-Rivera and Jacks, 2015; Peters et al., 2016). CRISPR-Cas9 genome-wide screens can be adapted to identify novel factors that confer either resistance or sensitivity to DNA replication inhibitors. Knowledge of these factors may inform future therapeutic strategies to design new drug classes or enhance the efficacy of current therapies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Two Family B DNA Polymerases From *Aeropyrum pernix*, Based on Revised Translational Frames

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Living organisms are divided into three domains, Bacteria, Eukarya, and Archaea. Comparative studies in the three domains have provided useful information to understand the evolution of the DNA replication machinery. DNA polymerase is the central enzyme of DNA replication. The presence of multiple family B DNA polymerases is unique in Crenarchaeota, as compared with other archaeal phyla, which have a single enzyme each for family B (PolB) and family D (PolD). We analyzed PolB1 and PolB3 in the hyperthermophilic crenarchaeon, *Aeropyrum pernix*, and found that they are larger proteins than those predicted from the coding regions in our previous study and from public database annotations. The recombinant larger PolBs exhibited the same DNA polymerase activities as previously reported. However, the larger PolB3 showed remarkably higher thermostability, which made this enzyme applicable to PCR. In addition, the high tolerance to salt and heparin suggests that PolB3 will be useful for amplification from the samples with contaminants, and therefore it has a great potential for diagnostic use in the medical and environmental field.

Keywords: Archaea, DNA replication, DNA polymerase, heat-stable enzyme, PCR

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INTRODUCTION

DNA polymerase catalyzes phosphodiester bond formation between the terminal 3′-OH of the primer and the α-phosphate of the incoming triphosphate to extend the DNA strand based on the template DNA sequence. Numerous DNA polymerases have been reported since *Escherichia coli* DNA polymerase I was first identified in 1956 (Bessman et al., 1956). DNA polymerases are now classified into seven families, A, B, C, D, E, X, and Y, based on the amino acid sequence similarity (Braithwaite and Ito, 1993; Cann and Ishino, 1999; Ohmori et al., 2001; Lipps et al., 2003). For example, *E. coli* has five DNA polymerases. Pol I, II, and III belong to families A, B, and C, respectively, and Pol IV and Pol V are classified in family Y, which mostly includes the DNA polymerases for translesion synthesis (TLS). The bacterial PolIII and the eukaryal Pols α, δ, and ε, the essential replicases for the DNA replication process, are family C and family B enzymes, respectively. The eukaryotic enzymes for TLS, including Pol η, ι, and κ, belong to family Y, as do those in the bacterial domain. The fundamental ability to incorporate a deoxymononucleotide into the DNA strand is generally conserved among the DNA polymerases. However, the specific properties, such as processivity, fidelity, and substrate nucleotide selectivity, vary depending on the families (Ishino and Ishino, 2013).

The distribution and functional sharing of DNA polymerases in Archaea, the third domain of life, have been analyzed since the early 1990s. It was exciting to find a gene encoding a eukaryotic

-like family B DNA polymerase in Archaea (Perler et al., 1992; Pisani et al., 1992; Uemori et al., 1993), and furthermore, two different family B DNA polymerases were identified in the archaeal genome of *Pyrodictium occultum* (Uemori et al., 1995). These findings, reported before the total genome sequencing, provided strong motivation to study DNA replication in Archaea, because the DNA replication system consisting of multiple family B DNA polymerases may be conserved between Archaea and Eukarya. However, some archaea have only one family B DNA polymerase, and as an alternative, their genomes encode an archaea-specific DNA polymerase, which has never been found in Bacteria and Eukarya (Uemori et al., 1997; Ishino et al., 1998). This DNA polymerase, originally discovered in *Pyrococcus furiosus*, consists of two proteins, DP1 and DP2, and their deduced amino acid sequences are not conserved in the DNA polymerase families. Therefore, this DNA polymerase was proposed to be PolD, from the family D (Cann and Ishino, 1999). PolD has now been found in the genomes of all of the archaeal phyla, except for Crenarchaeota (Ishino and Ishino, 2014). Based on our current knowledge about the distribution of DNA polymerases in Archaea, one PolB and one PolD are present in most archaeal cells, but two PolBs, without PolD are probably working in crenarchaeal cells. Therefore, one of the questions to be clarified in archaeal DNA replication is how the two PolBs share the functions in Crenarchaeota.

After our finding of two family B DNA polymerases in *P. occultum*, as described above (Uemori et al., 1995), we also found two DNA polymerase activities in the cell extracts of *Aeropyrum pernix*, another hyperthermophilic crenarchaeon, and their gene sequences revealed that both of these DNA polymerases belong to family B (Cann et al., 1999). The remarkable difference was their aphidicolin sensitivity and heat stability *in vitro*. Interestingly, a third gene encoding a family B DNA polymerase was found in the *Sulfolobus solfataricus* genome, and the three enzymes were designated as PolB1, B2, and B3 (Edgell et al., 1997). Only PolB1 and PolB3 contain the conserved exonuclease and polymerase motifs (Rogozin et al., 2008). The two enzymes, designated as PolI and PolII from *P. occultum* and *A. pernix* in our previous reports, were PolB1 and PolB3, respectively, and the euryarchaeal single PolBs belong to PolB3 (Rogozin et al., 2008).

The functions of these family B DNA polymerases in the crenarchaeal cells have not been elucidated, and thus further studies are required. In this study, we analyzed PolB1 and PolB3 in *A. pernix* cells, and found that both enzymes are produced as longer peptide chains than those predicted from their coding regions shown in our previous report. We prepared the longer PolB1 and PolB3 as recombinant proteins, and characterized them *in vitro*.

MATERIALS AND METHODS

Sequence Analysis

The *A. pernix* genome DNA sequence is available in GenBank (accession number: BA000002). Homologs of PolB1 and PolB3 were retrieved from the reference sequence database at the National Center for Biotechnology Information (NCBI), using BlastP with *A. pernix* PolB1 (APE0099) and PolB3 (APE2098.1) as queries. Among the 71 complete genome sequences of crenarchaeal organisms, we searched for possible initiation sites (GTG or TTG) in the nucleotide sequences upstream of the annotated ORFs of PolB1 and PolB3 in 24 species. Multiple alignments were performed with the MAFFT online service (Katoh et al., 2017), and the conserved motifs were manually identified.

Cloning of the DNA Polymerase Genes From *A. pernix*

The five genes were amplified by PCR directly from *A. pernix* genomic DNA, using the primer sets PolB1LF/PolB1R, PolB1MF/PolB1R, PolB1SF/PolB1R, PolB3LF/PolB3R, and PolB3SF/PolB3R for PolB1L, PolB1M, PolB1S, PolB3L, and PolB3S, respectively (Supplementary Table 1). *P. furiosus* DNA polymerase B (PfuPolB) was prepared as described previously (Komori and Ishino, 2000). Each fragment amplified by PfuPolB was digested by NdeI and NotI (New England Biolabs), and ligated into the corresponding sites of the pET-21a(+) expression vector (Novagen) by T4 DNA ligase (New England Biolabs), and the inserted sequences were confirmed.

Preparation of the Recombinant Proteins

Each recombinant protein was produced in *E. coli* BL21-CodonPlus (DE3)-RIL cells (Agilent). The transformed *E. coli* cells were grown in LB medium, containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol, at 37°C until the culture attained an OD_{600} of 0.5. Isopropyl β-D-thiogalactopyranoside was then added to a final concentration of 0.5 mM, and the cells were further grown for 18 h at 25°C. The cells were harvested and disrupted by sonication in buffer A (50 mM Tris-HCl, pH 8.0, 0.5 mM DTT, 0.1 mM EDTA, and 10% glycerol) containing 0.5 M NaCl. The crude protein samples, containing each overproduced DNA polymerase, were subjected to a western blot analysis. For further purification of ApePolB1L and ApePolB1S, the soluble cell extract was heated at 60°C for 20 min. The heat-resistant fraction was treated with 0.15% polyethyleneimine to remove the nucleic acids. The soluble proteins were precipitated by 80%-saturated ammonium sulfate. The precipitate was resuspended in buffer A containing 1.4 M $(NH_4)_2SO_4$ and subjected to chromatography on a HiTrap Phenyl HP column (GE Healthcare), which was developed with a 1.4–0 M $(NH_4)_2SO_4$ gradient in buffer A. The fraction containing ApePolB1 was dialyzed against buffer B (50 mM Tris-HCl, pH 8.6, 0.5 mM DTT, 0.1 mM EDTA, and 10% glycerol) containing 0.1 M NaCl. The dialysate was loaded onto a HiTrap Q HP column (GE Healthcare), which was developed with a 0.1–1 M NaCl gradient in buffer B. For the purification of ApePolB3L and ApePolB3M, the soluble cell extract was heated at 75°C for 20 min. The heat-resistant fraction was then treated with 0.15% polyethyleneimine. The soluble proteins were precipitated by 80%-saturated ammonium sulfate. The precipitate was dialyzed against buffer A containing 0.1 M NaCl and applied to a HiTrap SP HP column (GE Healthcare), which was developed with a 0–1 M NaCl gradient in buffer A. The protein concentrations were calculated by measuring the absorbance

at 280 nm, with theoretical extinction coefficients of 121,590, 118,610, 126,630, and 125,140 $M^{-1}cm^{-1}$ for ApePolB1L, ApePolB1S, ApePolB3L, and ApePolB3S, respectively, based on the tryptophan and tyrosine contents (Gasteiger et al., 2003).

Western Blot Analysis

The anti-PolB1 and anti-PolB3 antisera were prepared by immunizing rabbits with the recombinant PolB1S and PolB3S proteins, respectively. *A. pernix* K1 was cultivated as described previously (Sako et al., 1996). The cells (0.1 g) were disrupted by sonication in 2 ml buffer, containing 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 1 mM EDTA. The whole cell extracts and the crude recombinant proteins were subjected to SDS-12% PAGE, and the gel was run for a long duration to separate each band. The proteins were transferred onto PVDF membranes (Bio-Rad), which were incubated with the anti-PolB1 and anti-PolB3 antisera. The protein bands were reacted with Immobilon (Millipore), and detected with an LAS-3000mini image analyzer (FUJIFILM).

Nucleotide Incorporation Assay

The nucleotide incorporation assay was performed basically as described previously (Uemori et al., 1995). The reactions, containing 25 mM Tris-HCl, (pH 8.6 for PolB1 and pH 8.0 for PolB3), 60 mM NaCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1 mg/ml BSA, 2 mM $MgCl_2$, 2 mM DTT, 0.2 mM each dNTP, 10 μCi/ml [methyl-^{3}H]dTTP, 0.2 mg/ml activated salmon sperm DNA, and 20 nM DNA polymerase, were incubated at 70 and 50°C. Afterwards, 10 μl of each reaction mixture was spotted onto DE81 filters (GE Healthcare). The filters were washed with a 5% Na_2HPO_4 solution thrice, and were dried. The incorporated radioactivity was measured with a scintillation counter (Aloka). Reactions without enzyme were performed as negative controls.

Polymerase Chain Reaction

PCR performances under various conditions were assessed, using λ phage DNA (Takara Bio) as the template and the primers to generate 1-kbp products (5′-dGAGTTCGTGTCCG TACAACTGGCGTAATCATGGCC-3′ and 5′-dCTTTTCAGC CTGGCCCTTTCCTTTACC-3′). The basal PCR solution for ApePolB3 contained 100 mM Tris HCl, pH 8.8, 2 mM $MgSO_4$, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1 % Triton X-100, 0.1 mg/ml BSA, 0.2 mM dNTPs, and 0.4 μM each primer in a final volume of 50 μl. The basal PCR solution for PfuPolB contained 20 mM Tris HCl, pH 8.8, 2 mM MgSO4, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1 % Triton X-100, 0.1 mg/ml BSA, 0.2 mM dNTPs, and 0.4 μM each primer in a final volume of 50 μl. The modified conditions are described in each section. Cycles of PCR, consisting of denaturing at 98°C (10 s), annealing at 55°C (0.5 min), and extension at 72°C (1.5 min), were performed. The products were fractionated by 0.8% agarose gel electrophoresis and stained with ethidium bromide. The PCR fidelity assay was performed using the modified pTV119N plasmid (Takara Bio), containing the *lacZα* gene. The NcoI- and XhoI-recognition sequences were introduced to the outside edges of the *lacZα* gene and the original NcoI-recognition site was replaced with an NdeI-recognition site. The resultant plasmid was designated as pTV119NNX. The 483-bp PCR target region was amplified using 0.4 μM each of NF (5′-dCTGGCACGACAGGTTTCCATGGTGG-3′) and XR (5′-dCGTCATCACCGAAACGCTCGAGACG-3′) as the primer set (NcoI and XhoI sites are underlined) and 5 ng of pTV119NNX as the template. Twenty cycles of PCR, consisting of denaturing at 98°C (10 s), annealing at 56°C (20 s), and extension at 72°C (30 s), were performed, using 20 nM ApePolB3 and 20 nM PfuPolB in each basal PCR solution. Each amplified fragment was excised by NcoI and XhoI and inserted into the corresponding sites of pTV119NNX. *E. coli* JM109 cells were transformed with each ligation mixture, and were spread onto LB agar plates containing 50 μg/ml ampicillin, 1 mM IPTG, and 100 μg/ml X-gal. White and pale blue colonies were counted as mutated products while darker blue colonies were considered as intact products. In the amplified region, the 345 bp-fragments corresponded with the expression and function of *lacZα*. As a background control, excised fragments from pTV119NNX were re-ligated into the corresponding sites of pTV119NNX. White colonies were randomly subjected to colony PCR using the NF/XR primers, to confirm the insertion of the fragment.

Exonuclease Assays

A primed DNA was prepared by annealing 5′-Cy5-labeled 5′-d CGAACTGCCTGGAATCCTGACGACATGTAGCG-3′ and 5′-dTGAGGTGATCGTTCGCTACATGTCGTCAGGATTC CAGGCAGTTCG-3′, in 20 mM Tris–HCl, pH 8.0 and 2 mM $MgCl_2$. The ApePolB3 and PfuPolB nuclease reactions were performed with 10 nM substrate in each basal PCR solution, without dNTPs. The reaction mixture was incubated at 70°C and terminated by adding an equal volume of stop solution (98% formamide, 20 mM EDTA, and 0.01% orange G). Aliquots were subjected to 12% PAGE containing 8 M urea in TBE. The gel images were visualized with a Typhoon Trio + (GE Healthcare) image analyzer.

RESULTS

Search for the Open Reading Frames of *polB1* and *polB3*

In our previous work, two different DNA fragments were amplified from *A. pernix* K1 by PCR, using the degenerate primers based on motif A (SLYPSII) and motif C (VIYGDTD), which are conserved in the family B DNA polymerases, for the forward and reverse sequences, respectively. Approximately 400-bp fragments were amplified from *A. pernix* genomic DNA, and further genomic walking provided the entire structural gene for each *pol*. We deduced the longest in-frames to be the coding regions from their nucleotide sequences, with ATG as the initiation codon. After our analyses, the total genome sequence of *A. pernix* was published and 2,700 ORFs were predicted (Kawarabayasi et al., 1999). Since then, the ORFs were reannotated to 1,871 genes in 2000 (Natale et al., 2000), and furthermore re-evaluated to 1,610

genes in 2004 (Guo et al., 2004). In addition to these re-evaluations, proteome analyses using 2D-gel electrophoresis and mass spectrometry assigned 704 proteins (Yamazaki et al., 2006). One surprising finding was that many proteins (52%) use TTG, rather than ATG (28%) and GTG (20%), as the translational initiation codon (Yamazaki et al., 2006). However, neither of the two DNA polymerases was included in those studies. Each DNA polymerase has multiple candidates for its translational initiation codon, and these structural genes were still unclear even after the annotation accuracy was improved. We searched for the upstream sequences of the *polB1* and *polB3* genes. As shown in **Figure 1**, we found TTG and GTG, which were in-frame with our original ATG codon for the *polB1* gene. The TTG and GTG extended the ORFs with 58 and 36 amino acids, respectively. For the *polB3* gene, one TTG was found to be in-frame, extending it by 19 upstream amino acids. The amino acid-sequence alignment revealed that the N-terminal portions of the PolB1 homologs had divergent lengths, as well as sequences. The length of the extended PolB1 from *A. pernix* corresponds with those from closely related species (Supplementary Figure 1).

Identification of the Native PolB1 and PolB3 in the *A. pernix* Cell Extract

The recombinant proteins were prepared from *E. coli* cells in our previous study. To identify PolB1 and PolB3 in the *A. pernix* cell extract, anti-PolB1, and anti-PolB3 antibodies were prepared by immunizing rabbits with these purified proteins. The prepared antibodies were used for the western blot analysis to detect the native PolB1 and PolB3 bands from the *A. pernix* cell extract. Three and two ORFs were predicted for PolB1 and PolB3, respectively, as described above. All of these ORFs were cloned and expressed in *E. coli* to prepare each recombinant protein, designated as PolB1L (long), B1M (middle), B1S (short), B3L (long), and B3S (short). To compare the sizes of the bands detected by the western blot analysis, total cell extracts from each recombinant *E. coli* strain were used in parallel with the *A. pernix* cell extract. As shown in **Figure 2**, the protein bands corresponding to PolB1 and PolB3 were detected in the *A. pernix*

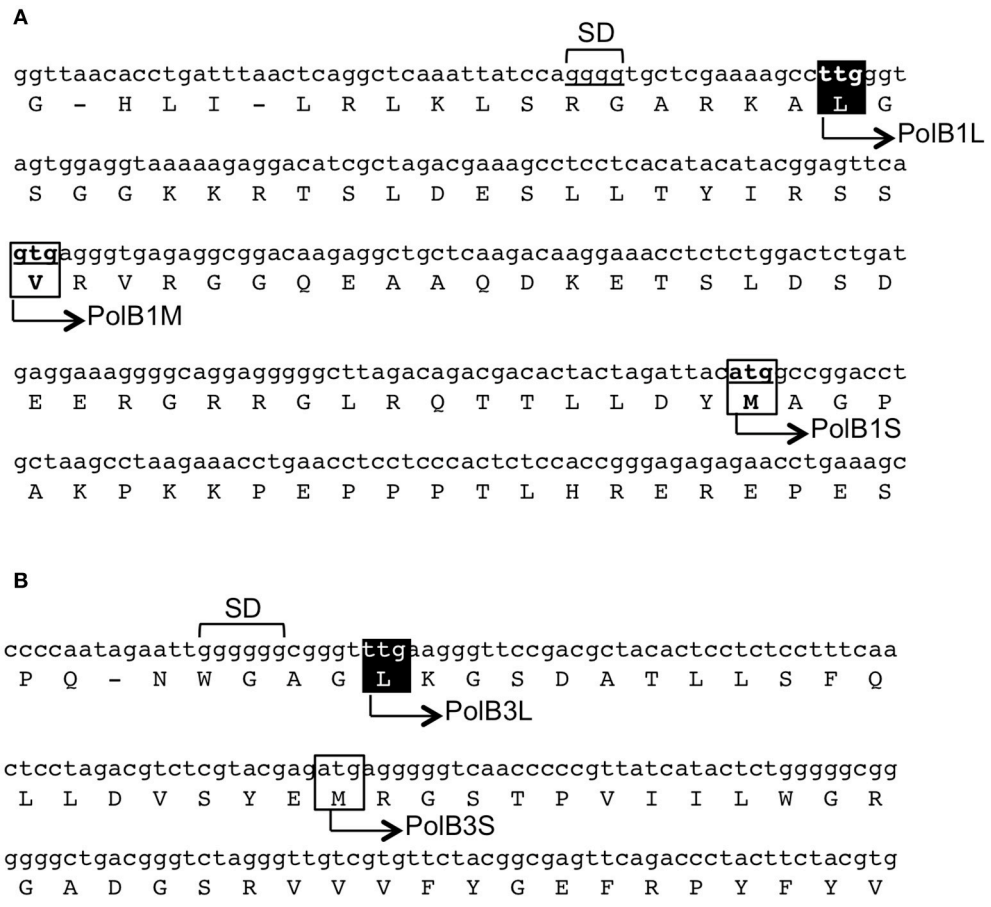

FIGURE 1 | Regions upstream of the initiation sites of the *polB1* **(A)** and *polB3* **(B)** genes from *A. pernix*. The nucleotide sequences of the genome (GenBank, BA000002.3) with the corresponding amino acids are shown. The boxes show the initiation codon candidates. The names of the recombinant proteins in this report are shown on the sequence. The identified initiation sites in this report are indicated in white over a black background. SD indicates the putative Shine–Dalgarno sequences.

cell extract with sizes equivalent to the recombinant PolB1L and PolB3L, respectively. These results suggest that the native PolB1 and PolB3 are produced by the expression of the longest ORFs in *A. pernix* cells.

Purification of Recombinant Proteins Based on the Predicted ORFs

We tried to purify all of the recombinant proteins for the two DNA polymerases. As shown in **Figure 2**, all five of the proteins were produced in *E. coli* cells. However, these proteins were mostly insoluble, and especially, PolB1M was not obtained in the soluble fraction at all. Therefore, we proceeded with the purifications of PolB1L, PolB1S, PolB3L, and PolB3S from each soluble fraction, and finally purified these recombinant proteins to homogeneity, as shown in **Figure 3**.

Comparison of the Specific Activities

The activities of the purified recombinant PolB1 and PolB3 proteins were compared using a nucleotide incorporation assay, as described in the Materials and Methods. As shown in **Figure 4**, the DNA polymerase activity was the same between the long and short proteins for both PolB1 and PolB3. These results suggest that the N-terminal extended portions are not directly involved in the catalytic activities of both PolB1 and PolB3.

Comparison of the Heat Stabilities

To investigate the functions of the N-terminal extended portions, the heat stability was compared between the long and short proteins for both PolB1 and PolB3. The heat-dependent decrease of the DNA polymerase activity was mostly the same between PolB1L and PolB1S, and both completely lost the activity upon an incubation at 80°C for 30 min (**Figure 5A**). However, PolB3L showed remarkable thermo-tolerance, and it retained full activity even after an incubation at 100°C for 30 min, in contrast to PolB3S, which gradually lost the activity with increasing temperature (**Figure 5B**). These results clearly indicated that the N-terminal 19 amino acids are critically important for the stable folding of the PolB3 protein.

Salt Tolerance

The remarkable thermo-tolerance of PolB3L, as described above, suggested that this DNA polymerase may be applicable for PCR. DNA polymerases, including practically used PCR enzymes, are generally sensitive to salt, and the activity decreases with increasing concentrations of NaCl in the reaction mixture *in vitro*. To investigate the salt sensitivity of PolB3L, we compared its DNA polymerase activity with that of *P. furiosus* PolB (a well-known commercial PfuDNA polymerase), which is also highly heat stable, with increasing concentrations of NaCl. As shown in **Figure 6**, PolB3L showed much higher tolerance to NaCl than PfuPolB.

PCR Performance of PolB3L

The above properties suggested that PolB3L may be suitable for PCR using dirty DNA samples. We used PolB3L from *A. pernix* (ApePolB3) for a standard PCR to amplify a 1 kb DNA fragment. Its PCR performances were compared with those of PfuPolB. As shown in **Figures 7A,B**, ApePolB3 amplified the target DNA in the presence of salt concentrations ranging from 0 to 100 mM NaCl and 0 to 120 mM KCl. In contrast, the amplification by PfuPolB was obviously inhibited from 40 mM of either NaCl or KCl. Furthermore, ApePolB3 was much more tolerant to heparin as compared with PfuPolB (**Figure 7C**). The apparent mutation frequencies were measured by the amplification of the *lacZα* gene in the plasmid. The apparent error rates of ApePolB3 and PfuPolB were $(2.6 \pm 0.5) \times 10^{-5}$ and $(1.3 \pm 1.6) \times 10^{-6}$, respectively (**Table 1**).

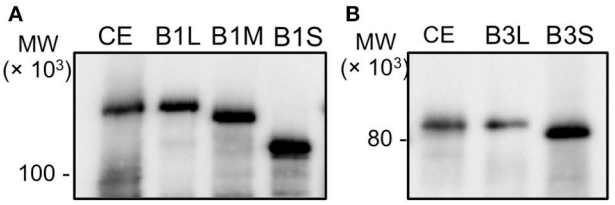

FIGURE 2 | The band mobilities of PolB1 and PolB3 between the cell extracts and the recombinant proteins. Western blotting analyses using anti-PolB1 **(A)** and anti-PolB3 **(B)** are shown. CE indicates crude extracts from 75 μg *A. pernix* cells. B1L, B1M, and B1S indicate the recombinant PolB1 proteins. B3L and B3S indicate the recombinant PolB3 proteins. Pre-stained protein size markers were run in the same gels, and their sizes are indicated on the left.

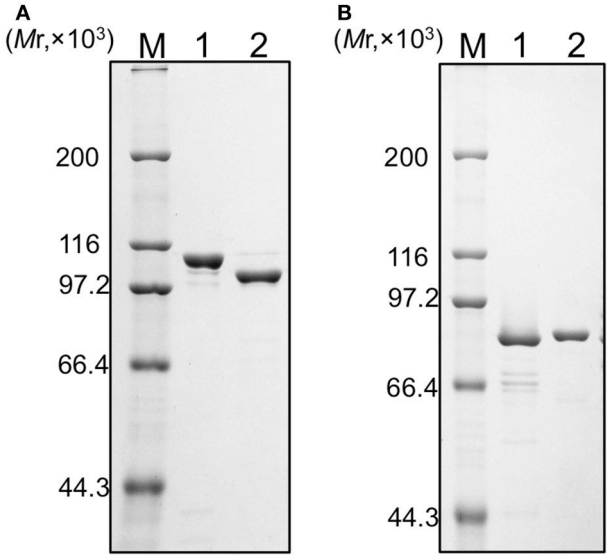

FIGURE 3 | Purification of the recombinant proteins. **(A)** PolB1 proteins (M, protein marker; 1, PolB1L; 2, PolB1S), **(B)** PolB3 proteins (M, protein marker; 1, PolB3S; 2, PolB3L). Purified proteins (1 μg each) were subjected to SDS-10% PAGE followed by Coomassie Brilliant Blue staining. Protein size markers were run in lane M, and their sizes are indicated on the left of the gels.

Exonuclease Activity of PolB3L

To analyze the difference in the apparent error rates between ApePolB3 and PfuPolB in more detail, the 3′-5′ exonuclease activities with ApePolB3 and PfuPolB were compared under

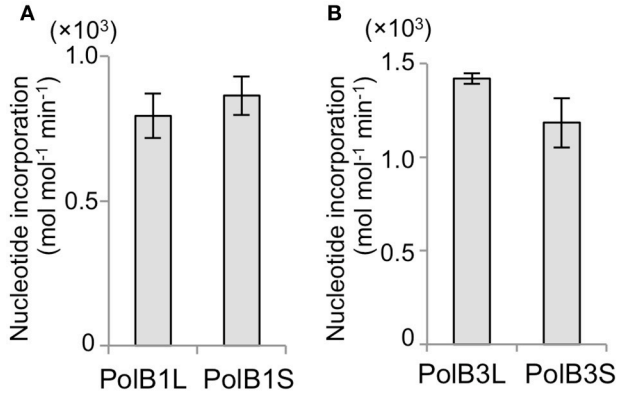

FIGURE 4 | Nucleotide incorporation activities. **(A)** The nucleotide incorporations by 20 nM PolB1 were measured at 50°C for 3 min. Error bars indicate the SEM from three replicates. **(B)** The PolB3 proteins (10 nM) were incubated at 70°C for 3, 6, and 9 min. Error bars indicate the SEM from three replicates.

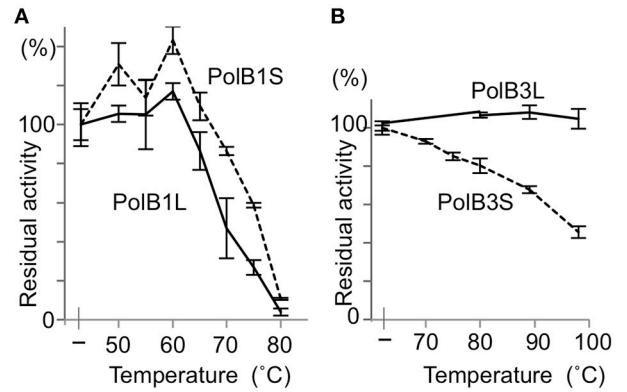

FIGURE 5 | Heat tolerance of DNA polymerases from *A. pernix*. **(A)** The PolB1 proteins were incubated at the indicated temperature for 5 min. **(B)** The PolB3 proteins were incubated at the indicated temperature for 30 min. The residual activities were measured by the nucleotide incorporation assay, using 20 nM PolB1 at 50°C for 2.5 min, and 10 nM PolB3 at 70°C for 5 min. Error bars indicate the SEM from three replicates.

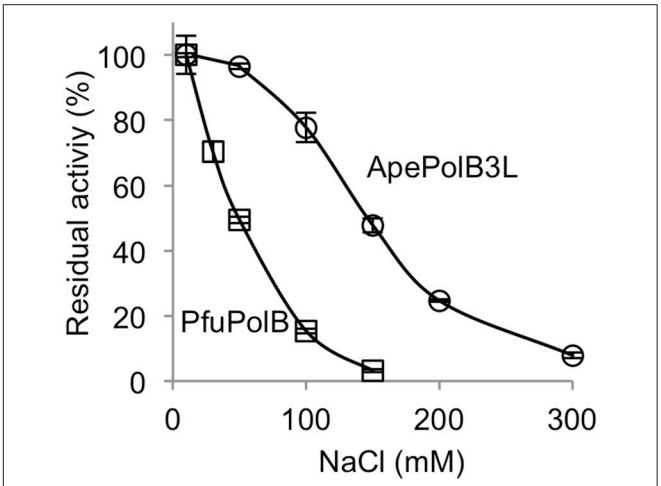

FIGURE 6 | Salt tolerance of DNA polymerases from *A. pernix* and *P. furiosus*. Nucleotide incorporation assays were performed in the presence of various concentrations of NaCl. ApePolB3L and PfuPolB (each 20 nM) were incubated at 70°C for 5 min. Error bars indicate the SEM from three replicates.

each PCR condition, using a 5′-labeled primer and a template substrate. As shown in **Figure 8**, 20 nM PfuPolB degraded the substrate completely in 1 min. In contrast, 20 nM ApePolB3 only degraded a few nucleotides in 5 min. This result is consistent with the difference in the apparent error rates between the two DNA polymerases. It is currently unknown why the exonuclease activity of ApePolB3 is distinctly weak, as compared with that of PfuPolB, even though the exonuclease motifs are well conserved (Supplementary Figure 2B; Blanco et al., 1991).

DISCUSSION

Aeropyrum pernix K1 is an hyperthermophilic crenarchaeon that was isolated from Kagoshima, Japan, in 1996 (Sako et al., 1996). It aerobically grows at 95°C, and we were interested in how this organism replicates its genomic DNA at such a high temperature. We predicted an ATG, which provided the longest ORF, as the initiation codon to produce recombinant proteins for both DNA polymerases before the genomic era (Cann et al., 1999). Here, we deduced the initiation codons for the translation of these genes more precisely. There is broad variation in both the transcriptional and translational signals in Archaea. In contrast to bacteria, many archaea show wide variations in their consensus Shine–Dalgarno (SD) sequences and frequently use GUG and UUG start codons. The structural genes are generally preceded by SD motifs between positions −15 and −4 in *A. pernix* (Torarinsson et al., 2005). In addition, the significant enrichment of G at position −3 is characteristic for *A. pernix* (Torarinsson et al., 2005). These features are also found in these two structural genes (**Figure 1**). As described above, *A. pernix* K1 is the first example of an organism, in which TTG is the most predominant translational initiation codon (Yamazaki et al., 2006), as in the two *pol* genes. The precise molecular mechanism of the archaeal translation initiation has not been elucidated, and it is still an interesting question why TTG is most frequently used as the initiation codon in *A. pernix*. The isolation and characterization of the initiation $tRNA_i$ will provide some clues for this answer.

What are the functions of the N-terminal portions in PolB1 and PolB3? No difference in the specific activities was detected between PolB1L and PolB1S, and also between PolB3L and PolB3S, indicating that the N-terminal portions are not directly involved in the catalytic function. A remarkable result in this study is that the heat stability of PolB3 was drastically increased with the addition of the N-terminal 19 amino acids (PolB3L), as compared with the protein from ORF from ATG (PolB3S). This difference suggests that the N-terminal 19 amino acids contribute to the stable folding of the PolB3 protein.

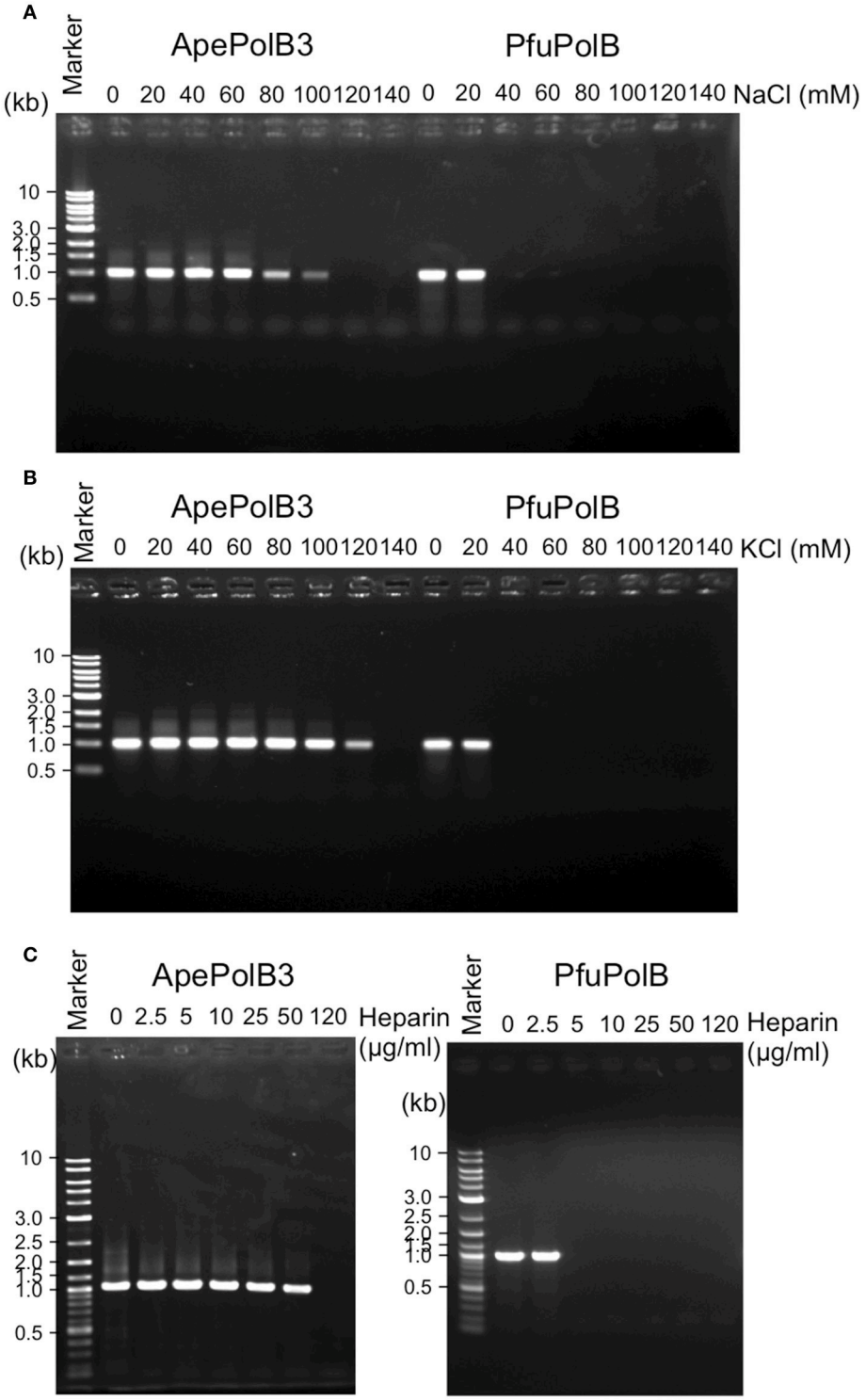

FIGURE 7 | PCR performance of ApePolB3 and PfuPolB. **(A)** and **(B)** Thirty cycles of PCR under each basal condition without any salt including 20 nM DNA polymerase and 0.8 ng λ DNA were performed. The final concentrations of NaCl **(A)** and KCl **(B)** in the reaction solutions are indicated. **(C)** Inhibitory effects of heparin on PCR were assessed with the indicated concentrations of heparin. Thirty cycles of PCR under each basal condition including 30 nM DNA polymerase and 0.8 ng λ DNA were performed.

It is well known that archaeal family B DNA polymerases have the uracil pocket, which functions to stall synthesis four bases ahead of uracil in the template DNA (this is called the read-ahead function) (Fogg et al., 2002). The amino acids involved in the uracil pocket are highly conserved in these DNA polymerases. The completely conserved Tyr, which is important for the read-ahead function, is present in PolB3L but not in PolB3S (Supplementary Figure 2A), supporting the proposal that PolB3L is the native PolB3 in *A. pernix*.

The lengths and sequences of the N-terminal portions of the PolB1 homologs are especially divergent (Supplementary Figure 1), and their function is currently unknown. A recent report showed that *S. solfataricus* PolB1 is a heterotrimeric enzyme, by the identification of two associated proteins, PBP1 and PBP2 (Yan et al., 2017). The *S. solfataricus* PolB1 complex with these proteins is more heat-stable than PolB1 alone. Their structural analysis showed that PBP1 interacts with the N-terminal and Exonuclease domains, and PBP2 interacts with Thumb domain, located in the C-terminal region. This report described that *A. pernix* also has PBP1 and PBP2 homologs in the genome. However their homologies to these *S. solfataricus* proteins are not obvious, probably because the N-terminal regions of the PolB1 proteins are highly divergent. The heat labile property of *A. pernix* PolB1L may be compensated by PBP1 and PBP2. Furthermore, it is possible that the remarkably long N-terminal region of *A. pernix* PolB1L plays a role in interacting with some other protein factors.

TABLE 1 | Apparent PCR fidelity of ApePolB3 and PfuPolB.

	Total colonies	Mutant colonies	Mutant frequency (mf)[a]	Error rate[b]
ApePolB3-1	9,810	966	0.0896	2.0×10^{-5}
ApePolB3-2	2,422	234	0.0881	2.0×10^{-5}
ApePolB3-3	2,065	361	0.1488	3.4×10^{-5}
ApePolB3-4	2,167	280	0.1144	2.6×10^{-5}
ApePolB3-5	2,923	396	0.1193	2.7×10^{-5}
PfuPolB-1	2,019	51	0.0246	4.5×10^{-6}
PfuPolB-2	1,929	23	0.0118	1.4×10^{-6}
PfuPolB-3	2,376	17	0.0071	2.5×10^{-7}
PfuPolB-4	2,900	20	0.0068	1.9×10^{-7}
PfuPolB-5	2,159	16	0.0074	3.1×10^{-7}
Background	3,599	22	0.0061	

The data of each DNA polymerase are from five independent assays. [a]Mutation frequency (mf) is the ratio of the number of mutant colonies vs. the total number of colonies. [b]Error rate was determined using the equation ER = (mf_sample − mf_background)/(bp × d), where mf is the mutation frequency, bp is the lacZα target size (345 bp), and d is the number of template doublings (12.0) Template doublings were calculated using the equation 2^d = (amount of PCR product)/(amount of starting target).

DNA polymerase is one of the most widely used enzymes in genetic engineering. Especially, thermostable DNA polymerases are valuable for applications to PCR-related technologies (Terpe, 2013; Ishino and Ishino, 2014). High heat resistance is required for PCR enzymes, and only the DNA polymerases from extreme thermophiles or hyperthermophiles, but not moderate thermophiles, can be used for this purpose. There are many commercial DNA polymerases from *Thermococcales* in Euryarchaeota, but no crenarchaeal DNA polymerase has been practically used for PCR so far, although several enzymes from *Pyrobaculum* and *Ignicoccus* are reportedly applicable (Kähler and Antranikian, 2000; Ali et al., 2011; Seo et al., 2014). The heat-stability of ApePolB3 is actually sufficient for applications to PCR, and this enzyme is more tolerant to salt and heparin. PolB3S is not applicable for PCR. However, we measured the nucleotide incorporation activity of PolB3S in the presence of 50 mM and 100 mM NaCl, and found the same tolerance as that of PolB3L. Therefore, this property seems to be derived from PolB3S, but not from addition of the N-terminal region. This property is especially advantageous to amplify target DNA fragments from environmental and medical samples with various contaminants. Our simple fidelity test revealed that ApePolB3 is less accurate

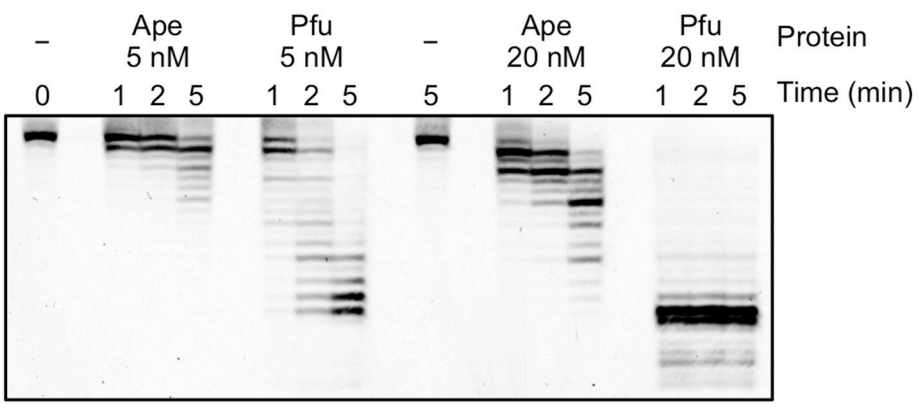

FIGURE 8 | Exonuclease activities of ApePolB3 and PfuPolB. Time course experiments were performed, using 10 nM Cy5-labeled primed DNA. The indicated amounts of ApePolB3 (Ape) and PfuPolB (Pfu) were incubated in the PCR solution without dNTPs at 70°C. For each time point, aliquots were removed from the reaction and quenched by 20 mM EDTA and formamide. The samples were subjected to 12% PAGE, containing 8 M urea, and the degradation products were visualized with a TyphoonTrio imager (GE Healthcare).

than PfuPolB, one of the most accurate PCR enzymes. However, the fidelity of DNA polymerases *in vitro* varies with differences in the reaction conditions, and the fidelity of ApePolB3 may increase under different conditions. For example, fidelity of DNA polymerase from *P. abyssi* varied depending on the reaction condition (Dietrich et al., 2002). Furthermore, the more important feature of the PCR enzymes for DNA typing is the efficient amplification of marker genes with precise lengths, and a single base substitution would not have a serious effect on judgment. Actually, the fidelity of *Taq* polymerase, commonly used for this purpose, is much lower than $(2.6 \pm 0.5) \times 10^{-5}$ shown for PolB3L in this study.

In conclusion, our discovery of PolB3 with native length in *A. pernix,* possessing the remarkable heat-stability and tolerance to salts and heparin, suggests its great potential for the application of this DNA polymerase as a PCR enzyme for diagnostic use from the samples in the medical field and various environments. Furthermore, this study of Family B DNA polymerases from *A pernix* will contribute to understanding of the DNA polymerase active site. In addition, this study may aid in designing of nucleoside inhibitors as therapeutics against viral DNA polymerases and mutants that are resistant to inhibitors.

AUTHOR CONTRIBUTIONS

YI and SI: Conception and design of study; KD, SI, NI, SN, TY, and HM: Acquisition of data; KD, SI, and YI: Analysis and/or interpretation of data; SI and YI: Preparation of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2018.00037/full#supplementary-material

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REVIEW
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Therminator DNA Polymerase: Modified Nucleotides and Unnatural Substrates

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A variant of 9°N DNA polymerase [Genbank ID (AAA88769.1)] with three mutations (D141A, E143A, A485L) and commercialized under the name "Therminator DNA polymerase" has the ability to incorporate a variety of modified nucleotide classes. This Review focuses on how Therminator DNA Polymerase has enabled new technologies in synthetic biology and DNA sequencing. In addition, we discuss mechanisms for increased modified nucleotide incorporation.

Keywords: DNA sequencing, xenonucleic acids, aptamers, functionalized DNA, unnatural base pairs

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DNA polymerases have evolved to efficiently and faithfully replicate DNA. To maintain replication fidelity, DNA polymerases have evolved mechanisms for exquisite selectivity to insert the correct nucleotide across its complementary templating base. In all families of DNA polymerases, DNA polymerization occurs via a well-studied generalized mechanism for activity (Steitz, 1999). After DNA template binding, a DNA polymerase binds a correct deoxynucleotide (dNTP) to induce a slow rate-limiting step. This slow step is proposed to represent a conformational change from an open to a closed state, positioning the enzyme active site, DNA template and dNTP in the correct conformation for catalysis. A rapid chemical reaction catalyzes phosphoryl transfer of the bound dNTP to the DNA template and produces one inorganic pyrophosphate. Finally, the closed state reverts back to an open relaxed state, inorganic pyrophosphate is released and the complex either dissociates or translocates along the DNA, poised to add the next nucleotide (Xia and Konigsberg, 2014).

Family A and Family B DNA polymerases active sites are arranged to stabilize the correct incoming dNTP in the proper geometry for catalysis [Reviewed in (Steitz, 1999)]. The dNTP deoxyribose moiety assumes a favorable 3′-endo-sugar conformation. This conformation is constrained by hydrogen bonds between the 3′-OH and a main chain amide (corresponding to Vent DNA Polymerase position 412) and a non-bridging β-phosphate oxygen. Nucleotide α, β, and γ-phosphates are further stabilized by direct or water-mediated hydrogen bonds with active site residues (Gardner and Jack, 2002). The absence of the 3′-OH on modified nucleotides (such as ddNTPs or 3′-*O*-azidomethyl-dNTPs) disrupts hydrogen bonding with the β-phosphate (and main chain amide), potentially increasing the activation energy required to orient the α-phosphate for phosphoryl transfer. Because of this intricate network of active site interactions between amino acid side chains and the correct dNTP, any structural modification to the nucleotide structure disrupts this active site network of interactions and leads to discrimination against unnatural nucleotides. Therefore, to efficiently incorporate modified nucleotides, the DNA polymerase active site needs to be engineered to accommodate a variety of nucleotide structural variants. This review examines one engineered DNA polymerase called Therminator DNA Polymerase and discusses recent applications and mechanisms for enhanced modified nucleotide incorporation.

THERMINATOR DNA POLYMERASE

Early studies using a DNA polymerase from *Thermococcus litoralis* (Vent DNA Polymerase exo-), demonstrated that mutating an active site alanine 488 to a larger, more bulky side chain increased the efficiency of modified nucleotides including ddNTPs, rNTPs, and 3′-dNTPs (Cordycepin) (Gardner and Jack, 1999). Similar increases in modified nucleotide incorporation efficiencies were demonstrated in related DNA polymerases by increasing the equivalent alanine position to larger amino acid side chains, Pfu exo-/A486Y (Evans et al., 2000); KOD exo-/A485L (Hoshino et al., 2016); Tgo exo-/A485L (Pinheiro et al., 2012)]. The Vent/A488L mutation was transferred to a similar exonuclease deficient (exo-) hyperthermophilic DNA polymerase from *Thermococcus* sp. 9°N resulting in the commercial Therminator DNA Polymerase. *Thermococcus* sp. 9°N is an anaerobic hyperthermophilic euryarchaeon isolated from scrapings of a deep sea volcanic smoker chimney collected at the 9°N East Pacific Rise vent site, 500 miles south of Acapulco, Mexico at a depth of 2500 meters (Southworth et al., 1996). Specifically, Therminator DNA Polymerase is derived from the Family B *Thermococcus* sp. 9°N DNA Polymerase (GenBank: AAA88769.1) (Southworth et al., 1996) and contains mutations in the conserved exonuclease domain (separate from the polymerase active site domain) (D141A/E143A) and a mutation (A485L) in the conserved polymerase active site Region III (**Figure 1**). The D141A/E143A mutations inactivate the 3′-5′ exonuclease activity so that any modified nucleotide that is incorporated is not subsequently removed by the 3′-5′ exonuclease proofreading activity.

A Model for Therminator DNA Polymerase for Increased Modified Nucleotide Incorporation

Three-dimensional structural information provides limited clues as to the positioning and mechanism of the Therminator DNA Polymerase A485L mutation. In the parental 9°N DNA Polymerase crystal structure, alanine 485 is located on the O-helix Finger domain facing away from the incoming nucleotide in the active site [PDB ID: 5OMV (Rodriguez et al., 2000; Bergen et al., 2013; Kropp et al., 2017)] (**Figure 1**). Therefore, mutations at 485 will not directly contact the incoming nucleotide and likely acts indirectly by reducing steric barriers for nucleotide analog binding or facilitating a conformational change during polymerization. Alternatively, since the Finger domain undergoes a conformational change upon nucleotide binding, the A485L mutation may alter the equilibrium between an open and closed active site which may decrease discrimination for modified nucleotides. Kinetic data demonstrated that in Vent DNA Polymerase, the alanine to leucine mutation (A488L) modestly (∼2-4-fold) increased both k_{pol} and modified nucleotide binding (K_D). Unfortunately, the direct mechanism for reduced discrimination against modified nucleotides by Therminator DNA Polymerase is not completely understood. Additional research is needed to definitively determine how the mutation affects the conformational change rate and if modulation of the conformational change by the mutation is important for nucleotide analog discrimination.

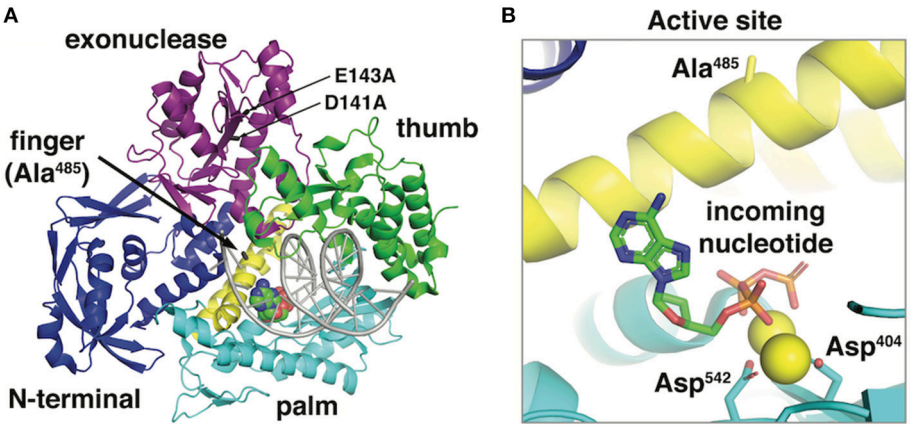
APPLICATIONS OF THERMINATOR DNA POLYMERASE

Engineered DNA polymerases capable of synthesizing modified nucleic acids have enabled emerging and foundational technologies, including synthetic biology (Houlihan et al., 2017a,b), aptamer therapeutics (Lapa et al., 2016) and DNA sequencing (Slatko et al., 2018). This Review focuses on how Therminator DNA Polymerase has been used as a tool to label DNA, synthesize modified substrates, for example containing an expanded genetic alphabet, and has enabled DNA sequencing-by-synthesis and genotyping methodologies (schematically depicted in **Figure 2**). Incorporation of a variety of modified nucleotides by Therminator DNA polymerase depicted in **Figure 3** will be discussed below.

SYNTHESIS OF RNA USING THERMINATOR DNA POLYMERASE

Ribonucleotide triphosphates (rNTPs, used for RNA synthesis) occur at a much higher concentration than deoxyriboncleotide triphosphates (dNTPs) inside cells (Williams and Kunkel, 2014). Therefore, DNA polymerases have evolved mechanisms to ensure selection of the correct nucleotide (dNTPs) in order to maintain the integrity of DNA. The DNA polymerase "steric gate" excludes rNTPs by a clash between bulky amino acids and the rNTP 2′-OH. Steric gate amino acids are well studied in Family A and B DNA polymerases (Brown and Suo, 2011). In 9°N DNA Polymerase, a Family B DNA polymerase, the conserved steric gate residue is Y409. Vent DNA Polymerase discriminates against rCTP incorporation via a 16-fold reduced binding affinity ($K_D = 1100\,\mu M$) and a 400-fold slower rate of incorporation ($k_{pol} = 0.160\ s^{-1}$) compared to dCTP (Gardner et al., 2004). When Y409 is mutated to a smaller amino acid such as valine, up to five successive ribonucleotides can be incorporated (Gardner and Jack, 1999).

Therminator DNA Polymerase retains the wildtype Y409 steric gate amino acid. However, the A485L mutation reduces discrimination for rNTPs and allows incorporation of up to twenty ribonucleotides (Gardner and Jack, 1999; Gardner et al., 2004). In addition, the equivalent mutation in Vent DNA Polymerase (A488L) incorporated rCTP more efficiently than wild-type due to a higher binding affinity ($K_D = 360\,\mu M$) and faster rate of incorporation ($k_{pol} = 0.7\ s^{-1}$) (Gardner et al., 2004). Presumably, RNA synthesis is limited to short products due to additional fidelity checkpoints and constraints that favor the synthesis of correct B-form DNA. Additional mutations engineered into the equivalent Therminator DNA Polymerase mutant (A485L) from *Thermococcus gorgonarius* (Tgo exo-/Y409G/A485L/E665K), dramatically increased ribonucleotide incorporation up to a 1.7 kb RNA product (Cozens et al., 2012). These additional mutations permit synthesis of A-form RNA:DNA molecules and may be used to create long RNAs.



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DNA polymerase		Region III		Reference
Therminator	484	RLIKILANSFY	495	Gardner and Jack, 2002
Thermococcus sp. 9°N	484	RAIKILANSFY	495	Southworth et al., 1996
Pyrococcus furiosus	483	RAIKILANSFY	492	Uemori et al., 1993
Thermococcus kodakarensis	484	RAIKILANSYY	495	Hashimoto et al., 2001
Thermococcus litoralis (Vent)	487	RAIKILANSYY	497	Kong et al., 1993
RB69	557	INRKLLINSLY	567	Franklin et al., 2001

FIGURE 1 | (A) The 9°N DNA polymerase exo- crystal structure [PDB ID: 5OMV (Bergen et al., 2013)] in complex with a primer:template (gray) and incoming nucleotide (spacefill) is shown. The position of D141A/E143A mutations in the exonuclease domain and the Therminator DNA Polymerase mutation (A485L) are highlighted. **(B)** The 9°N DNA polymerase active site shows the incoming nucleotide and catalytic aspartates (D404 and D542) stabilizing the nucleotide triphosphates. Alanine 485 in the 9°N DNA Polymerase exo- structure points away from the active site and does not directly interact with the incoming nucleotide. The Therminator DNA Polymerase mutation (A485L) increases the side chain size at position A485 but this structure has not yet been solved. **(C)** DNA polymerase Region III conserved active site residues in hyperthermophilic archaea and bacteriophage RB69 (Kong et al., 1993; Uemori et al., 1993; Southworth et al., 1996; Franklin et al., 2001; Hashimoto et al., 2001; Gardner and Jack, 2002) were aligned using Clustal Omega (Sievers and Higgins, 2018). Therminator DNA polymerase (D141A/E143A/A485L) is derived from the parental DNA polymerase from *Thermococcus* sp. 9°N (GenBank: AAA88769.1). The position of the Therminator DNA polymerase mutation (A485L) is highlighted in red.

SYNTHESIS OF LABELED DNA USING THERMINATOR DNA POLYMERASE

Many applications label DNA using modified nucleotides as detection reagents. Dye-derivatized nucleotides and analogs are commonly used for fluorescent detection during automated DNA sequencing (Prober et al., 1987), detection of single nucleotide polymorphisms (Chen and Kwok, 1999) and genome-wide mapping (Xiao et al., 2007). DNA polymerases from Families A and B have different preferences for labeled nucleotides based on linker arm length, dye charge and structure (Brandis, 1999; Gardner and Jack, 2002). For example, Therminator DNA Polymerase incorporates some dye-labeled nucleotides more efficiently than others suggesting that the dye structure can affect polymerase binding and incorporation. For example, modified nucleotides labeled with rhodamine dyes (TAMRA, ROX, R6G, and FL-12) are incorporated more efficiently than Cyanine-3 or Cyanine-5 dyes (Gardner and Jack, 2002). In addition to dye-labels, Therminator DNA Polymerase incorporates fluorescent analogs for use in fluorescence spectroscopy studies such as 8-vinyl-dGTP and 8-styryl-dGTP (Holzberger et al., 2012).

In addition to fluorescent labeling, nucleotides derivatized with a variety of reporter molecules act as important detection reagents. Therminator DNA Polymerase incorporates an amino functionalized analog [pyrrolidine ddNTP (prNTPs)] that enables conjugation without modifying the structure of the nucleobase (Gade et al., 2016). Matyasovky et al. designed base modified 2-allyl- and 2-proparylamino-dATP to introduce modifications into the minor groove of DNA via thiol-ene addition or CuAAC click chemistry (Matyašovsk et al., 2018). Another modified nucleotide designed by Marx and colleagues conjugated an oligonucleotide barcode to the base (ODNs) (Baccaro et al., 2012). Despite the very large base modification, Therminator DNA Polymerase synthesizes ODNs to form oligonucleotide barcoded substrates that could be used as hybridization probes for subsequent readout (Baccaro et al., 2012).

Synthesis of long modified DNAs may require high processivity yet Therminator DNA Polymerase is distributive, incorporating less than 20 nucleotides per binding event (Williams et al., 2008). To increase processivity and synthesize longer molecules, researchers attached two biotinylated peptide "legs" to Therminator DNA Polymerase and formed a complex with streptavidin beads. Then the modified Therminator DNA Polymerase-streptavidin bead complex bound DNA and improved processivity from less 20 nucleotides to several thousand nucleotides per binding event (Williams et al., 2008).

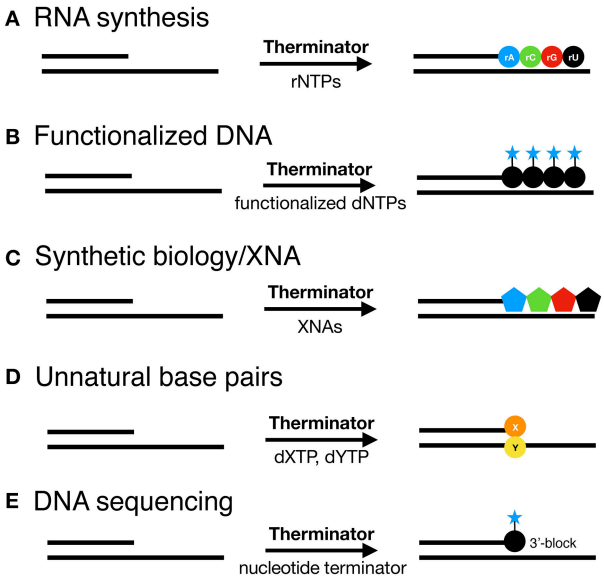

FIGURE 2 | Therminator DNA Polymerase incorporates a variety of modified nucleotide to synthesize on a DNA template **(A)** RNA, **(B)** Functionalized DNA, **(C)** XNA for synthetic biology, **(D)** Unnatural base pairs and enables **(E)** DNA sequencing methods.

SYNTHESIS OF MODIFIED FUNCTIONAL POLYMERS AND APTAMERS USING THERMINATOR DNA POLYMERASE

Therminator DNA Polymerase has been used to polymerize unnatural nucleotides to expand the functionality of DNA and alter its physical and chemical properties. Functionalized nucleic acids resistant to cellular nucleases may be used as aptamer therapeutics to inhibit protein targets (Lapa et al., 2016). Introducing chemical modifications of nucleotides increases the chemical diversity of synthesized polymers [reviewed in (Anosova et al., 2016)]. Hoshino and colleagues demonstrated incorporation of amphiphilic dNTP analogs by Therminator DNA Polymerase to create highly modified DNA molecules (Hoshino et al., 2016). Locked nucleic acids (LNAs) contain a ribofuranose ring in a locked, single conformation leading to duplex stabilization (Vester and Wengel, 2004). Alternatively, "unlocked" nucleic acids (UNAs) synthesized by Therminator DNA Polymerase offer additional substrate functionalization (Dubois et al., 2012). Other examples of functionalized DNAs made by Therminator DNA Polymerase using modified nucleotides include 2′-fluoro-NTPs (Kasuya et al., 2014), glyceronucleotides (gNTPs) (Chen et al., 2009), 7′,5′-Bicyclo-NTPs (Diafa et al., 2017), 3-phosphono-L-Ala-dNMPs (Yang and Herdewijn, 2011; Giraut et al., 2012), 3′-2′-phosphonomethyl-threosyl-NTPs (Renders et al., 2007, 2008), 5′-3′-phosphonomethyl-dNTPs (Renders et al., 2007, 2008), 2′-deoxy-2′-isonucleoside (iNTPs) (Ogino et al., 2010), 3′-deoxyapionucleotide 3′-triphosphates (apioNTPs) (Kataoka et al., 2008, 2011), 5-trifluoromethyl-dUTP (Holzberger and Marx, 2009) and 4′-C-aminomethyl-2′-O-methyl-TTP (Nawale et al., 2012). Kinetic studies of gNTP incorporation by Therminator DNA Polymerase demonstrate that while the rate of gNTP incorporation (k_{cat} = 0.8–4.7 s^{-1}) is similar to dNTPs (k_{cat} = 2–4.2 s^{-1}), gNTPs are bound with >350-fold lower affinity. For example, the K_m for TTP is 0.35 μM while the K_m for gTTP is 129 μM. Each of these modifications introduce functional diversity that may be useful for generating novel aptamers.

In addition to incorporating modified nucleotides on a natural DNA template, Therminator DNA Polymerase accommodates modifications in the template strand. For example, Xiao et al. demonstrate high fidelity synthesis of dATP across from mirror image L-thymidine in the template strand using Therminator DNA Polymerase (Xiao et al., 2017). Therefore, the ability of Therminator DNA Polymerase to synthesize modifications on both the primer and template strands will increase the diversity of modifications that can be introduced into substrates.

Xenonucleic acids [XNAs; reviewed in (Anosova et al., 2016)] are artificial genetic polymers that may be used to store genetic information in non-natural templates. New genetic scaffolds of modified nucleotides have the potential of building biosafe substrates for synthetic biology (Schmidt, 2010). Threose nucleic acid (TNAs: α-L-threofuanosyl-(3′-2′) nucleic acid) is an unnatural genetic polymer that is nuclease resistant (Horhota et al., 2005; Ichida et al., 2005a,b; Yu et al., 2013; Zhang and Chaput, 2013; Zhang et al., 2013; Dunn et al., 2015; Chim et al., 2017). In TNAs, the natural 5′-carbon ribose sugar is replaced with a four carbon threose sugar while phosphodiester bonds occur at the 2′ and 3′ positions. Threose has been found in pre-biotic reactions and on meteorites suggesting that the sugar can form spontaneously (Zhang and Chaput, 2013). Therminator DNA Polymerase can copy a 90 nucleotide DNA template into TNA using threose-NTPs (tNTPs) at a rate only four times slower than natural dNTPs (Dunn et al., 2015). For example, the observed rate (k_{obs}) of dCTP incorporation is 4.2 s^{-1} compared to 0.9 s^{-1} for tCTP (Dunn et al., 2015). Incorporation of TNA with Therminator DNA Polymerase enables further study of the structure and function of TNAs. Continued engineering of Therminator DNA Polymerase has increased the efficiency, fidelity and range of a variety of XNAs (Pinheiro et al., 2012; Chim et al., 2017).

SYNTHESIS OF UNNATURAL BASE PAIRS USING THERMINATOR DNA POLYMERASE

In theory, new synthetic base pairs enable site-specific introduction of new chemistries in the genetic code and are at the core of efforts to create semi-synthetic organisms that can store and retrieve increased genetic information [reviewed in (Malyshev and Romesberg, 2015)]. To truly create semi-synthetic organisms, DNA polymerases must maintain high fidelity incorporation of synthetic base pairs. Currently, novel base pair structures are being paired with new generations of engineered DNA polymerases to reach this goal. To expand the genetic alphabet, synthetic base pairs have been designed and successfully and specifically incorporated by Therminator

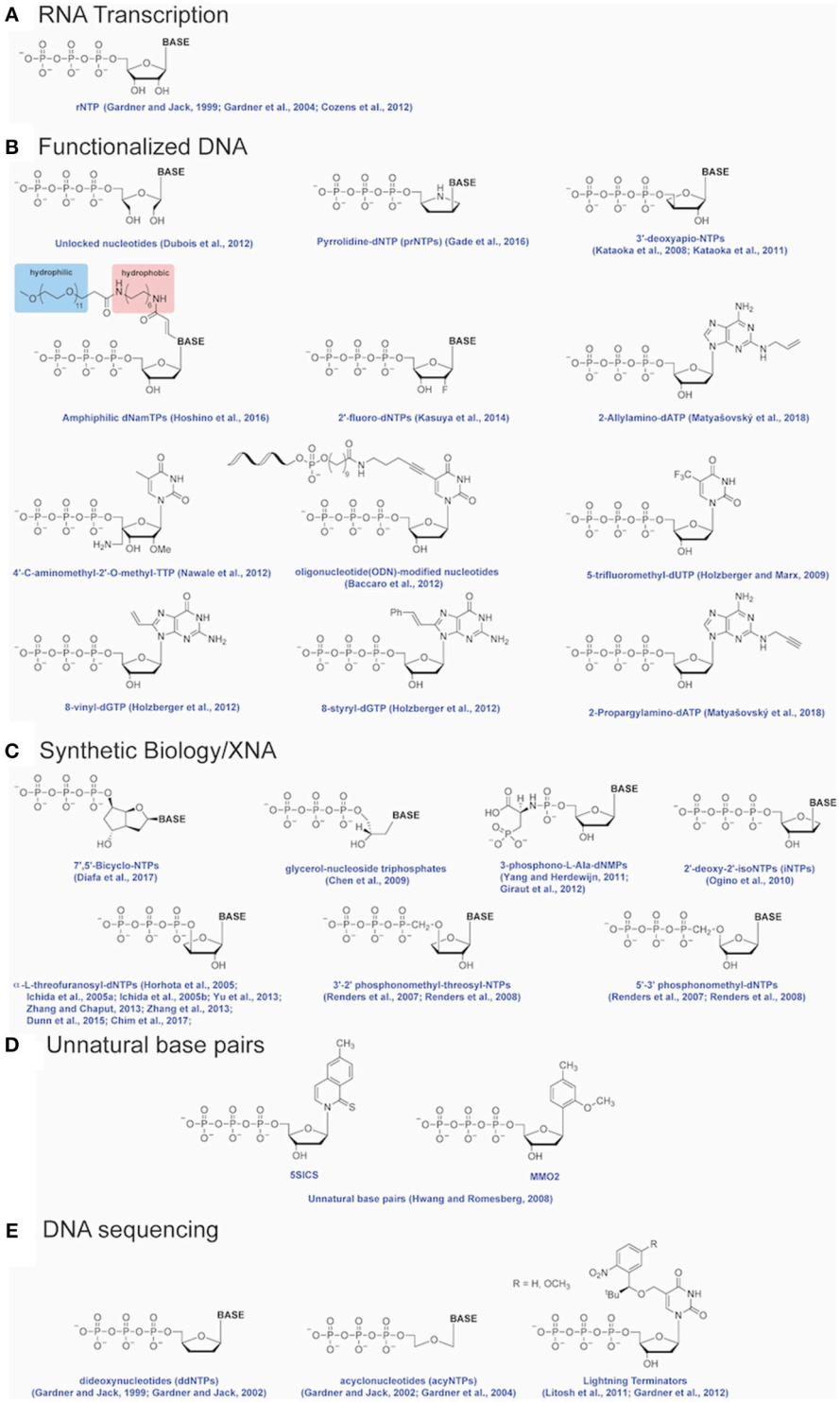

FIGURE 3 | Modified nucleotides. Modified nucleotides incorporated by Therminator DNA Polymerase for applications including **(A)** RNA transcription, **(B)** Functionalized DNA, **(C)** Synthetic Biology/XNA, **(D)** Unnatural base pairs and **(E)** DNA sequencing. References for each modified nucleotide are provided.

DNA Polymerase. For example, Therminator DNA Polymerase incorporates d5SICS:dMMO2 *in vitro,* however with low fidelity (Hwang and Romesberg, 2008). The next goal in the field is to increase functionalities in cells by introducing unnatural pairs into genomes and replicating with DNA polymerases such as Therminator DNA polymerase.

DNA SEQUENCING AND GENOTYPING WITH THERMINATOR DNA POLYMERASE

Sanger Sequencing

Sanger sequencing was the first method to sequence DNA (Sanger et al., 1977). In Sanger sequencing, a primer is extended by a DNA polymerase using a mixture of dNTPs and dye-labeled ddNTPs. After ddNTPs are incorporated, synthesis is stopped because the next nucleotide cannot be added due to the lack of the required 3′ hydroxyl group for dNMP phosphodiester bond formation. As a result, fragments labeled with different color dye-ddNTPs are produced and separated and analyzed via automated capillary electrophoresis (i.e., ABI 3730 Genetic Analyzer) [reviewed in (Slatko et al., 2011)].

Automated Sanger sequencing methods depend on engineered DNA polymerases that efficiently incorporate ddNTP terminators. The Therminator DNA Polymerase mutation was first discovered during studies focused on Vent DNA Polymerase exo- discrimination against ddNTPs for use in Sanger sequencing (Gardner and Jack, 1999). Mutating an active site alanine at position 488 to a larger amino acid (C,S,L,V, I or F) increased ddNTP incorporation by up to 15-fold compared to wild-type (Gardner and Jack, 1999). The analogous mutation in *Pyrococcus furiosus (Pfu)* (A486Y) had a similar increase in ddNTP utilization (Evans et al., 2000).

An alternative terminator, acyclonucleotides (acyNTP) substitutes a 2-hydroxyethoxymethyl group for the 2′-deoxyribofuranosyl sugar normally present in dNTP (Gardner and Jack, 2002). The increased ability to incorporate acyNTP vs. ddNTP is a distinguishing feature of hyperthermophilic archaeal DNA polymerases. DNA polymerases from *Thermococcus litoralis, Thermococcus* sp. *9°N7, Pyrococcus* sp. *GB-D,* and *Pfu* prefer acyNTP over ddNTPs by over 30-fold (Gardner and Jack, 2002). In addition, Therminator DNA Polymerase incorporates acyNTP 10-fold more efficiently than wild-type (Gardner et al., 2004). The catalytic incorporation efficiency of acyCTP (k_{pol} / K_D = 0.54) is similar to natural dCTP (k_{pol} / K_D = 0.72) suggesting that Therminator DNA Polymerase does not significantly discriminate between acyclonucleotides and natural nucleotides. As a result of high incorporation efficiency, dye-labeled acyNTP with Therminator DNA Polymerase were used in the LiCor/NEN Model 4200 Global IR2-automated DNA sequencer (Gardner and Jack, 2002). Despite its utility with dye-acyNTPs, Sanger sequencing technologies using Therminator DNA Polymerase remained far behind those using ThermoSequenase or TaqFS such as the ABI sequencing systems.

Next Generation Sequencing (NGS)

As next generation sequencing (NGS) technologies evolve, new sequencing-by-synthesis (SBS) methods use dye-labeled reversible terminators in an iterative cyclic fashion (Metzker, 2010; Slatko et al., 2018). Each sequencing cycle is comprised of nucleotide incorporation, fluorescence imaging and cleavage. In the first step, an engineered DNA polymerase incorporates one fluorescently modified reversible terminator complementary to the template base. Since reversible terminators block extension of the next base, only one base is incorporated per cycle. Following incorporation and washing, imaging of the dye-terminator determines the base added. After imaging, a cleavage step removes both the terminating group and the fluorescent dye and the unblocked 3′ end is ready for the next round of incorporation, imaging and cleavage. Because wild-type DNA polymerases discriminate strongly against incorporation of nucleotides modified at the 3′-position, all SBS methods depend on engineered DNA polymerases to efficiently incorporate dye-labeled reversible terminators (Fuller et al., 2009; Chen et al., 2013; Chen, 2014).

Sequencing-by-synthesis methods use 3′-blocked reversible nucleotide terminators such as 3′-*O*-azidomethyl-dNTPs or 3′-*O*-NH-dNTPs (Hutter et al., 2010). Therminator DNA Polymerase discriminates strongly against larger modifications at the 3′ position (such as 3′-*O*-azidomethyl or 3′-*O*-NH) and incorporates these nucleotides very inefficiently with little or no detectable incorporation (Gardner et al., 2012). Therefore, researchers have introduced other mutations into the Therminator DNA Polymerase backbone to increase 3′-*O*-azidomethyl-dNTPs or 3′-*O*-NH-dNTPs incorporation efficiency and to take advantage of the underlying Therminator DNA Polymerase activities (Smith et al., 2005; Guo et al., 2008).

In contrast to 3′-modified reversible terminators, LaserGen developed a novel sequencing chemistry based on a 2-nitrobenzyl-modified 3′-OH unblocked HOMe-dNTP nucleotide that terminates synthesis after one incorporation (Litosh et al., 2011; Gardner et al., 2012). After a single incorporation, the 2-nitrobenzyl group is removed by light to reverse termination and allow the next round of synthesis to proceed. These terminators are called Lightning Terminators™ and form the basis of the LaserGen/Agilent sequencing by synthesis technology. A variety of DNA polymerase incorporate Lightning Terminators but because the 3′-OH is unblocked, many DNA polymerases (such as Vent exo- or Klenow fragment exo-) continue synthesis rather than terminate (Litosh et al., 2011). Therminator DNA Polymerase has the unusual property of both incorporating Lightning Terminators and terminating synthesis despite an unblocked 3′-OH (Litosh et al., 2011). In addition, since the mismatch incorporation rate of Lightning Terminators is extremely slow, synthesis with Lightning Terminators increases incorporation fidelity compared to natural nucleotide (Gardner et al., 2012).

OUTLOOK FOR FURTHER THERMINATOR DNA POLYMERASE ENGINEERING

Engineered DNA polymerases will continue to play key roles as molecular tools for synthesizing and copying synthetic DNA substrates and as the basis of DNA sequencing-by-synthesis techniques. To reach the potential, future DNA polymerase engineering must balance efficient incorporation of modified nucleotides with high fidelity, ideally combining high modified nucleotide incorporation efficiency with high fidelity. Currently, many of the studies described in this Review lack data on incorporation kinetics and fidelity which can provide insights into mechanisms for incorporation and discrimination.

Therefore, more studies are needed to fully understand DNA polymerase mechanisms for modified nucleotide incorporation.

Improving Modified Nucleotide Incorporation Fidelity

DNA polymerases, contain compact active sites that promote correct nucleotide binding and incorporation, and prevent misincorporation of incorrect or bulky nucleotides. Typically, DNA polymerases favor incorporation of the correct nucleotide by at least 1,000-fold (Johnson, 2010). Due to the A485L mutation, Therminator DNA Polymerase incorporates a variety of modified nucleotides but is also is prone to misincorporation of nucleotides. For example, Dunn et al. observed that the rate of correct tNTP incorporation is only approximately 10-fold faster than the incorrect tNTP (Dunn et al., 2015). Similarly, with natural nucleotides, Therminator DNA Polymerase incorporates TTP across from a correct dA in the template with a k_{pol} of 170 ± 4 and K_D of 73 ± 3 μM while TTP is incorporated across from an incorrect dC template is only 2-fold slower (k_{pol} = 70 ± 1) and has 2-fold weaker binding affinity (K_D of 150 ± 8 μM) (Gardner et al., 2012). Therefore, the Nucleotide Selectivity $[(k_{pol}/K_D)_{correct}/(k_{pol}/K_D)_{mismatch}]$ for correct vs. mismatch incorporation is only 4.9-fold.

Recent advances in DNA polymerase engineering have improved the fidelity of modified nucleotide incorporation compared to Therminator DNA Polymerase. In one example, the same A485L mutation in an alternative DNA polymerase scaffold increased fidelity of amphiphilic dNTPs. Hoshino, et al. (Hoshino et al., 2016) measured fidelity by omitting a single dNamTP and testing if a DNA polymerase will halt in the absence of a correct nucleotide. In the absence of a single dNamTP, Therminator DNA Polymerase misincorporated and continued synthesis while a related DNA polymerase from *Thermococcus kodakarensis* (Tko exo-/A485L) stalled synthesis rather than misincorporate the wrong nucleotide. In addition, the Chaput group has improved incorporation fidelity of tNTP incorporation from 70 errors per 1,000 using Therminator DNA Polymerase to 4 errors per 1,000 with a related DNA polymerase KOD-RI (Tko A485R/E143A) (Horhota et al., 2005; Chim et al., 2017).

The modified nucleotide structure also influences their incorporation efficiency and fidelity. For example, Lightning Terminators are a class of reversible nucleotide terminators used in sequencing-by-synthesis methods that contain base rather than ribose modifications. Surprisingly, Lightning Terminators were incorporated with higher fidelity compared to natural nucleotides due to the 1,000-fold lower rate of incorporation of the wrong Lightning Terminator (Gardner et al., 2012). Therefore, we expect that a combination of additional DNA polymerase mutations and new nucleotide structural designs will continue to improve incorporation fidelity of modified nucleotides.

Improving Modified Nucleotide Incorporation Efficiency

Even though many modified nucleotides can be incorporated by Therminator DNA Polymerase into short synthesis products, a continuing challenge is the synthesis of longer highly modified DNA polymers. For example, after each sequencing cycle of incorporation, imaging and unblocking during sequencing-by-synthesis methods, residual linker structures remain attached to the 3′ base. As sequencing progresses, these molecular scars accumulate and alter the structure of DNA making it a less efficient template for further DNA polymerase synthesis [(Fuller et al., 2009; Metzker, 2010; Chen et al., 2013; Chen, 2014)]. Therefore, engineered DNA polymerase that can efficiently and accurately synthesize from these highly modified DNAs are needed.

Even though the Therminator DNA Polymerase mutation (A485L) increases a variety of modified nucleotide classes, more recent studies have added additional mutations to boost incorporation efficiency and fidelity of specific modified nucleotides (Dunn et al., 2016). These secondary mutations are specifically engineered for the modified nucleotide being studied. For example, the A485L mutation combined with a second mutation (E665K) switches *Thermococcus gorgonarius* (Tgo exo-/Y409G/A485L/E665K) DNA polymerase into an engineered RNA polymerase capable of synthesizing 1.7 kb of RNA (Cozens et al., 2012). Similarly Pinheiro and colleagues identified additional mutations in the Tgo exo-/A485L that enabled efficient synthesis of XNAs (Pinheiro et al., 2012). Secondary mutations have also been introduced in Therminator DNA Polymerase to make it a more efficient DNA sequencing enzyme (Smith et al., 2005). It is likely that future engineered DNA polymerases will continue to use the A485L mutation as an important scaffold for modified nucleotide incorporation but will require additional secondary mutations that will further improve polymerase substrate specificities and fidelity.

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AFG researched and wrote the paper. KJ, MB, JB, AMG, KZ, JO, and WJ researched and edited the paper. IC and VP made Figures and edited the paper.

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REVIEW
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Inhibiting DNA Polymerases as a Therapeutic Intervention against Cancer

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Inhibiting DNA synthesis is an important therapeutic strategy that is widely used to treat a number of hyperproliferative diseases including viral infections, autoimmune disorders, and cancer. This chapter describes two major categories of therapeutic agents used to inhibit DNA synthesis. The first category includes purine and pyrmidine nucleoside analogs that directly inhibit DNA polymerase activity. The second category includes DNA damaging agents including cisplatin and chlorambucil that modify the composition and structure of the nucleic acid substrate to indirectly inhibit DNA synthesis. Special emphasis is placed on describing the molecular mechanisms of these inhibitory effects against chromosomal and mitochondrial DNA polymerases. Discussions are also provided on the mechanisms associated with resistance to these therapeutic agents. A primary focus is toward understanding the roles of specialized DNA polymerases that by-pass DNA lesions produced by DNA damaging agents. Finally, a section is provided that describes emerging areas in developing new therapeutic strategies targeting specialized DNA polymerases.

Keywords: DNA polymerases, chemotherapy, nucleoside analogs, DNA damaging agents, cancer

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BIOLOGICAL ROLES OF DNA POLYMERASES

DNA Synthesis

DNA replication is an essential biological pathway that produces two identical copies of an organism's genome (Garg and Burgers, 2005). In eukaryotic cells, chromosomal replication is catalyzed by a multiprotein complex termed the replicase (Kunkel and Burgers, 2008). DNA sythesis is catalyzed by DNA polymerases that incorporate mononucleotides into a primer using DNA or RNA as the template to guide each polymerization step (**Figure 1**). During this process, the sequence of the template varies. As such, DNA polymerases must be remarkably flexible to recognize four distinct pairing combinations of A:T, C:G, T:A, and G:C. Despite this flexibility, polymerases must also remain stringent to ensure faithful duplication of the template.

Multiple Polymerases Are Involved in Processing Nucleic Acid

Humans possess at least 15 different DNA polymerases that play essential and distinct roles in chromosomal and mitochondrial replication, DNA repair, and translesion DNA synthesis, a biological process that involves the replication of damaged DNA (Hubscher et al., 2002; Shcherbakova et al., 2003). There are five DNA polymerases that participate in chromosomal DNA synthesis. These polymerases obey canonical Watson–Crick base pairing rules to catalyze both

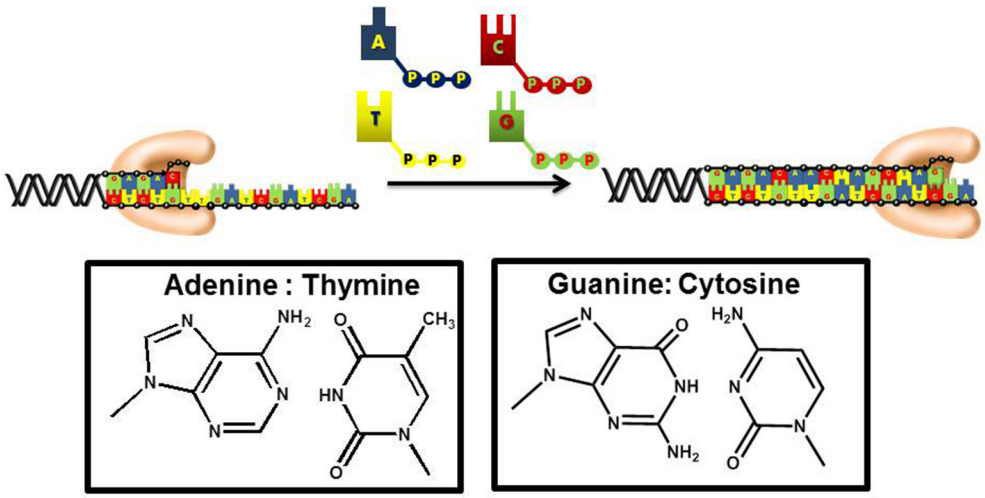

FIGURE 1 | DNA polymerases use a common mechanism to synthesize DNA. During the polymerization process, a nucleotide is covalently attached to the 3′-OH group of a preexisting DNA chain serving as a primer. With most DNA polymerases, DNA is used as the template to guide each incorporation event. However, telomerase and other reverse transcriptases use or RNA as the template. Correct polymerization results in the synthesis of a DNA chain that is complementary to the template strand of DNA.

efficient and faithful DNA polymerization. In general, replicative DNA polymerases synthesize nucleic acid at incredibly high rates that approach 1,000 nucleotides per second while making only one mistake in a million opportunities (Kunkel and Bebenek, 2000; Joyce and Benkovic, 2004). Pol δ and pol ε are the two DNA polymerases most closely associated with chromosomal DNA synthesis. However, these polymerases also participate in various DNA repair pathways such as base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR; Downey et al., 1990; Kunkel and Burgers, 2008; Pursell and Kunkel, 2008). Pol α is a primase that synthesizes short pieces of RNA that serve as primers during the initiation of leading and lagging strand DNA synthesis (Kuchta and Stengel, 2010). Telomerase is the only eukaryotic polymerase that functions as a reverse transcriptase during the replication of telomeric regions of the chromosome (Prescott and Blackburn, 1999). Pol γ participates in the replication and repair of the mitochondrial genome (Bailey and Anderson, 2010). Pol γ, pol δ, and pol ε all possess a rigorous 3′ → 5′ exonuclease proofreading activity which contributes to the maintenance of genomic fidelity.

Several DNA polymerases are involved in completing the repair of damaged DNA. As mentioned earlier, replicative polymerases including pol δ and pol ε participate in BER, NER, and MMR. However, pol β is the primary DNA polymerase involved in BER and gap-filling synthesis during NER (Beard and Wilson, 2000). Pol λ and pol μ participate in non-homologous end joining which allows double-strand DNA breaks to be repaired (Lieber et al., 2010). Finally, B- and T-cell possess a unique DNA polymerase terminal deoxynucleotidyl transferase (TdT) that incorporates deoxynucleotides in a random fashion at double-strand DNA breaks formed during V(D)J recombination (Gucalp et al., 1991; Marshall et al., 1998). In contrast to pol δ and pol ε, DNA polymerases involved in DNA repair such as pol β, pol μ, pol λ, and TdT do not possess a 3′ → 5′ exonuclease activity.

The final group of DNA polymerases are classified as "specialized" polymerases as they are capable of replicating distinct forms of damaged DNA. Members of this family include pol η, pol ι, pol κ, pol θ, pol ψ, pol σ, pol ξ, and Rev1. Specialized DNA polymerases are similar to repair polymerases as both do not possess 3′ → 5′ exonuclease activity. The lack of proof reading activity makes these polymerases error-prone, especially when replicating undamaged DNA. Surprisngly, the majority of these polymerases are remarkably faithful when replicating damaged nucleic acid. Of all the specialized DNA polymerases identified to date, the biological function of pol η has been the most extensively characterized at the cellular and biochemical level. This polymerase is responsible for accurately replicating naturally occurring crosslinked DNA lesions such as thymine dimers (Johnson et al., 1999; Yuan et al., 2000). As described later, pol η is also very efficient at replicating DNA lesions generated by chemotherapeutic agents such as cisplatin (Alt et al., 2007). Pol ι replicates several types of modified purines (Washington et al., 2004; Nair et al., 2006; Pence et al., 2009) while Rev1 preferentially insert dCMP opposite abasic sites and most DNA lesions that involved modifications to guanine (Haracska et al., 2001, 2002b). Pol κ incorporates nucleotides opposite bulky adducts such as N^2-acetylaminofluorene-G lesions and N^2-benzo(a)pyrene diolepoxide-G lesions (Ohashi et al., 2000; Zhang et al., 2002). In addition, pol κ extends beyond base pairs formed by other specialized DNA polymerases during TLS (Haracska et al., 2002a). Pol ξ is similar to pol κ as that it works together with other specialized DNA polymerases to extend beyond mispairs formed by other specialized DNA polymerases (Haracska et al., 2003). The biological function and activity of other specialized

DNA polymerases such as pol θ, ψ, and σ have yet to be unambiguously determined.

Structural Features of DNA Polymerases

Despite having different biological functions, the overall three-dimensional structures of all DNA polymerases determined to date are remarkably similar. In general, all DNA polymerases characterized to date resemble a "right hand" possessing subdomains corresponding to a palm, thumb, and fingers (**Figure 2**; Steitz, 1999; Johnson and Beese, 2004; Kretulskie and Spratt, 2006). In general, the palm subdomain is highly conserved amongst all polymerases and contains two aspartates and/or glutamates that function to coordinate metal ions in the active site which are necessary for catalysis. The fingers domain plays an essential role in achieving proper nucleotide selection by interacting with the incoming dNTP and the templating base. The thumb domain is important for correctly positioning duplex DNA in the polymerization active site as well as for assisting in translocating the polymerase to the next templating base.

While structurally similar, DNA polymerases display subtle differences which significantly influence their biological functions ate the cellular level. For instance, polymerases that catalyze chromosomal replication generally possess fingers that are longer and more extended compared to specialized DNA polymerases which tend to have shorter fingers (Doublié et al., 1998; Franklin et al., 2001; Hsu et al., 2004). The longer finders of chromosomal polymerase are proposed to be important for achieving higher replication fidelity and processivity during DNA synthesis (Ling et al., 2003). In contrast, shorter fingers present of specialized DNA polymerase are believed necessary to better accommodate structurally diverse DNA lesions (Washington et al., 2003; Fleck and Schär, 2004; Steitz and Yin, 2004).

Kinetic Mechanism of DNA Polymerases

Figure 3 provides a generalized kinetic model mechanism that applies to most DNA polymerases (Mizrahi and Benkovic, 1988; Berdis, 2009; Johnson, 2010). The first step is the binding of DNA substrate to the "open" conformation of the DNA polymerase. However, dNTP binding to the "open" polymerase:DNA complex (step 2) is generally considered to be the first control point for ensuring high catalytic efficiency and polymerization fidelity during normal DNA synthesis. After binding the correct dNTP, the fingers subdomain rotates to form a "closed" conformation that orients the incoming dNTP opposite the templating base (step 3). The formation of this "closed" conformation aligns the bound dNTP into a correct geometrical orientation that allows chemistry to occur (step 4). With most high-fidelity DNA polymerases, misaligned intermediates that form as a consequence of binding an incorrect dNTP change the geometry of the polymerase's active site and causes the rate constant for the conformational change step to be reduced significantly. Lowering this rate constant provides an opportunity for the incorrect dNTP to dissociate from the Pol:DNA complex rather than to proceed through the phosphoryl transfer step. Collectively, the overall catalytic efficiency (k_{pol}/K_d) for the steps involved in correct

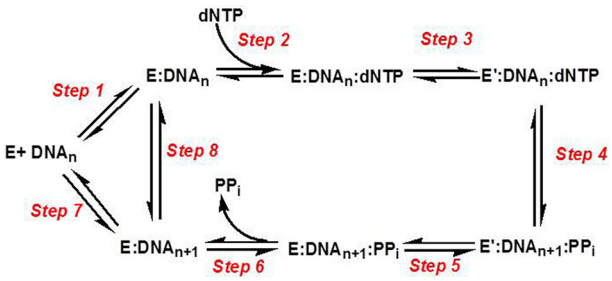

FIGURE 3 | Kinetic mechanism for DNA polymerases. Individual steps along the pathway for DNA polymerization are numbered and identified as described in the text. E, polymerase; DNA_n, DNA substrate; E′, conformational change in DNA polymerase; PP_i, inorganic pyrophosphate; DNA_{n+1}, DNA product (DNA extended by one nucleobase).

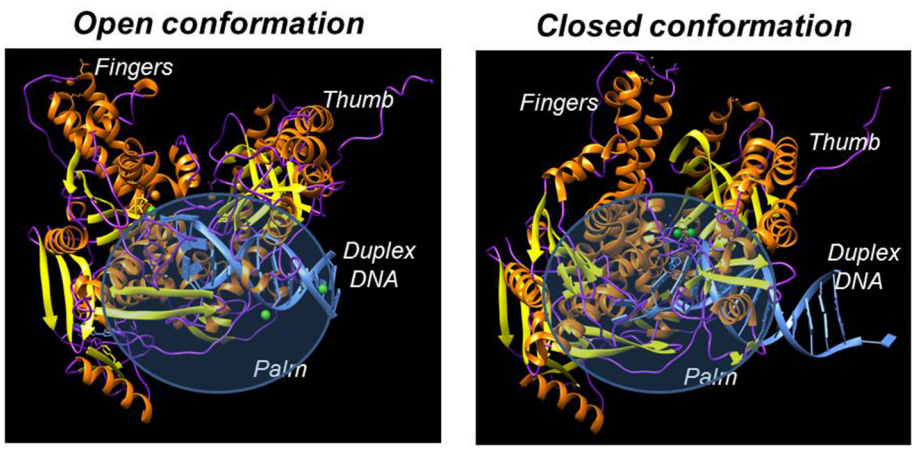

FIGURE 2 | X-ray crystallographic structures of DNA polymerases reveal common structural motifs representing the palm, fingers, and thumb subdomains that play important roles in nucleotide binding and phosphoryl transfer. The left panel displays the structure of a high-fidelity DNA polymerase (bacteriophage RB69) in the "open" conformation while the right panel displays the structure of the polymerase in the "closed" conformation.

polymerization is very large at $\sim 10^7$ M^{-1} s^{-1}. As expected, k_{pol}/K_d values for forming mismatches (i.e., misinsertion of dATP opposite C) are typically lower by several orders of magnitude, and this reduction is caused by decreases in the binding affinity of the incoming dNTP (step 2) coupled with decreases in the rate constant for the conformational change step (step 3).

Another conformational change (step 5) occurs after phosphoryl transfer, and this step allows pyrophosphate to be released (step 6) which is coordinated with the translocation of the polymerase along DNA to the next templating position. After the translocation step, the polymerase can dissociate from the extended primer (step 7) to begin DNA synthesis on another primer template or remain bound to the elongated DNA to proceed with subsequent rounds of DNA synthesis (step 8). The ability to incorporate multiple nucleotides without dissociating from DNA defines the processivity of the polymerase. Polymerases involved in chromosomal DNA synthesis usually display high processivity as they are required to replicate thousands of base pairs per binding event. Specialized polymerases differ as they are far less processive since their involvement in replicating damaged DNA requires that they only by-pass unrepaired DNA lesions that occur sporadically throughout the genome.

In addition to polymerization activity, most high-fidelity polymerases contain an exonuclease proofreading domain that can erase potentially pro-mutagenic mismatches. The overall excision reaction is complicated since the DNA substrate must partition between the polymerase and exonuclease active sites (Reha-Krantz, 1998). After placement of primer in the exonuclease active site, the terminal nucleotide is hydrolyzed in a reaction that is generally Mg^{2+}-dependent. After excision, the enzyme partitions the primer back into the polymerization domain which allows for correct DNA synthesis to be renewed without a requirement for polymerase dissociation and rebinding. This activity is important for chemotherapeutic intervention as it represents a potential mechanism of drug resistance by removing chain-terminating nucleotides from DNA.

CHAIN-TERMINATION WITH NUCLEOSIDE ANALOGS

An important therapeutic approach to inhibit DNA replication is to commandeer the high catalytic efficiency of the chromosomal DNA polymerases into using a "suicide" nucleotide that terminates DNA synthesis (**Figure 4**). This "Trojan Horse" strategy is considered the major paradigm toward the rational design of nucleoside analogs that display activity as anti-cancer agents. This strategy provides a polymerase with a nucleotide analog that contains simple alterations to the deoxyribose moiety. In most instances, the 3′-OH moiety that needed to elongate DNA is substituted with non-reactive functional groups such as hydrogen (-H), halogens (Cl, F, Br, etc.), or azide (N_3). Recently, newer approaches have generated analogs in which the entire deoxyribose moiety is replaced with an arabinose sugar that also contains a halogen in the 2′ or 3′-position. In general, the nucleobase component is left unmodified which allows the polymerase to form Watson-Crick base pairs with the templating base. As a result, the "suicide" analog is efficiently incorporated into DNA like its natural counterpart. However, the analog is devoid a usable 3′-OH group and thus produces a

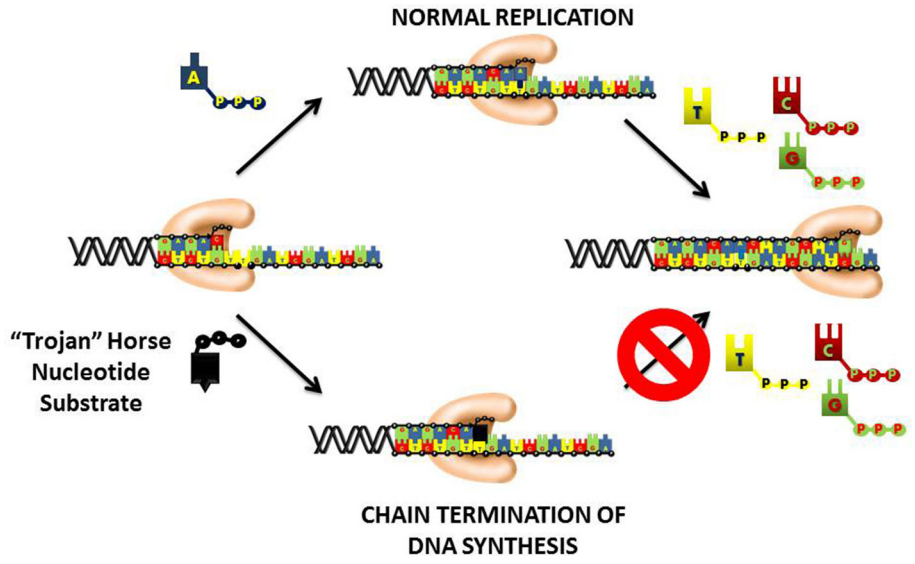

FIGURE 4 | "Trojan Horse" strategy of using nucleoside analogs to inhibit DNA polymerization. The polymerase is provided with a modified nucleotide in which the 3′-OH group required for DNA elongation is missing, replaced with a halogen, or altered in configuration from a normal ribose sugar. Since the nucleobase component is left unmodified, the polymerase incorporates the nucleotide analog into DNA as efficiently as its natural counterpart. After incorporation, the nucleotide lacking a usable 3′-OH group is refractory to elongation causing the induction of apoptosis by the termination of DNA synthesis.

nucleic acid substrate that cannot be efficiently elongated. The termination is DNA synthesis causes replication fork stalling to induce apoptosis.

These types of "suicide" nucleotides are termed anti-metabolites and represent the largest class of antineoplastic agents used clinically (Peters et al., 1993, 2000; García et al., 2008). Currently, there are 11 nucleoside analogs that are FDA approved, and these collectively represent about 20% of all drugs used in chemotherapy (Parker, 2009). **Figure 5** provides the chemical structures of several analogs that are widely used in chemotherapy. For comparison, the structures of their natural counterparts are provided as well. The most commonly used purine nucleoside analogs are fludarabine (9-β-D-arabinoside-2-fluoroadenine), cladribine [2-chlorodeoxyadenosine (2-CdA)], clofarabine [2-chloro-9-(2′deoxy-2′-fluoroarabinofuranosyl)adenine], and pentostatin (2′-deoxycoformycin). These nucleosides produce almost exclusive cytotoxic effects against hematological malignancies, most notably chronic lymphoblastic leukemia (CLL), non-Hodgkin's lymphomas, and cutaneous T-cell lymphoma (Robak et al., 2006). Commonly used pyrimidine analogs include gemcitabine and ara-C which are used to treat hematological malignancies and some solid tumors (Moysan et al., 2013).

In general, the cytotoxic effects produced by these nucleoside analogs is caused by the incorporation of their corresponding nucleoside triphosphates into DNA which results in chain termination of DNA synthesis to activate apoptosis. Discussions below focus on fludarabine (Fludara) and gemcitabine (Gemzar) as these are the two most widely used nucleoside analogs employed against cancer.

FLUDARABINE

The mechanism for the incorporation of the triphosphate form of fludarabine (designated F-ara-ATP) has been extensively studied with several human DNA polymerases (Tseng et al., 1982; White et al., 1982; Parker and Cheng, 1987; Parker et al., 1988; Huang et al., 1990; Gandhi et al., 1997). While polymerases such as pol α, pol β, pol γ, and pol ε incorporate F-ara-ATP, *in vitro* studies demonstrate that the IC_{50} value for F-ara-ATP varies considerably across these enzymes. For example, F-ara-ATP inhibits pol α and pol ε most potently with *in vitro* IC_{50} values of 1.6 and 1.3 μM, respectively. The potency for F-ara-ATP is 10-fold worse with the mitochondrial polymerase, pol γ, and the DNA repair polymerase, pol β, with IC_{50} values of 44 and 24 μM, respectively. The higher potency displayed against the chromosomal DNA polymerases suggests that fludarabine exerts its therapeutic effects by inhibiting DNA synthesis during S-phase of the cell cycle.

As expected for a competitive substrate, the inhibitory effects of F-ara-ATP can be effectively overcome through increasing concentrations of the natural nucleotide substrate, dATP. Once incorporated opposite thymine, most DNA polymerases poorly elongate beyond the modified nucleotide and this causes

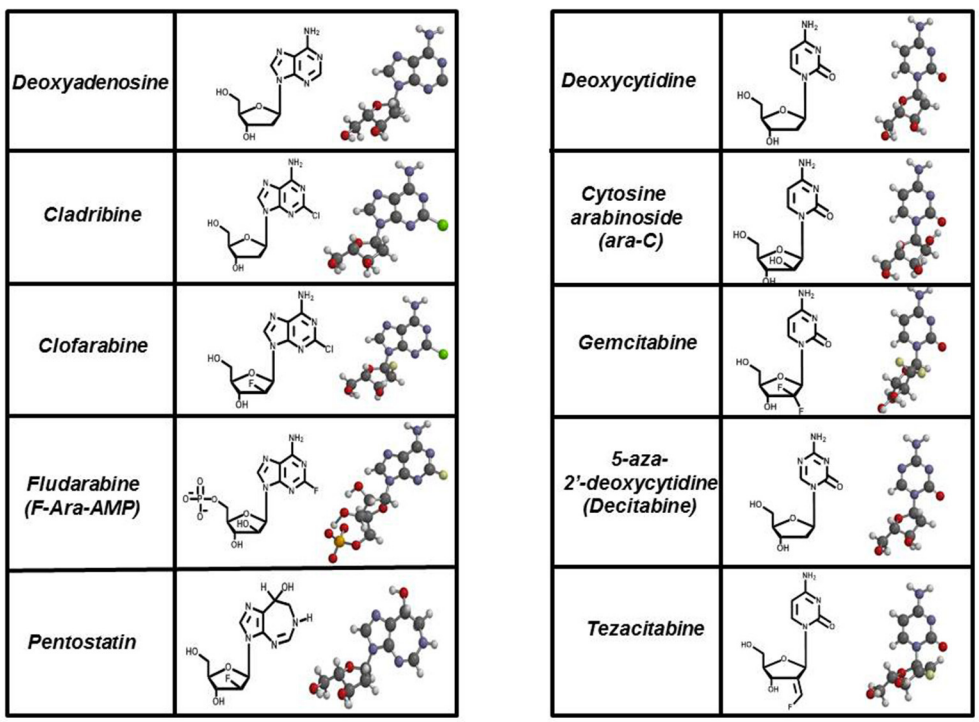

FIGURE 5 | Structures of FDA approved nucleoside analogs. Purine-like nucleosides include cladribine (2-chlorodeoxyadenosine), clofarabine [2-chloro-9-(2′deoxy-2′-fluoroarabinofuranosyl)adenine], fludarabine (9-β-D-arabinoside-2-fluoroadenine), and pentostatin (2′-deoxycoformycin). Pyrimidine-like nucleosides include cytarabine [1-β-D-arabinofuranosylcytosine (Ara-C)], gemcitabine [2′,2′-difluorodeoxycytidine (dFdC)], 5-aza-deoxycytidine, and tezacitabine.

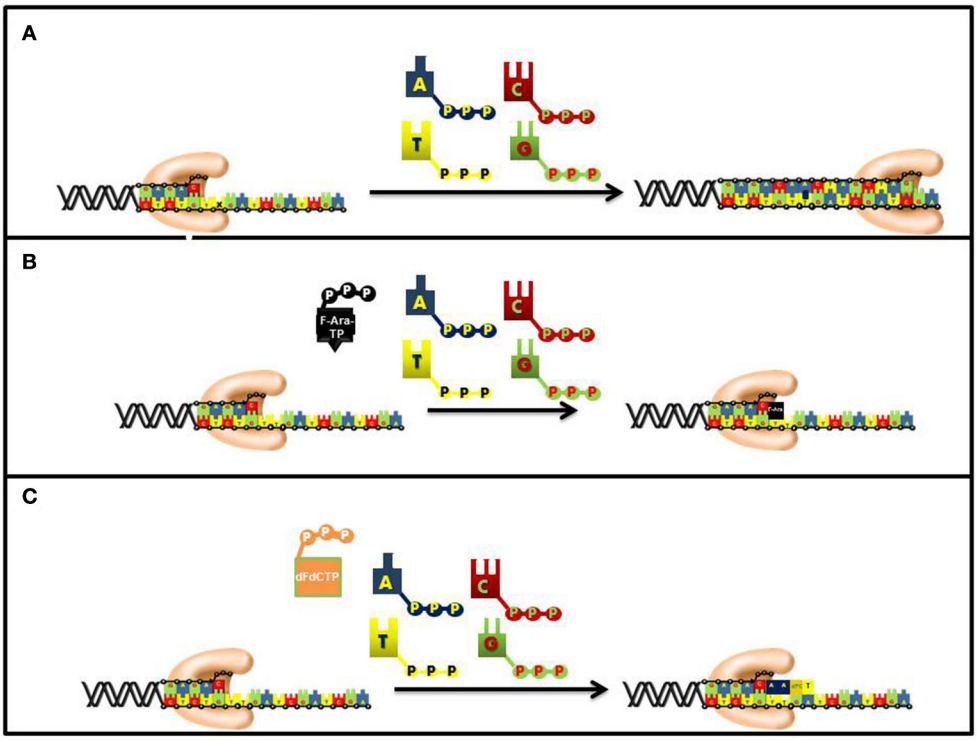

FIGURE 6 | Differences in the mechanism of chain termination by gemcitabine and ara-C. After incorporation into DNA, ara-CTP terminates DNA synthesis directly at the site of incorporation while gemcitabine can be elongated by one additional nucleotide. The placement of gemcitabine at the penultimate position is termed "masked chain termination" since the terminal nucleotide masks detection and removal of gemcitabine by exonucleases or DNA repair enzymes. **(A)** Normal DNA synthesis. **(B)** Inhibition by fludarabine. **(C)** Inhibition by gemcitabine.

subsequent chain termination (**Figure 6**). Indeed, quantitative analyses of DNA extracted from cells incubated with tritiated nucleoside analog demonstrate that it is present at terminal positions of DNA (Spriggs et al., 1986).

Fludarabine is currently the most effective purine analog used to treat several hematological cancers including chronic lymphocytic leukemia and indolent B-cell malignancies (Hallek, 2004). Standard doses of fludarabine range between 25 and 30/mg/m^2 given over 30 min for five consecutive days. Under these conditions, a plasma concentration of 3 μM for the analog is achieved within 30 min. Peak concentrations of the active metabolite, F-ara-ATP, are about 4 h post-infusion (Malspeis et al., 1990).

GEMCITABINE

The synthetic pyrimidine analog, gemcitabine [2′, 2′-difluorodeoxycytidine (dFdC)], differs from deoxycytidine by the addition of two fluorine atoms in the geminal configuration at the 2′-position of sugar (**Figure 5**). Gemcitabine produces a wide spectrum of anti-cancer activities against hematological cancers and solid tumors. The triphosphate form of gemcitabine, dFdCTP, functions as a substrate for a number of DNA polymerases involved in chromosomal replication, DNA repair, and translesion DNA synthesis (Huang et al., 1991; Jiang et al., 2000). For example, the IC_{50} values for dFdCTP are 11 and 14 μM for pol α and pol ε, respectively. Likewise, DNA primer extension assays performed using *in vitro* analyses show that there is direct competition between FdCTP and dCTP for insertion opposite guanine. After dFdC is incorporated, the modified pyrimidine can be elongated one additional nucleotide before DNA synthesis is terminated (**Figure 6**). This unique activity contrasts that of F-ara-TP which typically terminates DNA synthesis directly at the site of its incorporation. The unique method of inhibiting DNA synthesis by dFdCMP is coined "masked chain termination" as the addition of an extra nucleotide essentially hides the incorporated dFdCTP from various enzymes that could excise the pyrimidine analog from DNA to reverse its effect of DNA synthesis (Plunkett et al., 1995).

Gemcitabine is used as a monotherapeutic agent in the treatment of certain leukemias, lymphomas, and metastatic pancreatic cancer (Eckel et al., 2006). However, gemcitabine is more frequently combined with platinum drugs such as cisplatin and oxaliplatin (Hoff and Fuchs, 2003; Ozols, 2005; Sehouli, 2005; Chua and Cunningham, 2006; Richardson et al., 2008) to treat solid cancers such as non-small-cell lung, bladder, ovarian, and breast cancers (Lorusso et al., 2005; Silvestris et al., 2008). The reason for combining gemcitabine with platinum agents is based on cell-based data demonstrating that the combination of drugs produces a synergistic cell-killing effect. At the clinical level, treatment with oxaliplatin can cause serious complications such

as peripheral neurotoxicity and nephrotoxicity (Meliani et al., 2003). In contrast, gemcitabine is a well-tolerated drug as it produces mild side effects such as moderate myelosuppression, asthenia, and nausea/vomiting (Teusink and Hall, 2010). As a result, gemcitabine is used to sensitize the effects of platinum drugs so that lower doses of platinum-based DNA damaging agents can be administered acutely and cumulatively to avoid serious side effects.

DNA DAMAGING AGENTS

Another major strategy in chemotherapy is to use DNA damaging agents to inhibit processive DNA polymerases. Since DNA damaging agents are very electrophilic, they effectively react with nucleophilic moieties on DNA to significantly modify the hydrogen-bonding potential and structure of nucleic acid. In most instances, the formed DNA lesion acts as a physical barrier and hinders the movement of a DNA polymerase to inhibit DNA synthesis. In other cases, the change in hydrogen-bonding information on DNA tends to increase the frequency of misincorporation events to subsequently enhance the occurrence of pro-mutagenic DNA synthesis. The mismatches that are formed become excellent substrates for enzymes involved in various DNA repair pathways which can either correct the damaged DNA or cause cell death. The cellular effects of temozolomide (TMZ), a monofunctional alkylating agent, represent an excellent example of this phenomenon. TMZ produces cytostatic and cytotoxic effects primarily through the non-enzymatic methylation of DNA. Specifically, TMZ creates a number of DNA lesions including N^3-methyladenine, O^6-methylguanine, and N^7-methylguanine, the most commonly formed DNA adduct (Gates et al., 2004). Methylation at the N7 position of guanine produces a more toxic DNA lesion, termed an abasic site, which forms by the spontaneous depurination of the methylated base (Friedman et al., 2000). Since abasic sites lack Watson-Crick coding information, they are classified as non-instructional DNA lesions and typically inhibit the synthetic activity of most high-fidelity DNA polymerases (Shcherbakova et al., 2003). In contrast, alkylation of the O^6 position of guanine changes its hydrogen-bonding potential which increases the frequency of misincorporation events (Woodside and Guengerich, 2002). The resulting mispair that results from the misincorporation of dTMP opposite O^6-methylguanine activates the MMR pathway to ultimately induce apoptosis (Koç et al., 1996).

There are a large number of chemotherapeutic agents that exert their effects by damaging DNA as well. For example, one of the most used therapeutic modalities against solid tumors is ionizing radiation which creates radicals that inflict damage on nucleic acid (Santivasi and Xia, 2014). Doxorubicin is classified as a tetracycline antibiotic which intercalates into DNA to produce a variety of cellular effects (Pommier et al., 2010). First, the interaction with DNA inhibits the progression of topoisomerase II, an enzyme involved in relaxing supercoiled DNA that forms during replication and transcription. In addition, the quinone moiety of doxorubicin enhances free radical production in an oxygen-dependent manner to cause DNA damage. In both cases, the end result is the production of double-strand DNA breaks (DSBs) which inhibit DNA synthesis. Etoposide is similar in function as it forms a complex with DNA and topoisomerase II. The formation of this ternary complex inhibits the ability of topoisomerase II to re-ligate DNA, and this ultimately creates DSBs (Meresse et al., 2004). Camptothecin, a natural product isolated from the tree, Camptotheca acuminate, is a quinolone alkaloid that also creates DSBs by inhibiting the activity of topoosiomerase I (Liu et al., 2000). Unfortunately, camptothecin produces a number of adverse side effects in cancer patients and as such is not widely used clinically. However, two modified analogs of camptothecin (topotecan and irinotecan) display more favorable pharmacodynamic behavior and are used to treat several types of solid tumors (Mathijssen et al., 2002). Similar to the parental compound, topotecan and irinotecan exert their cytotoxic effects by generating DSBs.

With all of these agents, the DSBs that are formed directly inhibit DNA synthesis since these non-instructional lack Watson-Crick coding information (Boulton et al., 2000). DSBs can be repaired by non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ and HR use different DNA polymerases to efficiently and completely repair formed DSBs. Cisplatin, chlorambucil and cyclophosphamide represent another type of DNA damaging agent that is widely used to treat hematological and solid tumors (Passerini and Ponticelli, 2003; Anders et al., 2006). These agents are bifunctional alkylating agents that can create crosslinks and/or bulky adducts that produce physical barriers which inhibit DNA synthesis. By stalling DNA synthesis, these lesions generate single-stranded DNA breaks (SSBs) and DSBs that can cause cell death if left unrepaired.

COMBINATION THERAPIES

There is substantial clinical evidence supporting a strategy for combining nucleoside analogs with DNA damaging agents. As indicated earlier, gemcitabine is frequently combined with platinum drugs such as cisplatin and oxaliplatin to treat ovarian and pancreatic cancer (Hoff and Fuchs, 2003; Ozols, 2005; Sehouli, 2005; Chua and Cunningham, 2006). Several pre-clinical studies have examined the underlying mechanism for how gemcitabine synergizes the cytotoxic effects of platinum-based drugs. Using the ovarian cancer cell line, A2780, as a model, Jensen et al. showed that gemcitabine combined with cisplatin caused an increase in the amount of platinum-DNA adducts compared to cisplatin treatment alone (Jensen et al., 1997). The higher number of DNA adducts appeared to result from a decrease in DNA repair that was caused by the inhibition of cellular exonucleases such as excision repair cross-complementation group 1 (ERCC1). However, other models such as the inhibition of specialized DNA polymerases by gemcitabine have also been invoked (Chen et al., 2008). This model is based on evidence showing that pol η-deficient cells are more sensitive to the combination of gemcitabine and cisplatin compared to normal fibroblast that are pol η-proficient. In addition, pol η-deficient cells are ∼10-fold more sensitive to the combined treatment

of gemcitabine and cisplatin compared to treatment with cisplatin alone.

Surprisingly, attempts to combine other nucleoside analogs such as fludarabine (Fludara) and cladrabine (Leustatin) with DNA damaging agents have proven unsuccessful. For example, a study performed by Rai et al. was discontinued since patients receiving fludarabine and chlorambucil showed evidence for excessive hematological toxicity with no improvement in overall response compared to fludarabine monotherapy (Rai et al., 2000). A similar study using chlorambucil with escalating doses of fludarabine in patients with CLL also showed high levels of hematological toxicity (Weiss et al., 1994). Identical complications have been experienced in patients receiving cladribine and chlorambucil (Tefferi et al., 1994). The reason for the onset of these hematological toxicities may reflect a lack of selectivity exhibited by these purine nucleosides. In this case, the higher potency of fludarabine against replicative DNA polymerases may cause non-specific killing by placing a high burden on DNA replication and DNA repair in healthy cells.

DRUG RESISTANCE CAUSED BY TRANSLESION DNA SYNTHESIS

Although, cells possess several DNA repair pathways, there are situations in which DNA lesions are not detected and persist to block DNA synthesis catalyzed by high-fidelity DNA polymerases. To avoid this, cells use the unique activity of various specialized DNA polymerases to replicate unrepaired lesions in a process termed translesion DNA synthesis (TLS). As expected, the coordination of TLS activity at the cellular level is remarkably complex, and much of this complexity arises from the number of DNA polymerases that can replicate the various types of DNA lesions produced by endogenous and exogenous agents. Despite these complexities, there are two general models that describe how DNA polymerase activities are coordinated during TLS (**Figure 7**; Friedberg et al., 2005; McCulloch and Kunkel, 2006). In one model, a replicative DNA polymerase encounters a DNA lesion and incorporates a nucleotide opposite the DNA lesion. Since replicative polymerases display high-fidelity, they generally do not extend beyond the DNA lesion. This causes stalling of the replication fork which serves as a signal to enlist the activity of a specialized DNA polymerase such as pol κ or pol ξ to incorporate nucleotides beyond the lesion. Once the damaged DNA is by-passed, the specialized DNA polymerase is displaced by the replicative enzyme to continue DNA synthesis on the remainder of undamaged DNA. This model could occur with DNA lesions such as O^6-methylguanine and 8-oxo-guanine.

The second model differs slightly as the replicative polymerase is unable to incorporate a nucleotide opposite the lesion. Instead, one or more specialized DNA polymerases are used to incorporate a dNTP opposite the lesion. Depending upon the

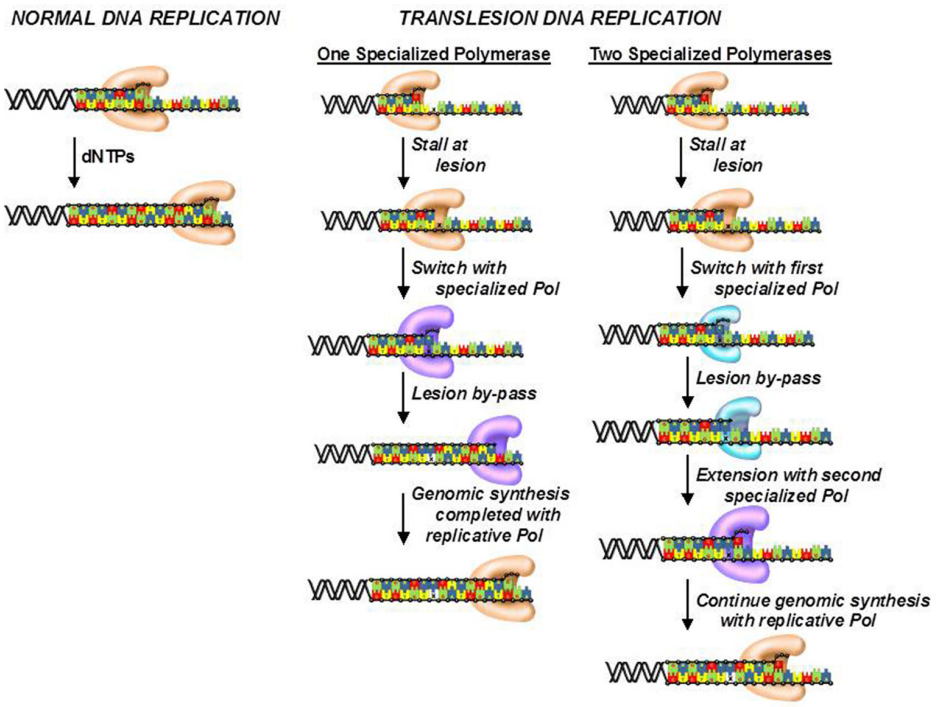

FIGURE 7 | Models for the efficient bypass of DNA lesions during translesion DNA synthesis. After encountering a DNA lesion, a replicative DNA polymerase incorporates a nucleotide opposite it but is unable to extend beyond the lesion. A specialized DNA polymerase extends beyond the lesion. Once the lesion is bypassed, the specialized polymerase is replaced by the replicative polymerase to resume processive DNA synthesis. In some cases, the replicative polymerase stalls at the DNA lesion and is unable to incorporate a nucleotide opposite the adduct. As a result, a specialized polymerase is recruited to the DNA lesion to incorporate a nucleotide opposite the lesion. A different specialized polymerase is then recruited for extension beyond the DNA lesion.

nature of the damaged DNA, the specialized DNA polymerase can extend beyond it as well. However, a different specialized polymerase such as pol ξ is often recruited to further elongate beyond the DNA lesion. Once the lesion is effectively by-passed, a replicative DNA polymerase replaces the extender polymerase and continues processive DNA synthesis on the remainder of the undamaged DNA. This scenario likely occurs with crosslinked or large bulky DNA lesions such as thymine dimers and cisplatinated DNA.

A number of retrospective clinical trials have been recently performed to examine possible correlations between patient responses to DNA damaging agents with the expression level of certain specialized DNA polymerases. Several studies have identified a group of distinct DNA polymerases that play important roles in modulating patient responses to certain chemotherapeutic agents. In particular, overexpression of DNA polymerases such as pol β, pol η, pol λ, and pol ι is observed in many different types of tumors (Albertella et al., 2005; Tan et al., 2005; Yoshizawa et al., 2009).

These specialized DNA polymerases also play important roles in defining how patients respond to certain chemotherapeutic agents. For example, pol η can extend beyond cisplatin-DNA lesions, and overexpression of this specialized DNA polymerase causes resistance to cisplatin in cancer cell lines whereas downregulation causes increased cellular sensitivity to cisplatin (Nivard et al., 2005; Chen et al., 2006). Higher mRNA expression of pol η is associated with poor outcomes in patients with non-small-cell lung cancer and is also associated with shorter survival times in patients receiving platinum drugs (Ceppi et al., 2009). Similar observations are seen with other specialized DNA polymerases such as pol ι which is overexpressed in breast cancer cells and found to be upregulated in ∼30% of glioma tumors (Yang et al., 2004) Overexpression of pol ι also appears to be clinically relevant as patients with pol ι-positive gliomas had shorter survival rates (Yang et al., 2004).

EMERGING AREAS

These examples suggest that selectively inhibiting one or more specialized DNA polymerases may provide a new strategy to combat clinical complications associated with unregulated TLS activity. In fact, inhibiting the replication of DNA lesions produced by anti-cancer agents may generate a number of positive effects in cancer patients that undergo chemotherapy. First, inhibiting TLS activity would likely increase the cytotoxic effects of DNA damaging agents and potentiate their effectiveness, especially in cancer cells that are defective in DNA repair. The benefit of potentiation is that lower doses of DNA damaging agents could be administered, thus reducing the risk of potential side effects. In addition, inhibiting TLS activity would combat drug resistance caused by the replication of damaged DNA. Finally, preventing pro-mutagenic DNA synthesis could hinder cancer recurrence caused by mutagenesis.

Efforts in our laboratory have focused on developing artificial nucleosides that are efficiently utilized by specialized DNA polymerases during the replication of lesions generated by DNA damaging agents. One DNA lesion that plays an important therapeutic role is the abasic site, a non-instructional form of DNA damage that is produced by several anti-cancer agents including TMZ and cyclophosphamide. To inhibit the replication of this lesion, we generated an artificial nucleotide, designated 3-ethynyl-5-nitroindolyl-2′-deoxyriboside triphosphate (3-Eth-5-NITP), that is a more efficient substrate than dATP, the natural nucleotide that is preferentially utilized by several human DNA polymerases during TLS (Motea et al., 2012). *In vitro* kinetic approaches compared the ability of pol δ, the high-fidelity polymerase involved in chromosomal replication and pol η, a specialized DNA polymerase, to incorporate dATP and 3-Eth-5-NITP opposite an abasic site (Choi et al., 2017). Our studies showed that pol η is 500-fold more efficient at incorporating dATP opposite the non-instructional lesion compared to the high-fidelity polymerase, pol δ. This large difference verifies that pol η contributes significantly to the error-prone replication of this lesion. More importantly, we demonstrated that pol η utilizes 3-Eth-5-NITP ∼30-fold more efficiently than dATP when replicating an abasic site. Furthermore, this artificial analog blocks extension beyond the lesion and terminates pro-mutagenic DNA synthesis. Cell-based studies demonstrate that the corresponding artificial nucleoside (3-Eth-5-NIdR) potentiates the effects of certain DNA damaging agents that produce abasic sites (Choi et al., 2017). Using acute lymphoblastic leukemia (ALL) cells as a model, we showed that co-treatment with TMZ and sub-lethal doses of 3-Eth-5-NIdR results in a synergistic increase in cell death. This synergism in apoptosis was caused by inhibiting TLS activity, and this was confirmed as the levels of 3-Eth-5-NITP in genomic DNA were higher in cells treated with 3-Eth-5-NIdR and DNA damaging agent compared to cells treated with 3-Eth-5-NIdR alone. We are currently testing the efficacy of combining 3-Eth-5-NIdR with TMZ in several xenograft mouse models of human cancer to demonstrate proof-of-concept for this strategy. Preliminary data from these *in vivo* studies look very promising, and the theranostic capabilities of 3-Eth-5-NIdR could usher in a new strategy in precision-based therapies against cancer.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Off-Target Effects of Drugs that Disrupt Human Mitochondrial DNA Maintenance

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Nucleoside reverse transcriptase inhibitors (NRTIs) were the first drugs used to treat human immunodeficiency virus (HIV) the cause of acquired immunodeficiency syndrome. Development of severe mitochondrial toxicity has been well documented in patients infected with HIV and administered NRTIs. *In vitro* biochemical experiments have demonstrated that the replicative mitochondrial DNA (mtDNA) polymerase gamma, Polg, is a sensitive target for inhibition by metabolically active forms of NRTIs, nucleotide reverse transcriptase inhibitors (NtRTIs). Once incorporated into newly synthesized daughter strands NtRTIs block further DNA polymerization reactions. Human cell culture and animal studies have demonstrated that cell lines and mice exposed to NRTIs display mtDNA depletion. Further complicating NRTI off-target effects on mtDNA maintenance, two additional DNA polymerases, Pol beta and PrimPol, were recently reported to localize to mitochondria as well as the nucleus. Similar to Polg, *in vitro* work has demonstrated both Pol beta and PrimPol incorporate NtRTIs into nascent DNA. Cell culture and biochemical experiments have also demonstrated that antiviral ribonucleoside drugs developed to treat hepatitis C infection act as off-target substrates for POLRMT, the mitochondrial RNA polymerase and primase. Accompanying the above-mentioned topics, this review examines: (1) mtDNA maintenance in human health and disease, (2) reports of DNA polymerases theta and zeta (Rev3) localizing to mitochondria, and (3) additional drugs with off-target effects on mitochondrial function. Lastly, mtDNA damage may induce cell death; therefore, the possibility of utilizing compounds that disrupt mtDNA maintenance to kill cancer cells is discussed.

Keywords: nucleoside reverse transcriptase inhibitors, mitochondrial DNA polymerase gamma, human immunodeficiency virus (HIV), mitochondrial diseases, cancer, antiviral ribonucleosides, mitochondrial DNA (mtDNA)

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THE ORIGIN OF MITOCHONDRIA AND OFF-TARGET EFFECTS OF ANTIBIOTICS

Mitochondria are best known for their role in generating energy by oxidative phosphorylation (OXPHOS), the process of coupling substrate oxidation to the production of the energy-rich molecule adenosine triphosphate (ATP). In addition to generating the bulk of the cell's energy supply mitochondria are important sites of calcium homeostasis, nucleotide and amino acid metabolism and biosynthesis of heme, iron-sulfur clusters, and ubiquinone. Mitochondria are

eukaryotic organelles that share bacterial features such as a double-membrane structure and a circular multi-copied genome or mitochondrial DNA (mtDNA). The endosymbiotic theory hypothesizes mitochondria descended from an ancient alpha (α)-proteobacteria that developed a symbiotic relationship with an ancient nucleated cell (Gray, 2017). Support for the endosymbiotic hypothesis comes from striking similarities revealed between the mitochondrial and the *Rickettsia prowazekii* genomes (Andersson et al., 1998). Over time mitochondria lost most of their proto-bacterial genome to the nucleus. One thousand one hundred and forty-five nuclear-encoded mitochondrial gene products must be imported into mitochondria following translation on cytoplasmic ribosomes and estimates place the total mitochondrial proteome at ∼1,500 gene products (Lopez et al., 2000; Calvo et al., 2016). Currently, there are ∼170 known mitochondrial disease genes associated with ∼500 clinical phenotypes suggesting that most medical specialists could see patients with mitochondrial disease (Scharfe et al., 2009; Turnbull and Rustin, 2016). The α-proteobacterial endosymbiont origin of mitochondria is supported by observations that certain antibiotics have off-target effects on mitochondrial ribosomes. Similar to bacterial translation, mitochondrial translation is initiated with an *N*-formylmethionine and mitochondrial but not cytoplasmic translation is sensitive to bacterial antibiotics such as chloramphenicol (CAP) and aminoglycosides (Wallace et al., 1975; Oliver and Wallace, 1982; Wallace and Chalkia, 2013). Additionally, mitochondrial ribosomes are resistant to inhibitors of eukaryotic translation such as emetine and cycloheximide (Oliver and Wallace, 1982).

MITOCHONDRIAL DISORDERS AND THE IMPORTANCE OF mtDNA MAINTENANCE IN HUMAN HEALTH

The haploid human nuclear genome consists of ∼3 billion base pairs (bp) of DNA and contains ∼20,000 protein-coding genes and ∼23,000 non-coding genes. Examples of non-coding genes include transfer RNA (tRNA), ribosomal RNA (rRNA), micro RNA (miRNA), miscellaneous RNA (miscRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), small cytoplasmic RNA (scRNA), and long non-coding RNA (lncRNA). In comparison, the mitochondrial genome harbors only 13 genes for polypeptides, 2 genes for rRNA, and 22 genes for tRNA on ∼16,600 bp and mutations associated with maternally-inherited mitochondrial disorders have been identified in all 37 open reading frames. Similar to practically all prokaryotic genes, human mtDNA genes lack introns. *The 13 polypeptide-encoded genes code for subunits of the mitochondrial inner membrane (MIM) OXPHOS machinery*. While the size and coding capacity of mtDNA is much less than the nuclear genome our maternally inherited genome is critical to cellular viability as exemplified by the numerous disease mutations associated with it and by observations that knocking out mtDNA maintenance genes results in embryonic lethality in various mouse models (Park and Larsson, 2011). Currently, greater than 660 mtDNA mutations are associated with disease phenotypes (www.mitomap.org). The most common encephalopathies caused by mtDNA point mutations include Leigh Syndrome, Leber Hereditary Optic Neuropathy, MERRF (myoclonic epilepsy with ragged red fibers), MIDD (maternally inherited diabetes and deafness), MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes), non-syndromic hearing loss, and NARP (neuropathy, ataxia, retinitis pigmentosa) (Pinto and Moraes, 2014). Maintenance of the mitochondrial genome is also required to avoid apoptosis induced by mtDNA damage (Santos et al., 2003; Tann et al., 2011).

Molecules of mtDNA associate with various DNA-binding proteins on the matrix-side of the MIM and form protein-mtDNA structures known as nucleoids (Bogenhagen et al., 2008; Brown et al., 2011; Hensen et al., 2014; Young et al., 2015). Utilizing live-cell fluorescence microscopy or immunocytochemistry, nucleoids can be visualized as foci or puncta. Furthermore, a single cell can contain several thousand copies of mtDNA which are distributed within hundreds of individual mitochondria or throughout an elaborate mitochondrial reticular network (Miller et al., 2003; Spelbrink, 2010; Archer, 2013; Young et al., 2015). Localization of mtDNA at the MIM is likely important to coordinate mtDNA replication and transcription with mitochondrial translation, cytoplasmic translation, and mitochondrial protein import and assembly (Iborra et al., 2004; Spelbrink, 2010). Nuclear-encoded mitochondrial transcription machinery is imported into the organelle to transcribe mtDNA genes. Nuclear-encoded mitochondrial ribosomal subunits assemble with mtDNA-encoded rRNAs following protein import to form the translation machinery necessary to synthesize the 13 mtDNA-encoded polypeptides. Therefore, the MIM OXPHOS energy-generating process is strictly dependent on mtDNA maintenance and pharmacological blocks to mitochondrial genome replication would be devastating to this energy-generating process.

MITOCHONDRIAL REACTIVE OXYGEN SPECIES (ROS) AND BASE EXCISION REPAIR (BER)

Aberrant electron leakage from the OXPHOS machinery to molecular oxygen (O_2) can generate reactive oxygen species (ROS) which, if not detoxified, cause damage to intracellular molecules such as DNA, RNA, lipids, and proteins (Wallace, 2005). The close proximity of mtDNA-containing nucleoids to the OXPHOS machinery generating ROS has been suggested to inflict more damage on the mitochondrial genome than on the nuclear genome (Tann et al., 2011). ROS-induced DNA damage includes a large quantity of mutagenic oxidized bases and the mutation rate of human mtDNA has been estimated to be 20–100-fold higher relative to nuclear DNA. Nuclear-encoded base excision repair (BER) machinery is imported into the mitochondrion to assist with mending abnormal and oxidized base lesions. During mitochondrial short-patch BER, an oxidized base may first be excised by a monofunctional DNA glycosylase such as UNG1 or MUTYH, **Figure 1A**. DNA glycosylase cleaves

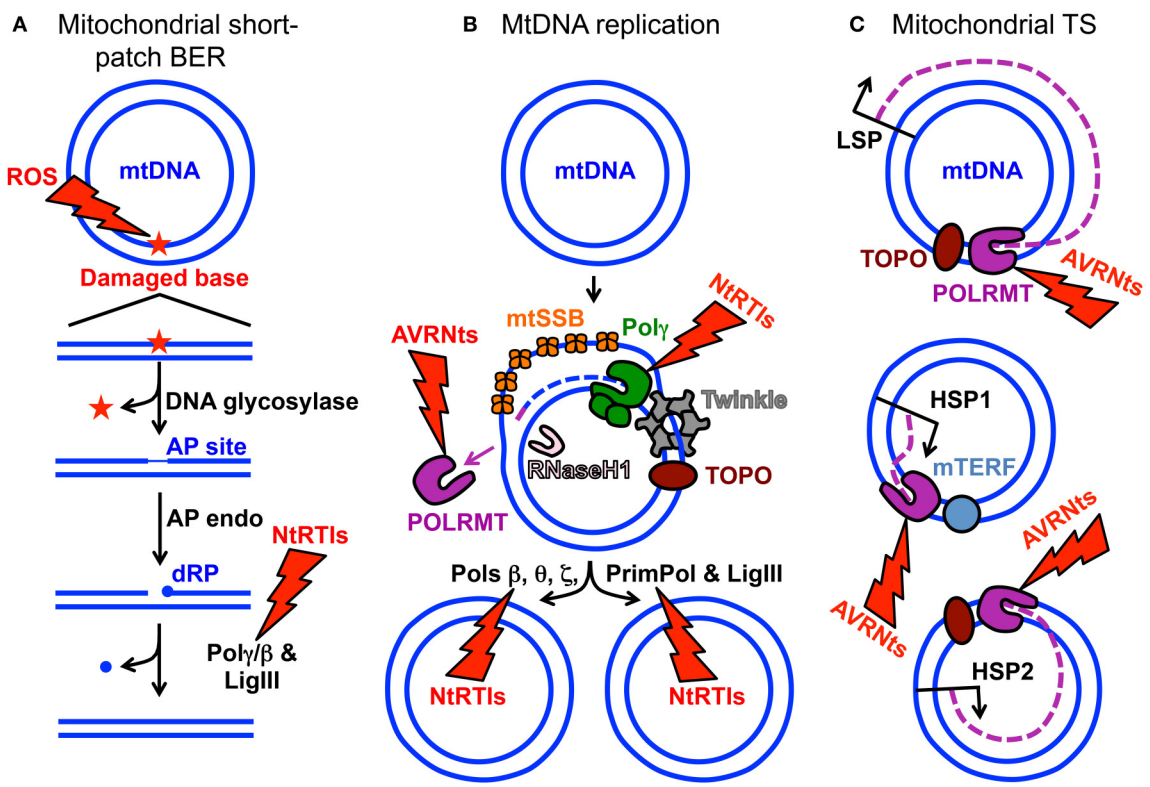

FIGURE 1 | MtDNA maintenance and mitochondrial gene expression. **(A)** Mitochondrial short-patch base excision repair (BER) initiated with a monofunctional DNA glycosylase. The ROS lightning bolt represents reactive oxygen species-induced mtDNA damage generating an oxidized base lesion (star) that is subsequently removed and repaired by the BER machinery. Two blue circles represent the double-stranded circular mitochondrial genome. A region of the damaged mtDNA is shown below the circular genome to emphasize the BER pathway steps. AP site, apurinic/apyrimidinic site; AP endo, AP endonuclease; dRP, 5′-deoxyribose phosphate; Polγ/β the replicative mtDNA polymerase gamma or DNA polymerase beta; LigIII, mitochondrial DNA ligase III. The NtRTIs lightning bolt represents nucleotide reverse transcriptase inhibitors blocking Polγ or β. **(B)** Key components of the mtDNA replication and repair machinery. The small purple line represents an RNA primer while the blue dashed line represents newly synthesized mtDNA. TOPO, topoisomerase; Twinkle, Twinkle mtDNA helicase; POLRMT, mitochondrial RNA polymerase and primase; RNaseH1, Ribonuclease H1; mtSSB, mitochondrial single-stranded DNA binding protein. DNA polymerase beta, theta, zeta, and the DNA *prim*ase and translesion DNA *pol*ymerase are represented by Pols β, θ, ζ, and PrimPol respectively. These polymerases likely assist Polγ with overcoming mtDNA damage. The AVRNts lightning bolt represents antiviral ribonucleotides blocking POLRMT activity. **(C)** Polycistronic mitochondrial transcription. Mitochondrial transcription (TS) occurs from three promoters: (1) LSP, light-strand promoter, (2) HSP1, heavy-strand promoter 1, and (3) HSP2, heavy-strand promoter 2. Three purple dashed lines represent transcripts synthesized from the promoters. Although not visualized in the cartoon, mitochondrial TS initiation requires mitochondrial TS factor A (TFAM) and either of mitochondrial TS factors B1 or B2 (TFB1M or TFB2M). It is generally accepted that TFB2M is the primary factor for TS initiation (Shutt et al., 2010). Mitochondrial TS termination factor is represented by mTERF.

the damaged base *N*-glycosidic bond generating an abasic or apurinic/apyrimidinic (AP) site then this site is cleaved by an AP endonuclease to generate a 3′-OH and non-ligatable 5′-deoxyribose phosphate (dRP) moiety. Next, the catalytic subunit of the replicative mitochondrial 5′-3′ DNA polymerase gamma (Polγ) fills in the gap via its DNA polymerase activity and removes the dRP group via its 5′-deoxyribose phosphate (dRP) lyase activity leaving a 5′-phosphate. Lastly, DNA ligase III seals the nick and the damage is repaired (Longley et al., 1998). Alternatively, a bifunctional DNA glycosylase harboring an intrinsic lyase activity can cleave the *N*-glycosidic bond and incise the AP site; however, the ends generated by the incision are non-ligatable and must be processed by either AP endonuclease or polynucleotide kinase 3′-phosphatase then Polγ can fill the gap and ligase can seal the nick (Bebenek and Kunkel, 2004; Alexeyev et al., 2013). **Figure 1A** is a simplified cartoon of short-patch BER. Details regarding mitochondrial short-patch and long-patch BER pathways have been thoroughly reviewed (Alexeyev et al., 2013; Copeland and Longley, 2014; Van Houten et al., 2016).

POLγ AND THE REPLISOME

Human Polγ is the replicative mitochondrial DNA polymerase that harbors 3′-5′ exonucleolytic proofreading activity and participates in mtDNA repair (Young and Copeland, 2016). Polγ is a heterotrimer consisting of one 140-kDa catalytic subunit, p140 encoded by the nuclear *POLG* gene, and a 110-kDa homodimeric processivity subunit, p55 encoded by the nuclear *POLG2* gene. MtDNA disorders can be caused by genetic defects in nuclear genes, and a class of genes specifically linked to instability of mtDNA has emerged over the last

16 years which includes *POLG* and *POLG2*, **Table 1** (Young and Copeland, 2016). Nuclear mitochondrial disease genes are associated with a complex spectrum of early onset and late onset type phenotypes. One subclass of disorders, mtDNA depletion syndromes, may arise due to defects in genes encoding mtDNA replication machinery (ex. *POLG*, Alpers-Huttenlocher syndrome) or enzymes required for nucleotide synthesis (ex. *TK2*). MtDNA depletion syndromes in of themselves are variable and clinical manifestations may include myopathy, encephalomyopathy, neurogastrointestinal, or hepatocerebral phenotypes (Stiles et al., 2016). In addition to the 5′-3′ DNA polymerase, 3′-5′ exonuclease, and 5′ dRP lyase activities mentioned above the p140 catalytic subunit harbors reverse transcriptase (RT) activity (Murakami et al., 2003; Kaguni, 2004; Graziewicz et al., 2006). The RT activity or RNA-dependent DNA polymerase activity is similar to viral enzymes such as human immunodeficiency virus RT (HIV-RT). Unfortunately, as will be discussed below, biochemical experiments have demonstrated that Polγ is sensitive to inhibition by metabolically active forms of anti-HIV nucleoside reverse transcriptase inhibitors (NRTIs) known as nucleotide reverse transcriptase inhibitors (NtRTIs). Treatment of HIV-infected patients with NRTIs is accompanied by loss of mitochondrial function and NRTI toxicity mimics mitochondrial genetic diseases and induces similar symptoms such as mtDNA depletion (Graziewicz et al., 2006). One explanation as to why Polγ harbors RT activity may be to replicate past ribonucleotides (ribonucleoside monophosphates) that are evenly distributed between the two strands of mtDNA (Murakami et al., 2003; Berglund et al., 2017). The homodimeric Polγ p55 subunit imparts high processivity onto the holoenzyme by increasing the binding affinity to DNA (Lim et al., 1999; Young et al., 2015). Processivity is a measurement of the extent of Polγ DNA synthesis during a primer-template binding event. Polγ functions in conjunction with several replisome components including: (1) topoisomerase, (2) mitochondrial single-stranded DNA binding protein (mtSSB), (3) Twinkle mtDNA helicase, (4) RNaseH1, (5) mitochondrial RNA polymerase (POLRMT), and (6) mitochondrial DNA ligase III, **Figure 1B**. Additional factors critical for mitochondrial genome maintenance include: the multifunctional mitochondrial transcription factor A (TFAM) with significant roles in mtDNA replication and packaging, the RecB-type mitochondrial genome maintenance 5′-3′ exonuclease 1 (MGME1), the RNA and DNA 5′ flap endonuclease (FEN1), and the helicase/nuclease, DNA2 (Kalifa et al., 2009; Kornblum et al., 2013; Ngo et al., 2014). MGME1, FEN1, and DNA2 have all been implicated in mtDNA BER (Copeland and Longley, 2014). Furthermore, DNA2 has been shown to stimulate Polγ activity and to co-localize with Twinkle in the mitochondrial nucleoid, which suggests an important role in the replisome (Zheng et al., 2008). Some of the genes encoding components of the mtDNA replication machinery may have been acquired as part of a protomitochondrial genome, in the form of integrated phage genes from a T-odd lineage, which were then transferred to the eukaryotic nucleus (Shutt and Gray, 2006). This hypothesis is based on the shared conservation of primary protein amino acid sequences of T-odd bacteriophages with mitochondrial Polγ, POLRMT, and Twinkle helicase.

In agreement with the requirement for mtDNA replication re-initiation between embryonic day (E)6 and 7.5 (Stewart and Larsson, 2014), p140 in animal cells was shown to be essential using *POLG* knockout (KO) mice. The *POLG* KO results revealed embryonic lethality at E7.5–8.5 with subsequent depletion of mtDNA (Hance et al., 2005). Comparatively, several studies have illustrated the essential role of p55 in mtDNA replication: (i) two separate null mutations in the *Drosophila melanogaster*

TABLE 1 | Nuclear genes identified in mitochondrial patients that affect mtDNA stability[a].

Gene	Disorder[b]	Chromosomal locus	Function
MtDNA REPLICATION AND REPAIR			
POLG	PEO/Alpers/ataxia	15q25	Polγ catalytic subunit
POLG2	PEO	17q	Polγ processivity subunit
Twinkle	PEO/ataxia	10q24	MtDNA helicase
RNASEH1	PEO/ataxia	2p25	Mitochondrial and nuclear RNaseH1 (Reyes et al., 2015)
DNA2	PEO	10q21.3-22.1	Mitochondrial and nuclear helicase/nuclease (Ronchi et al., 2013)
MGME1	PEO, mtDNA depletion	20p11.23	RecB type exonuclease
TFAM	Neonatal liver failure mtDNA depletion	10q21.1	Mitochondrial transcription factor A (Stiles et al., 2016)
MAINTAINING dNTP POOLS			
ANT1	PEO	4q35	Adenine nucleotide translocator
TP	MNGIE	22q13.33	Thymidine phosphorylase
DGUOK	MtDNA depletion	2p13	Deoxyguanosine kinase
TK2	MtDNA depletion	16q22-23.1	Mitochondrial thymidine kinase
SUCLA2	MtDNA depletion	13q14.2	ATP-dependent Succinate-CoA ligase
SUCLG1	MtDNA depletion	2p11.2	GTP-dependent Succinate CoA ligase
RRM2B	MtDNA depletion	8q23.1	p53-Ribonucleotide reductase, small subunit
MPV17	MtDNA depletion and deletion	2p23.3	Mitochondrial inner membrane protein
ABAT	MtDNA depletion	16p13.2	4-aminobutyrate aminotransferase (Besse et al., 2015)
MITOCHONDRIAL HOMEOSTASIS AND DYNAMICS			
OPA1	Dominant optic atrophy	3q29	Dynamin-related GTPase
MFN2	Recessive optic atrophy	1p36.22	Mitofusin 2 (Rouzier et al., 2012)
FBXL4	MtDNA depletion, Encephalopathy	6q16.1-16.3	Mitochondrial LLR F-Box protein

[a] *The table is an updated version of Table 1 found in reference (Young and Copeland, 2016) and is reproduced with permission.*

[b] *PEO, progressive external ophthalmoplegia; MNGIE, mitochondrial neurogastrointestinal encephalomyopathy.*

POLG2 gene lead to lethality in the early pupal stage of fly development (Iyengar et al., 2002), (ii) homozygous *POLG2* KO mice are embryonic lethal at E8–8.5 (Humble et al., 2013), and (iii) in a porcine oocyte knockdown model, oocyte maturation requires *POLG2* (Lee et al., 2015). Mouse RNaseH1$^{-/-}$ embryos are null at E8.5 and have decreased mtDNA content leading to apoptotic cell death (Cerritelli et al., 2003). A mouse model of Twinkle deficiency has been generated by transgenic expression of a Twinkle cDNA with an autosomal dominant mutation found in patients (Tyynismaa et al., 2005; Tyynismaa and Suomalainen, 2009). These mice developed progressive respiratory chain deficiency at 1 year of age in cerebellar Purkinje cells, hippocampal neurons, and skeletal muscle. The affected cells accumulated multiple mtDNA deletions. These "Deletor" mice recapitulate many of the symptoms associated with PEO and represent a useful research model.

NEWLY IDENTIFIED HUMAN DNA POLYMERASES LOCALIZING TO MITOCHONDRIA - POLS β, θ, ζ, AND PRIMPOL

Prior to 2013 human Polγ was the only polymerase out of the 17 known cellular DNA polymerases demonstrated to localize to human cell mitochondria; however, mounting evidence suggests it is not the only one. Recently, DNA polymerase beta (Polβ) was detected in mitochondrial extracts prepared from human embryonic kidney cells (HEK-293T) and from various tissues obtained from mice (Sykora et al., 2017). Analysis of mouse tissue extracts revealed Polβ in brain and kidney mitochondria while none was detectable in heart, liver, or muscle. As a key member of the nuclear BER machinery Polβ provides the majority of the required 5′-dRP lyase activity in the nucleus; therefore, Polβ may participate in mitochondrial BER. In a short-patch BER scenario, following the actions of a monofunctional DNA glycosylase and an AP endonuclease Polβ could insert a nucleotide onto the 3′-OH then remove the 5′-dRP group using its dRP lyase activity followed by the nick sealing action of DNA ligase (**Figure 1A**). As mentioned above mitochondrial ribosomes are sensitive to CAP (CAP^{S}). MtDNA can develop resistance to CAP (CAP^{R}) through mutation of the mtDNA 16S rRNA gene changing the specificity of CAP for the mitochondrial ribosome and inhibiting its binding (Blanc et al., 1981; Kearsey and Craig, 1981; Wallace and Chalkia, 2013; Sykora et al., 2017). In two HEK-293T Polβ KO cell lines very few CAP^{R} cells could be isolated relative to the parental cell line when plated at high cell density. This finding suggests that Polβ may mediate mtDNA mutational events. Utilizing *in vitro* biochemistry Polβ was also demonstrated to interact with the mitochondrial Twinkle helicase and this interaction facilitated Polβ strand displacement. Enhanced strand displacement suggests Polβ may participate in the mitochondrial long-patch BER pathway (Sykora et al., 2017). As many *Twinkle* gene-disease mutations result in protein variants with partial helicase defects (Longley et al., 2010) it would be interesting to investigate strand displacement using recombinant Twinkle disease variants and Polβ to provide insight into possible mechanisms of *Twinkle*-related mitochondrial disease. Besides BER, other roles of Polβ in mtDNA maintenance remain to be elucidated. Polβ is not likely a replicative mtDNA polymerase as this enzyme lacks 3′-5′ exonuclease proofreading activity, has low processivity, incorporating few nucleotides each time it binds a primer-template, and has a high error rate relative to the proofreading proficient Polγ (Bebenek and Kunkel, 2004). However, *POLG*-related disease mutations that abolish p140 activity and are associated with late age of onset may argue in favor of redundant DNA polymerase function(s) in human cell mitochondria (Sykora et al., 2017). Polβ$^{-/-}$ mouse embryos survive the course of development but die immediately at the perinatal stage suggesting the cause of death is a neonatal respiratory defect (Sugo et al., 2000).

The DNA *prim*ase and translesion DNA *pol*ymerase, PrimPol, has been identified in mitochondria isolated from HEK-293T cells (García-Gómez et al., 2013). Translesion DNA polymerases are specialized enzymes that pass through DNA damage. However, PrimPol is likely only required for mtDNA repair and not for mtDNA replication, as *PRIMPOL*$^{-/-}$ KO mice are viable. Like Polβ PrimPol is localized to both the nucleus and the mitochondrion and lacks proofreading activity. Of note to human genetic disease, mutation of *PRIMPOL* is associated with the ocular disorder high myopia (Zhao et al., 2013; Keen et al., 2014). DNA polymerase theta (Polθ) was recently identified in mitochondria isolated from human cells (Wisnovsky et al., 2016). Polθ is a proofreading-deficient and error-prone polymerase capable of translesion DNA polymerization (Arana et al., 2008). In the nucleus, Polθ is implicated in double-strand DNA break repair, non-homologous end joining and maintenance of DNA replication timing. The translesion DNA polymerase zeta (Polζ) is composed of two subunits the catalytic subunit Rev3 and the structural subunit Rev7. To date, no evidence for Rev7 localization to human cell mitochondria has been described but the Rev3 subunit has been reported to localize to the organelle and may play a role in protecting mtDNA from ultraviolet radiation-induced DNA damage (Singh et al., 2015). Compared to Polγ, Pols θ and ζ localize to both the nucleus and the mitochondrion, have low fidelity, lack proofreading activity and have only moderate processivity (Bebenek and Kunkel, 2004; Arana et al., 2008; Lee et al., 2014); therefore, their main roles are likely in assisting the core replisome in overcoming mtDNA damage. Polθ KO mice are viable whereas Polζ KO mice are embryonic lethal with a block in embryo development not beyond 8–8.5 days *post-coitus* (Esposito et al., 2000; Shima et al., 2004). Details regarding the evidence supporting mitochondrial localization of the aforementioned human DNA polymerases have been reviewed (Krasich and Copeland, 2017).

NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS, NRTIs

NRTIs were the first drugs used to treat HIV, the cause of acquired immunodeficiency syndrome (AIDS). NRTIs remain effective today for treating HIV when combined with other drugs. Highly active antiretroviral therapy (HAART) uses multiple drugs to act on different HIV life-cycle stages. For patients

with HIV infection, HAART regimens include treatment with NRTIs in combination with non-nucleoside reverse transcriptase inhibitors (NNRTIs) or protease inhibitors, PIs (Nolan and Mallal, 2004). NNRTIs and NRTIs primarily block HIV genome replication by inhibiting the HIV-RT from transcribing the viral single-stranded RNA genome into DNA. FDA-approved NRTIs used to treat HIV infection include: ddC (zalcitabine), 3TC (Epivir®, lamivudine), AZT (Retrovir®, zidovudine), ddI (Videx-EC®, didanosine), PMPA (Viread®, tenofovir DF), d4T (Zerit®, stavudine), ABC (Ziagen®, abacavir), and FTC (emtricitabine, Emtriva®), **Table 2**. NRTIs may be administered to patients in fixed-dose combinations: Combivir® (Retrovir + Epivir), Descovy® (tenofovir alafenamide + Emtriva), Epzicom® (Epivir + Ziagen), Trizivir® (Retrovir + Epivir + Ziagen), and Truvada® (Viread + Emtriva), https://www.hiv.va.gov/patient/treat/NRTIs.asp. The history of antiretroviral drugs and the currently used antiretroviral therapies have been reviewed (Pau and George, 2014). A discussion of what is currently known regarding NRTIs with off-target effects on mtDNA replication is discussed below.

Nucleoside analogs, including NRTIs, are taken up by cells then phosphorylated to active nucleotide analogs by intracellular kinases (Zhu et al., 2000). Nucleoside kinases such as DCK, CMPK1, and nucleoside diphosphate kinases (NME) act on NRTIs like ddC and perform the first, second, and third phosphorylation steps respectively generating the active NtRTI in the cytoplasm ex. ddCppp, where ppp represent the triphosphate (Liyanage et al., 2017). NtRTIs can then be imported into mitochondria and could compete with native nucleotides at DNA polymerase active sites to inhibit mtDNA replication through chain termination and persistence in the mitochondrial genome. Unlike natural deoxyribonucleotide triphosphate substrates, and with the exception of FIAU, NtRTIs are chain terminators that lack the 3′ hydroxyl group and therefore cannot be extended by a polymerase once incorporated into DNA. Therefore, if these analogs are not removed from DNA, replication will stall (**Figure 1B**).

CLINICAL EVIDENCE FOR NRTI DISRUPTION OF mtDNA REPLICATION

In clinical trials drugs that showed promise in AIDS therapy, such as fluoro-dideoxyadenosine (FDDA), or in the treatment of chronic hepatitis B infection, such as FIAU, toxicity was reported affecting peripheral nerves, liver, skeletal, and cardiac muscle (Lewis et al., 2001). Toxicity to mitochondria was so severe that hepatic failure and death in some patients necessitated discontinuation of their use (McKenzie et al., 1995). One long-term AZT use study of HIV-positive patients concluded that AZT treatment caused toxic mitochondrial myopathy (Dalakas et al., 1990). In a follow-up study investigating mtDNA content in muscle biopsies, mitochondrial genome depletion was discovered in all HIV-positive patients who were treated with AZT and who displayed myopathy and ragged-red fibers in comparison to controls (Arnaudo et al., 1991). Another study investigated HIV-positive patients who developed neuropathy 6–10 weeks after starting ddC and this investigation found mitochondrial alterations and significantly reduced mtDNA copy number in nerve biopsy specimens (Dalakas et al., 2001). These and other observations led to the Polγ dysfunction hypothesis. Hypothetically, poisoning of Polγ would lead to decreased mtDNA, increased mitochondrial stress due to compromised OXPHOS (as OXPHOS subunits are encoded by mtDNA), increased cellular energy depletion (due to diminished ATP pools), and acquired mitochondrial disease phenotypes (Koczor and Lewis, 2010). Key side effects of NRTIs are summarized in **Table 2** and (Koczor and Lewis, 2010). Support for the Polγ dysfunction hypothesis comes from cell culture and biochemical work discussed below.

EVIDENCE FOR POLγ-MEDIATED NRTI TOXICITY FROM BIOCHEMICAL STUDIES

Polγ-mediated NRTI mitochondrial toxicity requires that analogs be metabolized to NtRTIs, imported into mitochondria then incorporated into mtDNA and persist there to block further genome replication events. Support for NRTI toxicity caused by inhibition of Polγ DNA polymerase activity comes from extensive biochemical evidence. Pre-steady and steady-state enzyme kinetic analyses have demonstrated that Polγ is able to incorporate various anti-retroviral NtRTIs (Martin et al., 1994; Johnson et al., 2001; Lim and Copeland, 2001; Brown et al., 2011). NtRTIs that have been tested *in vitro* for incorporation into nascent DNA by Polγ include: ddCppp, ddTppp, d4Tppp, ddAppp (the active form of ddI), (+) and (−)3TCppp, PMPApp (PMPA triphosphate), AZTppp, CBVppp (the active form of ABC), and FIAUppp. These biochemical studies agree that Polγ incorporates NtRTIs during DNA replication; however, the efficiency of analog incorporation is variable among the NtRTIs that have been examined. Polγ incorporates ddCppp, ddAppp (ddI), and d4Tppp analogs most efficiently while 3TCppp, PMPApp, AZTppp, and CBVppp (ABC) are modestly incorporated into DNA. Steady-state and pre-steady-state kinetics have also demonstrated that FIAUppp is strongly incorporated by Polγ (Lewis et al., 1996; Johnson et al., 2001). Mitochondrial toxicity, therefore, may be acquired due to a block in mtDNA replication if chain-terminating NtRTIs cannot be removed. Indeed, biochemical evidence has shown that Polγ does not efficiently proofread NtRTIs incorporated into DNA. Pre-steady-state measurements have demonstrated that a ddCp (ddC monophosphate) incorporated into the 3′-end of a DNA oligonucleotide annealed to a DNA template essentially cannot be removed by Polγ proofreading activity even after 12-h incubations with the DNA duplex (Johnson et al., 2001). The remaining NtRTIs analyzed for exonucleolytic removal had slow rates of excision and it has been estimated that the half-life of the reaction to remove (+)3TCp or (−)3TCp is ∼1 min (Feng et al., 2001). The rate of NtRTI excision could be detrimental *in vivo* by slowing the mtDNA replication machinery. If Polγ dissociates from mtDNA prior to cleaving an incorporated nucleotide analog then replication would be terminated. When PMPA-terminated DNA substrate was tested for excision in the presence of trap

TABLE 2 | NRTIs with off-target effects on human DNA polymerases that localize to mitochondria.

Drug	Target	Potential off-target[a]	Mode of action	Side effects/toxicity/other notes	Experimental evidence for off-target effect	References
ddC, 2′,3′-dideoxycytidine, zalcitabine, hivid	HIV-RT	Polγ, PrimPol, Polβ	Deoxycytidine analog, chain-terminator	Peripheral neuropathy, sensorineural deafness, hypertrophic cardiomyopathy; according to the FDA ddC is no longer marketed	MtDNA depletion in various human cell lines; efficiently incorporated by Polγ and PrimPol, *in vitro* (Polγ 14-fold reduction in dCTP/ddCppp discrimination relative to PrimPol); Polβ incorporates and sensitive to ddCppp inhibition *in vitro*	Martin et al., 1994; Pelletier et al., 1994; Johnson et al., 2001; Lewis et al., 2001; Lim and Copeland, 2001; Birkus et al., 2002; Ashley et al., 2005; Setzer et al., 2005; Rocher et al., 2008; Jemt et al., 2015; Mislak and Anderson, 2015
ddI, 2′,3′-dideoxyinosine, didanosine, Videx-EC®	HIV-RT	Polγ, PrimPol, Polθ?	Deoxyadenosine analog, chain-terminator	Peripheral neuropathy, pancreatitis, hypertrophic cardiomyopathy, diabetes mellitus, hepatocellular failure, lactic acidosis; ddI is metabolized to ddAppp	Aberrant cristae and decreased mtDNA copy number in human cell lines; ddAppp (active form of ddI) incorporated efficiently by Polγ and incorporated by PrimPol *in vitro* (Polγ 233-fold reduction in dATP/ddAppp discrimination relative to PrimPol)	Medina et al., 1994; Johnson et al., 2001; Lewis et al., 2001; Birkus et al., 2002; Setzer et al., 2005; Mislak and Anderson, 2015; Zahn et al., 2015
d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine, stavudine, Zerit®	HIV-RT	Polγ, Polβ	Thymidine analog, chain-terminator	Peripheral neuropathy, pancreatitis, hepatocellular failure, lactic acidosis, lipodystrophy; no longer recommended for administration	Aberrant cristae and decreased mtDNA copy number in human cell lines; incorporated efficiently by Polγ *in vitro*; Polβ incorporates and sensitive to d4Tppp inhibition *in vitro*	Martin et al., 1994; Vázquez-Acevedo et al., 1995; Johnson et al., 2001; Lewis et al., 2001; Lim and Copeland, 2001; Birkus et al., 2002; Setzer et al., 2005; Koczor and Lewis, 2010
3TC, 2′,3′-dideoxy-3′-thiacytidine, lamivudine, Epivir®	HIV-RT	Polγ, Polβ	Zalcitabine/cytosine analog (see above), chain-terminator	Peripheral neuropathy, lactic acidosis, hepatomegaly with steatosis	Kinetic analysis with HeLa Polγ, modest inhibition of Polγ *in vitro*; Polβ has a 9-fold reduction in dCTP/L-3TCppp discrimination in comparison to Polγ *in vitro*	Johnson et al., 2001; Lewis et al., 2001; Lim and Copeland, 2001; Koczor and Lewis, 2010; Brown et al., 2011
PMPA, (*R*)-9- (2-phosphonylmethoxypropyl)adenine, TFV, tenofovir	HIV-RT	Polγ, Polβ	Deoxyadenosine monophosphate analog, chain-terminator	Mitochondrial nephrotoxicity, kidney dysfunction; Viread®/TDF is a prodrug of PMPA	Modest inhibition of Polγ *in vitro*; Polβ has a 270-fold reduction in dATP/PMPApp discrimination in comparison to Polγ *in vitro*	Johnson et al., 2001; Koczor and Lewis, 2010; Brown et al., 2011
AZT, 3′-azido-2′,3′-dideoxythymidine, zidovudine, ZDV, Retrovir®	HIV-RT	Polγ, PrimPol, Polβ	Thymidine analog, chain-terminator, decreases levels of pyrimidines	Myopathy including ragged red fibers, decreased muscle mtDNA, bone marrow suppression, hypertrophic cardiomyopathy, sideroblastic anemia, pancytopenia, hepatocellular failure, lactic acidosis	Decreased mtDNA in cell culture, biochemical defects with Polγ *in vitro*; modestly incorporated by Polγ; Polβ has an ∼3,850-fold reduction in dTTP/AZTppp discrimination relative to Polγ and PrimPol has an ∼60-fold reduction in dTTP/AZTppp discrimination relative to Polγ *in vitro*.	Johnson et al., 2001; Lewis et al., 2001; Lim and Copeland, 2001; Brown et al., 2011; Mislak and Anderson, 2015; Fernández-Moreno et al., 2016

(Continued)

TABLE 2 | Continued

Drug	Target	Potential off-target[a]	Mode of action	Side effects/toxicity/other notes	Experimental evidence for off-target effect	References
CBV, (−)-cis-2-amino-1,9-dihydro-9-(4-hydroxymethyl)-(2-cyclopenten-1-yl)-6H-purin-6-one, carbovir active form of abacavir, ABC, see below	HIV-RT	Polγ, PrimPol	Deoxyguanosine analog, chain-terminator	See below	Strongly incorporated by PrimPol *in vitro* and modest inhibition of Polγ *in vitro*	Johnson et al., 2001; Lewis et al., 2001; Lim and Copeland, 2001; Mislak and Anderson, 2015
ABC, [(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9-purinyl]-1-cyclopent-2-enyl]methanol, abacavir, Ziagen®	HIV-RT	Polγ, PrimPol	Deoxyguanosine analog, chain-terminator	Increased myocardial infarction and congestive heart failure; Note: following intracellular phosphorylation ABC monophosphate is converted to CBV monophosphate by cytosolic enzymes then to CBVppp by cellular kinases	See CBV above	Koczor and Lewis, 2010; Mislak and Anderson, 2015
FIAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil, fialuridine, fluoroiodoarauridine	Hepatitis B, herpes virus DNA pols	Polγ	Uridine analog, not a chain terminator as it contains a 3′ OH, but impairs DNA elongation at adenosine tracts	Severe lactic acidosis, liver failure, and steatosis, kidney failure, myopathy, peripheral neuropathy; discontinued use due to severe hepatotoxicity and death	Inhibition of Polγ *in vitro*, cytotoxic to human Molt-4 cells, aberrant mitochondrial structures	Martin et al., 1994; McKenzie et al., 1995; Lewis et al., 1996, 2001; Johnson et al., 2001
FTC, 5-fluoro-1-[(*2R*,*5S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine, RCV, emtricitabine, Emtriva®, coviracil, racivir	HIV-RT	Polγ, Polβ	Deoxycytidine analog, chain termination	Lactic acidosis, hepatomegaly with steatosis	Polβ has a 100-fold reduction in dCTP/FTCppp discrimination in comparison to Polγ *in vitro*	Koczor and Lewis, 2010; Brown et al., 2011

[a] *The Polθ carboxyl-terminal polymerase domain has been crystalized inserting ddAppp opposite a template abasic site (Zahn et al., 2015).*

DNA, no Polγ exonuclease activity was detected (Johnson et al., 2001). This finding suggests that NtRTI-containing duplex DNA is released from Polγ prior to NtRTI excision and perhaps a similar mechanism could happen *in vivo* with many copies of mtDNA. Similar findings of slow rates of NtRTI excision were observed utilizing steady-state analyses. Additionally, Polγ exonuclease activity was inhibited at *in vivo* concentrations of the AZTppp phosphorylated intermediate AZT monophosphate, AZTp (Lim and Copeland, 2001). Perhaps *in vivo* intracellular levels of AZTp allow for binding of the analog to the exonuclease active site and lower Polγ's fidelity by blocking proofreading.

In 2015 crystal structures of Polγ-DNA replication complexes separately bound to ddCppp or to the natural substrate dCTP were solved (Szymanski et al., 2015). Within the DNA polymerase active site the side chain of the p140 Y951 residue stacks with the incoming ddCppp nearly identically to the natural dCTP substrate. The ribose sugar moieties of both nucleotides are located 3.5 Å from the p140 Y951 hydroxyl group. In support of the p140 Y951 residue being the likely cause of ddCppp toxicity, a biochemical study demonstrated that mutation of Y951 to phenylalanine maintains DNA polymerase activity but renders p140 Y951F almost completely incapable of incorporating ddCppp, CBVppp, 3TCppp, and d4Tppp (Lim et al., 2003). The p140 Y951F had a 2,400-fold increase in dCTP/ddCppp discrimination relative to wild-type p140. Therefore, the substitution of the smaller phenylalanine side chain in the p140 Y951F variant must influence the structure such that ddCppp is excluded from the DNA polymerase active site and not readily incorporated into DNA.

Variability in mtDNA depletion has been observed in HIV-positive patients treated with NRTIs and may result from a difference in treatment times or from genetic variations that have increased susceptibility to NRTIs or both. A homozygous mutation encoding p140 R964C was identified in a 34-year-old HIV-infected woman with a history of lactic acidosis induced by d4T treatment (Yamanaka et al., 2007). Recombinant p140 R964C displays 14% polymerase activity relative to WT p140. Additionally, a patient-derived p140 R964C lymphoblastoid cell line (LCL) cultured with d4T displays mtDNA depletion relative to a WT LCL suggesting p140 R964C is associated with severe lactic acidosis induced by NRTI use. A pre-steady state analysis of Polγ holoenzyme harboring the p140 R964C variant determined that the substitution caused a 33% reduction in dTTP incorporation efficiency and a 3-fold decrease in dTTP/d4Tppp discrimination relative to WT suggesting p140 R964C has a higher propensity to incorporate d4Tppp (Bailey et al., 2009). The p140 R964 residue is located in close proximity to the DNA polymerase active site. One explanation for the mechanism of increased d4Tppp incorporation is that the p140 R964C substitution modulates active site access increasing binding to d4Tppp. Also, a heterozygous mutation (C>T 2857/p140 R953C) was identified in an HIV-infected patient undergoing antiretroviral therapy who displayed mitochondrial toxicity and mtDNA depletion (Li et al., 2016). The recombinant R953C Polγ holoenzyme displayed an 8-fold weakened ability to bind to dCTP and a 4-fold decrease in its ability to discriminate between dCTP and (−)-3TCppp relative to WT. Molecular modeling revealed that a cysteine substitution at position 953 in p140 could abolish interactions between p140 side chain residues in the active site thereby reducing the binding of an incoming nucleotide. In another case-control study examining the relationship between p140 E1143D/G substitutions, lipodystrophy, and d4T treatment it was concluded that HIV-infected patients harboring an E1143D/G variant are 4-fold more likely to develop lipodystrophy and if treated with d4T the risk of developing lipodystrophy increased (Chiappini et al., 2009).

EVIDENCE FOR NRTI DISRUPTION OF mtDNA REPLICATION FROM CELL CULTURE AND ANIMAL STUDIES

Support for intracellular NRTI mitochondria toxicity mediated by disruption of mtDNA replication comes from observations that primary and immortalized cell lines undergo mtDNA depletion upon exposure to various NRTIs. **Table 3** lists examples of human cell lines exposed to various nucleoside analogs in tissue culture. In some reports, mtDNA depletion was so severe cell lines became rho zero completely lacking mtDNA. These findings are similar to what has been reported with LA9 mouse cells exposed to ddC (Brown and Clayton, 2002) and with treating human cell lines with the mtDNA replication inhibitor ethidium bromide, EtBr (King and Attardi, 1996). Low concentrations of EtBr either partially or completely inhibit maintenance of the negatively supercoiled circular mitochondrial genome but not nuclear DNA (nDNA). EtBr binds better to negatively supercoiled substrates than to positively supercoiled ones and might enhances topoisomerase-mediated cleavage of negatively supercoiled DNA; therefore, EtBr may act as a topoisomerase topological poison (Gentry et al., 2011). In agreement with Polγ biochemical analyses, treatment of human cell lines with several nucleoside analogs typically duplicate the finding that ddC causes the most severe inhibition of mtDNA replication as indicated by mtDNA depletion. In an animal study investigating AZT exposure by administering the drug in drinking water to rats, transmission electron microscopy revealed widespread mitochondrial alterations in the heart following 35 days of treatment with 1 mg/ml AZT (Lewis et al., 1991). In another 4-month study investigating the treatment of BALB/C mice with ddI, d4T, AZT, or 3TC, and with the exception of liver tissue from mice treated with 3TC, mtDNA depletion was reported in liver, muscle, and cortical neurons. Also, cortical neurons isolated from mice treated with ddI, d4T, and 3TC were reported to harbor an increased level of mtDNA deletions (Zhang Y. et al., 2014).

OTHER POTENTIAL MECHANISMS OF NRTI TOXICITY

Other mechanisms of NRTI toxicity include increased frequency of mtDNA mutations (perhaps from an altered Polγ function), enhanced oxidative stress, and competition with endogenous nucleotides for kinases required to phosphorylate and activate them thereby lowering the *in vivo* concentrations of nucleotides

TABLE 3 | NRTIs that disrupt mtDNA maintenance in human cell lines.

Cell line	Source of cell line	Nucleoside analog or agent studied[a]	Effect on mtDNA maintenance	Treatment time	References
Molt-4	T lymphoblast	AZT, d4T, FLT, 935U83, FIAU, 524W91, 3TC, ddC, ddI	ddC and FLT, mtDNA depletion and cell death; d4T caused mtDNA depletion; FIAU did not alter ratio of mtDNA to nDNA but was cytotoxic; 524W91, AZT, 935U83 no detectable affect on mtDNA or cell growth	5 days (FIAU and ddC), 6 days (d4T), rest 7 days	Martin et al., 1994
HepG2	Hepatocellular carcinoma	PMPA, 3TC, ABC, ddC, ddI, d4T, and AZT	PMPA, 3TC, and ABC had no detectable effects on mtDNA levels; ddC > ddI > d4T > AZT depletion of mtDNA	9 days	Birkus et al., 2002
Primary SkMC	Skeletal muscle cells	PMPA, 3TC, ABC, ddC, ddI, d4T, and AZT	PMPA, 3TC, ABC, AZT had no detectable effects on mtDNA levels; ddC > ddI > d4T depletion of mtDNA	9, 18, and 21 days	Birkus et al., 2002
Primary RPTECs	Renal proximal tubule epithelial cells	PMPA, ddC, ddI, d4T, and AZT	PMPA & AZT had no detectable effects on mtDNA levels; ddC > ddI > d4T depletion of mtDNA	12 and 21 days	Birkus et al., 2002
Lymphocytes	Primary peripheral blood lymphocytes	ddC, ddI, d4T, AZT	ddI > ddC > d4T deplete mtDNA; AZT did not affect mtDNA but increased lactic acid production and reduced cell counts	10 days	Setzer et al., 2005
Lymphoblastoid cell line	Blood lymphocytes transformed with the Epstein Barr Virus	ddC	MtDNA depletion down to 20% of untreated cells	15 days	Rocher et al., 2008
HCA2-htert	Fibroblast cell line immortalized by over-expression of human telomerase	ddC	Extreme mtDNA depletion	8 days	Ashley et al., 2005
KP hMSC	Immortalized mesenchymal/stromal cell line	EtBr, AZT, d4T	MtDNA depletion EtBr > d4T > AZT	10 days	Fernández-Moreno et al., 2016
3a6 hMSC	Immortalized mesenchymal/stromal cell line	EtBr, AZT, d4T	MtDNA depletion d4T > EtBr; AZT no detectable mtDNA depletion	6 (d4T), 9 (AZT), or 10 (EtBr) days	Fernández-Moreno et al., 2016
HeLa	Cervical cancer cells	ddC	MtDNA depletion	3 days	Jemt et al., 2015
CEM	Leukemia cell line	ddC, d4T, ddI	MtDNA depletion, potencies in reducing cell viability, mtDNA content and normal mitochondrial morphology were ddC > d4T > ddI	4 days	Medina et al., 1994

[a] *FLT, 3′-fluoro-3′-deoxythymidine; 935U83, 3′-fluoro-2′,3′-dideoxy-5-chlorothymidine; 524W91, [(−) FTC], (−)-β-L-2′,3′-dideoxy-5-fluoro-3′-thiacytidine; EtBr, ethidium bromide.*

available to replicate mtDNA (McKee et al., 2004). The recent discovery of other cellular DNA polymerases localizing to human mitochondria also has implications for NRTI toxicity as these enzymes may incorporate analogs. Purified Polβ is considerably sensitive to NtRTIs including d4Tppp and ddCppp (Martin et al., 1994; Pelletier et al., 1994) and compared to Polγ is less selective for and can incorporate AZTppp, PMPApp, L-FTCppp, and L-3TCppp (**Table 2** and Brown et al., 2011). In the nucleus Pols alpha (α), delta (δ), and epsilon (ε) harbor strong nucleotide selection mechanisms and are less likely to incorporate NtRTIs (Brown et al., 2012). Incorporation of NtRTIs by Polβ within the organelle would be complicated by (1) the sensitivity of analog incorporation by Polγ and (2) the lack of Polβ proofreading activity, which would likely contribute to NtRTI persistence within mtDNA. **Figures 1A,B** highlight key steps in BER and mtDNA replication that could be negatively affected by NtRTIs. Finally, the mitochondrial localization of DNA repair polymerases with flexible active sites could allow for accommodation of nucleotide analogs and contribute to unwanted insertion of chain terminators. Pre-steady-state analyses of PrimPol NtRTI incorporation kinetics revealed effective incorporation of CBVppp, followed

by ddCppp > ddAppp > AZTppp while d4Tppp, 3TCppp, PMPApp, and FTCppp were not readily incorporated. From this study, it was determined that CBVppp is actually a better substrate for PrimPol than for HIV-RT which may help to explain life-threatening sensitivity to this analog in some patients (Mislak and Anderson, 2015).

EVIDENCE FOR POLRMT-MEDIATED AVRN TOXICITY FROM BIOCHEMICAL AND CELL CULTURE STUDIES

POLRMT directs polycistronic transcription from three promoters the heavy-strand promoter 1 (HSP1), the HSP2, and the light-strand promoter, LSP (Lodeiro et al., 2012; **Figure 1C**). The two mtDNA strands are named heavy (H) and light (L) based on the ability to separate them on alkaline cesium chloride buoyant density gradients (Kasamatsu and Vinograd, 1974). RNA polymerase enzymes known as primases synthesize RNA primers required for initiation of DNA replication. Evidence supporting the role of human POLRMT as the mtDNA primase comes from the identification of primers located adjacent to nascent H-strands isolated from human KB cell mitochondria (Chang and Clayton, 1985), from *in vitro* experiments demonstrating that POLRMT has primase activity (Wanrooij et al., 2008), and from the observation that replicating mtDNA obtained from mouse embryonic fibroblasts, and lacking RNaseH1, retain unprocessed primers at origins of replication (Holmes et al., 2015). The 5′-end of RNA primers that have been mapped to the LSP therefore likely serve to initiate synthesis of nascent H-strand mtDNA (Chang and Clayton, 1985; **Figure 1B**). Consequently, mtDNA replication is likely dependent on mitochondrial transcription.

Sofosbuvir is an antiviral uridine analog inhibitor of hepatitis C virus (HCV) RNA-dependent RNA polymerase (HCV non-structural protein 5B, NS5B) currently approved for use to treat patients with HCV infections. A number of reports have described the potential use of other antiviral ribonucleosides (AVRNs) as anti-viral and anti-cancer agents; however, many of these AVRNs have had adverse toxic effects when administered to patients and did not pass clinical trials or gain FDA approval (Arnold et al., 2012a,b). For example, the AVRN analog BMS-986094 developed to treat HCV infection did not pass phase II development after nine patients became hospitalized and one died (Mislak and Anderson, 2015). Utilizing a POLRMT *in vitro* biochemical system to measure substrate utilization a panel of more than 10 AVRN analogs were investigated that contained moieties found in past and lead anti-HCV non-obligate chain terminators (Arnold et al., 2012a). Non-obligate chain terminators are AVRNs containing a 3′-OH yet prevent viral RNA elongation. Except for one analog, all AVRN triphosphates (AVRNts) investigated were readily utilized by POLRMT as off-target substrates and five analogs were strong non-obligate chain terminators of POLRMT RNA elongation. Utilizing the human hepatoma cell line, Huh-7, the panel of AVRNs were metabolized to active triphosphates, presumably by intracellular kinases, and the levels of the triphosphate forms varied from less than 0.15 μM to 3.5 mM. Cellular evidence for AVRNs being used as substrates by POLRMT was demonstrated using Huh-7 cells pre-treated for 24 h with EtBr to suppress mitochondrial transcription then cells were exposed to AVRNs for 1, 2, and 3 days. Mitochondrial transcription was impaired in cells exposed to 2′-C-methyladenosine, 6-methylpurine-riboside, and 4′-azidocytidine. This study demonstrated that toxic effects of AVRNs might result from inhibition of the mitochondrial transcription machinery and mtDNA gene expression (Arnold et al., 2012a). Due to the close coupling of mitochondrial transcription and mtDNA replication, prolonged exposure to AVRNs might also affect mtDNA maintenance, **Figures 1B,C**.

OTHER REPORTS OF DRUGS WITH OFF-TARGET EFFECTS ON mtDNA MAINTENANCE

Four human cellular topoisomerases localize to mitochondria: TOP1mt, TOP2α, TOP2β, and a TOP3α long isoform (Zhang H. et al., 2014; Pommier et al., 2016). Tamoxifen a drug used to prevent breast cancer, tacrine a drug used to treat Alzheimer's disease, and a fluoroquinolone broad-spectrum antibiotic, have all been hypothesized to have off-target effects on mitochondrial topoisomerases (Lawrence et al., 1996; Mansouri et al., 2003; Larosche et al., 2007; Nadanaciva et al., 2010; Begriche et al., 2011). Mice separately treated for 28 days with tamoxifen and tacrine displayed mtDNA depletion and both of these drugs were demonstrated to inhibit *in vitro* topoisomerase-mediated plasmid DNA relaxation (Mansouri et al., 2003; Larosche et al., 2007). The fluoroquinolone ciprofloxacin, an inhibitor of bacterial type II topoisomerase DNA gyrase, was reported to induce double-strand mtDNA breaks when mouse L1210 cells were exposed to various concentrations of the drug (Lawrence et al., 1996). The pyrrole alkaloid lamellarin D and the chemotherapy drug doxorubicin have both been shown to poison mitochondrial and nuclear topoisomerases (Pommier et al., 2016).

Menadione (vitamin K3, VK3) has been demonstrated to inhibit the growth of human cancer cell lines derived from various tissues and induces an increase in ROS leading to apoptosis. In an *in vitro* biochemical assay VK3 selectively inhibited Polγ DNA polymerase and RT activities but did not inhibit the activity of other DNA polymerases tested including Pols α, β, δ, ε, eta (η), iota (ι), kappa (κ), and lambda (λ). The authors proposed that suppression of mtDNA replication and repair could trigger ROS production leading to apoptotic cell death (Sasaki et al., 2008). Although the neurotoxicant 1-methyl-4-phenylpyridinium ion (MPP+) does not directly inhibit the catalytic activity of Polγ, MPP+ was reported to cause mtDNA depletion by destabilizing the mtDNA displacement-loop, a mtDNA replication intermediate, thereby inhibiting mitochondrial genome replication (Umeda et al., 2000). Acetaminophen (APAP or paracetamol) is a commonly used over the counter drug used for fever and pain relief. Mice treated with 300 mg/kg of acetaminophen had mtDNA depletion as quantitated using a slot blot hybridization technique (Cover et al., 2005). The depletion is likely due to mtDNA

stand breaks caused by the production of ROS, reactive nitrogen species (RNS), and other reactive metabolites followed by rapid degradation of damaged mtDNA by endogenous mitochondrial endonucleases (Begriche et al., 2011). Troglitazone is an anti-inflammatory and anti-diabetic drug that was withdrawn from the market due to serious hepatotoxicity. Primary human hepatocytes exposed to troglitazone had increased mtDNA depletion, decreased ATP production, and decreased cellular viability (Rachek et al., 2009). ROS and oxidative stress were hypothesized to be the source of mtDNA depletion causing mtDNA strand breaks and cytotoxicity and treatment with *N*-acetyl cysteine (NAC), a known ROS scavenger, reduced the troglitazone-induced cytotoxicity. Cisplatin is a platinum-based FDA-approved chemotherapeutic known to damage nDNA by forming inter-strand crosslinks. Patients treated with platinum-based compounds often display peripheral neuropathy, which may result from damage to dorsal root ganglion neuronal mtDNA (Cline, 2012). *In vitro* work has demonstrated cisplatin or oxaliplatin block Polγ DNA synthesis. Furthermore, cisplatin has been demonstrated to inhibit rat neuronal mtDNA replication and mitochondrial transcription (Vaisman et al., 1999; Podratz et al., 2011; Cline, 2012).

TARGETING mtDNA MAINTENANCE TO KILL CANCER CELLS

Cancer cells display uninhibited DNA replication; therefore, DNA polymerases and DNA repair proteins have been exploited as therapeutic targets to combat certain types of cancer (Lange et al., 2011; Somasagara et al., 2017). NRTI-sensitive mitochondrial DNA polymerases afford a unique opportunity to target cancer cell mitochondria as certain cancers have an increased reliance on OXPHOS and nDNA polymerases are less sensitive to NRTI inhibition (Martin et al., 1994; Liyanage et al., 2017). In a study comparing normal hematopoietic cells to a panel of 542 primary acute myeloid leukemia (AML) samples, it was recently discovered that 55% of the AML samples had increased mtDNA biosynthesis gene expression. Upregulated genes included *POLG*, *POLG2*, *POLRMT*, *Twinkle*, *TFAM*, *SSBP1*, *DGUOK*, *TK2*, nucleotide transporters (*SLC25A33*, *SLC25A36*, and *SLC29A3*) and nucleoside kinases (*CMPK1* and *NME1-NME2*). When treated with ddC AML cells preferentially activated the analog and blocked mtDNA replication and OXPHOS in comparison to hematopoietic cells. Cytotoxicity was preferentially activated in NRTI-treated AML cells and an AML animal model treated with low doses of ddC (35 and 75 mg/kg/day over 11 days) resulted in decreased mtDNA, decreased mtDNA-encoded cytochrome oxidase subunit 2 (COX II), and induced tumor regression without apparent toxicity (Liyanage et al., 2017).

Targeting mtDNA maintenance has also been exploited to treat cancer cell lines with a mitochondrial-targeted cisplatin (Marrache et al., 2014). Nucleotide excision repair (NER) machinery repairs cisplatin-nDNA adducts; however, mitochondria lack NER machinery to deal with this type of damage. Most cancer cells have an increased mitochondrial membrane potential relative to non-cancer cells and triphenylphosphonium (TPP) cations are targeted to mitochondria due to their size, lipophilic properties, and delocalized positive charge. An engineered TPP-tagged cisplatin, Platin-M, caused increased cytotoxicity relative to cisplatin only treatment in several cancer cell models: cisplatin-resistant A2780/CP70 ovarian cancer, prostate cancer PC3 (inherently resistant to cisplatin therapy), and SH-SY5Y neuroblastoma cells. Furthermore, encapsulating Platin-M into specialized nanoparticles enhanced cytotoxicity. SH-SY5Y cells treated with Platin-M and Platin-M encapsulated in nanoparticles were annexin V-positive and propidium iodide-negative, indicative of early apoptosis. Treatment with both Platin-M and Platin-M encapsulated in nanoparticles weakened mitochondrial citrate synthase activity and diminished bioenergetic parameters: spare respiratory capacity, coupling efficiency, and basal respiration. PC3 cells treated separately with cisplatin, Platin-M, and Platin-M encapsulated inside of nanoparticles were subjected to subcellular fractionation then platinum concentrations in various fractions were quantified. Cells treated with Platin-M and Platin-M encapsulated in nanoparticles, contained platinum-mtDNA adducts while cells treated with cisplatin contained mostly platinum-nDNA adducts. These findings support that cisplatin is likely released from Platin-M within mitochondria then binds to mtDNA and inhibits replication.

CONCLUSIONS

Evaluation of antibiotic and antiviral mitochondrial exposures using biochemistry and human cell line and animal models is an important consideration for determining drug toxicity because the complex mitochondrial network harbors multiple copies of OXPHOS complexes and mtDNA that may cause a slow response to these agents. Chronic exposures to drugs may result in long-term mtDNA and OXPHOS depletion. NRTIs may have tissue-specific toxicities such as skeletal- and cardio-myopathies, peripheral neuropathy, and others (**Table 2** and Lewis et al., 2001). Side effects may limit NRTI use in some individuals, cause organ failure and death in others or may only result in minor discomfort (Koczor and Lewis, 2010). Gene variations (like those seen in *POLG* encoding p140 R964C, R953C, and E1143D/G) may exacerbate mitochondrial disease-like phenotypes in HIV-infected patients treated with NRTIs. Also, valproic acid has been demonstrated to induce liver failure in autosomal recessive *POLG* disease but may not be as toxic in autosomal dominant disease. However, due to the potential for valproic acid to cause death by liver toxicity experts recommend avoiding this drug (Saneto and Naviaux, 2010). *POLG* is a highly polymorphic gene and the association between disease-causing and non-disease causing substitutions are often unclear and dependent on other complex factors. How other drugs or environmental factors interact with various genetic variant backgrounds (so-called ecogenetic single nucleotide variants, ESNVs) and contribute to mitochondrial disease manifestation is poorly understood (Saneto and Naviaux, 2010; Zolkipli-Cunningham and Falk, 2017). Do ESNVs predispose individuals to mitochondrial dysfunction via pharmacological

or environmental toxicants while individuals harboring other polymorphism remain resistant? ESNV-environment interaction is an important area for future mitochondrial disease and mtDNA maintenance research. Evidence suggests mitochondria are targets of environmental toxicants that disrupt mtDNA maintenance and chemical exposures may cause increased and decreased mtDNA copy number. At low doses, oxidative stress stimulates mtDNA replication but at high doses mtDNA depletion. Polycyclic aromatic hydrocarbons cause more damage to mtDNA than to nDNA and a compilation of studies comparing nDNA to mtDNA damage following chemical exposure has been reviewed (Meyer et al., 2013).

Evidence suggests five DNA polymerases localize to human cell mitochondria: Polγ, Polβ, PrimPol, Polθ, and Pol ζ. *In vitro* biochemistry measuring substrate binding and incorporation lends strong support to Polγ, Polβ, and PrimPol being off-targets for nucleotide analogs (Pelletier et al., 1994; Szymanski et al., 2015). Additionally, the Polθ carboxyl-terminal polymerase domain has been crystallized in a translesion DNA synthesis mode inserting ddAppp opposite a template abasic site (Zahn et al., 2015). Comparative investigations of mtDNA polymerase enzyme kinetics and determination of crystal structures with and without lesions will assist in our understanding of the spectrum of mtDNA polymerase toxicity. The overarching goal is that these structure-function studies will assist with designing novel antiviral analogs with higher specificity to viral polymerases and less mitochondrial off-target effects. In mice, mitochondrial Polβ was undetectable in heart, liver, and muscle but was present in organelles obtained from brain and kidney (Sykora et al., 2017). Potential questions for future research include: (1) How are the other newly identified mtDNA polymerases distributed among human organs and tissues and do they associate with other components of the mtDNA repair or replication machinery? Knowledge of the distribution of mtDNA polymerases within human tissues may assist with the prediction of tissue-specific toxicant effects. (2) Could knowledge of mtDNA polymerases within different tissues be exploited to treat certain types of cancers with NRTIs? (3) What analogs and toxicants are incorporated by the newly identified mtDNA polymerases? And (4) Do ESNVs exist in any other genes of interest required for mtDNA maintenance? Current next-generation sequencing technologies and continued research utilizing *in vitro* biochemistry and model systems such as human cell lines and mice will be essential to answer these questions and will be necessary for future investigations of mitochondrial dysfunction and disease.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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DNA Sliding Clamps as Therapeutic Targets

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Chromosomal DNA replication is achieved by an assembly of multi-protein complexes at the replication fork. DNA sliding clamps play an important role in this assembly and are essential for cell viability. Inhibitors of bacterial (β-clamp) and eukaryal DNA clamps, proliferating cell nuclear antigen (PCNA), have been explored for use as antibacterial and anti-cancer drugs, respectively. Inhibitors for bacterial β-clamps include modified peptides, small molecule inhibitors, natural products, and modified non-steroidal anti-inflammatory drugs. Targeting eukaryotic PCNA sliding clamp in its role in replication can be complicated by undesired effects on healthy cells. Some success has been seen in the design of peptide inhibitors, however, other research has focused on targeting PCNA molecules that are modified in diseased states. These inhibitors that are targeted to PCNA involved in DNA repair can sensitize cancer cells to existing anti-cancer therapeutics, and a DNA aptamer has also been shown to inhibit PCNA. In this review, studies in the use of both bacterial and eukaryotic sliding clamps as therapeutic targets are summarized.

Keywords: β-clamp, DNA clamp, DNA sliding clamp, proliferating cell nuclear antigen, PCNA, therapeutic

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INTRODUCTION

DNA polymerases that replicate chromosomal DNA are not processive by themselves and polymerize only a few nucleotides at a time. In all organisms, processive replication is achieved by additional factors including a protein referred to as a "sliding clamp". The sliding clamp is a ring-shaped protein that encircles duplex DNA, binds to the DNA polymerase and tethers it to the DNA template, preventing its dissociation and providing high processivity. The sliding clamp does not assemble itself around DNA, but is loaded onto DNA in an ATP-dependent mechanism by a "clamp loader" complex. In all organisms, the sliding clamps and clamp loaders are essential for cell viability. In addition to their role in chromosomal DNA replication, the sliding clamps also play essential roles in DNA repair, recombination, and cell cycle progression and control (Kelman and O'Donnell, 1995; Jeruzalmi et al., 2002; Vivona and Kelman, 2003). In both bacteria and eukarya, many proteins interact with sliding clamps and these interactions regulate their biochemical properties (Kelman and Hurwitz, 1998; Vivona and Kelman, 2003).

Proliferating cell nuclear antigen (PCNA) is the eukaryotic sliding clamp (Kelman, 1997; Moldovan et al., 2007) and plays an essential role in chromosomal DNA replication, repair and recombination as well as other cellular processes such as translesion synthesis (Yang and Gao, 2018). PCNA forms a stable homotrimer. Replication factor C (RFC) utilizes the energy from ATP hydrolysis to assemble trimeric PCNA around duplex DNA at the primer-template junction on the lagging strand. PCNA interacts with the two replicative polymerases, DNA polymerases δ and ε (Polδ and Polε), proteins involved in Okazaki fragment maturation [i.e., DNA ligase and flap endonuclease-1 (FEN-1)], proteins needed for DNA repair [Apurinic/apyrimidinic endonuclease 1 and Xeroderma pigmentosum G, cell cycle regulators (i.e., p21)] and many other cellular factors. The DNA repair PCNA proteins are also often post-translationally modified, and the type of post-translational modification directs PCNA towards different signaling processes (Wang, 2014).

DNA polymerase III (Pol III) is the replicative polymerase in bacteria. The β-subunit of Pol III is the bacterial sliding clamp, also called the β-clamp (Kuriyan and O'Donnell, 1993; Kelman and O'Donnell, 1995). It forms a stable dimer and requires the τ-complex for assembly around the primer-template junction in an ATP-dependent manner. Similar to eukaryal PCNA, the β-clamp interacts with the replicative polymerase, Pol III, as well as proteins involved in DNA repair (i.e., Pol II and Pol IV), the cell cycle regulator, DnaA, and other proteins.

DNA sliding clamps from all organisms share a common architecture. They are multi-domain, multimeric proteins that form a toroidal structure with an ∼35 Å diameter central pore large enough to accommodate duplex DNA that is lined with positively charged side chains, primarily Lys and Arg. PCNA is a trimeric protein, while the β-clamp is dimeric. Although there is low sequence identity between PCNA and β-clamp (<15%), their three-dimensional structures are nearly superimposable. This comes about from the similar structure of the domains that comprise each chain. These domains consist of two 4-stranded β-sheets that are located on the outside of the DNA clamp, and two α-helices that when assembled, line the core of the clamp. There are three domains in each monomer of the β-clamp dimer, and two domains in each monomer of the PCNA trimer, creating a pseudo-hexameric symmetry that is present in all DNA clamps (Kelman and O'Donnell, 1995) (**Figure 1**).

The face of the DNA clamp that points in the direction of DNA synthesis is known as the front face, and is the interaction site for many binding partners. The interaction sites on DNA clamps are largely hydrophobic and are located near domains I and II in PCNA and domains II and III in β-clamp (**Figure 1**). Most proteins that interact with PCNA do so via a conserved motif referred to as the PCNA interacting peptide (PIP) motif (Warbrick, 1998; Warbrick et al., 1998). These PIP motifs are short protein segments that are usually located at the C-terminal end of the interacting proteins. For example, the p21 protein functions as an inhibitor of the cyclin-dependent protein kinases that control the initiation of the cell cycle S phase and DNA replication. PCNA interactions with p21 or the C-terminal peptide of p21 can inhibit other proteins binding to PCNA and affect PCNA activities (Gibbs et al., 1997). The C-terminal peptide of p21 contains a PIP motif and binds to the PIP site (Gulbis et al., 1996). The PIP motif is a weak consensus of Qxxhxxaa, where "h" is a hydrophobic amino acid (isoleucine, leucine or methionine) and "a" is an aromatic residue (tryptophan, tyrosine, or phenylalanine). Although the PIP motif is the most common interaction sequence among PCNA-interacting proteins, other motifs have also been reported to bind PCNA. For example, the AlkB homolog 2 PCNA-interacting motif (APIM) is commonly found in DNA repair enzymes (Gilljam et al., 2009). The APIM is a five residue motif, (K/R)(F/Y/W)(L/I/V/A)(L/I/V/A)(K/R), which binds at the PIP site in a similar conformation to PIP peptides (Sebesta et al., 2017). Much of the research on therapeutics to PCNA is focused on its role in DNA repair often in combination with other therapeutics (Gederaas et al., 2014; Inoue et al., 2014).

The analogous binding sequence to bacterial DNA clamps is a five residue linear motif with a canonical sequence of QL(S/D)LF (Dalrymple et al., 2001) and is called β-clamp binding motif (CBM). The peptide sequences that bind to the bacterial and eukaryal DNA clamps share a few similarities, namely an N-terminal Q and two hydrophobic, often aromatic amino acids at the peptide C-terminus. However, the total number of amino acids in the clamp binding sequences is different (eight for PCNA and five for the β-clamp) and there is no similarity between the remaining residues. It is no surprise, then, that the peptide pockets at each of the DNA clamps are significantly different. As such, peptides that bind to the β-clamp do not bind to PCNA and vice versa (Flores-Rozas et al., 1994).

Since DNA clamps operate as a binding "hub" with many interacting proteins (Kelman and Hurwitz, 1998), they show a certain amount of binding site promiscuity. Specificity to the binding pocket depends on conserved residues of the peptide motif binding to target receptor "hot spots" (Yin et al., 2013). Any effective DNA clamp inhibitor must bind tightly in order to inhibit DNA synthesis (Wolff et al., 2011) or repair. Initial leads for compounds that bind to the peptide pockets on sliding clamps are often discovered using high-throughput screening of compound libraries to identify molecules that bind to the interaction site. Further modification of these lead compounds to optimize binding to the pocket is measured using cell assays, affinity measurements and structural details. DNA sliding clamps are essential for cellular replication and repair, and as such, are a prime target for the development of anti-proliferatives and antibacterial drugs. The current world-wide emergence of antibacterial resistance and prevalence of cancer make these efforts current and crucial. In this review, studies on DNA sliding clamps as drug targets are summarized. Previous reviews related

Abbreviations: PCNA, proliferating cell nuclear antigen; RFC, replication factor C; FEN-1, flap endonuclease 1; Polδ, DNA polymerase δ; Polε, DNA polymerase ε; Pol III, bacterial DNA polymerase III; PIP, PCNA interacting peptide; APIM, AlkB homolog 2 PCNA-interacting motif; CBM, β-clamp binding motif; IDCL, interdomain connector loop; Cha, cyclohexyl-alanine; GM, Griselimycin; CGM, Pro-8-cyclohexyl Griselimycin; NSAIDs, non-steroidal anti-inflammatory drugs; MIC, minimal inhibitory concentration; caPCNA, cancer-associated PCNA; α-PCNA, DNA aptamer of PCNA; TIP, Thermococcales inhibitor of PCNA.

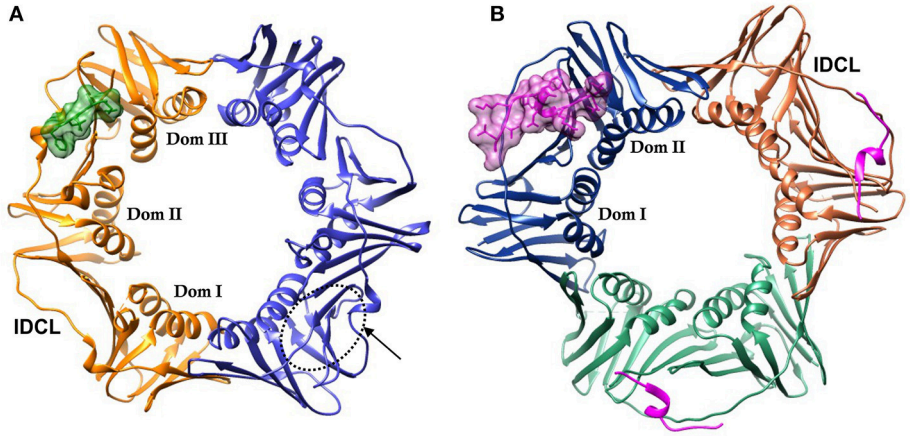

FIGURE 1 | DNA sliding clamps showing pseudo-hexameric symmetry and the central hole of the ring structure that accommodates double stranded DNA. **(A)** The *Escherichia coli* β-clamp dimer with one monomer colored blue and the other monomer orange. The three similar domains in each monomer are labeled Dom I, II, and III. One of the four IDCL loops is labeled. The peptide AcQADLF with its surface colored green shows the location of one of the binding pockets. The second binding pocket, notated by an arrow and dotted line is empty in this structure [PDBID: 4K30 (Zhao et al., 2013)]. **(B)** The human PCNA trimer with one monomer in orange, one in green and the third monomer colored blue. The two domains in one of the monomers are labeled Dom I and II. One of the three IDCLs is labeled. The FEN-1 PIP peptides are drawn in purple, with one of the ligands shown in molecular surface representation and the other two ligands as ribbons [PDBID: 1U7B (Bruning and Shamoo, 2004)]. Molecular rendering was made using Chimera (Pettersen et al., 2004).

to the subject include (Bruck and O'Donnell, 2001; Kontopidis et al., 2005; Wang, 2014; Choe and Moldovan, 2016).

THE β-CLAMP

The bacterial β-clamp is a homo-dimer of ∼82 kDa. As described above, each monomer of β-clamp is composed of three similar globular domains resulting in an overall pseudo-hexameric symmetry (Kong et al., 1992). The domains of each monomer have extensive interactions along the neighboring β-strands and α-helices. In addition, there are four flexible inter-domain connector loops (IDCLs) between the domains, two for each monomer. The dimer interface consists of β-strand contacts in a head to tail arrangement (**Figure 1A**). The peptide binding pocket for CBMs is located near the IDCL of domains II and III, and consists of two subsites: subsite 1 between domains II and III that is ∼8.5 Å deep, and subsite 2 in domain III that is narrower and shallower at ∼4.5 Å deep. Using the numbering 1 through 5 to refer to the five canonical residues of the CBM ($Q_1L_2(S/D)_3L_4F_5$), peptide ligand residues L_4 and F_5 bind to subsite 1, while residues Q_1 and L_2 bind to subsite 2 (Bunting et al., 2003; Burnouf et al., 2004; Georgescu et al., 2008). Most of the interactions between the peptide and the binding pocket are hydrophobic. However, there are several β-clamp side chains that make ionic contacts to the peptide ligand as well as a few backbone amides and carboxyl oxygens that form hydrogen bonds to the peptide. An example of a linear peptide in the binding pocket is shown in **Figure 2A** [PDBID: 4K30 (Zhao et al., 2013)]. This structure contains the peptide sequence Ac-QADLF, but it is clear that the binding pocket can also accommodate the more canonical L_2 in the hydrophobic pocket in subsite 2. Many bacterial proteins bind to β-clamp at the CBD through similar interactions.

PEPTIDES AS INHIBITORS OF β-CLAMP

Peptides from the C-terminus of the Pol III δ subunit that bind to the β-clamp compete effectively with binding of the intact δ subunit (Yin et al., 2013). Peptides are synthesized using known chemistry and as drugs can have low toxicity and good efficacy, however they are often quickly metabolized. It was shown that this problem can be overcome by conjugating a fatty acid to the amino acid sidechain of a short peptide. The fatty acid is a ligand to stable blood-serum proteins such as albumin, and when the fatty acid binds to albumin it helps protect the peptide-drug in circulation in the body. These new, soluble "chimera ligands" bind tightly to human albumin (K_D ∼ 40 nM) and can be attached to peptide drugs using standard synthesis (Zorzi et al., 2017).

Several β-clamp inhibitors were designed that are modified CBM peptides. The goal was to maintain the structure of the canonical peptide backbone in the binding pocket, while increasing the affinity to β-clamp by varying the side chains with other moieties and non-natural amino acids. A commonly used modification is the acetylation of Q_1 (to form Ac-$Q_1L_2D_3L_4F_5$) that improves binding by about 30-fold compared to the non-acetylated peptide, due to hydrogen bond formation to an arginine residue in subsite 2 (Yin et al., 2013). When F_5 is replaced with a 3,4-dichlorophenyalanine, the resulting Ac-$Q_1L_2D_3L_4$(3,4)ClF_5 peptide binds with three times higher affinity (**Table 1**), due to improved hydrophobic and van der Waal's contacts from the halogen groups to subsite 1. The combination of the Ac-Q_1 and (3,4)ClF_5 substitutions results in

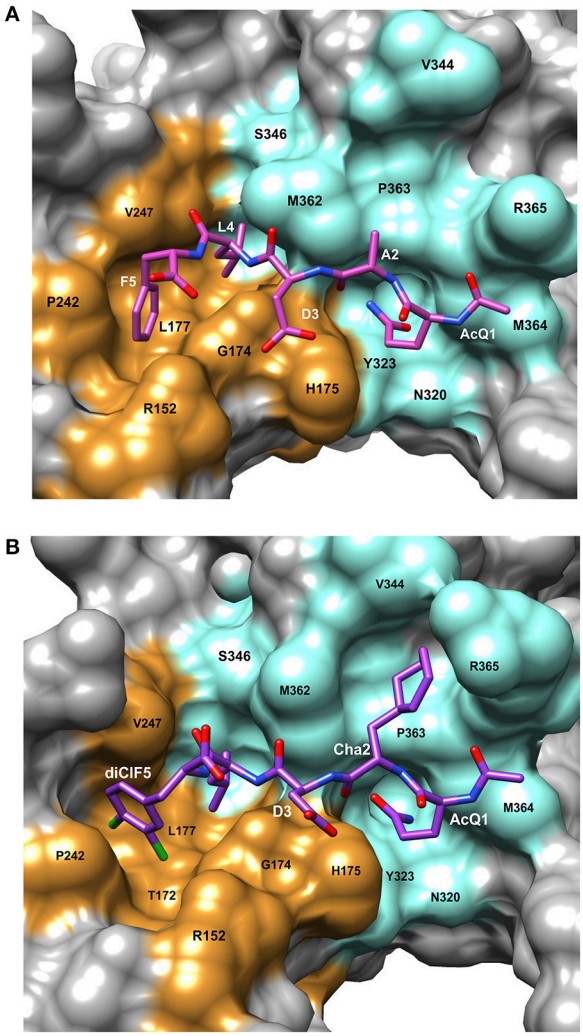

FIGURE 2 | Details of the peptide binding site on β-clamp. The surface residues in subsite 1 are colored in orange and subsite 2 in cyan. Residue labels in white are for the CBM peptide and labels in black are the β-clamp binding site residues. **(A)** Surface representation of the peptide binding pocket and stick figure of the AcQADLF peptide bound to *E. coli* β-clamp [PDBID: 4K3O (Zhao et al., 2013)]. **(B)** Surface representation of the *E. coli* β-clamp peptide binding pocket with a modified peptide competitive inhibitor, Ac-$Q_1Cha_2D_3L_4(3,4)ClF_5$ bound in the peptide pocket [PDBID: 3Q4L (Wolff et al., 2011)]. Molecular rendering was made using Chimera (Pettersen et al., 2004).

110-fold tighter binding for this modified penta-peptide inhibitor (Wijffels et al., 2011).

Another example of β-clamp inhibitor design based on the native peptide started with the polymerase IV CBM peptide, $R_0Q_1L_2V_3L_4G_5L_6$ (called P1). The first peptide modifications were acetylation of Q_1 (described above) and use of the consensus peptide (P6) which improved affinity to β-clamp. Modification of L_2 to a cyclohexyl-L-alanyl (Cha) residue and F_5 to 3,4-dichlorophenylalanine improved binding by an additional 15-fold (Wolff et al., 2011). Overall, this modified peptide inhibitor, Ac-$Q_1Cha_2D_3L_4(3,4)ClF_5$ (called P14), bound 100 times tighter than the native P1 (**Table 1**). The structure of P14 bound to the β-clamp (**Figure 2B**) shows the backbone is in a similar conformation as the native peptide (Wolff et al., 2011). The tighter affinity is achieved because the Cha residue extends further into the hydrophobic pocket and makes additional subsite 1 interactions. In addition to stronger hydrophobic contacts from the halogen-substituted phenylalanine, the meta-chlorine forms a halogen bond with the hydroxyl oxygen of a threonine providing further enhanced binding (Wolff et al., 2011).

SMALL MOLECULES AS INHIBITORS OF β-CLAMP

One of the first β-clamp inhibitors reported was identified using library screening for compounds that inhibited *in vitro* DNA synthesis by Pol III and competed for binding to β-clamp. This compound, called RU7, contains a di-brominated aromatic ring and also had different inhibitory effects on Pol II, Pol III, and Pol IV. The structure of the RU7-β-clamp complex showed that RU7 was bound to subsite 1 in the binding site, but had fewer overall contacts to the pocket than the native CBM peptide (Georgescu et al., 2008). Another small molecule inhibitor that was identified from an *in silico* screen of the $D_3L_4F_5$ tripeptide motif, is a biphenyloxime ether peptide mimetic, called "compound 4" (**Table 1**) (Wijffels et al., 2011). Another set of inhibitors was designed using structurally based fragment screening and other *in silico* methods. A resulting lead, called "compound 8," is a tetrahydrocarbazole and inhibited both gram-negative and gram-positive bacteria (**Table 1**). Improved efficacy was achieved by increasing the number of contacts within subsite 1 of the β-clamp (Yin et al., 2014a).

A NATURAL PRODUCT AS AN INHIBITOR OF β-CLAMP

Griselimycin (GM) is a bacterial derived natural product that was isolated from *Streptomyces* and has antibacterial activity against *Mycobacteria*. GM is a partially cyclic peptide with the sequence $V_1P_2T_3L_4P_5L_6V_7P_8L_9G_{10}$ where the cyclization is between T3 and G10. Initially it was not fully developed into a drug product because of a short half-life upon oral administration, but it was revisited in order to address drug-resistant strains of *Mycobacterium tuberculosis* (Kling et al., 2015). Metabolic stability profiling experiments of natural analogs showed that the eighth residue in GM, a proline, is the site of metabolic degradation and the cause of instability. Two modifications of Pro_8 at the Cδ atom on GM caused it to be more resistant to degradation without affecting the binding affinity: one is addition of a methyl group resulting in methyGM and the other is a cyclohexanyl group, forming cyclohexylGM (CGM). A crystal structure of CGM bound to β-clamp of *M. tuberculosis* showed that it binds in the CBM peptide interaction site, inhibits the interaction of β-clamp with the Pol III δ subunit and may also lead to reduced polymerase processivity. Most of the interactions between CGM and the β-clamp are hydrophobic, with only two hydrogen bonds. Interestingly, low frequency resistance to GM

TABLE 1 | Inhibitory constants of β-clamp ligands[a,b,c].

Ligand	Molecule	IC_{50} (μM)	$K_{i,d}$ (μM)	Method	References
NATURAL PEPTIDE					
Pol IV peptide (P1)	$R_0Q_1L_2V_3L_4G_5L_6$	8.85	0.15	SPR to β-clamp	Wolff et al., 2011
Consensus	$Q_1L_2D_3L_4F_5$	63.2	29.5	FP to β-clamp	Yin et al., 2013
MODIFIED PEPTIDES					
Ac-consensus	Ac-$Q_1L_2D_3L_4F_5$	1.9	0.9	FP to Pol III β-clamp	Yin et al., 2013
Ac-consensus	Ac-$Q_1L_2D_3L_4F_5$	0.07	n.r.	α-subunit plate binding	Wijffels et al., 2011
Ac-consensus (P6)	Ac-$Q_1L_2D_3L_4F_5$	1.12	1.2	SPR to β-clamp	Wolff et al., 2011
Ac-cons.+diClF_5	Ac-$Q_1L_2D_3L_4$(3,4)ClF_5	0.021	n.r.	α-subunit plate binding	Wijffels et al., 2011
P14	Ac-$Q_1Cha_2D_3L_4$(3,4)ClF_5	0.077	i, 17	SPR to β-clamp	Wolff et al., 2011
SMALL MOLECULES					
RU-7	RU-7	n.r.	i, 10	Pol III replication assay	Georgescu et al., 2008
O-8	compound 8	115	i, 64	screen, x-ray structure	Yin et al., 2014a
Compound 4	compound 4	40	n.r.	α-subunit plate binding	Wijffels et al., 2011
NATURAL PRODUCT					
CGM	Pro-8-cylohexanyl GM	n.r.	0.66	SPR	Kling et al., 2015

[a] *FP, fluorescence polarization; SPR, surface plasmon resonance.*
[b] *n.r., value not reported.*
[c] *Dissociation constants are reported in the original literature sometimes as Ki and in others as Kd. Kd's are listed except where noted as Ki.*

was seen in *M. tuberculosis* and *Mycobacterium smegmatis* that was attributed to up-regulation of several genes. One of these genes is the gene encoding for β-clamp, the *dnaN* gene. It was found that the overexpression of the *dnaN* gene in low-frequency resistance is due to a point mutation in the *dnaN* promotor resulting in an elevated level of β-clamp (Kling et al., 2015). This suggests that Mycobacterial resistance to GM is mediated by over expression of β-clamp.

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDS) AS INHIBITORS OF β-CLAMP

Several non-steroidal anti-inflammatory drugs exhibited suppression of *Escherichia coli* Pol III β-clamp (Yin et al., 2014b). An assay using minimal components (β-clamp, the clamp-loader complex, Pol III α subunit and single-stranded binding protein), showed that inhibition of β-clamp-mediated interactions by these NSAIDs directly affected *E. coli* DNA replication *in vitro*. Crystal structures of three NSAID-β-clamp complexes showed that carprofen, bromfenac, and vedaprofen bound to subsite 1 on β-clamp. These molecules bury a hydrophobic group into subsite 1 and an aromatic group into an adjacent region. The lack of interaction with subsite 2 is likely the reason for the relatively weak interaction with β-clamp (minimal inhibitory concentration, MIC > 1,250 μM) and relatively weak inhibition as compared to antibiotics such as ampicillin (MIC = 125 μM) and Chloramphenicol (MIC = 1.37 μM). Nevertheless, these results suggest that NSAID drugs may be used as a promising starting point for the design of new antibiotic drugs (Yin et al., 2014b).

CONSIDERATIONS FOR THERAPEUTICS TARGETED TO β-CLAMP

Different ligands that bind at the protein-protein interaction site on β-clamp were discussed above. Although a consensus sequence has been identified, the CBM of various bacterial proteins have somewhat different sequences and number of residues [for example see: (Patoli et al., 2013)]. The overall structure of the β-clamp does not change in many of these binding events, as the root-mean-square deviation (r.m.s.d.) between bound and ligand free structures is between 1 and 3 Å. There are local changes, however, in the β-clamp binding pocket to accommodate the ligand. In the *E. coli* β-clamp, there is a rotation of the M_{362} and S_{346} sidechains that opens a pathway between subsites 1 and 2 upon ligand binding. In addition, the β-clamp sidechain R_{365} shifts position and opens a hydrophobic platform for the canonical L_3 residue (Wijffels et al., 2011). Any effective inhibitors that are designed to this pocket should interact with both subsites.

In addition to the design of inhibitors that have higher affinity than the natural ligand, specificity of the drug to its molecular target and also to the bacterial species is important to determine the activity spectrum of the drug. For example, differences were found between GM analogs and their interaction with different strains of *Mycobacteria*. GM also has lower binding to *E. coli* β-clamp and does not interact with eukaryotic PCNAs (Kling et al., 2015). These results imply that it may be possible to target drugs to an individual bacterial strain. A study assessing the different modes of peptide binding to the β-clamps from various bacteria found that there are differences in the binding thermodynamics of the peptides to their cognate clamps, and that small modifications can greatly affect the affinities (Wolff et al., 2014). Development

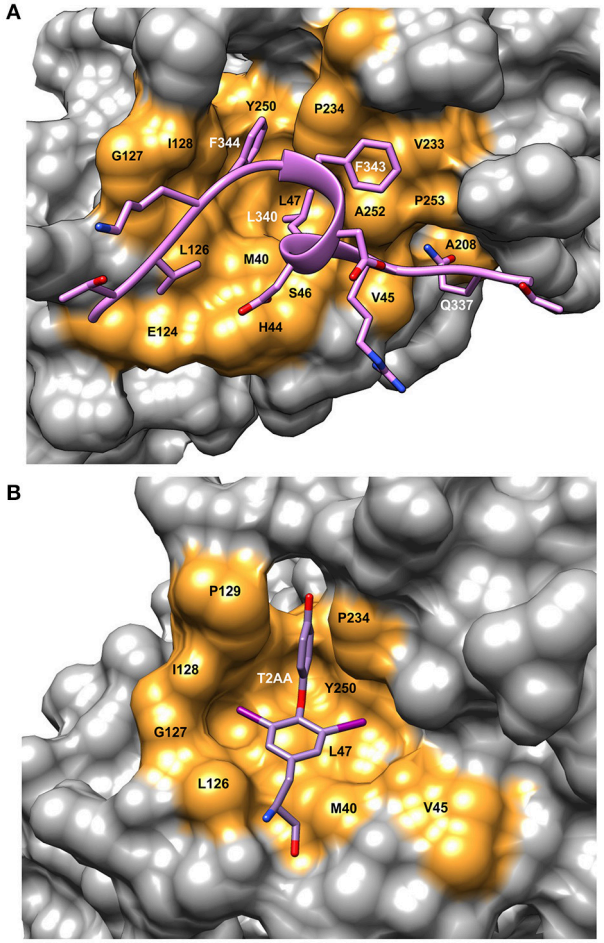

FIGURE 3 | Details of the binding site on PCNA showing ligand interactions. **(A)** Surface representation of FEN-1 peptide bound to human PCNA [PDBID:1U7B (Bruning and Shamoo, 2004)]. Residue labels in white refer to the FEN-1 peptide and labels in black indicate PCNA binding site residues. **(B)** Surface representation of the PIP binding pocket of mono-ubiquitinated human PCNA with the small molecule inhibitor, T2AA bound [PDBID: 3WGW (Inoue et al., 2014)]. This inhibitor binds 2:1 to PCNA with the second binding site at the interface between the subunits (detail not shown). Molecular rendering was made using Chimera (Pettersen et al., 2004).

of a new, successful antibacterial drug will likely require a combination of multiple approaches, including those discussed above.

PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA)

PCNA is a homo-trimeric protein of ∼86 kDa. The two domains in each monomer are connected with a flexible loop referred to as the IDCL as in the β-clamp. The PIP sequence interacts with a hydrophobic pocket on the front face of the PCNA protein near the IDCL (Gulbis et al., 1996) (**Figure 1B**). The PIP peptide binding pocket on PCNA consists of a "Q" pocket in which hydrogen bonds form between the conserved Q sidechain and PCNA residues, which is then followed by a hydrophobic patch (**Figure 3A**). The PIP peptides generally form a single turn of a 3–10 helix that begins at the hydrophobic fourth residue and places this residue next to the final two hydrophobic, aromatic residues (7 and 8). These three residues fit into the hydrophobic pocket in an orientation resembling a plug, as shown for a PCNA-interacting protein FEN-1 (Bruning and Shamoo, 2004) (**Figure 3A**). Most of these interactions are conserved among PIP peptides and proteins that bind to PCNA.

PCNA was originally identified as a nuclear antigen in highly proliferating cells hence the name proliferation cell nuclear antigen (Miyachi et al., 1978; Bravo and Celis, 1980). Therefore, PCNA is a target for the development of anti-proliferation and anti-cancer drugs. In cancer treatment, chemotherapy can be genotoxic to normal cells as well as cancer cells. By specifically targeting proliferative cancer cells, the toxicity of these treatments is decreased. In its role in translesion synthesis, PCNA is post-translationally modified (Wang, 2014) and is not associated with chromatin. It is possible that developing therapeutics to these modified PCNA molecules will lead to more specific targeting of diseased cells. Many PCNA inhibitors bind at the PIP-site and prevent other protein partners from binding thus inhibiting DNA replication. However, not all PCNA inhibitors bind at the PIP pocket. One of the small molecule inhibitors, called PCNA-I1, was shown to bind instead to the interface between PCNA monomers resulting in reduced PCNA binding to chromatin (Tan et al., 2012).

PEPTIDES AS INHIBITORS OF PCNA

PCNA function and its interaction with APIM are important during cellular stress as they play a role in the repair of damaged DNA. Therefore, inhibiting this interaction can affect the survival of cells undergoing stress induced by chemotherapeutic drugs (Gederaas et al., 2014). Over-expression of APIM-containing peptides caused cancer cells to be hypersensitive against various chemotherapeutics. ATX-101 is a cell-penetrating APIM-containing peptide (Muller et al., 2013) and was shown to increase the anticancer efficacy of the drug mitomycin C that creates inter-strand crosslinks in DNA, as well as with bleomycin and gemcitabine in bladder cancer cells (Gederaas et al., 2014). Similarly, ATX-101 induced rapid caspase-dependent apoptosis and increased the cytotoxic effect of melphalan over several days in multiple myeloma cells. Treatment with ATX-101 induced apoptosis in all phases of the cell cycle. This is different from the activity of two small molecule PCNA inhibitors of cancer cell growth that disrupt PCNA during replication, T2AA and PCNA-I1 (Punchihewa et al., 2012; Tan et al., 2012), and rely on the high proliferation rate of these cells (Muller et al., 2013; Choe and Moldovan, 2016).

Another modified PCNA binding peptide has shown promise in breast cancer. Analysis of breast cancer tissue showed increased PCNA expression over normal tissue nearby and this type of cancer was also correlated with shorter survival (Smith et al., 2015). DNA replication in malignant breast cancer cell lines and tumor tissue is more error-prone than in non-malignant

tissue (Sekowski et al., 1998). A unique form of PCNA, termed cancer-associated PCNA (caPCNA) was identified in these cells that is different from PCNA in normal breast cells due to a post-translational modification, specifically methyl esterification of glutamic and aspartic acids residues (Hoelz et al., 2006). Using a polyclonal antibody that specifically recognizes the caPCNA isoform, a peptide sequence was identified, called R9-cc-caPeptide (cc is a linker from R9 to the caPeptide), and was synthesized to mimic this region. The nine arginines (R9) were added to facilitate uptake across the cell membrane. The designed peptide part of the sequence, caPeptide, corresponds to residues 126–133 of PCNA, which is part of the IDCL. The caPeptide inhibits proteins that bind to PCNA that are necessary for DNA replication and repair, resulting in eventual cellular death. The R9-cc-caPeptide was cytotoxic to several breast cancer cell lines as well as pancreatic cancer and lymphoma. Because the R9-cc-caPeptide is specific to cancer-associated PCNA, it is targeted to cells containing only this isoform and thus has less of an effect on normal cells (Smith et al., 2015).

A SMALL MOLECULE AS AN INHIBITOR OF PCNA

T2AA is a small molecule analog of triiodothyronine (T3) that inhibits translesion DNA synthesis (Punchihewa et al., 2012). T2AA was found to inhibit PCNA interaction with p21 and Polδ by binding in the PIP site (**Figure 3B**). From the crystal structure, it was found that two T2AA molecules bind to each monomer of PCNA with the second molecule binding at the interface between the trimer subunits near K_{164} (Inoue et al., 2014). This lysine residue is a known site for mono-ubiquitination, which is a key factor in regulating how cells respond to DNA damage (discussed in more detail in Choe and Moldovan, 2016). It was reported that T2AA inhibits protein-protein interactions between mono-ubiquitinated PCNA and a polη fragment containing a PIP-box. Inter-strand DNA cross-links are repaired by TLS and monoubiquitinated PCNA. Cells that were treated with T2AA as well as with the cancer therapeutic cisplatin, showed lower survival and an increase in double-strand breaks due to cisplatin. Thus, it is possible that mono-ubiquitinated PCNA could be a drug target for chemo-sensitization with cancer therapeutics (Inoue et al., 2014).

A DNA APTAMER THAT INHIBITS DNA POLYMERASE δ AND ε

Another direction for the development of anti-cancer drugs is to make use of nucleic acid aptamers. A short DNA aptamer, called α-PCNA, was designed specifically to bind human PCNA that inhibited Polδ and Polε at nanomolar concentrations *in vitro* (Kowalska et al., 2018). The α-PCNA aptamer adopts a β-form helical DNA conformation by itself, but shows some secondary structural changes when bound to PCNA. The PCNA protein itself does not change conformation when α-PCNA binds. It was proposed that an α-PCNA aptamer-PCNA-DNA polymerase complex is not accessible to the primer-template junction on the lagging strand (Kowalska et al., 2018). If the hypothesis is correct than this is a unique mechanism for inhibition of PCNA dependent functions and has potential for future anti-cancer therapy.

TARGETING PCNA IN ANTI-INFLAMMATORY TREATMENT

A less explored function of PCNA is its function in inflammatory diseases *via* the role it plays in neutrophil survival. Mature neutrophils are non-proliferating cells and PCNA is found in the cytosol. The mechanism involved is not well-understood, but it has been shown that PCNA binds to several procaspases, preventing their activation and inhibiting apoptosis (Dibbert et al., 1999; Witko-Sarsat et al., 2010). One of the characteristics of cystic fibrosis is an intense pulmonary inflammation that involves neutrophils (Chiara et al., 2012). Neutrophils from patients with this disease experience delayed apoptosis. As an infectious disorder, treatment of cystic fibrosis lung disease involves antibiotics and mucolytics, but this treatment is often marginally successful (Pier, 2012). Anti-inflammatory drugs appear to somewhat delay disease progression. The C-terminal p21 peptide which contains a PIP motif and binds at the PIP site on PCNA, also causes neutrophil apoptosis and subsequent PCNA breakdown (Witko-Sarsat et al., 2010). It has been suggested that targeting PCNA to modulate delayed neutrophil apoptosis, in combination with anti-inflammatory and anti-infectious therapies could be beneficial, but requires more development and research (Chiara et al., 2012).

CONSIDERATIONS FOR THERAPEUTICS TARGETING PCNA

Human PCNA is a very stable structure that does not change much when PIP or APIM ligands bind, as the structures are nearly super-imposable with or without ligands. The r.m.s.d. between Cα atoms is <1 Å for the bound and non-bound structures (Bruning and Shamoo, 2004). Because the IDCL interacts with the PIP peptides, its structure varies with different peptides in the pocket. Most of the PCNA inhibitors reported to bind at the PIP-site do not affect the structure of PCNA. A small protein that can break the trimeric ring of PCNA was reported in the archaeon *Thermococcus kodakarensis* (Li et al., 2014). The protein, referred to as TIP, contains a non-canonical PIP motif that is followed by a 17-residue amphipathic helix that binds on the surface of PCNA near the IDCL. The structural effect of TIP binding to the individual PCNA domains is small, but enough to break apart the PCNA trimeric structure (Altieri et al., 2016). It is not known if a similar protein is expressed in eukarya. PCNA is expressed in all cells and therefore PCNA inhibitors can be toxic not only to the malignant cells but also to the healthy cells. Targeting cytosolic PCNA or specific PCNA variants are likely to be more successful as therapeutics. In their review, de March and de Biaisio (De March and De Biaisio, 2017) discuss structural details of their work on the inner

sliding surface of PCNA and suggest targeting these interactions has the potential for new therapeutics. Targeting PCNA in its role in DNA repair and post-translational signaling is likely to enhance specificity to cells involved in disease states (Wang, 2014). PCNA is post-translationally modified by ubiquitination, sumoylation, acetylation, and phosphorylation among others and these modifications can be used as drug targets. PCNA responds to DNA damage by providing an error-free pathway and so adding inhibitors designed to target modified PCNAs could be used in conjunction with anti-cancer therapeutics (Zhu et al., 2014).

CONCLUDING REMARKS

The DNA sliding clamps are essential for cell viability and thus are targets for anticancer and antibacterial drugs. Although several peptides and small molecules that inhibit the sliding clamps have been reported, to date none have reached the clinic. One of the main issues with PCNA inhibitors is potential toxicity to healthy cells due to lack of specificity to malignant cells. Future studies may identify new mechanisms to direct drugs only to malignant cells (i.e., antibody-drug conjugates or to post-translational modifications on PCNA in DNA repair). Work on the inhibition of bacterial β-clamps has provided more detailed information and is very promising for the development of new anti-bacterial drugs. Although the overall structures of PCNA and the β-clamps are similar, their amino acid sequences and binding sites are substantially different. Thus, inhibitors that bind the bacterial clamps are not likely to affect eukaryotic PCNA function. Though the research summarized here has moved us forward in DNA clamp inhibition and the design of new anti-bacterial and anti-cancer therapeutics, future work toward a better understanding of the specific mechanisms behind these interactions and related processes will provide much needed insight. It is clear that fundamental research into the structure and binding of therapeutic drugs to PCNA and β-clamp has led to promising advancements in the areas of infectious disease and cancer. Further work towards a better understanding of the specific mechanisms behind the interactions and related processes of sliding clamps promises to provide important applications.

AUTHOR CONTRIBUTIONS

AA reviewed the literature, summarized the findings, and wrote the paper. ZK suggested the review topic and wrote the paper.

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REVIEW
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New Insights Into DNA Helicases as Druggable Targets for Cancer Therapy

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Small molecules that deter the functions of DNA damage response machinery are postulated to be useful for enhancing the DNA damaging effects of chemotherapy or ionizing radiation treatments to combat cancer by impairing the proliferative capacity of rapidly dividing cells that accumulate replicative lesions. Chemically induced or genetic synthetic lethality is a promising area in personalized medicine, but it remains to be optimized. A new target in cancer therapy is DNA unwinding enzymes known as helicases. Helicases play critical roles in all aspects of nucleic acid metabolism. We and others have investigated small molecule targeted inhibition of helicase function by compound screens using biochemical and cell-based approaches. Small molecule-induced trapping of DNA helicases may represent a generalized mechanism exemplified by certain topoisomerase and PARP inhibitors that exert poisonous consequences, especially in rapidly dividing cancer cells. Taking the lead from the broader field of DNA repair inhibitors and new information gleaned from structural and biochemical studies of DNA helicases, we predict that an emerging strategy to identify useful helicase-interacting compounds will be structure-based molecular docking interfaced with a computational approach. Potency, specificity, drug resistance, and bioavailability of helicase inhibitor drugs and targeting such compounds to subcellular compartments where the respective helicases operate must be addressed. Beyond cancer therapy, continued and new developments in this area may lead to the discovery of helicase-interacting compounds that chemically rescue clinically relevant helicase missense mutant proteins or activate the catalytic function of wild-type DNA helicases, which may have novel therapeutic application.

Keywords: helicase, DNA repair, replication, genomic instability, small molecule, therapy, cancer

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INTRODUCTION

Targeting the DNA damage response and DNA repair to combat cancer became an attractive hypothesis with the original discoveries made by Thomas Helleday, Alan Ashworth and colleagues that chemicals which inhibit the DNA damage sensor poly(ADP-ribose) (PAR) polymerase 1 (PARP-1) could be used to kill breast cancer cells that are defective in the tumor suppressor genes encoding homologous recombination (HR) repair proteins BRCA1 or BRCA2 (Bryant et al., 2005; Farmer et al., 2005). As elaborated below, there has been much interest in the mechanisms of PARP inhibitors as well as topoisomerase inhibitors used in preclinical and clinical settings, and the

progress made in these areas have prompted biomedical researchers to investigate these and other potential therapeutic DNA repair proteins as targets to enhance the effects of chemotherapy drugs or ionizing radiation to eradicate cancer cells but spare normal cells, thereby minimizing toxicity usually associated with the DNA damaging treatments. An important aspect of small molecule drugging of at least some DNA repair protein targets involves trapping the enzyme on its DNA substrate resulting in a poisonous protein complex, which will be discussed as a possible precedent for a new class of chemical inhibitors that target DNA helicases, the subject of this review.

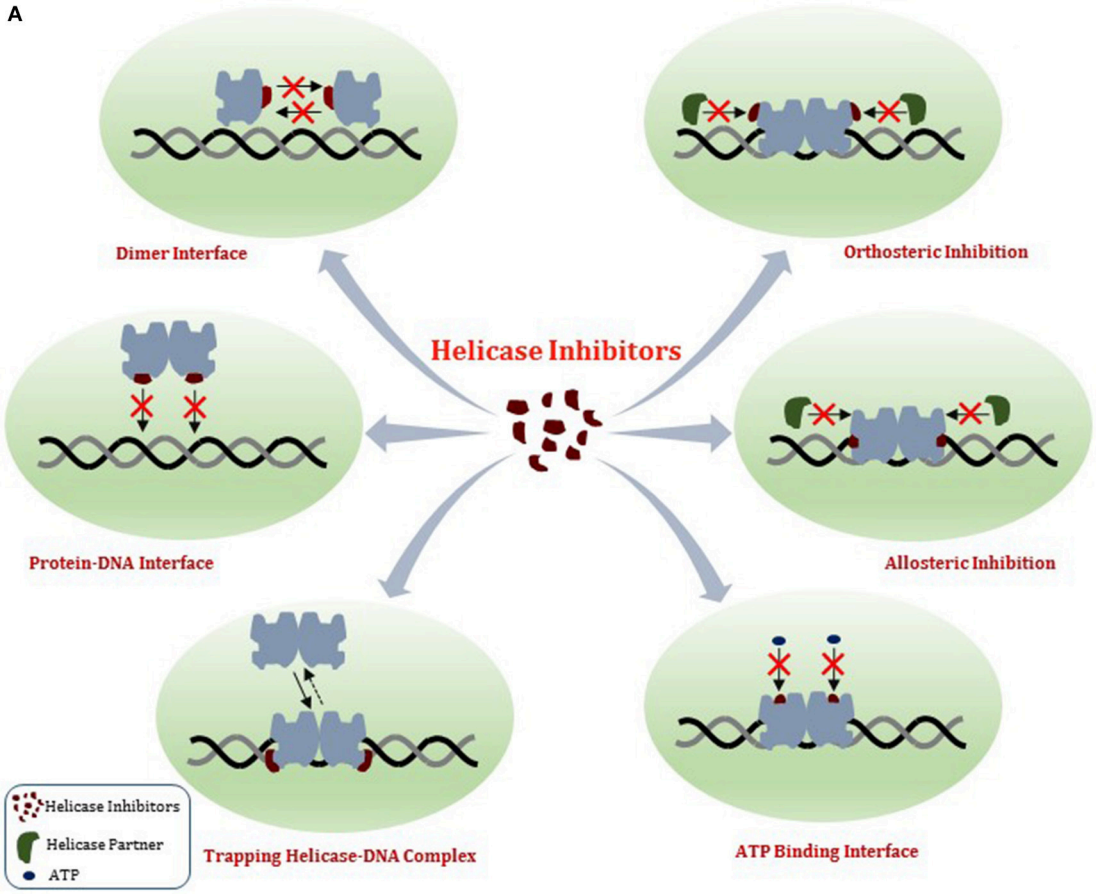
DNA helicases are ubiquitous enzymes found in all domains of life and involved in every aspect of nucleic acid metabolism (Crouch and Brosh, 2017). As molecular motors, they utilize the energy derived from binding and hydrolysis of nucleoside triphosphate (typically ATP) to translocate on DNA and disrupt the many hydrogen bonds between complementary strands of the DNA double helix. In addition, certain DNA helicases unwind alternate DNA structures such as triplexes or G-quadruplexes and/or displace proteins bound to single-stranded or double-stranded DNA. DNA helicases play instrumental roles in cellular DNA replication, transcription, DNA repair, and other processes to preserve genomic integrity and maintain cellular homeostasis. Their vital functions are illustrated by the fact that mutations in a number of helicase genes are either linked to hereditary diseases characterized by chromosomal instability or associated with various cancers (Brosh, 2013).

The molecular differences among helicases are of interest as they may provide opportunities for targeting specific helicases in anti-cancer therapies. DNA helicases are broadly categorized according to the grouping (Superfamily (SF)/Family) to which they belong based on sequence homology within conserved motifs in the helicase core domain as well as auxiliary domains residing in the N- or C-terminal regions of the protein (Umate et al., 2011). Many of the human DNA repair helicases belong to SF2, and the two most prominent families are the RecQ helicases and Iron-Sulfur (Fe-S) helicases which have opposite polarities of single-stranded DNA translocation (Estep and Brosh, 2017). The 3′ to 5′ RecQ helicases (RECQL1, WRN, BLM, RECQL4, RECQL5) share prominent roles in replication fork remodeling, double-strand break (DSB) repair, and regulation of gene expression mediated by their nucleic acid structure-specific binding and catalytic properties as well as their protein interactions. Molecular defects in the RecQ helicases give rise to genomic instability, and mutations in WRN, BLM, and RECQL4 are linked to hereditary diseases characterized by accelerated aging or associated with cancer (Croteau et al., 2014). All five human RecQ helicases are up-regulated in various cancers, suggesting their specialized requirement in rapidly dividing cells to repair replicative lesions or elicit an appropriate response in cell cycle checkpoint or gene expression (Brosh, 2013). As discussed further in the review, structural characterization of various RecQ helicases has provided new insight to the functional importance of key structural elements within the helicase core as well as auxiliary regions that may lead to the design of small molecules which target specific domains.

Given their crucial roles in DNA replication, repair, and genomic stability, DNA metabolic proteins with a characteristic Fe-S cluster have attracted interest from both the basic science and clinical perspectives. Apart from several DNA repair proteins (e.g., DNA glycosylases) and DNA polymerases, certain DNA helicases and helicase-nuclease enzymes possess a conserved Fe-S cluster domain (Wu and Brosh, 2012). The presence of a Fe-S cluster in DNA helicase enzymes was first discovered in XPD, the founding member of a group of DNA repair helicases (DDX11, RTEL-1, FANCJ) that unwind duplex DNA with 5′-3′ polarity and are implicated in human chromosomal instability disorders (Rudolf et al., 2006). Research from several labs established that the Fe-S cluster is essential for DNA unwinding by XPD and other Fe-S helicases (Estep and Brosh, 2017), including FANCJ (Wu et al., 2010).

In addition to the SF2 helicases, the SF1 Pif1 helicase is thought to play an important role in nuclear DNA replication (Budd et al., 2006), telomere replication/repair (Geronimo and Zakian, 2016), and mitochondrial DNA synthesis (Lahaye et al., 1991; Pinter et al., 2008). Pif1 may serve to aid the 5′ to 3′ Twinkle hexameric ring-like helicase (SF4) as it generates single-stranded DNA template through difficult-to replicate sequences (Korhonen et al., 2003). For nuclear DNA replication, the ring-like 3′ to 5′ helicase complex constituted by the MCM2-7 proteins (SF6) is essential (Chong et al., 2000). The ring-like structures of the replicative helicases Twinkle (Fernandez-Millan et al., 2015) and MCM complex (Zhai and Tye, 2017), combined with their accessory factors [e.g., mitochondrial single-stranded binding protein (Korhonen et al., 2003), Cdc45/GINS (Aparicio et al., 2009)], enhance the processivity of these helicases to fulfill unwinding of duplexes inherently longer than what is required for strand separation by the helicases implicated in stalled fork remodeling or DNA repair.

In this review, we will provide a framework for thoughtfully considering DNA helicases as a desirable new avenue to target for anti-cancer therapy. In **Figure 1**, we depict some potential modes of small molecule inhibition of DNA helicases, as well as chemically induced or genetic synthetic lethality that will be referred to in the text. Clearly, an intricate molecular knowledge of helicase conformational states, substrate specificities, protein interactions, pathways, etc. is required to screen for compounds which target helicases successfully *in vitro* and *in vivo* with optimal characteristics. We will discuss novel and emerging concepts and developments in anti-cancer therapy as they relate to proposed helicase targets, highly relevant to the prognosis of individuals suffering from many types of cancer that remain a major health risk and source of mortality. Moreover, the current anti-cancer strategies are still highly sub-optimal in many treatments due to the toxicity in normal cells and tissues imposed by chemotherapy drugs and radiation. With the advent of new helicase inhibitors discovered by both high-throughput *in vitro* assays and *in silico* compound screening approaches relying on



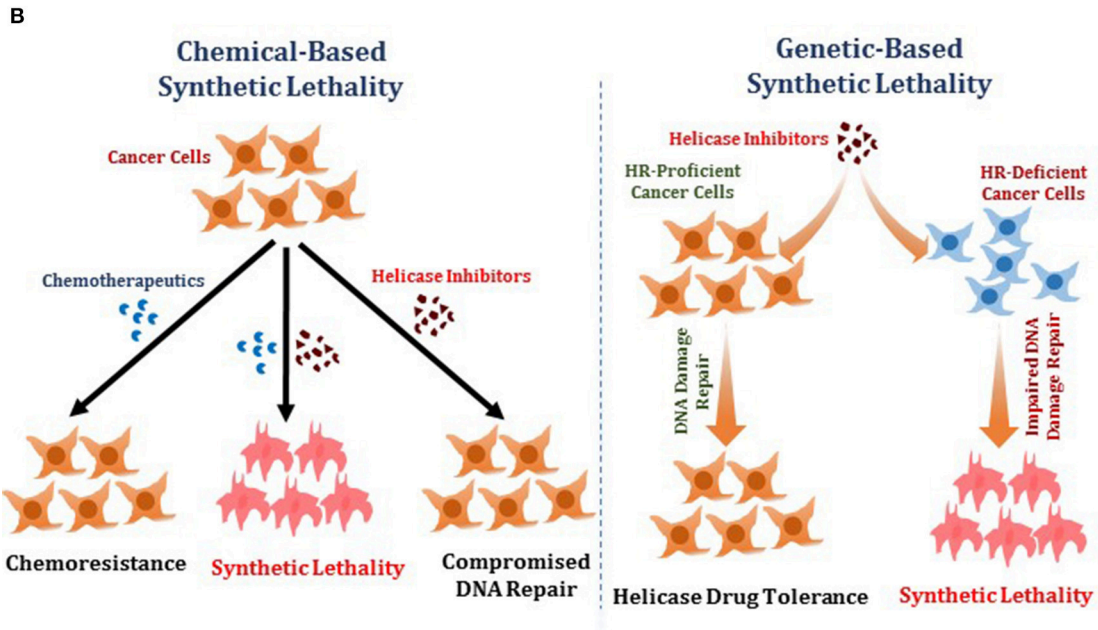


FIGURE 1 | Mechanisms of DNA helicase inhibitors and therapeutic strategies**. (A)** Small molecule helicase inhibitors may interfere with the catalytic activities of DNA helicase proteins and their molecular and cellular functions by a variety of mechanisms. A helicase-interacting compound may disrupt protein oligomerization, binding to DNA substrate, or compete with ATP binding. Small molecules may alter helicase interactions with other proteins (e.g., DNA repair/replication factors) by orthosteric

(Continued)

FIGURE 1 | or allosteric mechanisms. Helicase-interacting compounds may also cause the protein to become trapped on DNA, resulting in a toxic complex or lead to the hijacking of other proteins. **(B)** Two potential strategies for helicase inhibitors (that are not mutually exclusive) are (i) Chemical-based synthetic lethality whereby pharmacological helicase inhibition compromises the cancer cell to chemotherapy DNA damaging drugs or radiation; (ii) Genetic-based synthetic lethality whereby the defined genetic mutant background of the cancer cell is hypersensitive to pharmacological helicase inhibition. See text for details.

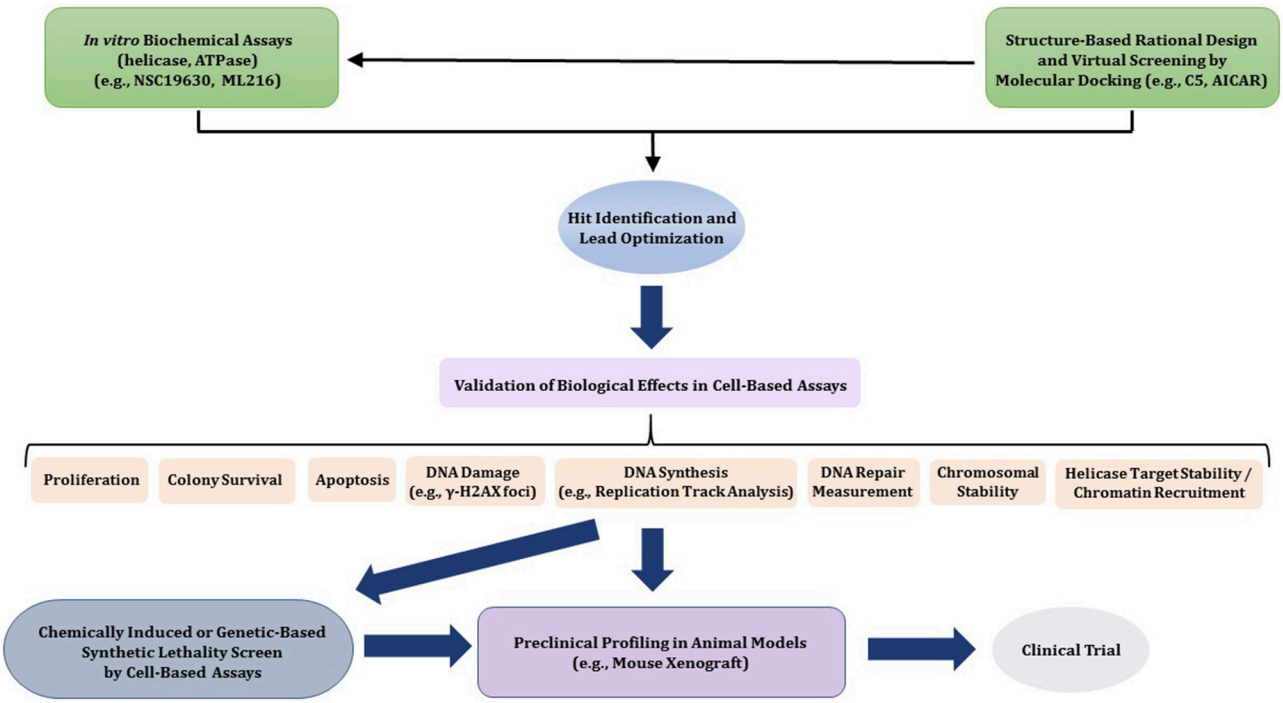


FIGURE 2 | Flow diagram for discovery, optimization, and validation of DNA helicase inhibitors. See text for details.

molecular docking, the stage is set to assess their efficacy using preclinical *in vivo* models (**Figure 2**).

DNA DAMAGE RESPONSE PROTEINS: TARGETS FOR CANCER THERAPY?

The concept of DNA repair or replication stress response modulation for therapeutic intervention has become a hot topic of research and in recent years, clinical pursuit. The field really got its start with the discovery of PARP inhibitors and topoisomerase inhibitors and has taken off with the identification and characterization of novel DNA repair targets. This discussion provides an excellent backdrop for consideration of DNA helicases as potential targets for chemical modulation. From a clinical perspective, personalized medicine has become prominent over the past decade or more. Understanding the genotype-phenotype relationships controlling tumor aggressiveness and their influence over the effectiveness of chemotherapy/radiation treatments has become of increasing importance to the emerging field of DNA damage signaling and DNA repair inhibitors (Velic et al., 2015; Hengel et al., 2017). As illustrated above by the discussion of PARP and topoisomerase inhibitors, their efficacy to combat cancer is dependent on the genetic background of the tumor.

Seminal Discovery of PARP Inhibitors

Over a decade ago, the concept of DNA repair inhibition emerged in the laboratory setting as a potential avenue for the development of DNA damage response or DNA repair inhibitors with the discovery of small molecules (<300 Da) that deter the molecular and cellular function of PARP (Bryant et al., 2005; Farmer et al., 2005). PARP inhibitors impair the enzyme's ADP-ribose modification function, which in turn suppresses its role in base excision repair, single-strand break repair, and more generally, DNA damage sensing (Cseh et al., 2017). In the case of PARP-1 inhibitors, studies were historically focused on familial and sporadic breast and ovarian cancers with bi-allelic mutations in the HR repair genes *BRCA1, BRCA2*, or *PALB2* (Hengel et al., 2016; Pommier et al., 2016). The rationale was that pharmacological inhibition of PARP-1 function in rapidly dividing cancer cells would result in an accumulation of single-strand breaks leading to broken replication forks that are essentially DSBs. These DSBs that normally would be repaired by HR with the sister chromatid duplex would be fixed inefficiently in certain cancers due to the absence of any

one of the three key HR repair proteins BRCA1, BRCA2, or PALB2. Although some success was achieved for treatment of ovarian cancer patients with the PARP-1 inhibitor olaparib, resistance to the drug has limited its therapeutic effectiveness (Murata et al., 2016), leaving researchers to continue exploring and developing new and better cancer therapies focused on PARP inhibitors targeting each member of the family and optimal co-treatment strategies of these compounds with other anti-cancer agents.

Development of Clinically Relevant Topoisomerase Inhibitors

Topoisomerase inhibitors are perhaps as high profile as the PARP inhibitors for potential clinical use (Pommier et al., 2010). The discovery and development of topoisomerase inhibitors that cause cytotoxicity in cancer cells has sparked tremendous interest in their suitability for anti-bacterial and anti-cancer applications. Much has been learned about the mechanism of action of topoisomerase-poisoning inhibitors, and it is postulated that many of these compounds act in a similar manner to certain PARP inhibitors by trapping the enzyme on DNA (see below).

Researchers are engaged in the quest to discover more effective topoisomerase (as well as PARP) inhibitors that can hit every cellular target, are chemically stable and behave optimally according to pharmacokinetic parameters. In addition, chemotherapy drug combinations need to be optimized. Aside from these challenges, the looming concerns for compounds that impair the functions of other DNA repair proteins is their effective targeted drug delivery and sub-optimal therapeutic index (Hengel et al., 2017). Further studies that elucidate the pathways whereby such inhibitors act in cells to exert their cytotoxicity and optimize tumor-specific delivery approaches are high priorities in the field. Moreover, combination therapies that exploit the genomic signature of a tumor may lead to the development of anti-cancer strategies which lower the cancer-killing drug doses, thereby sparing normal cells and tissues. Such efforts in precision medicine have become paramount (O'Connor, 2015).

Trapping PARP- and Topoisomerase-DNA Complexes Presents a Paradigm for New Anticancer Drugs

Foreshadowing the potential mechanism of action of DNA helicase inhibitors (discussed below), research has revealed that some chemical PARP inhibitors and topoisomerase inhibitors act by trapping the enzyme on DNA, thereby poisoning cells via the formation of toxic DNA-protein-drug complexes that have consequences beyond simply inhibiting catalytic function (Pommier et al., 2015). Yves Pommier and colleagues first used the term interfacial inhibitors to describe drugs that trap protein-DNA complexes by binding at their interfaces, and the concept has been expanded to include medicinal compounds that bind at protein-protein interfaces as well (Pommier and Cherfils, 2005). An excellent example of the former, highly germane to certain emerging DNA repair inhibitors, is represented by those compounds which inhibit topoisomerase action by binding to the very site where the enzyme interacts with DNA to cleave its phosphate backbone, resulting in a trapped topoisomerase inhibitor-stabilized cleavage complex (Pommier, 2013).

In parallel to such interfacial topoisomerase inhibitors, certain PARP-1 and PARP-2 inhibitors act by trapping PARP on DNA *in vitro*, helping to explain why cellular exposure to these PARP-binding drugs exerts a greater cytotoxicity than the absence of PARP altogether (Pommier et al., 2016). The development of PARP trapping assays using extracts of PARP inhibitor-treated cells to assess chromatin enrichment or employing purified PARP recombinant proteins and fluorescently or radioactively labeled oligonucleotide-based DNA substrates with site-specific damage (e.g., single-strand nick) incubated with PARP inhibitor has provided seminal evidence for DNA-bound PARP complexes. From a more clinical perspective, there is great interest in understanding the mechanisms for resistance to PARP inhibitors such as PARP expression, drug efflux, and changes in DNA damage response/DNA repair in response to PARP inhibitor exposure. In addition, ongoing efforts in anti-cancer therapy focus on developing successful combinations of PARP inhibitor with other DNA damaging treatments and/or DNA repair inhibitors. Studies of the interfacial PARP inhibitors and topoisomerase inhibitors will likely serve as models for the future investigations of DNA helicase inhibitors that behave according to a similar enzyme trapping mechanism; however, these mechanistic analyses are only in their infancy (see below).

New Targets in Genomic DNA Metabolism to Enhance Cancer Therapy

While topoisomerase and PARP inhibitors continue to attract interest as targets for anti-cancer therapy, in recent years other DNA damage response and DNA repair targets have emerged. For the small molecules that inhibit these proteins, some of the same basic idealized principles apply in which the chemical agents acting as a monotherapy or in combination with other chemotherapy treatments will target the cancerous cells and tissues, sparing normal ones by exploiting a therapeutic threshold index. Some examples of new DNA repair inhibitors that target HR repair proteins (e.g., RAD51, RAD52, RAD54), the structure-specific nuclease MRE11, and others are summarized in recent reviews (Huang and Mazin, 2014; Velic et al., 2015; Hengel et al., 2017). These relatively new targets, like the PARP- and topoisomerase-interacting drugs, may provide insights to mechanistic aspects of helicase inhibitors and their application in anti-cancer regimes. For example, small molecule inhibitor-induced trapping of DNA metabolic proteins may represent a more generalized mechanism with poisonous consequences applicable to compounds that target other DNA repair proteins such as DNA methyltransferases and DNA helicases.

DNA REPAIR INHIBITORS IDENTIFIED BY HIGH-THROUGHPUT SCREENS AND MOLECULAR DOCKING APPROACHES

As mentioned above, small molecule inhibitors of proteins implicated in DNA damage signaling and DNA repair identified by high-throughput screens (HTS) have been advanced by basic research efforts in part to develop anti-cancer strategies to enhance chemotherapy or radiation treatments. These assays can be broadly divided into two categories: (1) Biochemical screens which are used to directly assess modulation of protein function, be it enzymatic activity or ligand binding; (2) Cell-based assays used to investigate if a set of compounds influence a DNA repair pathway, ultimately with an outcome on cellular homeostasis and/or genomic stability. Often, once a compound is identified that is potent and specific for its target *in vitro*, chemists will optimize its structural and solubility properties for *in vivo* application.

In the following sections, we will highlight some examples of DNA repair inhibitors and DNA helicase inhibitors discovered by HTS and molecular docking approaches. Practically all the presently known helicase inhibitors have been shown to act synergistically in a genetic or chemical manner with druggable DNA repair targets in cell-based systems, and some of these synergistic combinations will be discussed.

Structure-Based Design of DNA Repair Inhibitors by Molecular Docking Approaches

Although significant advancement has been made in developing potential small molecule inhibitors targeting DNA repair machinery, only a few have reached the clinic so far. Hits primarily identified by HTS based on *in vitro* biochemical assays sometimes fail to exert their desired effect at the cellular level and are often non-specific. In general, the associated cost, time, assay complexities and screening quality are considered as major challenges in developing highly potent and specific drug-like molecules using experimental HTS approaches (Shoichet et al., 2002; Moitessier et al., 2008; Awate and Brosh, 2017). With the rapid advancement in computational methodologies coupled with the availability of high-resolution crystal structures of target proteins, structure-based virtual screening of large compound libraries has drawn significant attention in modern drug discovery research over the last two decades. The approach has been successfully used to identify highly accurate lead molecules in a time- and cost-effective manner (Kroemer, 2007; Meng et al., 2011). Among the various structure-based *in silico* compound screening methodologies, molecular docking technique is widely adopted and considered as the principal one. Given the three-dimensional structure of the target protein, this important computational tool allows the researchers to virtually screen a large set of small organic molecules and provides information about the binding mode and strength of the binding for individual protein-ligand complexes. Therefore, molecular docking is useful not only in identifying new hits but also in facilitating the further optimization of the pre-identified lead molecules to develop more potent analogs.

SMALL MOLECULE INHIBITORS OF DNA HELICASES

Although somewhat lagging behind in the field of small molecule DNA repair inhibitors, pharmacological inhibition of DNA helicases has begun to attract interest. A recent review summarizes experimental approaches to identify and characterize DNA helicase inhibitors by biochemical and cell-based assays (Banerjee et al., 2016). With the discovery of new helicase protein structures and a growing understanding of their molecular mechanisms, there has been increasing interest in small molecules that modulate helicase function. Below, we provide the reader a current assessment of the field. Given the number of both DNA and RNA helicases implicated in fundamentally important areas of nucleic acid metabolism in human cells, it seems likely that continued advances in pharmacological interventions will be made. These advances should provide unique tools to investigate the cellular functions of helicases, their biological pathways in nucleic acid transactions, and further development in pre-clinical models (**Figure 2**).

While some helicase inhibitor studies have focused on pharmacological modulation of human DNA helicases involved in DNA damage responses that would affect the efficacy of ionizing radiation or chemotherapy treatments, a number of inhibitors of viral helicases have been discovered over the past decade that may be useful for suppressing viral diseases (Shadrick et al., 2013). The clinical success of a herpes simplex virus (HSV) helicase-primase complex inhibitor known as Amenamevir (ASP2151) is a strong testament to viral helicases as potential druggable targets to deter viral pathogenesis (Chono et al., 2010; Katsumata et al., 2012; Tyring et al., 2012). ASP2151 has a broad anti-herpes virus spectrum including HSV-1, HSV-2, and varicella zoster virus as well as acyclovir-resistant thymidine kinase–deficient HSV strains (Chono et al., 2010; Himaki et al., 2012). Although not yet clinically proven, small molecule-based approaches showed significant progress in targeting other viral helicases such as human papilloma virus (HPV) and hepatitis C virus (HCV) helicases. By high-throughput screening and subsequent chemical optimization, a family of biphenylsulfonacetic acid-based small molecules was discovered that inhibit the ATPase and helicase activity of HPV6 E1 helicase *in vitro*, but their bioactivity remains elusive (Faucher et al., 2004). On the other hand, a novel class of small molecules that specifically antagonizes the physical interaction of HPV E1 helicase with E2 protein was found to be effective to inhibit HPV DNA replication in cell-based assays (White et al., 2003; Yoakim et al., 2003). This work serves as an excellent example of targeting viral helicase protein-protein interactions to develop potential antiviral therapies. As reviewed elsewhere, the hepatitis C virus NS3 helicase is considered a potential candidate for specifically targeted antiviral therapy (Belon and

Frick, 2009). Lead compounds (e.g., Soluble blue HT, Acridone-4-carboxylic acid derivatives, benzotriazoles) that inhibit HCV helicase activity and impair cellular HCV RNA replication hold immense potential. For a comprehensive review of new and developing antiviral and antibiotic small molecule inhibitors, see (Shadrick et al., 2013).

In Vitro and Cell-Based Properties of Known Compounds That Inhibit DNA Unwinding Catalyzed by Human Helicases

Table 1 lists recently identified small molecular inhibitors of human DNA helicases. Most of the small molecule helicase inhibitors were identified from *in vitro* helicase assays using purified recombinant DNA helicase proteins, oligonucleotide-based DNA substrates, and small molecule libraries. However, a virtual screen of FDA-approved drugs by a nuclease-based assay identified an inhibitory compound for the DNA2 helicase-nuclease implicated in Okazaki fragment processing during DNA replication (Liu et al., 2016). Of those tested in cell-based assays, small molecule inhibitors of DNA unwinding catalyzed by WRN (Aggarwal et al., 2011, 2013b), BLM (Nguyen et al., 2013), and DNA2 (Liu et al., 2016; Kumar et al., 2017) all negatively affect proliferation of cancer cells and induce DNA damage and/or chromosomal instability. Moreover, these helicase inhibitors behave synergistically with other compounds that induce DNA damage, inhibit DNA repair, or impose replication stress. Certain helicase inhibitors operate in a manner that is dependent on the presence of the DNA helicase target (Aggarwal et al., 2011, 2013b; Nguyen et al., 2013; Liu et al., 2016), suggesting that pharmacological inactivation of helicase function involves the interference of a genome maintenance pathway which is distinct from the effect imposed by the absence of the helicase altogether. Presumably, backup mechanisms are elicited in certain helicase-deficient backgrounds, whereas a helicase inhibitor complex with its target imposes uniquely deleterious effects, akin to those caused by a protein trapping mechanism discussed earlier. While an inhibitor of the RECQL1 helicase was identified from an *in vitro* helicase activity screen (https://pubchem.ncbi.nlm.nih.gov/bioassay/2708), no published advances in terms of effects of the small molecule on functionality or metabolism of human cells have been reported. In the following sections, we will discuss some key features of the human DNA helicase inhibitor studies to provide the reader a sense of the field's current advances and future directions with an eye on clinical applications.

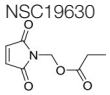
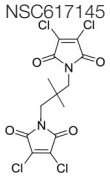
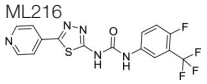
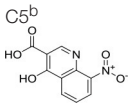
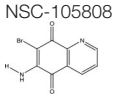
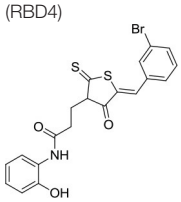
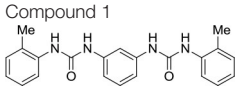
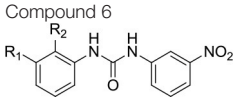
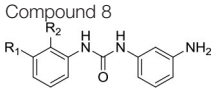
WRN

Bi-allelic mutations in the *WRN* gene result in Werner syndrome (WS), a progeroid disease considered by many to most closely resemble accelerated aging (Oshima et al., 2017). With the apparent exception of neurodegeneration, almost all clinical features associated with normal aging (e.g., heart disease, osteoporosis, diabetes, cataracts, etc.) are observed early in life (concomitant with the adolescent growth spurt) for those individuals diagnosed with WS. Although the *WRN* gene product has been studied by molecular biologists, biochemists, cell biologists, and clinicians for over 2 decades, it is still unclear the molecular defects of WRN that are responsible for the mutant cellular phenotypes which include chromosomal instability, replication and DNA damage response defects, and abnormal transcriptional regulation. The *WRN* gene encodes a protein that has both DNA helicase activity and DNA exonuclease activity. Moreover, WRN interacts with a large cast of proteins implicated in various DNA transactions important for DNA repair, the replicational stress response, and telomere capping, suggesting that it may have pleiotropic roles. Although WRN mutant mouse models, particularly those crossed with other mutant mice, have provided some clues to WRN's involvement in telomere metabolism (Chang et al., 2004; Du et al., 2004; Laud et al., 2005), the definitive molecular and cellular deficiencies underlying WS remain elusive.

To provide a fresh approach to studying WS, we conducted a search for small molecules that behaved as potent and specific WRN helicase inhibitors (Aggarwal et al., 2011). The *in vitro*-based compound screen using a conventional biochemical DNA unwinding assay was performed to identify inhibitors of purified recombinant human WRN helicase-catalyzed DNA unwinding and positive hits were tested in cell-based assays. These efforts led to the identification and characterization of the compound NSC 19630 from the National Cancer Institute (NCI) Diversity Set which inhibited WRN-catalyzed DNA unwinding of a forked duplex DNA substrate ($IC_{50} \sim 20\,\mu M$) in a specific manner based on the observation that other purified recombinant DNA helicase proteins tested (e.g., BLM, RECQL1) were either not inhibited or only modestly affected at much greater compound concentrations (**Table 1**). The inhibitory effect of NSC 19630 on WRN DNA unwinding was not mirrored by a similar effect on DNA binding or ATP hydrolysis, suggesting that the compound specifically interfered with WRN's strand separation activity. Biological studies with NSC 19630 and the human cervical cell line HeLa demonstrated that the WRN helicase inhibitor negatively affected cell proliferation and replication, as well as induced DNA damage and apoptosis in a WRN-dependent manner based on the observation that the same cells depleted of WRN by RNA interference were resistant to the effects of NSC 19630. Further studies showed that NSC 19630 behaved synergistically with the topoisomerase inhibitor topotecan, the PARP1 inhibitor KU0058948, or the G-quadruplex binding ligand Telomestatin for inhibiting proliferation and inducing DNA damage (Aggarwal et al., 2011), suggesting that pharmacological inhibition of WRN helicase activity under conditions of replicational stress or DNA damage severely compromised the cellular response.

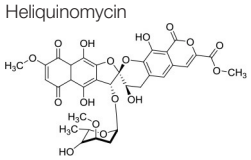
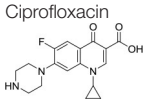
In subsequent work, a compound designated NSC 617145 that is structurally related to NSC 19630 was found to inhibit WRN helicase activity with even greater potency ($IC_{50} \sim 230$ nM) and render human cells deficient in the Fanconi Anemia (FA) pathway hypersensitive to the DNA cross-linking agent mitomycin C (MMC) in a WRN-dependent manner (Aggarwal et al., 2013b) (**Table 1**). Interestingly, NSC 617145 did not sensitize FA-deficient cells to hydroxyurea (HU) (which causes replication stress by depleting the deoxynucleotide phosphate pool), suggesting that DNA DSBs that accumulate when the FA

TABLE 1 | Small molecule inhibitors of human DNA helicases.

Helicase/compound	Screen/library	*In vitro* properties		Biological effects	References
		Biochemical effects	Specificity[a]		
WRN NSC19630 	Helicase screen; NCI Diversity Set	$IC_{50} \sim 20\,\mu M$; Inhibits DNA unwinding	Helicase-specific	Bioactive at 1–3 μM; Inhibits proliferation; Induces DNA damage; Synergistic with PARP inhibitor, CPT, or TMS; WRN-dependent	Aggarwal et al., 2011
WRN NSC617145 	Helicase screen; NCI Diversity Set	IC_{50} ~230 μM; Inhibits DNA unwinding	Helicase-specific	Bioactive at 0.125–1 μM; Inhibits proliferation; Induces DNA damage; Synergistic with MMC in FA mutant background; WRN-dependent	Aggarwal et al., 2013b
BLM ML216 	Helicase screen; MLSMR	$IC_{50} \sim 3\,\mu M$; Impairs DNA binding, helicase	Inhibits WRN helicase	Bioactive at 50 μM; Inhibits proliferation; Elevates SCE; Synergistic with aphidicolin; BLM-dependent	Nguyen et al., 2013
DNA2 C5[b] 	Virtual screen of FDA drugs; Nuclease screen; NCI DTP Set	$IC_{50} \sim 20\,\mu M$; Impairs DNA binding, helicase, nuclease	Nuclease-specific; DNA2 helicase not assessed	Bioactive at 7–70 μM; Inhibits proliferation, fork resection, and recombination; Alters fork restart in BRCA2 / BOD1L mutant background; Synergistic with PARP inhibitor; DNA2-dependent	Liu et al., 2016
DNA2 NSC-105808 	Nuclease screen; NCI DTP Set	$IC_{50} \sim 2\,\mu M$ (yeast DNA2); $IC_{50} \sim 1.49\,\mu M$ (human DNA2); Inhibits nuclease activity	Nuclease-specific; Helicase not assessed	Bioactive at 0.25–2 μM; Inhibits HR repair, DSB end resection and suppresses proliferation of cancer cells	Kumar et al., 2017
DDX3 Rhodamine-based derivative 4 (RBD4) 	Virtual screen	Inhibits DDX3 ATPase activity ($IC_{50} = 5.4\,\mu M$)	Helicase not assessed	Inhibits HIV-I (III_B) replication in MT-4 leukemia cells ($EC_{50} = 86.7\,\mu M$)	Maga et al., 2008
DDX3 Compound 1  Compound 6  Compound 8 	Virtual screen	Inhibits DDX3 ATPase (compound 1, $IC_{50} = 17 \pm 2\,\mu M$; compound 6, $IC_{50} = 20 \pm 3\,\mu M$; compound 8, $IC_{50} = 40 \pm 0.5\,\mu M$) and helicase activities (Compound 1, $IC_{50} = 65 \pm 5\,\mu M$; compound 6, $IC_{50} = 1 \pm 0.2\,\mu M$; compound 8, $IC_{50} = 5 \pm 0.6\,\mu M$)	Helicase not assessed	Suppresses HIV-1 replication in [b]PBMCs (compound 6, $EC_{50} = 10\,\mu M$; compound 8, $EC_{50} = 15\,\mu M$)	Radi et al., 2012

(Continued)

TABLE 1 | Continued

Helicase/compound	Screen/library	*In vitro* properties		Biological effects	References
		Biochemical effects	Specificity[a]		
Mcm4/6/7 Heliquinomycin 	Helicase screen	$IC_{50} \sim 2.4\ \mu M$; Inhibits DNA unwinding	Helicase-specific	Bioactive at 2–14 μM; Inhibits proliferation of cultured cancer cells	Ishimi et al., 2009; Toyokawa et al., 2011
Mcm2-7 Ciprofloxacin 	Helicase screen	$IC_{50} \sim 632\ \mu M$; Inhibits DNA unwinding	Helicase-specific	Bioactive at 520–670 μM (yeast) and 160–350 μM (human cells); Inhibits proliferation of yeast and human cells	Simon et al., 2013

[a] *Specificity determined by its effect on other DNA helicases.*
[b] *DNA2 nuclease, but not helicase activity, was assessed.*

pathway is crippled in its ability to respond to interstrand cross-links (ICL) are particularly problematic in the face of poor WRN helicase activity as opposed to simply stalled forks. Perhaps WRN may aid in the repair of such DSBs by mediating HR. Supporting the hypothesis that the interaction of the helicase inhibitor with WRN causes the formation of a toxic ternary complex with genomic DNA that interferes with normal DNA repair, NSC 617145 treatment enriched WRN in the chromatin fraction of human cells (Aggarwal et al., 2013b). It remains to be seen if (or what) other DNA replication/repair factors are sequestered with WRN in the chromatin fraction due to the presence of the drug. This is particularly relevant as protein hijacking may contribute to the cytotoxicity of the WRN helicase inhibitor.

Further studies with NSC 617145 revealed that the drug is even more toxic in human cells that are doubly deficient in the FA pathway and DNA Protein Kinase C (PK_{cs}), a DNA damage sensor and phosphorylating enzyme which is implicated in non-homologous end-joining (NHEJ) pathway of DSB repair (Aggarwal et al., 2013a). This finding suggests that the toxicity imposed by WRN helicase inhibition in the context of ICL-induced DNA damage does not derive from a self-imposed deleterious NHEJ pathway. Moreover, the results provide further evidence that WRN helicase inhibitors can induce synthetic lethality via a genetic-based and/or chemically induced mechanism. These results, along with those from other studies in the DNA damage response/DNA repair field, continue to spark interest in the development of anti-cancer strategies that exploit the genetic background of a tumor as well as drug combinations that together might overwhelm that resistance of tumors to mono-therapies. Moving toward a pre-clinical application is a priority in the DNA repair community, which will require advancement through model genetic systems and *in vivo* applications.

It seems likely that small molecule helicase inhibitors like those directed against WRN will operate in a manner that is dependent on the genetic background of the tumor. WRN, like other human RecQ helicases, is typically up-regulated in its expression in various cancer cell lines; moreover, their down-regulation by RNA interference has been shown to cause decreased proliferation (Brosh, 2013). Therefore, inhibition of WRN function may represent a useful strategy to compromise rapidly dividing cancer cells dependent on WRN to deal with replicative lesions. Analysis of the NCI 60 cancer cell database did not show a strong correlation between WRN protein level and sensitivity to NCS 19630 (Aggarwal et al., 2011), indicating a more complex scenario. Nonetheless, non-cancerous breast epithelial cells or normal fibroblasts were found to be resistant to NSC 19630 (Aggarwal et al., 2011), suggesting that a therapeutic threshold for the WRN inhibitor may come into play. Interestingly, it was reported that the susceptibility of breast cancer cells to killing by camptothecin (CPT) correlated with CPT-induced WRN degradation (Shamanna et al., 2016). Exposure to the WRN helicase inhibitor NSC 617145 was also observed to cause WRN degradation (Aggarwal et al., 2013b), suggesting that the anti-proliferative effects of compounds that target WRN or protein partners with which it interacts (e.g., topoisomerase I) is a causative factor. Further studies in this area may help to elucidate strategies to target tumors by exploiting their genetic background and negatively affecting the activity as well as the stability of DNA repair protein targets with small molecules.

BLM

Bi-allelic mutations in the *BLM* gene result in Bloom's syndrome (BS) characterized by a pronounced predisposition to all types of cancer and certain features of accelerated aging (de Renty and Ellis, 2017). A prominent form of chromosomal instability used to clinically diagnose BS is elevated sister chromatid exchange (SCE) that is attributed to defects in recombinational repair and a poor replication stress response. The BS helicase (BLM) shares sequence homology within the conserved ATPase/helicase core domain of WRN and RecQ orthologs. In addition, BLM (as well as WRN) contains a conserved RecQ C-terminal (RQC) region that bears Zn^{2+}-binding and winged helix (WH) domains and a

Helicase RNase D-like C-terminal (HRDC) domain (see below). The RQC is implicated in structure-specific DNA binding and protein interactions of WRN and BLM (Estep and Brosh, 2017), and the HRDC is implicated in ligand-induced conformational changes in BLM (Newman et al., 2015).

To gain greater insight to BLM's molecular and cellular roles in DNA metabolism, a HTS of greater than 350,000 compounds from the Molecular Libraries Small Molecule Repository (MLSMR) was undertaken to identify inhibitors of BLM-catalyzed helicase activity on a fluorometric-labeled forked duplex DNA substrate (Nguyen et al., 2013). For this assay, a recombinant truncated version of BLM containing the helicase core domain but lacking 119 amino acids in the N-terminus and 635 residues in the C-terminus was used. Of the positive hits for BLM helicase inhibition, one compound was optimized for its medicinal chemistry properties (e.g., solubility, cell permeability), leading to ML216 (**Table 1**) as a lead candidate for further studies. The IC_{50} for ML216 inhibition of helicase activity catalyzed by the BLM helicase domain fragment or full-length BLM was determined to be in the low micromolar range. Interestingly, ML216 was much less effective in inhibiting branch-migration of synthetic Holliday Junction (HJ) or mobile D-loops DNA substrates, as well as a G-quadruplex DNA substrate, with an IC_{50} value of $\sim 50\,\mu M$ (Nguyen et al., 2013). The differential effect of ML216 on BLM catalytic activity with these different DNA substrates would suggest that the compound affects BLM in a unique manner as it unwinds forked duplex DNA, and that BLM operates by distinct DNA structure-specific mechanisms during DNA unwinding and branch-migration, as suggested by previous studies (Mazina et al., 2012). Although ML216 displayed specificity for helicase inhibition based on results from assays with UvrD, RECQL1, and RECQL5 helicases ($IC_{50} > 50\,\mu M$), the small molecule inhibited forked duplex DNA unwinding by a WRN helicase domain fragment or full-length WRN at significantly lower drug concentrations (Nguyen et al., 2013). The IC_{50} value for inhibition of forked duplex DNA unwinding by full-length WRN was only 1.7-fold greater than IC_{50} value for inhibition of unwinding by full-length BLM on the same partial duplex DNA substrate. This raises the possibility that ML216 binds to both BLM and WRN through their conserved ATPase/helicase core, RQC, or HRDC domains; however, biophysical mapping studies are required to address this. ML216 was shown to inhibit BLM binding to single-stranded DNA or forked duplex DNA (Nguyen et al., 2013), suggesting that the compound inhibits BLM-catalyzed DNA unwinding by interfering with its DNA binding function, but an analysis of ML216's effect on WRN DNA binding was not reported in the study.

A structurally related analog of ML216 (5-(pyridin-4-yl)-1,3,4-thiadiazol-2-amine derivative, designated as compound 33) was achieved through medicinal chemistry efforts focused on structure-activity relationships (SAR) (Rosenthal et al., 2013). Like ML216 (Nguyen et al., 2013), compound 33 inhibited BLM helicase activity and single-stranded DNA binding, consistent with its non-ATP competitive inhibition of DNA-dependent BLM ATPase activity (Rosenthal et al., 2013). Compound 33 showed a greater selectivity for inhibition of BLM helicase activity over WRN compared to ML216. Although compound 33 also inhibited single-stranded DNA binding by WRN, its effect was not quite as potent as that observed for BLM DNA binding.

From a preclinical perspective, an initial litmus test for the medicinal development of a small molecule DNA repair inhibitor is its activity in cell-based models. ML216 was observed to inhibit proliferation of human SV40-transformed skin fibroblasts in a BLM-specific manner, i.e., the presence of BLM in the isogenic cell line was required for ML216 (12.5 or $50\,\mu M$) to inhibit proliferation in the 48 or 72-h time-period (Nguyen et al., 2013). Because elevated SCE is such a pronounced phenotype of BS, the effect of BLM small inhibitors ML216 (Nguyen et al., 2013) and compound 33 (Rosenthal et al., 2013) on this form of chromosomal instability in human cells was assessed. Both BLM inhibitors induced SCE in BLM-positive, but not BLM-negative cells, consistent with a BLM-dependent effect. Pre-exposure to $50\,\mu M$ ML216 for 24-h sensitized human cells to the DNA polymerase inhibitor aphidicolin in a BLM-dependent manner (Nguyen et al., 2013). A 48-h pre-exposure to ML216 caused a greater frequency of γ-H2AX foci (a marker of DSBs) induced by MMC in a BLM-dependent manner as well. Thus, the results suggest that the BLM helicase inhibitor ML216 as well as compound 33, like the WRN helicase inhibitors NSC 19630 and NSC 617145, behave in a dominant-negative fashion, relying on their helicase target to cause anti-proliferative and DNA damage-inducing effects. However, both ML216 (Nguyen et al., 2013) and compound 33 (Rosenthal et al., 2013) impaired DNA binding by BLM [whereas, the WRN inhibitor NSC 19630 did not appreciably affect WRN DNA binding at drug concentrations in which significant helicase inhibition was observed (Aggarwal et al., 2013b)], suggesting that the BLM inhibitors may not trap BLM helicase protein on genomic DNA in cells. This is contrasted to NSC 617145, which was reported to enrich WRN's association with chromatin (Aggarwal et al., 2013b).

DNA2

DNA2 is a protein with dual helicase and endo-/exo-nuclease activities originally discovered in yeast to play an important role in processing of DNA replication intermediates (Budd et al., 1995, 2000). These advancements laid the foundation for studies of DNA2 in human cells, which also revealed its importance in DNA metabolism. Emerging evidence indicates that in addition to DNA2's involvement in Okazaki fragment processing (Kang et al., 2010b), the helicase-nuclease is important for DNA end-processing as an early step in DSB repair (Symington, 2016) and nucleolytic processing of stalled or regressed forks that arise during replication stress (Thangavel et al., 2015). These findings, coupled with observations that DNA2 is overexpressed in various cancers, has made DNA2 an attractive candidate for inhibition as a strategy for cancer therapy (Jia et al., 2017). Indeed, several groups have reported that DNA2 depletion by RNA interference causes the reduced proliferation of cancer cells (Jia et al., 2017). Thus, DNA2 may be suitable for chemical inhibition by small molecules that inhibit its catalytic nuclease and/or helicase function.

A HTS with a fluorometric DNA substrate and yeast DNA2 was employed to search for inhibitors of the enzyme's nuclease

activity (Kumar et al., 2017). From this screen of ∼50,000 compounds, a couple of compounds (NSC 5195242, NSC 105808) were identified that could inhibit yeast and human DNA2 nuclease in a specific manner (by its negligible effect on other nucleases tested) (**Table 1**). However, these compounds were not tested on nuclease-dead versions of DNA2 to assess if they affected DNA2 helicase activity. It was found that NSC 105808 did not affect DNA2 ATPase (Kumar et al., 2017). NSC 105808 negatively affected proliferation of human bone osteosarcoma U2OS cells, and its anti-proliferative effect was suppressed by ectopic expression of DNA2 at a level 1.5–2.0-fold greater than endogenous DNA2, leaving the authors to propose that DNA2 is the target of the compound (Kumar et al., 2017). However, NSC 105808 was not tested on DNA2-deficient or DNA2-depleted cells, so a comparison to the mechanisms of action for the reported WRN- or BLM-specific helicase inhibitors which impaired cell proliferation in a manner that was dependent on the presence of either RecQ helicase in human cells has not been done. Nonetheless, it may be speculated that a small molecule which causes the trapping of a helicase on the DNA, such as WRN inhibitor NSC 617145 (Aggarwal et al., 2013b), may behave quite differently from a DNA2 nuclease inhibitor such as NSC 105808. Further studies are warranted to characterize the cell-based effects of chemical DNA2 nuclease inhibitors.

Both DNA2 nuclease inhibitors NSC 105808 and NSC 5195242 inhibited DNA end processing in a reconstituted system (Kumar et al., 2017). Moreover, NSC 105808 was observed to diminish DNA end-resection and HR in human cells. In addition, NSC 105808 suppressed the sensitivity of FANCD2 −/− cells to cisplatin, like the effect of DNA2 depletion (Kumar et al., 2017), suggesting that the compound targets DNA2. In several different cancer cell models, the DNA2 inhibitor suppressed proliferation of cancer cells with oncogene-induced replication stress (Kumar et al., 2017), suggesting a potential avenue of further exploration for cancer therapy. Studies with DNA2 inhibitors applied to genetic organisms and mouse xenografts that serve as good preclinical models will help to address the usefulness of these compounds for further development.

Mcm2-7

The mini-chromosome maintenance protein 2-7 (Mcm2-7) is a well conserved hexameric DNA helicase that plays an essential role in DNA replication by unwinding the parental duplex strands to be copied (Abid Ali and Costa, 2016). The observations that certain mutations in the Mcm helicase subunits are associated with cancer and that Mcm is over-expressed in cancer cells supports the idea that this hexameric helicase complex is a suitable target for cancer therapy (Neves and Kwok, 2017). Currently, only very limited work has been done to identify inhibitors of DNA unwinding by Mcm complexes. It was determined that helicase activity catalyzed by the Mcm467 subcomplex was inhibited by heliquinomycin (Ishimi et al., 2009); furthermore, this compound decreased proliferation of cancer cells grown in culture (Toyokawa et al., 2011) (**Table 1**). More recent efforts in this area led to the identification of a fluoroquinoline antibiotic known as ciprofloxacin (previously shown to deter the catalytic function of topoisomerase II) as an inhibitor of the Mcm2-7 helicase (Simon et al., 2013) (**Table 1**). Ciprofloxacin was shown to inhibit the growth of yeast cultures, and one of the mcm mutant strains tested was resistant to the compound, suggesting that Mcm2-7 is a target of the drug.

SUCCESSFUL VIRTUAL SCREENS TO DISCOVER HELICASE INHIBITORS

We expect these seminal studies to be followed by new compound inhibitors designed by molecular docking with recently solved DNA helicase structures. Most of the docking programs perform two basic operations, "docking" and "scoring." Ligands are docked into the protein structure to predict most possible conformations of the protein-ligand complexes, particularly the conformations of the ligands bound to the binding pockets of the target protein. In the second operation, using a scoring function, the binding affinities of the individual ligands to the target protein in each conformational state are calculated and thus multiple ligands are ranked according to their respective docking score. In the following sections, we will discuss advances to identify helicase inhibitors by virtual screening.

DDX3

Resistance of HIV-1 to the commonly used anti-HIV drugs is often due to drug-induced acquired mutations in the viral enzymes. Targeting host cell cofactors holds immense therapeutic potential because they are less susceptible to drug-induced mutability compared to the viral enzymes (Kwong et al., 2005). Cellular RNA helicases (e.g., RNA Helicase A (RHA), RNA Helicase 116 (RH116), DEAD-box helicases DDX1 and DDX3) play crucial roles in HIV-1 replication inside the host cells and may represent good targets, provided that cytotoxicity is not a factor. Cell-based screening of a series of ring-expanded nucleoside (REN) analogs identified a potent small molecule inhibitor (CID 44586781) of DDX3, an ATP-dependent RNA helicase required for exporting HIV-1 RNA from the nucleus to cytoplasm (Yedavalli et al., 2008). CID 44586781 was effective in suppressing HIV-1 replication in macrophages and T cells without imparting significant cytotoxicity *in vivo*. Notably, DDX3 was one of the first helicases subjected to the structure-based design of small molecular inhibitors using a molecular modeling approach (Maga et al., 2008). Using the crystal structure of DDX3 bound with AMP, a potential inhibitor of the enzyme's ATPase activity, designated RBD4 (**Table 1**), was identified by pharmacophoric modeling and subsequent molecular docking-based virtual screening of compound libraries. In cell-based assays, the small molecule inhibitor was found to be effective in inhibiting HIV-1 replication, thereby strengthening the power of the docking approach. Optimized inhibitors that interfere with DDX3 RNA binding and helicase activity were also identified by precise homology modeling followed by high-throughput molecular docking (Radi et al., 2012). Although there are other cellular helicases which could serve as potential anti-HIV1 drug targets, only DDX3 has been successfully targeted by small molecule inhibitors so far. Therefore, structure-based design and virtual screening approaches targeting additional helicases

involved in HIV-1 replication may aid in the development of more potent and effective inhibitors.

NS3

The Hepatitis C virus (HCV) NS3 helicase plays a key role in HCV replication and has been an attractive target for developing antiviral drugs (Frick, 2007). A potent inhibitor of NS3 helicase was successfully identified by exploiting its crystal structure using molecular docking-based virtual screening (Chen et al., 2009). The blue soluble HT dye that docked into the NS3 ATP binding site was found to inhibit its helicase activity as measured by a FRET-based assay (Chen et al., 2009). The co-crystal structure of the compound with NS3 was subjected to a small chemical fragment-based virtual screening search, leading to the discovery of a novel triphenylmethane derivative (Compound 12) that suppressed HCV replication in host cells (Chen et al., 2009). More recently, an *in silico* small molecule docking screen was used to identify an anti-helminthic drug (ivermectin) as a potent inhibitor of NS3 helicase activity; furthermore, ivermectin suppressed replication of common flaviviruses in cultured cells (Mastrangelo et al., 2012).

DNA2

The crystal structure of murine DNA2 bound to a short (15 nt) single-stranded DNA molecule revealed a unique mechanism of nucleolytic processing of DNA strand in which single-stranded DNA threaded through a central tunnel where it is bound by both the nuclease and helicase domains (Zhou et al., 2015). Although this murine DNA2 crystal structure has not yet been exploited for molecular docking of small molecules, an alternative virtual screening approach was used (Liu et al., 2016). The researchers employed the crystal structure of yeast Upf1-RNA U15 complex and human Upf1-ADP complex (because they share high sequence identity with DNA2) to generate a stable homology model of human DNA2 bound to single-stranded DNA and then predicted potential druggable sites on the protein surface by docking a set of FDA-approved drug molecules. The most favorable docking pocket with the maximum score and DNA binding affinity was then subjected to HTS using a large NCI Developmental Therapeutics Program (DTP) library of small molecules. The approach led to the successful discovery of a lead compound (C5) that was predicted to bind to the DNA binding sites within the helicase domain.

The helicase domain-interacting molecule, designated C5 (**Table 1**), impaired nuclease, ATPase, and helicase activities of DNA2 (Liu et al., 2016). C5 inhibited proliferation of multiple cancer cell lines originating from breast, colon, prostate, or lung. Depletion of DNA2 in the breast cancer cell line MCF7 suppressed the anti-proliferative effect of C5, suggesting that DNA2 is the target of the compound *in vivo*. Consistent with this finding, embryonic stem cells from dna2$^{-/-}$ mice were also resistant to C5. The authors did not assess if C5 caused sequestration of DNA2 on DNA, but this would be a worthwhile experiment to address the cytotoxicity of the DNA2-interacting compound.

In further cell-based assays, C5 was shown to inhibit single-stranded DNA annealing and HR, and this effect was likely due to a negative effect on DNA2-mediated end resection because RPA foci formation was reduced after CPT exposure in the C5-treated cells (Liu et al., 2016). To assess the effect of C5 on DNA2's involvement in fork stabilization/restart, DNA fiber assays were performed with cells exposed to the replication inhibitor HU or low levels of CPT. The results from these assays indicated that C5 prevents normal restart of stalled replication forks. Furthermore, C5 prevented over-resection of stalled forks, suggesting the compound prevents DNA2's catalytic activities from processing stalled or regressed forks. Finally, it was shown that C5 sensitized cancer cells lines to various chemotherapeutic agents including a PARP inhibitor and CPT.

NEW DNA HELICASE STRUCTURES PROVIDE FUTURE TARGETS FOR MOLECULAR DOCKING

Recent discoveries of DNA helicase crystal structures have been informative from a mechanistic perspective and suggest that the development of specific helicase inhibitors using rational drug design approaches will accelerate in the future. Given the importance of RecQ helicases in genomic stability and their proposed differences and overlap in function, efforts to dock compounds on functionally distinct and less conserved domains of RecQ helicases is warranted and may provide useful tools to not only explore RecQ biological functions but also develop chemotherapy drugs against the helicase targets. In the following sections, we will discuss some key structural features of RecQ helicases and a Fe-S helicase (XPD) which may be exploited for drug development.

RECQL1

Like the other RecQ helicases, RECQL1 possesses two conserved RecA motor domains positioned such that the nucleotide binds within the cleft (Pike et al., 2009, 2015; Lucic et al., 2011) (**Figure 3**). High-resolution X-ray crystal structures of human RECQL1 bound to DNA and biochemical studies by the Gileadi and Vindigni labs revealed that the conserved WH domain (adjacent to the Zn^{2+} binding domain) bears a prominent β-hairpin structure with a tyrosine residue (Y564) at the tip which acts as a unique strand separating pin; this β-hairpin is also found in WRN and BLM helicases, but theirs are considerably shorter than the one located in RECQL1 (Pike et al., 2015). The intimate interaction of the RECQL1 strand-separating β-hairpin with the DNA branchpoint of the single-stranded DNA-double-stranded DNA junction suggests a relevant target for molecular docking by small molecules. Nonetheless, conservation of the β-hairpin among other DNA helicases may compromise its utility as a drug target due to specificity issues. However, RECQL1's strand-separating pin is buttressed by a protein dimer interface required for optimal duplex DNA unwinding; furthermore, RECQL1 also forms a tetramer that is implicated in HJ branch-migration. We conjecture that small molecules which dock at sites of critical contact points for oligomerization (e.g., dimer interface) might effectively modulate RECQL1's assembly state that would have dramatic consequences for biochemical and cellular function

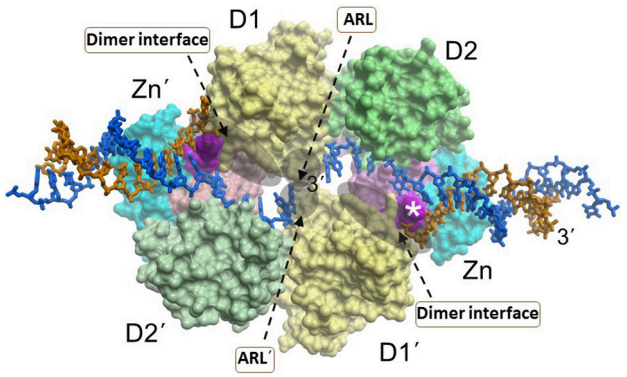


FIGURE 3 | A dimer of RECQL1 molecules, bound to two forked DNA molecules. The first RECQ1 monomer is marked with standard lettering, the second monomer is with primes. The two RecA binding domains (D1, D2) and Zn^{2+} binding domain (Zn) are indicated. The white asterisk denotes the strand-separating beta hairpin. 3' end of DNA strand is indicated. The locations of the conserved aromatic-rich loops (ARL) implicated in the coupling of single-stranded DNA binding to ATP hydrolysis are indicated by the gray shadow. The region comprising the dimer interface between RECQL1 monomers is indicated by gray shadow. Docking small molecules at the ARL or dimer interface may provide a strategy to modulate RECQL1's catalytic function. Image was modified from one kindly provided by Dr. Opher Gileadi, University of Oxford.

(**Figure 3**). In addition, RECQL1 has a conserved aromatic-rich loop (ARL) within the ATPase/helicase core domain that couples single-stranded DNA binding to ATP hydrolysis; the critical nature of RECQL1's ARL for its helicase activity, as revealed by site-directed mutagenesis studies (Banerjee et al., 2015), suggests another structural target to pharmacologically modulate its catalytic activity (**Figure 3**). However, the fact that all five human RecQ helicases possess the conserved ARL (Estep and Brosh, 2017) raises doubt if interaction specificity of a small molecule would be easily achievable unless mitigating factors are addressed with sophisticated molecular docking approaches (see below).

BLM

A recent BLM-DNA crystal structure solved by the Gileadi lab provided fresh insight to its DNA unwinding mechanism, suggesting a base-flipping action that is critical for duplex strand separation (Newman et al., 2015) (**Figure 4**). The mobility of the WH domain evident from the BLM structures suggests that a small molecule which docks in an allosteric site controlling the relative orientation may alter BLM's DNA unwinding mechanism. In addition, a new significance to the auxiliary HRDC domain found only in the human BLM and WRN helicases was ascribed. The HRDC domain was previously implicated in specialized DNA substrate recognition/binding and protein interaction for BLM and WRN [for review, see (Estep and Brosh, 2017)]. Moreover, the BLM HRDC domain is functionally important in double HJ dissolution, a reaction catalyzed by a BLM-topoisomerase complex that is believed to help suppress SCE, a characteristic feature of Bloom's syndrome (Wu et al., 2005). The new BLM structural data indicated a close residence of the HRDC domain to the nucleotide-binding pocket formed by the cleft between the two RecA domains (Newman et al., 2015), suggesting a structural arrangement that might be affected by HRDC-interacting small molecules (**Figure 4**). Compounds that interfere with the interaction of the HRDC with the RecA cleft would be predicted to disrupt the overall catalytic ATPase cycle of BLM, which in turn would affect its helicase activity. Thus, the BLM-DNA structure provides a framework for rational design of BLM-specific inhibitors that should deeply perturb its mechanism of action in cells.

RECQL4

Hereditary mutations in *RECQL4* result in three genetically distinct diseases known as Rothmund-Thomson syndrome, Baller-Gerold syndrome, and RAPADILINO syndrome (Lu et al., 2017). While the *RECQL4* gene product is a DNA helicase, the unwinding activity catalyzed by the purified recombinant RECQL4 helicase protein measured *in vitro* on conventional duplex DNA substrates is relatively weak (Macris et al., 2006; Xu and Liu, 2009). The limited unwinding activity of RECQL4 may be due to its strong annealing activity and its protein architecture as it lacks the classical Zn^{2+} binding domain and WH domain found in most other RecQ helicases (**Figure 5**). However, as revealed by structural and biochemical studies from the Kisker lab, the C-terminus of RECQL4 contains a unique Zn^{2+} binding domain (R4ZBD) and a region sharing homology to two winged helices that are distinct from the RQC WH in other RecQs (Kaiser et al., 2017). The unique identity of RECQL4's C-terminal region, which was found to be important for DNA unwinding (Kaiser et al., 2017), suggests a potential site for molecular docking of small molecules in the upper or lower half of the R4ZBD-WH to modulate its catalytic function (**Figure 5**).

RECQL5

The most recently solved structure of a human RecQ helicase was that of RECQL5 (Newman et al., 2017) (**Figure 6**). This work from the Gileadi lab showed that RECQL5 binds Zn^{2+} via the conserved domain found in RECQL1, WRN, and BLM and that RECQL5 possesses a unique adjacent α-helix with positively charged residues on its surface not found in the other human RecQ helicases. The unique RECQL5 α-helix is proposed to operate as a wedge analogous to the β-hairpin in RECQL1, WRN, and BLM, suggesting a potential RECQL5-specific domain to target with small molecules (**Figure 6**). From a molecular docking perspective, it is quite interesting that RECQL5 was demonstrated to exist in two distinct conformations (open and closed) that are regulated by nucleotide binding. Further studies may identify interfacial small molecule inhibitors that bind within the inter-domain cleft and lock it into the open or closed conformation (**Figure 6**). Screening for small molecules that affect the nucleotide-induced conformational switch of RECQL5 may be informative for further understanding mechanism. The aforementioned α-helix was implicated in DNA binding and site-specific mutagenesis revealed that it plays an important role in helicase activity. RECQL5-interacting compounds that affect the conformational freedom of the α-helix and other key structural

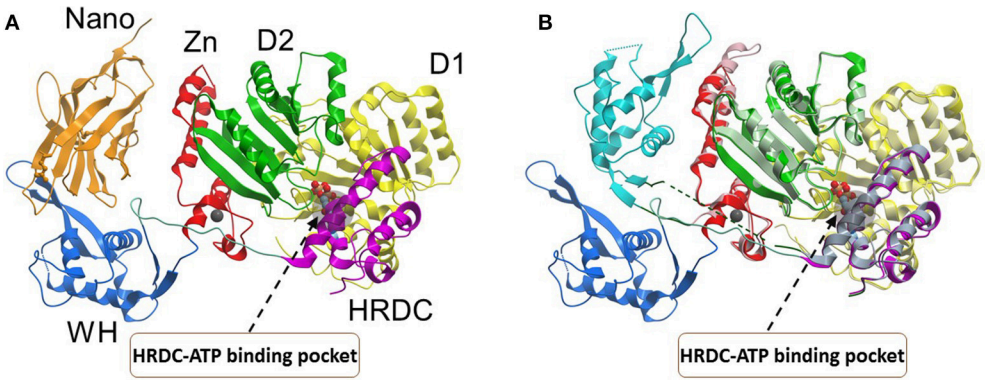


FIGURE 4 | Conformations of the BLM helicase core and winged helix domains**. (A)** A co-crystal structure with a nanobody (orange), which shifts the WH domain out of its helicase-active position (PDB:4CDG). **(B)** A superposition of the nanobody-bound conformation (WH domain in blue, nanobody omitted), with a DNA-bound conformation (WH domain in cyan, DNA omitted; PDB:4CGZ). The RecA domains (D1, D2) and the conserved HRDC are indicated with a region of the HRDC residing close to the ATP binding pocket shown by gray shadow. Small molecules which bind to the HRDC may modulate ATP binding and/or hydrolysis by BLM. Image was modified from one kindly provided by Dr. Opher Gileadi, University of Oxford.

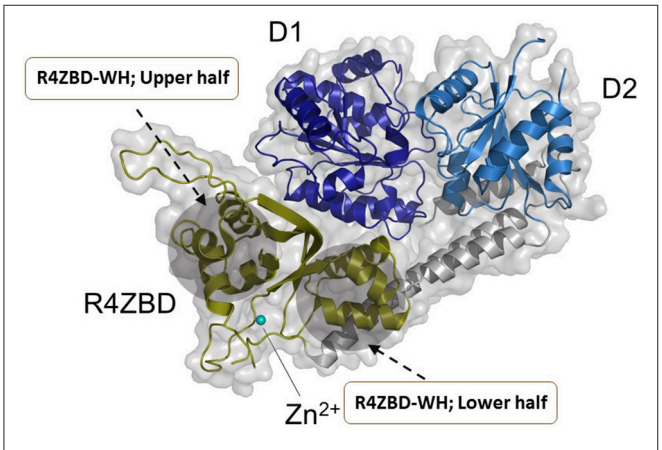


FIGURE 5 | Structure of human RECQL4 (residues 449-1111). The ATPase domain, comprising HD1 and HD2, are shown in dark blue and light blue, respectively. RECQL4 features a structurally unique domain, termed RECQL4-Zn^{2+}-binding domain (R4ZBD), shown in olive. The R4ZBD coordinates a Zn^{2+}-ion (cyan sphere). The gray shadow regions represent the upper and lower halves of the R4ZBD that may be suitable for molecular docking of small molecules to the unique Zn^{2+} binding domain of RECQL4. RECQL4 harbors the Sld2-homology domain at its N-terminus (not shown). Image was modified from one kindly provided by Drs. Sebastian Kaiser and Caroline Kisker, University of Wuerzburg.

elements would be anticipated to impact its catalytic functions and potentially modulate its functions in cells.

XPD

Eukaryotic XPD is a component of the general transcription factor (TF)IIH complex that is implicated in both cellular transcription and nucleotide excision repair (NER) of damaged DNA (Kraemer et al., 2007). Mutations in the *XPD* gene are linked to genetic diseases characterized by premature aging and/or cancer predisposition including Trichothiodystrophy (TTD), Xeroderma pigmentosum, and Cockayne's syndrome (Lehmann, 2001). High-resolution crystal structures of XPD helicase and the data obtained from the associated biochemical and mutational studies over the past decade provided significant mechanistic insights about the function of this important class of Fe-S helicases (Liu et al., 2008; Wolski et al., 2008; Kuper et al., 2012; Abdulrahman et al., 2013). Crystal structures of archaeal homologs of XPD revealed that in addition to two canonical RecA motor domains, the structure contains a Fe-S cluster and Arch domain. In its proper conformational state, the XPD Fe-S cluster remains tightly connected to the ATP binding/hydrolysis domain. It has been proposed that the wedge-like structure formed by the Fe-S and Arch domains facilitates unwinding of duplex DNA during ATP-driven translocation of the enzyme. Furthermore, in addition to its essential role in helicase activity, mutational studies confirmed that Fe-S cluster is structurally important to maintain proper folding and stability of the XPD protein (Rudolf et al., 2006; Fan et al., 2008; Pugh et al., 2008).

More functional insights came from the crystal structure of XPD from *Thermoplasma acidophilium* (taXPD) in complex with DNA solved by the Kisker lab (Kuper et al., 2012) (**Figure 7**). The taXPD-DNA complex structure, combined with biochemical and mutational analyses from their lab (Kuper et al., 2012) and Spies' (Pugh et al., 2012), has begun to elucidate the underlying mechanism for XPD's DNA translocation polarity, thereby providing insight into the role of the helicase during NER. Apart from the Fe-S cluster domain, the Arch domain of XPD also has been shown to be critical for its DNA binding and strand separating activities. Introduction of a TTD-linked point mutation (XPD-C259Y) or deletion of the entire Arch domain (XPD-ΔARCH) was found to impair DNA binding and helicase activity of XPD (Abdulrahman et al., 2013). Collectively, these studies suggest that Fe-S cluster and Arch domain play key roles in the unwinding mechanism and governing XPD functions during DNA damage repair. Targeting the Arch and Fe-S domains of XPD with small molecules may be valuable (**Figure 7**);

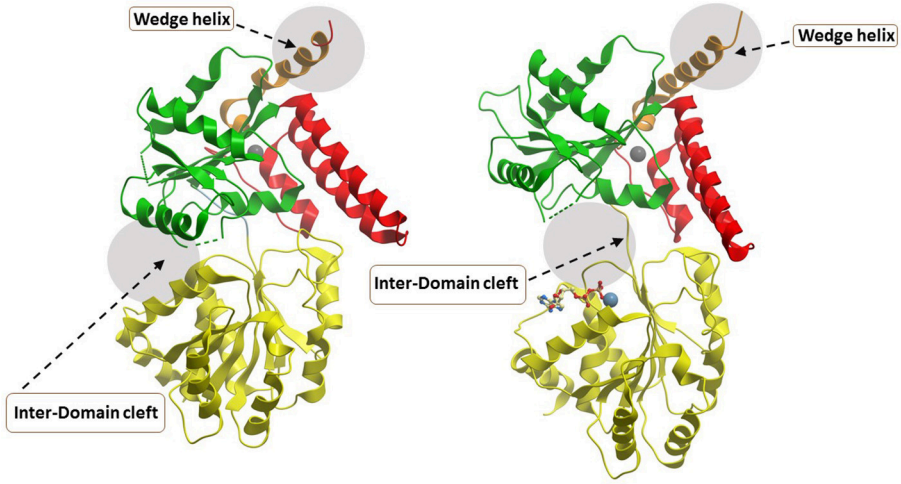


FIGURE 6 | Closed and open conformations of RECQL5 helicase domain**. (A)** Closed state, no nucleotide (PDB ID: 5LB8); **(B)** Open state, with Mg^{2+}-ADP (PDB: 5LB3). Note the absence of the WH and HRDC domains in the C-terminal region of the protein which can be found in WRN, BLM, and RECQL1. The unique "wedge" α-helix of RECQL5 critical for helicase activity is indicated and may be targeted by small molecules to modulate ATP-dependent strand separation. Also indicated by gray zone is the inter-domain cleft which widens significantly between the open and closed conformations. The inter-domain cleft may be a useful site for molecular docking of small molecule interfacial compounds that perturb open-closed conformational switches of RECQL5. Image was modified from one kindly provided by Dr. Opher Gileadi, University of Oxford.

however, these domains are conserved in other Fe-S helicases (FANCJ, RTEL1, DDX11), raising doubt if they would be specific. Nonetheless, given that DNA damaging chemotherapy drugs often introduce bulky lesions recognized by NER, it is reasonable to postulate that small molecule targeted inhibition of DNA unwinding by the XPD helicase (thought to be an early sensor or verifier of the DNA damage) would be useful to sensitize tumors to certain compounds used in anti-cancer treatments.

Further research in this area to solve structures of XPD with key DNA structural intermediates (e.g., DNA bubbles with site-specific damage), as well as structures of other Fe-S helicases, is likely to advance efforts in molecular docking and HTS for small molecules that modulate their functions. With the discovery of specific and potent inhibitors of Fe-S helicases, the co-crystal structures of helicase-DNA-small molecule complexes may elucidate key helicase interactions in DNA metabolic pathways. Molecular docking-based virtual screening should also be considered as a highly effective complementary approach toward the discovery of small molecule inhibitors of disease-linked DNA repair helicases.

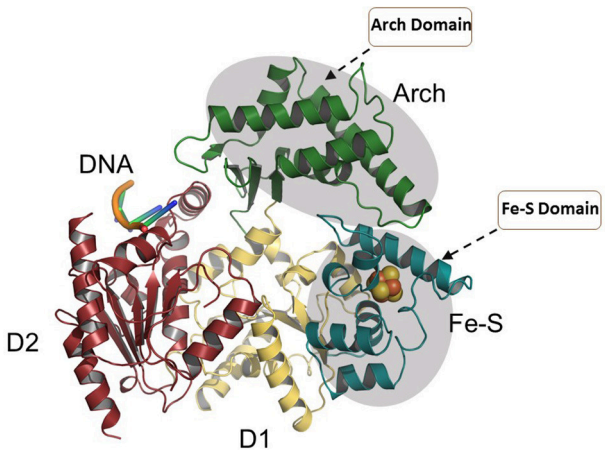


FIGURE 7 | Overall structure of the taXPD-DNA complex. Shown are the two RecA-like domains in yellow orange and ruby, the FeS cluster domain in deep teal, and the Arch domain in forest green. The DNA is shown in orange/yellow/blue. The Arch and Fe-S domains, both implicated in strand separation and only found in Fe-S helicases, are proposed sites for docking small molecules to modulate helicase function. Image was modified from one kindly provided by Drs. Jochen Kuper and Caroline Kisker, University of Wuerzburg.

CHALLENGES TO DEVELOPMENT AND APPLICATION OF HELICASE INHIBITORS

Although small molecule inhibitors of a few viral helicases showed preclinical and clinical success [e.g., the herpes simplex virus helicase-primase inhibitor Amenamevir (ASP2151) (Chono et al., 2010) and other drug candidates (Kleymann et al., 2002)], development of highly specific and pharmacologically effective helicase inhibitors is still challenging. In general, high throughput biochemical screening assays return very few hits and most often they fail to deliver expected biological effects in subsequent cell-based assays or display poor pharmacological outcomes [e.g., HPV helicase inhibitor (Faucher et al., 2004)]. As is the case for a significant number of small molecule inhibitors, bioavailability is likely to be one of the major obstacles to developing clinically effective DNA repair helicase inhibitors and target validation is

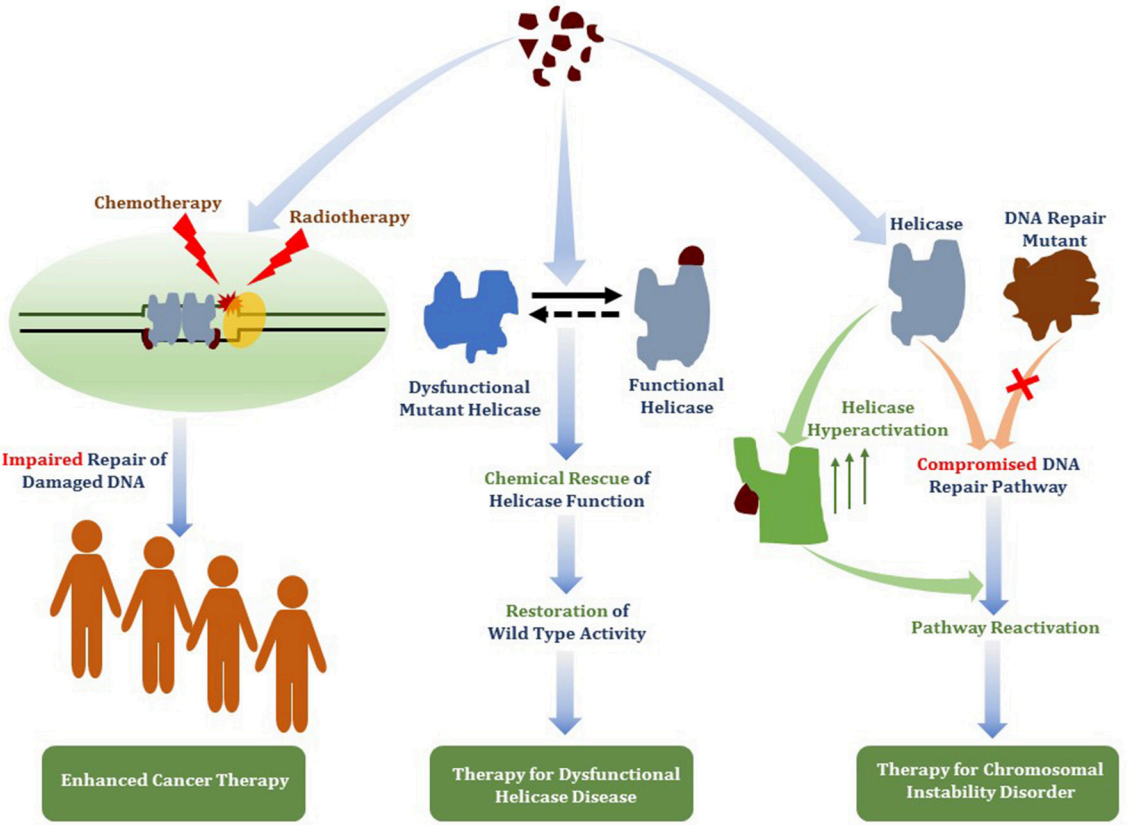


FIGURE 8 | Therapeutic avenues for helicase-interacting small molecules. Compounds that bind DNA helicase proteins may be useful for (i) targeted helicase inhibition to deter DNA damage response pathways to enhance chemotherapy drug or radiation strategies (ii) chemical rescue of catalytically defective and/or misfolded helicase proteins due to missense mutations linked to hereditary chromosomal instability disorders, or (iii) functional activation of a wild-type helicase that has an overlapping role with a DNA repair pathway that is defective due to a genetic inactivating mutation linked to a DNA repair disorder.

required (Bunnage et al., 2013). Therefore, cellular bioavailability parameters including inhibitor aqueous solubility, nonspecific binding to the cell membrane and extracellular matrix, cellular uptake, intracellular metabolic stability, and biotransformation to an inactive secondary metabolite should be taken into consideration for the successful therapeutic exploitation of a newly identified promising drug (Frye, 2010; Workman and Collins, 2010), such as a lead molecule against a helicase protein. For example, although BLM inhibitors ML216 and its analog 33 were found to exhibit good general pharmacokinetic properties including clogP (computational method for measurement of drug hydrophilicity and lipophilicity properties), microsomal stability, and plasma stability, both compounds displayed low solubility and permeability suggesting that further optimization of the lead compounds is required (Rosenthal et al., 2013). In this scenario, sensitive assays such as a HPLC-MS based method to determine bioavailability of a given compound inside cells may prove fruitful to assess cellular uptake of helicase inhibitors (Teuscher et al., 2017).

Given the precise functions of helicases in nucleic acid metabolism, another consideration is if the helicase-interacting compound reaches its desired subcellular localization (i.e., nucleus, cytosol, mitochondrion) to bind its desired target helicase inside the respective cellular compartment. In order to get the most desirable therapeutic effect and to minimize the negative side effects, it is also very important to ensure that the helicase inhibitors are delivered specifically to their sites of action within the cells. Nuclear targetted delivery of these small molecule inhibitors might be achieved using nanoparticles coated with nuclear localization signal (NLS) (Kang et al., 2010a). Similarly, clinically approved nanoparticles should be considered to deliver the helicase inhibitors selectively to the target tumor sites at sufficient concentration to attain therapeutic efficacy.

One of the prime challenges associated with the development of helicase inhibitors is their relative potency and specificity. The inhibitors should be potent enough to exert their biological effects at minimal concentration. For example, Mcm2-7 helicase inhibitor ciprofloxacin (Simon et al., 2013) and BLM helicase inhibitor ML216 (Nguyen et al., 2013) were found to exhibit their bioactivity at relatively high concentrations (160–350 μM and 50 μM, respectively; see **Table 1**). Therefore, structural optimization of these hit compounds is warranted to obtain more potent leads. Moreover, the effect of ML216 is not entirely specific to BLM helicase because the compound also inhibits DNA

unwinding by the sequence-related WRN helicase with a similar potency (Nguyen et al., 2013). Hence, promising hits identified for a target helicase from the initial screening should be further assessed for their potency as a function of drug concentration as well as for their specificity by determining their effects on other helicases. Substantial counterscreening of the newly identified potential helicase inhibitors is required before they can be reliably pursued in preclinical models.

A number of drug resistance mechanism are known to operate which contribute to tumor resistance (Holohan et al., 2013). These include: (i) drug efflux or alteration by activation or inactivation; (ii) alterations in the drug target by mutation or change in gene expression; (iii) repair of chemotherapy- or radiation-induced DNA damage; (iv) up-regulation or activation of compensatory signaling pathways; (v) cell death evasion (e.g., attenuated apoptosis). From the perspective of DNA helicase inhibitors, one of the basic principles is to compromise a helicase-dependent pathway of repair [(iii) above] to confer synthetic lethality; however, it is possible that another repair pathway or signaling pathway is elicited that compromises the efficacy of the helicase inhibitor. Certainly, it is plausible that even the functional redundancy between members of the RecQ or Fe-S helicases, for example, may contribute to helicase inhibitor resistance by a compensatory overlapping pathway. Other avenues of drug resistance, such as those mentioned above, may allow resistance to the anti-cancer effects of a helicase-directed drug inhibitor; however, little is known in this area because it is such a new field. The fact that tumors are often heterogeneous may allow cancer cell subpopulations survive under pressure from a cancer drug (Zahreddine and Borden, 2013), including one against a specific DNA helicase. These topics all deserve prioritized attention.

In terms of molecular docking approaches for the discovery of compounds that inhibit helicases and other DNA repair proteins, a significant challenge is the protein flexibility of the target in which intrinsic conformational states may compromise a good fit for the docking ligand (Tripathi and Bankaitis, 2017). Other mitigating factors including protein pocket architecture to accommodate the three-demonsional geometry of the ligand, ligand access (surface or protein interior), the role of structured water molecules in the ligand-target interaction, protonation, and ionization states of the protein: ligand system, and entropy considerations. Advances in artificial intelligence and machine learning algorithms provide new and innovative direction for structure-based drug design. These efforts, combined with high-resolution structures of helicase proteins, provide excitement for anticipated progress.

SUMMARY AND FUTURE DIRECTIONS

DNA helicases are often recruited to sites of DNA damage or stalled replication forks. The very nature of their catalytic function to separate complementary DNA strands is imperative to a wide variety of DNA transactions that play instrumental roles in cellular DNA replication, recombination, repair, and transcription. Therefore, chemical modulation of the molecular functions of DNA helicases provides an approach to alter not only the efficiency or fidelity of transactions in nucleic acid metabolism but also affect cellular homeostasis, including the division rate of cancerous cells. As detailed in this review, DNA helicases join a larger class of DNA metabolic proteins that are considered as potential targets to augment radiation and chemotherapy strategies to combat cancer. Small molecule-induced trapping of DNA helicases may represent a generalized mechanism exemplified by certain topoisomerase and PARP inhibitors that exert poisonous consequences, especially in rapidly dividing cancer cells. An area that remains underexplored is the synergism between compounds that modulate different DNA proteins. This is particularly interesting from the perspective of DNA helicase inhibitors that might be combined with compounds that deter the functions of other DNA repair enzymes to enact targeted anti-proliferative and lethal effects in various cancer types (**Figure 8**). Just as chemical and genetic synthetic lethality has become more widely appreciated and better understood, we expect that pharmacological modulation of helicase function will move to the forefront as molecular motor DNA unwinding enzymes play such pivotal roles in nucleic acid metabolism and cross-talk with many cellular pathways. With the increased knowledge of structure-activity relationships from the solution of helicase structures, we anticipate that molecular modeling will provide a more readily accessible and informed pathway for the discovery of novel helicase-interacting compounds. An important challenge in the field will be the utilization of helicase-modulating drugs in preclinical models that will accelerate their implementation in therapeutic approaches.

A unique aspect of this review is to detail potential strategies to target helicase with small molecules using a structure-design molecular docking approach (**Figure 2**). We believe that virtual screening of small molecule libraries to identify compounds predicted to modulate helicase function will become main-streamed as more helicase structures (and their conformational states induced by ligand binding) become solved and computational strategies advance. An illustrative example of this approach was recently provided by the Berger lab. Lawson et al. observed that the structural interactions of nucleic acid or the antibiotic bicyclomycin with the same binding site in the hexameric RNA translocase/helicase Rho are distinguished from each other by the closed-ring (translocase competent) vs. open-ring (RNA binding defective) conformations of Rho, respectively (Lawson et al., 2016). This work is significant because it showed that nucleic acid substrate loading by a helicase could be modulated by a small molecule via a conformational switch in the enzyme that altered its ring-closure dynamics. Furthermore, the Rho-bicyclomycin study leads to the anticipation that other helicase-interacting small molecules may be identified virtually from compound libraries using a molecular docking approach that would be highly selective and mechanistically driven.

Although molecular compound inhibitors of DNA helicases or more generally DNA repair enzymes are increasingly discussed, conversations and research directions could also be directed toward small molecule chemical rescue of

catalytically dysfunctional or misfolded helicase proteins as well as activation or up-regulation of wild-type helicase-catalyzed strand separation (**Figure 8**). Given the number of helicase missense mutations linked to hereditary disorders or associated with cancer (Suhasini and Brosh, 2013), and following the lead of other clinically relevant targets [e.g., chemical rescue of p53 missense mutations (Bullock and Fersht, 2001)], the prospect of finding treatments or cures for certain chromosomal instability disorders arising from catalytic deficiencies in helicase proteins is plausible. In another realm, helicase activation may have therapeutic value. For example, the possible functional overlap of DNA helicases [e.g., RecQ family members (Brosh, 2013; Croteau et al., 2014)] suggests that increased activity of one helicase may help to overcome the deficiency of another helicase or DNA repair protein (**Figure 8**). With molecular docking and high-throughput screens becoming more commonplace, the hypothesis that up-regulation of catalytic function by a helicase or DNA repair enzyme can rescue a helicase-deficient disease state may become testable in cell-based and pre-clinical models.

AUTHOR CONTRIBUTIONS

AD and RB both contributed to writing original component and editing of the manuscript.

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REVIEW
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The Human Replicative Helicase, the CMG Complex, as a Target for Anti-cancer Therapy

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DNA helicases unwind or rearrange duplex DNA during replication, recombination and repair. Helicases of many pathogenic organisms such as viruses, bacteria, and protozoa have been studied as potential therapeutic targets to treat infectious diseases, and human DNA helicases as potential targets for anti-cancer therapy. DNA replication machineries perform essential tasks duplicating genome in every cell cycle, and one of the important functions of these machineries are played by DNA helicases. Replicative helicases are usually multi-subunit protein complexes, and the minimal complex active as eukaryotic replicative helicase is composed of 11 subunits, requiring a functional assembly of two subcomplexes and one protein. The hetero-hexameric MCM2-7 helicase is activated by forming a complex with Cdc45 and the hetero-tetrameric GINS complex; the Cdc45-Mcm2-7-GINS (CMG) complex. The CMG complex can be a potential target for a treatment of cancer and the feasibility of this replicative helicase as a therapeutic target has been tested recently. Several different strategies have been implemented and are under active investigations to interfere with helicase activity of the CMG complex. This review focuses on the molecular function of the CMG helicase during DNA replication and its relevance to cancers based on data published in the literature. In addition, current efforts made to identify small molecules inhibiting the CMG helicase to develop anti-cancer therapeutic strategies were summarized, with new perspectives to advance the discovery of the CMG-targeting drugs.

Keywords: CMG, DNA helicase, DNA replication, cancer, therapeutic target

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DNA HELICASES, AN EMERGING TARGET FOR CANCER THERAPY

DNA helicases unwind duplex DNA in an ATP hydrolysis-dependent manner. *In silico* studies have identified 31 human DNA helicases that are functionally non-redundant (Umate et al., 2011). They are involved in a variety of DNA transactions including DNA replication, repair, recombination and telomere maintenance for the preservation of genome stability (Brosh, 2013; Bochman, 2014; Croteau et al., 2014). Failure of faithful duplication and repair of DNA leads to the loss of genome integrity, thereby causing cancers or other diseases which promote cancers. Many helicase-linked diseases have been identified (Bochman, 2014). Thus, DNA helicases have important roles as genome caretakers and can be considered as potential targets for treating life-threatening diseases including cancers.

The initial effort to discover potent helicase inhibitors had been put to control the replication of infectious microorganisms. Compounds that inhibit helicases have been explored to find alternative way to treat immunocompromised patients suffering from cold sores and genital herpes caused by herpes simplex virus (HSV) infection (Crute et al., 2002; Kleymann et al., 2002). Antiviral drugs targeting viral DNA polymerase (including guanosine, nucleotides or pyrophosphate analog) have been developed over the past 40 years and widely used. However, discovery of the drug-resistant isolates has required development of novel strategies to treat HSV patients (Jiang et al., 2016). The HSV helicase-primase complex is composed three subunits possessing 5′–3′ helicase, primase, and single stranded DNA (ssDNA)-dependent NTPase activities that are essential for DNA synthesis. Several compounds inhibiting the helicase-primase complex with notable efficacy have been identified, although viral resistance against some of these molecules have been reported (James et al., 2015). Two of them (Pritelivir and Amenamevir) have been applied for human clinical trials. Other viral DNA helicases including papillomavirus E1 and polyomavirus large tumor antigen (Tag) are also widely studied as drug targets (Shadrick et al., 2013).

The non-structural protein 3 (NS3) of Hepatitis C virus (HCV) is another example of DNA helicase as an antiviral drug target. NS3 RNA helicase, which also possesses DNA helicase activity, is responsible for viral genome replication (Pang et al., 2002). Nearly 200 million people are estimated to be infected by HCV worldwide and effective vaccine is not currently available (Jin et al., 2017). Several compounds that inhibit the NS3 helicase activity have been isolated and the most potent compound tested inhibited unwinding activity of NS3 by more than 50% at approximately 2 μM concentration (Jin et al., 2017).

The strategy to develop antibacterial drugs by targeting DNA helicases is also relatively novel concept in comparison to other types of antibiotics. The mechanistic studies of DNA replication with *in vitro* reconstitution systems have been extensively carried out using purified proteins from *Escherichia coli (E. coli)*. Isolation and biochemical analysis of *E. coli* DnaB replicative helicase enabled the reconstitution of *E. coli* replisome *in vitro* and has deepened our understanding on the prokaryotic DNA replication (Wright et al., 1973; LeBowitz and McMacken, 1986; Yao and O'Donnell, 2010). Inhibitors against DnaB could be used to treat pathogenic strains of *E. coli*. Several groups identified inhibitors of DnaB helicase of *Bacillus anthracis (B. anthracis)* and *Staphylococcus aureus (S. aureus)* through the use of high-throughput screenings (Head et al., 2016). The most potent and selective molecule inhibited DnaB helicase activities of *B. anthracis* and *S. aureus* with an IC_{50} of 0.2 μM (Li et al., 2013). *B. anthracis* as a potential biological warfare and methicillin-resistant *S. aureus* (MRSA) infections among patients in the emergency department, have made it urgent to develop novel therapies for the treatment of these bacteria (Shadrick et al., 2013; Head et al., 2016).

Malaria is one of the widespread human diseases cause by the parasite *Plasmodium falciparum (P. falciparum)*. Approximately, 212 million cases occurred worldwide in 2015, leading to 429,000 deaths (WHO, 2016). It was shown that some compounds inhibited activity of a *P. falciparum* helicase and the growth of the parasite (Tuteja, 2007). Genome analyses have revealed that *P. falciparum* contains several parasite-specific helicases in addition to homolog of human helicases. Development of inhibitors that specifically target the helicases unique to *P. falciparum* would help to cure malaria (Tuteja, 2017).

The similar idea has been applied to our own DNA helicases in order to curb uncontrolled growth of cancers. Our combats against cancers have come a long way and numerous kinds of drugs have been developed based on understandings of the mechanisms governing proliferation of normal and cancerous cells and tumor progression. These include a vast array of cellular processes such as cell cycle checkpoint, transcription, microtubule assembly, DNA replication and repair, signal transduction, angiogenesis, ubiquitin proteasome system, and immune checkpoint (Dickson and Schwartz, 2009; Priyadarshini and Keerthi, 2012; Vasudev and Reynolds, 2014; Weathington and Mallampalli, 2014; Gross et al., 2015; Sharma and Allison, 2015; Gavande et al., 2016; Zhang et al., 2016). Developing anti-cancer therapy by targeting DNA repair pathways has been an active area of recent cancer research with a promising future. One of the examples is olaparib, an inhibitor of poly(ADP-ribose) polymerase (PARP) which is involved in multiple DNA repair pathways by recruiting repair proteins (Gavande et al., 2016). Olaparib was approved by FDA in December 2014 as monotherapy for ovarian cancer. Finding potent and safe inhibitors against DNA processing enzymes, DNA binding proteins, DNA polymerases and DNA damage response kinases acting on DNA repair pathways are also under extensive investigations. DNA helicases belong to the category of the DNA processing enzymes.

Several model DNA repair pathways and related DNA helicases as prospective targets for treatment of cancers were discussed comprehensively in several reviews (Gupta and Brosh, 2008; Brosh, 2013; Suhasini and Brosh, 2013). Most well-known DNA helicases involved in the maintenance of genome integrity are RecQ family helicases. Human RecQ helicases belong to SF2 helicase superfamily, share three highly conserved protein domains, and unwind a variety of DNA structures resembling DNA repair intermediates *in vitro* (Croteau et al., 2014). The RecQ helicases are Janus-faced. Genetic defects in these helicases are linked to diseases prone to develop cancers. However, targeted depletion of RecQ helicases in cancer cells decreased cell proliferation. It is interpreted that in the absence of functional RecQ helicases, genome instability increases which leads to the development of cancer. At the same time, upregulation of RecQ helicase is required to properly deal with lesions occurred during DNA replication in rapidly dividing cancer cells and, thus, it ensures the sustained growth of cancers (Brosh, 2013). RecQ-type helicases WRN and RecQL1 are expressed highly in several cancers and anticancer effects of siRNA against these genes, based on mouse models, were reported (Futami and Furuichi, 2015). Small-molecule inhibitors of helicase activities of WRN and another RecQ helicase BLM have been identified and both displayed anti-proliferative activity synergistically in the presence of chemotherapy drugs (Aggarwal et al., 2011, 2013; Nguyen et al., 2013).

Traditional anti-cancer drugs target DNA directly to trigger replication stress. Alkylating agents such as nitrogen mustard originated from chemical warfare during World Wars I and II, and their selectivity to kill cancer cells was dependent on the quantitative differences in the cell division rate between normal and cancer cells. Subsequently, numerous DNA-interacting agents were developed for the treatment of cancers including DNA crosslinkers, intercalators, and double-stranded DNA breaking agents (Hurley, 2002). The strategies to increase replication stress did not depend only on the DNA-interacting chemicals. Competitive inhibitors targeting enzymes required to maintain the pool of dNTPs are another type of conventional approaches to increase replication stress in cancer cells. Emerging approaches include (i) interfering with ATR-Chk1 replication checkpoint signaling to cause stalling and collapse of replication forks, (ii) inhibition of Wee1 kinases, that produces incompletely DNA-replicated cells, leading to mitotic catastrophe, and (iii) inhibition of histone deacetylases that dysregulates DNA replication (Conti et al., 2010; Puigvert et al., 2016; Zhang et al., 2016). DNA replication *per se* could be targeted by inhibiting enzymes responsible for the assembly of DNA replication complex and the initiation and elongation of DNA synthesis. These includes DDK and CDK kinases, proliferating cell nuclear antigen (PCNA), topoisomerases, DNA polymerases, and replicative helicase MCM (or CMG as an activated complex) (Berdis, 2008; Rodriguez-Acebes et al., 2010; Kang et al., 2012; Simon and Schwacha, 2014; Huggett et al., 2016; Roskoski, 2016).

Unwinding of duplex DNA during eukaryotic DNA replication is catalyzed by the CMG (Cdc45/Mcm2-7/GINS) helicase complex composed of three replication factors: Cdc45 protein, the Mcm2-7 and the GINS complexes (Moyer et al., 2006). Expression levels of Mcm subunits are down-regulated in differentiated somatic cells in keeping with its function in cell proliferation (Gupta and Brosh, 2008). On the other hand, enhanced expression of Mcm proteins have been reported in many cancer cells derived from patients (Neves and Kwok, 2017). The roles of the Mcm proteins in cancer progression have been linked to at least two cancer hallmarks including enhanced proliferation and regulation of replicative stress. For its catalytic role during DNA replication, the Mcm helicase has been considered as an emerging target for cancer therapy. The Mcm2-7 complex consists of six different Mcm ATPase subunits, however, the complexes in higher eukaryotes are not active as helicases (Kang et al., 2012). The Mcm2-7 complex is activated when it forms a macromolecular complex with Cdc45 and the GINS complex (Ilves et al., 2010). Until now, a few molecules have been identified to inhibit helicase activities of the budding yeast Mcm2-7, which is active as a helicase *in vitro* in certain conditions and the human Mcm4/6/7 subcomplex which is also active, but different from the Mcm2-7 complex in helicase activity (Simon and Schwacha, 2014). It appears that further screening of potential candidate compounds is required using the human CMG complex *in vitro*. Other approaches identified small molecules modulating the level of Mcm proteins in cells instead of inhibiting *in vitro* helicase activity. In this review, we discuss our current knowledge and progress on the development of chemotherapeutic drugs that target the human replicative helicase CMG and propose perspectives for the future of cancer treatment.

CMG: MECHANISM AND FUNCTION IN DNA REPLICATION

DNA Replication Initiation and Identification of Replicative Helicase CMG

The CMG helicase complex consists of 11 polypeptides. Its assembly is cell-cycle regulated and takes place on DNA in order to unwind duplex DNA during DNA replication. DNA replication occurs once per cell cycle, and the entire process is tightly regulated by multiple processes participated by dozens of proteins. The framework of current DNA replication model was first suggested by Jacob et al. (1963). In the replicon model, an initiator made from chromosome acts on a *cis*-acting replicator sequence allowing the beginning of the replication which spreads along the chromosome. Around 30,000–50,000 replicators, which we now call replication origin, are activated at each cell division in humans (Méchali, 2010).

The initiation of protein assembly reaction on DNA replication origins is relatively well understood in a budding yeast *Saccharomyces cerevisiae (S. cerevisiae)*. During late mitosis and G1-phase, the heterohexameric origin recognition complex (ORC) binds to the origin and recruits Cdc6 and Cdt1 proteins (Siddiqui et al., 2013). The resulting complex facilitates loading of two Mcm2-7 complexes onto the protein-origin DNA complex in a head-to-head fashion forming the pre-replication complex (pre-RC) and this assembly is also known as replication licensing (Riera et al., 2017). Loading of other replication factors onto chromatin is governed by the two S-phase specific kinases, DDK and CDK (Tanaka and Araki, 2013). Sld3-Sld7-Cdc45 binds to the origin during G1 and this step is dependent on phosphorylation of Mcm2-7 by DDK. Essential CDK phosphorylation occurs on Sld3 and Sld2, making them have affinity to Dpb11. Dpb11 is a part of the pre-loading complex (pre-LC) composed of Dpb11-Sld2-GINS-Polymerase (Pol) ε. Therefore, CDK phosphorylation leads to the assembly of a multi-protein complex onto the chromosome making the complex ready to initiate DNA replication and the complex in this state is called the pre-Initiation complex (pre-IC). These multi-step processes ultimately lead to the formation of the CMG helicase complex from Cdc45, the Mcm2-7, and the GINS and Mcm10 protein activates the CMG helicase to create replication forks (Kanke et al., 2012; van Deursen et al., 2012; Watase et al., 2012). Two CMGs converted from double hexameric Mcm2-7 move in opposite directions with polymerases (Pol ε, Pol δ, Pol α/primase) elongating new DNA chains (Riera et al., 2017). Humans have orthologues of the replication proteins described above except for Sld7 (MTBP is a functional homolog of Sld7) (Boos et al., 2013). However, the mechanism of loading of replication factors are not as clear as that of the budding yeast.

The six subunits (Mcm2, 3, 4, 5, 6, and 7) of the hexameric Mcm2-7 were discovered from genetic screenings of mutants defective in artificial minichromosome maintenance (Mcm) in

attempts to isolate replication initiator proteins (Maine et al., 1984). All six members showed sequence similarity (20–30%) and shared a characteristic structural feature; AAA+ (ATPases associated with various cellular activities) domains flanked by the N- and C- terminal domains (NTD and CTD) (Tye, 1999; Parker et al., 2017). Six subunits are radially arranged in the hexameric Mcm2-7 complex, forming a central channel that DNA can access (Costa et al., 2011) (**Figure 1B**).

Cdc45 was originally identified as a cold-sensitive cell division cycle (cdc) mutant involved in the regulation of DNA replication and the *CDC45* gene was shown to have genetic interactions with *ORC2*, *MCM3*, *MCM5, MCM7* genes (Moir et al., 1982; Hardy, 1997). Chromatin loading of Sld3 and Cdc45 is mutually exclusive and experimental data suggested that phosphorylation of the Mcm2-7 by DDK enhances association of Sld3-Sld7-Cdc45 on chromatin in *S. cerevisiae* (Tanaka and Araki, 2013).

A subunit of the GINS was identified from the screening of synthetic lethal mutants with the temperature-sensitive *dpb11-1* allele in *S. cerevisiae* (Kamimura et al., 1998). One of the mutant isolated was named *sld5* (synthetic lethality with *dpb11-1 5*). Other three subunits of the GINS were identified from genetic screenings using *sld5* mutants and by immunoprecipitation assays, and they were named Psf (partner of Sld five) 1, Psf2, and Psf3 (Takayama et al., 2003). Independent of this, other screening efforts using temperature-induced degradation of target proteins and analyses of cell cycle progression defects also led to identification of the GINS subunits in *S. cerevisiae* (Kanemaki et al., 2003). Homologs of the yeast GINS were also identified in *Xenopus* and the ring-like structure of the four-subunit GINS was characterized (Kubota et al., 2003). The GINS is recruited to DNA in the CDK-dependent manner in S-phase as a part of pre-LC as described above.

Since identification of Mcms as factors required for replication licensing, their molecular functions at the chromatin have been investigated. The presence of ATPase motifs in the central domain of all Mcms, inhibition of DNA replication initiation in cells by nuclear microinjection of anti-human Mcm2 antibodies, and the presence of the stable complex containing all six Mcm subunits in cells raised the possibility that the Mcm complex is the replicative helicase that is responsible for progression of replication forks to generate template DNA during DNA replication (Todorov et al., 1994; Ishimi et al., 1996; Schulte et al., 1996). First evidence came from studies with human proteins. The human Mcm complexes were isolated from HeLa cells and it was found that the 3′–5′ DNA helicase activity was intrinsically associated with the non-canonical Mcm complex containing only Mcm4, 6, and 7 (Ishimi, 1997). Subsequently, it was shown that mouse Mcm4/6/7 complex reconstituted by baculovirus infection has intrinsic helicase activity (You et al., 1999). Fission yeast Mcm4/6/7 was shown to have a similar activity, while the hexameric Mcm2-7 complexes had not been found to have DNA helicase activity (Lee and Hurwitz, 2000). However, the predominant form of Mcm complex present in *Xenopus* egg extracts is a heterohexamer containing all six subunits and only the Mcm2-7 complex supported DNA replication in the extract, although *Xenopus* Mcm4/6/7, not Mcm2-7, has intrinsic helicase activity (Prokhorova and Blow, 2000; Ying and Gautier, 2005). Besides, the ATPase activity of the *Xenopus* Mcm2-7 is required for DNA unwinding (Ying and Gautier, 2005). Moreover, all Mcm2-7 proteins are essential for the elongation of chromosome replication (Labib et al., 2000), indicating that heterohexameric Mcm2-7 is the *in vivo* replicative helicase. Until now, only *S. cerevisiae* Mcm2-7 has been shown to have DNA helicase activity comparable to that of Mcm4/6/7 under certain conditions in a manner dependent on specific anions (Bochman and Schwacha, 2008). For these and other reasons as discussed below, screenings of inhibitor molecules targeting the Mcm complexes have been performed only with Mcm4/6/7 and *S. cerevisiae* Mcm2-7.

Cdc45 was determined as a factor required for DNA unwinding in *Xenopus* egg extract and was shown to physically interact with Mcm proteins (Mimura et al., 2000; Walter and Newport, 2000). Neutralizing antibodies against Cdc45 abolished chromosomal unwinding (Pacek and Walter, 2004). Furthermore, DNA helicase activity was detected with both anti-Mcm and anti-Cdc45 immunoprecipitates from chromatin replication reactions in *Xenopus* egg extracts and the complex containing Mcm2-7 and Cdc45 was partially purified, suggesting the possibility of activation of Mcm2-7 helicase activity by Cdc45 (Masuda et al., 2003). Finally, Moyer et al. (2006) isolated a high-molecular-weight complex containing Cdc45 from *Drosophila* embryo extracts, establishing that the eleven-subunit complex containing Mcm2-7, GINS, and Cdc45 (CMG) is an active helicase. The same group reconstituted the *Drosophila* CMG complex using purified proteins from the baculovirus expression system, and showed that the Mcm2-7 is activated by association with Cdc45 and the GINS (Ilves et al., 2010). Human CMG was also reconstituted similarly as described for *Drosophila* CMG, and *S. cerevisiae* CMG complex was isolated from yeast cells overexpressing all 11 subunits (Kang et al., 2012; Georgescu et al., 2014). All the CMG complexes purified up to date displayed 3′–5′ DNA helicase activities.

Structure of the CMG Complex

The CMG complex is the core of the replication protein complex and its near-atomic three-dimensional structure was revealed through the cryo-EM and X-ray crystallography of the sub-complexes. Overall structure of the Mcm2-7 is a toroid shaped by a regular arrangement of the six Mcm monomers (Costa et al., 2011) (**Figure 1A**). All six Mcm protomers display the same domain organization, suggesting they are derived from one ancestral gene and this is supported by the presence of homo-oligomeric Mcm complexes in archaea (Abid Ali and Costa, 2016). The N-terminal domain (NTD) of Mcm can be further divided into several subdomains; (i) a helical domain A involved in the DDK-mediated conformational switch and the activation Mcm helicase in archaea. This domain contains an oligonucleotide-binding (OB)-fold which makes contact with ssDNA and is required for Mcm oligomerization and the loading of the Mcm2-7 to replication origins (Fletcher et al., 2003; Slaymaker and Chen, 2012; Froelich et al., 2014). (ii) A Zinc (Zn)-finger domain, along with the yeast-specific Mcm5-7 inter-ring contact, provides an interface for the Mcm2-7 double hexamer formation on the chromatin (Li et al., 2015). (iii) Eukaryotic Mcms have N-terminal extensions (NTEs), which are

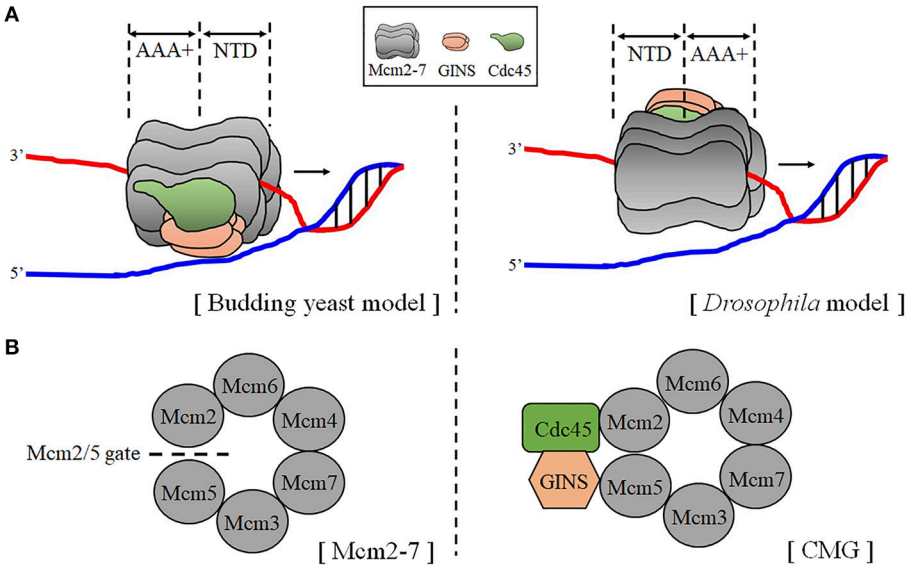

FIGURE 1 | Schematic structure of the CMG helicase. **(A)** Two transloaction models of the CMG helicase on DNA. The orientations of the CMG with respect to translocation on DNA are illustrated based on the findings from cryo-EM studies of budding yeast (left) and the *Drosophila* (right) CMG. The N-tier faces dupelx DNA in the budding yeast model, while the C-tier (the AAA+ plus CTD domains) faces duplex DNA in the *Drosophila* model. **(B)** Structural comparison of the Mcm2-7 and the CMG complex. The views from the NTD of the Mcm2-7 (left) and the CMG (right) are presented. The Mcm2/5 gate is open as indicated in the Mcm2-7, while the gate is closed by bound Cdc45 and the GINS in the CMG. According to Costa et al. (2011), Cdc45-GINS binding to the Mcm2-7 complex surface bridges Mcm2 and 5 and the discontinuity between Mcm2 and Mcm5 is sealed by a nucleotide, which is omitted in the structure for clarity.

especially long in Mcm2, Mcm4, and Mcm6 (Riera et al., 2017). These extensions play roles in the DDK-dependent regulation of replication initiation. In contrast, archaeal Mcms lacks the NTEs.

The AAA+ ATPase domain of Mcms consists of Walker A and B motifs which associates with an arginine finger from the adjacent subunit for its ability to hydrolyze ATP (Davey et al., 2003). Inter-subunit interactions in the ATPase domain requires four conserved hairpins, more specifically, loops from two adjacent subunits including the allosteric communication loop (ACL) and helix-2-insert (H2I) of the first subunit, and H2I and the pre-sensor1 (PS1) hairpin of the second subunit (Li et al., 2015). PS1 projects into the central cavity of the Mcm2-7 and is involved in helicase translocation (Miller et al., 2014; Petojevic et al., 2015). H2I loops from all six Mcm AAA+ modules form a right-handed spiral staircase in the channel of the Mcm2-7 and are suggested to be involved in the initial melting of origin DNA (Li et al., 2015). The atomic model of CMG-ssDNA (14-mer) obtained from cryo-EM revealed that six out of the 14 nucleotides interact with the OB loops of Mcm4 and Mcm7 in the N-tier ring, whereas remaining 8 nucleotides form a right-handed B-form spiral structure in the C-tier ring that interacts with PS1 and H2I loops of Mcm3, 5, 2, and 6 (Georgescu et al., 2017). The right-handed spiral staircase of H2I pore loops are also present in other hexameric helicases (Abid Ali and Costa, 2016). In the structure of ssDNA bound-E1 AAA+ domain of bovine papillomavirus, the ATP-bound active site of E1 has a pore loop on top of the spiral staircase, while the ATP-free site has the loop at the bottom. This observation had led to the suggestion of the rotary translocation mechanism of hexameric DNA helicases.

The CTD domain of Mcm adopts a winged-helix (WH) domain which is flexibly tethered to the AAA+ domain (Parker et al., 2017). The WH domain in the archaeal Mcm controls ATPase activity of Mcms allosterically (Wiedemann et al., 2015). WH domains of eukaryotic Mcm3 and Mcm6 have been shown to be required for pre-RC assembly through interactions with Cdc6 and Cdt1, respectively (Liu et al., 2012; Frigola et al., 2013). In the CMG complex, unlike in the Mcm2-7, the WH domains Mcm5 and Mcm6 are stacked inside the central channel restricting the diameter of the channel as discussed below (Yuan et al., 2016).

Cryo-EM analyses of a single Mcm2-7 complex have revealed it has spiral cracked-ring structure containing a gap between Mcm2 and Mcm5 (Costa et al., 2011; Lyubimov et al., 2012; **Figure 1B**). The cryo-EM analysis of the Cdt1-Mcm2-7 heptameric complex of *S. cerevisiae* revealed that Cdt1 wraps around NTDs of Mcm2, Mcm4, and Mcm6, contributing to stabilization of the complex (Yuan et al., 2017; Zhai et al., 2017). Interactions between Cdt1 and the three Mcms were also observed by cross-linking experiments (Frigola et al., 2017). The role of the Cdt1 suggested in the complex assembly was that it acts as a "brace" which maintains the open spiral structure of Mcm2-7 until ATP hydrolysis (induced by ORC-Cdc6-Cdt1-Mcm2-7 on DNA) triggers the Mcm2-5 gate to close, which in turn leads to the release of Cdt1. In keeping with this, another report also showed that ATP hydrolysis by Mcm2-7, which is coupled to the ring closure, releases the associated Cdt1 protein (Ticau et al., 2017). In the CMG structure, Cdc45 and the GINS

complex close the Mcm2-5 gate through binding at the side of the Mcm2-7, forming ATPase site essential for translocation on DNA (Abid Ali and Costa, 2016; **Figure 1B**). Further work is needed to unveil switching mechanism from double-hexameric Mcm2-7 to two single CMG complexes. The orientation of the CMG with respect to translocation on DNA was analyzed by cryo-EM of the CMG on a model DNA. However, studies on *S. cerevisiae* and *Drosophila* CMG gave rise to different results (Costa et al., 2014; Georgescu et al., 2017; **Figure 1A**). The *S. cerevisiae* CMG had an N to C-tier polarity of translocation which is opposite to the model described based on *Drosophila* CMG studies.

Unwinding Mechanism of the CMG Helicase

The authors of the paper above (Georgescu et al., 2017) proposed an interesting model of the initial melting of the origin by the CMG. The N-termini of the two Mcm2-7 complexes are facing each other in the double hexameric state (head-to-head), encircling dsDNA (**Figure 1A**). If the CMG complex has a N-tier to C-tier polarity in translocation, they must pass each other and it requires them to encircle opposite strands of the DNA. If this is the case, it is imperative that each CMG encircles ssDNA after activation. The cryo-EM structure of the double hexameric Mcm2-7 bound to DNA gave another insight into the initial melting of DNA. Based on the structure of yeast double-hexmeric Mcm2-7, two Zn-finger domains from each hexamer form two smaller rings, but large enough to accommodate duplex DNA in the interface of hexamers (Li et al., 2015; Abid Ali and Costa, 2016). However, two rings are not co-axial and even are twisted with respected to each other. This would make distortion of DNA resulting in the opening of the origin. The structure of double hexameric MCM2-7 bound to duplex DNA was analyzed by another group and the authors suggested that a lateral shift and tilt of the N-tier rings would help separate and extrude lagging strands through the Mcm2-Mcm5 gates (Noguchi et al., 2017).

Currently, two proposals are available to account for the mode of translocation of hexameric helicases on DNA (Costa and Onesti, 2009). According to the "strand extrusion (or side channel extrusion)" model, the CMG translocates encircling dsDNA and the unwound strand is extruded via a side channel formed between NTD and the AAA+ domain of the Mcm2-7. In the "steric exclusion" model, the CMG encircles only the leading-strand template while the lagging-strand template is excluded from the interior of the complex. The latter model is supported by biochemical data obtained by others (Langston and O'Donnell, 2017; Trakselis et al., 2017). The inside channel of the Mcm2-7 in the CMG was shown to be large enough to accommodate dsDNA (Costa et al., 2011; Sun et al., 2015). In contrast, it was found that the WH domain of Mcm5 protrudes into the interior axial channel of the CTD ring, rendering the pore size too narrow for dsDNA accommodation, and this finding was used as evidence to support the steric exclusion model (Yuan et al., 2016). However, unwinding studies have shown that CMG is able to move along the duplex DNA, suggesting that dsDNA can be accommodated in the central channel of the CMG (Kang et al., 2012). The steric exclusion model is supported by the results obtained from experiments using *Xenopus* egg extracts and DNA substrates containing roadblocks on selected DNA strands. The CMG stalled when the leading strand had obstructions, but did not when obstructions were in the lagging strand, suggesting that the lagging strand is excluded from the interior of the CMG (Fu et al., 2011). Similar *in vitro* experiments were performed with *S. cerevisiae* CMG. However, blocks on either strand resulted in inhibition of CMG unwinding, which made authors propose a modified steric exclusion model in which both strands enter the channel and the duplex unwinding occurs internally, followed by exclusion of the non-tracking strand (Langston and O'Donnell, 2017). And Botchan's group performed cross-linking of CMG with fork-structured DNA, revealing interactions of the leading strand with the inside channel while lagging strand contacts the Mcm2-7 external surface (Petojevic et al., 2015). The same study has led to a proposal that Cdc45 acts as a guardian of the Mcm2-Mcm5 gate which captures the leading strand in a 3′->5′ orientation.

Reconstitution of the CMG Activation and DNA Replication Reaction

Identification of replication factors, their interactions, and enzymatic functions allowed researchers to reconstitute DNA replication *in vitro*. Formation of active CMG in eukaryotes depends on multiple stepwise reactions that can be largely segregated into two stages as described above; (i) formation of the two Mcm2-7 on an origin and (ii) conversion of the double hexameric Mcm2-7 into two single CMG complexes.

Through the single molecule analysis using fluorescence tagged proteins, sequential loading of two hexameric Mcm2-7s on DNA was monitored (Ticau et al., 2015). The first Mcm2-7 was recruited by the ORC/Cdc6 followed by the recruitment of the second Mcm2-7 by the first Mcm2-7. Although this study proposed that single ORC is responsible for the loading of double hexameric Mcmc2-7s, the study published recently suggested that two ORC molecules are required for the loading of two Mcm2-7s (Coster and Diffley, 2017). The study has shown that relative orientation of the ORC binding sites was critical for the bidirectional loading of two Mcm2-7 molecules.

The *S. cereviseae* CMG was assembled with purified components and its assembly and activation was indirectly monitored by polymerase reactions (Yeeles et al., 2015). The *in vitro* replication initiation system has allowed detailed analyses of the CMG activation and functional interactions between the CMG and other replication factors (Deegan et al., 2016; Douglas and Diffley, 2016; Devbhandari et al., 2017; Frigola et al., 2017; Gan et al., 2017; Kurat et al., 2017; Looke et al., 2017; Yeeles et al., 2017). At the same time, incubation of purified *S. cereviseae* CMG with other factors such as polymerases and the scaffolding factor Ctf4 has made possible microscopic analysis of the minimal replisome and studies on the distribution of DNA polymerases on leading and lagging strands (Georgescu et al., 2014, 2015, 2017; Sun et al., 2015). Reconstitution of the CMG helicase assembly reaction *in vitro* can provide a platform to

screen and test candidate molecules that could inhibit the CMG formation.

THE CMG AS A POTENTIAL TARGET FOR CANCER THERAPY

The CMG and Cancer

The correlation between Mcm and cancer was first proposed 20 years ago by an observation of increased levels of nuclear Mcm7-positive cells in the malignant form of cutaneous keratinocytic tumors. This was further supported by subsequent studies that reported elevated levels of other Mcms (Mcm2 and Mcm5) in various types of cancers (Hiraiwa et al., 1997; Todorov et al., 1998; Freeman et al., 1999).

The CMG and Genome Instability

There is an excess amount of the Mcm2-7 on chromatin, playing a role in maintaining genome stability, particularly under replicative stress by activating dormant origins as backups during DNA replication (Ge et al., 2007; Ibarra et al., 2008). In accordance and compatible with this, some types of cancer are also caused by low activity of Mcms. A genetic screen for spontaneous chromosomal instability in mice using *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis followed by genetic mapping and sequencing identified a mutant allele of Mcm4 (Shima et al., 2003, 2007). The mutated Mcm4 (F345I) ($Mcm4^{Chaos3}$) in mice caused reduction in overall levels of Mcms, leading to high incidences of mammary adenocarcinomas (Shima et al., 2007). The phenylalanine at 345 in Mcm4 is well conserved, located downstream from a Zn-finer domain, and important for its interaction with other Mcms. The mutation at the corresponding residue of the budding yeast Mcm4 displayed a minichromosome loss phenotype. Mouse embryonic fibroblast cells (MEFs) with the Mcm4 (F345I) allele were more susceptible to chromosome breakage under replicative stress. One other group reported that this allele caused instability of Mcm2-7 without affecting helicase activity of the CMG *in vitro* (Kawabata et al., 2011). The same group also showed increases in dormant origin activation in $Mcm4^{Chaos3/Chaos3}$ MEFs and cells are arrested in M phase with increasing numbers of abnormal chromosomes. These results suggest that the impaired ability of cells under replication stress to activate the dormant origins leads to the loss of tumor suppression. A dominant allele of mouse Mcm4 (D573H) enhanced genome instability leading to increased incidence of cancer development (Bagley et al., 2012). Unlike the Mcm4 (F345I) allele, this mutant did not show any detectable change protein stability. However, it appears that Mcm4 (D573H) assemble into the Mcm2-7, leading to formation of the inactive holocomplex. The mutation at the corresponding residue of budding yeast Mcm4 did not support growths of mutant cells. When the expression levels of Mcm2 were reduced to one-third of wild-type levels, the average life span of mice decreased markedly due to high incidence of cancers, and the majority of them were T- and B-cell lymphoma (Pruitt et al., 2007). Decreased expression of Mcm2 led to elevated levels of Ser139 phosphorylated histone H2AX (γ-H2AX) foci in cultured muscle satellite cells, which is indicative of thigh incidence double-strand DNA breaks. These phenotypes are consistent with the proposed function of dormant origins as backups during DNA replication which would prevent the incidence of double-stand DNA breaks that could arise from stalled DNA replication forks.

The CMG as a Prognostic Marker for Cancers and a Target for Cancer Therapy

Most of the Mcm-related cancers have shown positive correlation between Mcm expression levels and the malignancy of cancers in keeping with the intrinsic proliferative function of Mcms. For example, Mcms were abnormally highly up-regulated in pancreatic cancer and gliomas, and this was closely related to rapid cancer progression (Hua et al., 2014; Peng Y. P. et al., 2016). The proliferative index of Mcm2 and Mcm3 can be used as a prognostic marker for survival of patients with small intestinal neuroendocrine neoplasm (SI-NEN) (Schimmack et al., 2016). The cancer types where increased levels of the CMG components were observed are summarized in the **Table 1**.

Mcm2 is extensively studied as a prognostic marker for cancer. Increased levels of Mcm2 expression have been detected in a variety of cancers (**Table 1**), and it is often accompanied with overexpression of Ki-67, a representative marker for cell proliferation (Meng et al., 2001; Going et al., 2002; Chatrath et al., 2003; Davidson et al., 2003; Yang et al., 2006; Guzinska-Ustymowicz et al., 2008, 2009; Szajerka et al., 2008; Vargas et al., 2008; Giaginis et al., 2009; Razavi et al., 2015; Scapulatempo-Neto et al., 2017). It has been shown that Mcm2 is a prognostic marker superior to Ki-67 in oral cavity squamocellular carcinoma and kidney tumor samples (Rodins et al., 2002; Szelachowska et al., 2006). The validity of Mcm2 as a prognostic marker is also dependent on the genetic background in gastric adenocarcinoma (Tokuyasu et al., 2008; Giaginis et al., 2010). Immunocytochemistry of Mcm2, together with p53, when added to the conventional cytological evaluation, increased sensitivity in the diagnosis of pancreaticobiliary adenocarcinoma (Abe et al., 2016). It has been shown that Mcm2 expression has positive correlation with malignancy grade in breast carcinoma and esophageal squamous cell carcinoma (Gonzalez et al., 2003; Kato et al., 2003; Wojnar et al., 2011). Association of Mcm2 with clinicopathological characteristics was also observed in other types of cancer including diffuse large B-cell lymphoma and muscle-invasive urothelial cancer (Korkolopoulou et al., 2005; Obermann et al., 2005).

Overexpression of Mcm3 was also detected in many cancers, for example, salivary gland epithelial tumors and papillary thyroid carcinoma (Igci et al., 2014; Zielinski et al., 2016). There was positive correlation between levels of Mcm3 expression and malignancy grade in ovarian cancers (Kobierzycki et al., 2013). Increased levels of Mcm3, along with Mcm2 and Mcm7, have shown poor prognosis (Hua et al., 2014).

Mcm4 expression also increased in diverse cancers. Elevated levels of Mcm4 were detected in non-small cell lung cancer (NSCLC) compared to neighboring normal bronchial epithelial cells (Kikuchi et al., 2011). Expression of Mcm4, along with

TABLE 1 | Overexpression of the CMG components in cancers.

Subunit	Cancer types	References
Mcm2	Adrenocortical dysplasia	Szajerka et al., 2008
	Anal carcinoma	Scapulatempo-Neto et al., 2017
	Breast carcinoma	Gonzalez et al., 2003; Wojnar et al., 2011
	B-cell lymphoma	Obermann et al., 2005
	Cervical cancer	Ishimi et al., 2003
	Colon cancer	Giaginis et al., 2009
	Colorectal cancer	Guzinska-Ustymowicz et al., 2008
	Dysplastic squamous oesophageal epithelium and Barrett's mucosa	Going et al., 2002
	Esophageal squamous cell carcinoma	Kato et al., 2003
	Gastric adenocarcinoma	Tokuyasu et al., 2008; Giaginis et al., 2011
	Glioma	Hua et al., 2014
	Laryngeal squamous cell carcinoma	Chatrath et al., 2003
	Meningioma	Saydam et al., 2010
	Merkel cell carcinoma	Gambichler et al., 2009
	Muscle-invasive urothelial cancer	Korkolopoulou et al., 2005
	Non-small cell lung cancer	Yang et al., 2006
	Oral squamocellular carcinoma	Szelachowska et al., 2006; Razavi et al., 2015
	Pancreatic cancer	Peng Y. P. et al., 2016
	Pancreaticobiliary adenocarcinoma	Abe et al., 2016
	Prosate cancer	Meng et al., 2001
	Renal cell carcinoma	Rodins et al., 2002
	Salivary gland tumors	Vargas et al., 2008
	Small intestinal neuroendocrine neoplasm	Schimmack et al., 2016
	Vulval intraepithelial neoplasia	Davidson et al., 2003
Mcm3	Breast cancer	Kwok et al., 2015
	Cervical cancer	Ishimi et al., 2003
	Dysplastic squamous oesophageal epithelium and Barrett's mucosa	Going et al., 2002
	Glioma	Hua et al., 2014
	Meningioma	Saydam et al., 2010
	Ovarian cancer	Kobierzycki et al., 2013
	Papillary thyroid carcinoma	Igci et al., 2014
	Salivery gland epithelial tumor	Zielinski et al., 2016
	Small intestinal neuroendocrine neoplasm	Schimmack et al., 2016
Mcm4	Breast cancer	Kwok et al., 2015
	Cervial cancer	Ishimi et al., 2003
	Esophageal cancer	Huang et al., 2005; Choy et al., 2016
	Meningioma	Saydam et al., 2010
	Merkel cell carcinoma	Gambichler et al., 2009
	Non-small cell lung cancer	Kikuchi et al., 2011
	Pancreatic cancer	Peng Y. P. et al., 2016
Mcm5	Breast cancer	Kwok et al., 2015
	Cervical dysplasia (or cancer)	Ishimi et al., 2003; Murphy et al., 2005
	Colon carcinoma	Freeman et al., 1999
	Bladder carcinoma	Freeman et al., 1999
	Esophageal cancer	Williams et al., 2004
	Gastric adenocarcinoma	Giaginis et al., 2011
	Meningioma	Saydam et al., 2010
	Prosate cancer	Dudderidge et al., 2010
	Urothelical cancer	Stoeber et al., 1999; Korkolopoulou et al., 2005
Mcm6	Breast cancer	Kwok et al., 2015
	Cervical cancer	Ishimi et al., 2003; Henderson et al., 2011
	Mantle cell lymphoma	Schrader et al., 2005
	Pancreatic cancer	Peng Y. P. et al., 2016

(Continued)

TABLE 1 | Continued

Subunit	Cancer types	References
Mcm7	Acute myeloid leukemia	Lee et al., 2017
	Breast cancer	Kwok et al., 2015
	Cervical cancer	Ishimi et al., 2003; Henderson et al., 2011
	Cutaneous keratinocytic tumor	Hiraiwa et al., 1997
	Endometrial carcinoma	Li et al., 2005
	Glioma	Facoetti et al., 2006a
	Head and neck squamous cell carcinoma	Cromer et al., 2004
	Meningioma	Saydam et al., 2010
	Merkel cell carcinoma	Gambichler et al., 2009
	Mesothelioma	Kimura et al., 2009, 2013
	Neuroblastoma	Shohet et al., 2002
	Oral squamous cell carcinoma	Tamura et al., 2010
	Prostate cancer	Padmanabhan et al., 2004
	Small lung adenocarnoma	Fujioka et al., 2009
Psf1	Breast cancer	Nakahara et al., 2010
	Hepatocellular carcinoma	Zhou et al., 2015
	Lung cancer	Zhang J. et al., 2015
	Non-small cell lung cancer	Kanzaki et al., 2016
	Prostate cancer	Tahara et al., 2015
Psf2	Breast cancer	Peng L. et al., 2016
	Cervical cancer	Ouyang et al., 2017
	Intrahepatic cholangiocarcinoma	Obama et al., 2005
Psf3	Colon carcinoma	Nagahama et al., 2010a
	Colorectal cancer	Sun et al., 2014
	Non-small cell lung carcinoma	Tane et al., 2015
	Lung adenocarcinoma	Hokka et al., 2013; Tauchi et al., 2016
Sld5	Bladder cancer	Yamane et al., 2016
	Breast cancer	Mohri et al., 2013
	Cervical cancer	Mohri et al., 2013
	Colon cancer	Mohri et al., 2013
	Gastric cancer	Mohri et al., 2013
	Liver cancer	Mohri et al., 2013
	Lung cancer	Mohri et al., 2013
	Prostate cacer	Mohri et al., 2013
Cdc45	Acute lymphoblastic leukemia	Pollok et al., 2007
	Acute promyelocytic leukemia	Pollok et al., 2007
	Acute T-cell leukemia	Pollok et al., 2007
	Breast carcinoma	Pollok et al., 2007
	Cervical cancer	Di Paola and Zannis-Hadjopoulos, 2012
	Chronic myelogenous leukemia	Pollok et al., 2007
	Glioblastoma	Pollok et al., 2007
	Histiocytic leukemia	Pollok et al., 2007
	Lung cancer	Tomita et al., 2011
	Osteosarcoma	Pollok et al., 2007
	Papillary thyroid cancer	Sun et al., 2017
	Tongue squamous cell carcinoma	Li et al., 2008

Mcm2 and Mcm7, has significantly increased in Merkel cell carcinoma (Gambichler et al., 2009). It appears that levels of Mcm4 is closely associated with pathological grade of esophageal cancer, and its overexpression has been shown to correlate with lymph node metastasis and poor survival in adenocarcinoma patients (Huang et al., 2005; Choy et al., 2016).

The fraction of Mcm5-immunostained cells can be used to determine the pathological grade in urothelial cancers (Stoeber et al., 1999). Detection of elevated levels of Mcm5 in gastric aspirates and in urine sediments was effective in the prediction of esophageal and prostate cancer, respectively, with high sensitivity and specificity (Williams et al., 2004; Dudderidge et al., 2010). Mcm5 expression was significantly elevated in both HPV (human papilloma virus) dependent and independent cervical dysplasia (Murphy et al., 2005). Levels of Mcm5 expression is tightly associated with clinicopathological characteristics and

patient survival in gastric adenocarcinoma and muscle-invasive urothelial cancer (Korkolopoulou et al., 2005; Giaginis et al., 2011).

Comparative analyses revealed that Mcm6 is a marker far superior to Ki-67 in predicting the outcome in mantle cell lymphoma (Schrader et al., 2005). Immunological assays using anti-Mcm6 and anti-Mcm7 antibodies were developed to efficiently detect cervical cancer (Henderson et al., 2011).

Expression levels of Mcm7 were also upregulated in a variety of cancer cells (**Table 1**; Freeman et al., 1999; Shohet et al., 2002; Cromer et al., 2004; Padmanabhan et al., 2004; Facoetti et al., 2006a). Conditional expression of Mcm7 in mice was shown to induce tumor formation (Yoshida and Inoue, 2003; Honeycutt et al., 2006). Mcm7, together with other cell proliferation markers, were used for differential diagnosis of reactive mesothelial cells and malignant mesothelioma cells (Kimura et al., 2009, 2013). The labeling index (LI) of Mcm7 in immunohistochemistry was more reliable than that of Ki-67 in several types of cancer and is strongly correlated to the tumor aggressiveness in astrocytoma and oral squamous cell carcinoma (Li et al., 2005; Facoetti et al., 2006b; Fujioka et al., 2009; Tamura et al., 2010; Choy et al., 2016). Mcm7 polymorphisms were shown to have a close relationship with relapse of acute myeloid leukemia (Lee et al., 2017). Mcm7, however, was not useful as a prognostic marker in colorectal cancer and as a risk factor for recurrence-free survival in patients with Duckes C colorectal cancer (Nishihara et al., 2008; Ishibashi et al., 2014).

It was shown that concurrent overexpression of all six Mcm subunits, rather than single individual Mcm subunits, is strongly correlated with poor survival in breast cancer patients (Kwok et al., 2015). It has been reported that expression of all Mcm2-7 subunits could lead to significant increases in meningiomas compared to arachnoidal tissue controls (Saydam et al., 2010). Oncogenic mutant p53 (mtp53) influenced chromatin enrichment of pre-RC components and protein-protein interactions were detected between mtp53 and Mcm subunits in triple negative breast cancer (TNBC) cells (Qiu et al., 2017). Enhanced expression of Mcms in total cellular proteins and in the chromatin-bound fractions were observed in cervical cancer cells (Ishimi et al., 2003). Immunocytochemistry for Mcm proteins has been shown to be more rapid and reliable than the traditional Papanicolaou test for cervical cancer (Mukherjee et al., 2007).

Overexpression of the GINS subunits were also reported in cancers. Psf1 was relatively highly expressed in breast tumor and lung cancer cells (Nakahara et al., 2010; Zhang J. et al., 2015). An increased ability of cancer cells to proliferate by Psf1 overexpression was observed by a mouse xenograft model (Nagahama et al., 2010b). Psf1 expression was found to be an independent prognostic marker for poor survival in NSCLC patients treated with surgery after preoperative chemotherapy or chemoradiotherapy (Kanzaki et al., 2016). Increased expression of Psf1 was associated with aggressiveness of hepatocellular carcinoma and prostate cancer (Tahara et al., 2015; Zhou et al., 2015). Overexpression of Psf2 was detected in intrahepatic cholangiocarcinoma, and tumorigenesis was promoted by elevated expression of Psf2 in early-stage cervical cancer (Obama et al., 2005; Ouyang et al., 2017). Interestingly, Psf2 knockdown in TNBC cells downregulated expression of matrix metallopeptidase 9 which is required for tumor invasion, thereby inhibited the migration and invasion of TNBC cells (Peng L. et al., 2016). However, it is unclear whether this regulatory role is CMG-related or independent of the Psf2 function. Psf3 expression was also increased in colon carcinoma compared to the neighboring normal mucosa (Nagahama et al., 2010a). The survival rate of the patient group with increased Psf3 expression was considerably lower than that of the reduced Psf3 expression group in NSCLC, lung adenocarcinoma, and colorectal cancer patients (Hokka et al., 2013; Sun et al., 2014; Tane et al., 2015; Tauchi et al., 2016). Finally, robust expression of Sld5 was detected from bladder cancer tissues and many cancer cell lines (Mohri et al., 2013; Yamane et al., 2016).

The level of the Cdc45 protein was upregulated in a variety of cancer-derived cell lines including carcinoma, sarcoma, leukemia, and lymphoma (Pollok et al., 2007; Tomita et al., 2011; Di Paola and Zannis-Hadjopoulos, 2012). The overexpression of Cdc45 closely correlated with tumor sizes and stages in papillary thyroid cancer (Sun et al., 2017). The expression of Cdc45 increased in tongue squamous cell carcinomas and the level of Cdc45 was shown to have positive correlation with grades of precancerous lesions in epithelial dysplasia (Li et al., 2008). Cdc45 overexpression phenocopied the proto-oncogne Myc-dependent phenotypes (Srinivasan et al., 2013). Thus, it was suggested that Cdc45 acts as a downstream effector of Myc-induced replication stress which is required for oncogenesis.

These results suggest that the proliferative function of CMG is generally required for the growth of tumors and, but to varying extents, depending on the type of cancer. Besides, overexpressions of CMG subunits are closely associated with malignancy. Therefore, CMG can act as not only a marker for cancer progression but also as a druggable target to treat cancers.

STRATEGIES TO TARGET THE CMG FOR ANTICANCER THERAPY

Background for Targeting the CMG for Cancer Therapy

Two theoretic models provide a rational basis to use Mcms as a target for treatment of cancers (Neves and Kwok, 2017). One model is involved with downregulation of Mcm in the hope of reducing tumor growth because sustained and elevated expression of Mcms would be essential for rapid proliferation of cancer cells. Consistent with this, knockdown of Mcm components by siRNA suppressed proliferation of cancer cells (Lau et al., 2010; Kikuchi et al., 2011; Toyokawa et al., 2011; Zhang X. et al., 2015). In addition, this holds true for Cdc45 and the GINS (Nakahara et al., 2010; Tane et al., 2015; Zhang J. et al., 2015; Sun et al., 2017). The precise mechanisms by which cancer cell proliferation is inhibited by depletion of the CMG components are still remains to be investigated. The siRNA-mediated knockdown of Mcm2, Mcm3, and Mcm7 inhibited medulloblastoma cell growth (Lau et al., 2010). However, the point of cell cycle arrest was not the same. For example,

knockdown of Mcm3 resulted in G1 arrest with reduced levels of cyclin A, while knockdown of Mcm2 or Mcm7 resulted in G2/M arrest without any detectable change of cyclin A levels. Therefore, it is inappropriate to simply attribute the growth inhibitory effect by Mcm downregulation to low amounts of functional replicative helicase.

The other model focuses on excess amounts of Mcms which is important for cancer cells to survive, with maintaining minimal genome stability, in the presence of high degree of replicative stress (Neves and Kwok, 2017). Reduction of Mcms to limited extents does not impair DNA replication in normal condition (Lei et al., 1996; Crevel et al., 2007; Ibarra et al., 2008). However, cells became highly sensitive to chemicals that interfere with DNA replication in the presence of low amounts of Mcms because of limited activation of backup origins (Woodward et al., 2006; Ge et al., 2007; Ibarra et al., 2008; Chuang et al., 2010).

Based on the two primary mechanisms described above, several strategies have been explored to inhibit the CMG functions in order to develop anti-cancer therapeutics. The molecules targeting Mcms are listed and summarized in **Table 2**.

Strategy I. Downregulation of Constituents of the CMG Complex

Knockdown of the CMG subunits could be achieved by identification of small molecules exerting its effect on transcription or post-transcriptional regulation (**Figure 2A**). Changes of target genes at transcriptional levels could occur through direct association of such molecules with transcriptions factors, through induction of DNA damages, or through the alteration of signaling pathways governing downstream gene transcription as described below.

It was shown that genistein, a natural, nontoxic dietary isoflavone and trichostatin A (TSA), a classical histone deacetylase (HDAC) inhibitor downregulated Mcm2 expression in prostate cancer (Majid et al., 2010). These molecules also decreased expression levels of protein factors required for loading and activation of Mcm2-7, which include Cdt1, Cdc7, and Cdk2. The same study showed that levels of microRNA (miR)-1296 expression was reduced in carcinoma samples and miR-1296 targeted Mcm2 mRNA probably by binding to the 3′ untranslated region (3′ UTR) of Mcm2. Expression of miR-1296 was enhanced by treatment of PC3 cells with genistein. Treatment with genistein, and TSA, or transfection of miR-1296 to PC3 cells all reduced the percentage of S-phase cells.

Other independent studies confirmed the inhibitory effect of TSA on Mcm2 expression in HCT116 colon cancer cells (Liu et al., 2013). Real-time PCR (RT-PCR) arrays of HCT116 cells revealed that 34 cell cycle-related genes were significantly changed with regard to mRNA expression level in the presence of TSA. Among them, the expression level of Mcm2 was considerably reduced. Treatment of TSA induced growth inhibition and apoptosis in human HCT116 cells and the same phenotype was induced by transfection of Mcm2-specific siRNA. It was reported that JNK signaling is activated by TSA (Han et al., 2010). Treatment of a JNK inhibitor, SP600125 in HCT116 cells restored TSA-induced decrease of Mcm2, suggesting that the JNK signaling pathway is responsible for downregulation of Mcm2 (Liu et al., 2013).

Widdrol, an aromatic compound derived from *Juniperus chinensis* induced G1 phase arrest and inhibited growth of some cancers including A549 lung carcinoma and HT29 colon adenocarcinoma (Hong et al., 2009; Kwon et al., 2010). IC_{50} of widdrol was lowest in HT29 cells among cell lines tested (Kwon et al., 2010). In A549 lung carcinoma cells, levels of p53 and p21, a Cdk inhibitor increased, while those of Cdk2, cyclin E, retinoblastoma protein (RB), PCNA, and all Mcm subunits decreased (Hong et al., 2009). In HT29 colon adenocarcinoma cells, ataxia-telangiectasia mutated kinase (ATM) and p21 were upregulated and Cdc25A, Cdk2, cyclin E, E2F7, PCNA and the Mcm2-7 were downregulated (Kwon et al., 2010; Yun et al., 2012). Chk2 and p53 were activated by DNA damages that were probably directly induced by widdrol treatment in HT29, which leads to upregulation of p21 and reduced phosphorylation of RB. This result suggests that upregulated p21 exerts an inhibitory effect on the Cdk2/cyclin E complex which is required for inactivating phosphorylation of RB. Activated RB prevents E2F-mediated transcription, thereby decreasing expression of downstream genes including Mcms. Decrease of Mcm4 was detected in early time points after treatment of widdrol in HT29.

Statin family drugs include inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase used for lowering plasma cholesterol levels (Zhang X. et al., 2015). However, statins have been considered as candidates for anti-cancer drug because of its ability to arrest cell cycle, to suppress tumor growth, and to induce apoptosis. It was shown that atorvastatin inhibited expressions of Mcm6 and Mcm7 in rat aortic vascular smooth muscle cells by inhibiting E2F promoter activity (Bruemmer et al., 2003). Treatment of lovastatin inhibited growth of NSCLC cells, arrested cell cycle at G1/S phase, and induced apoptosis (Zhang X. et al., 2015). Lovastatin activated the JNK signaling pathway involved in the downregulation of Mcm2 as observed with TSA treatment described above. Addition of the JNK inhibitor SP600125 to A549 and GLC-82 cells restored lovastatin-induced downregulation of Mcm2.

Although Metformin (N′,N′-dimethylbiguanide) was first developed to treat type2 diabetes, recent studies demonstrated that it has anticancer activity (Kim et al., 2017). Metformin was found to inactivate mTOR, thereby leading to activation of p53 and cell cycle arrest. Treatment of metformin to colorectal cancer cell lines downregulated expression of genes involved in cell cycle regulation and DNA replication including Mcms and PCNA in colorectal cancer cells resistant to 5-fluorouracil (5-FU, explained below).

BET is the abbreviation of bromodomain and extra-terminal family of proteins (Nicodeme et al., 2010). Proteins, which belong to this family, recognize acetylated histones for transcription of downstream target genes. I-BET (or BETi) is a synthetic compound developed to mimic acetylated histones in order to disrupt chromatin complexes responsible for expression of genes involved in inflammation. Several BETis were developed and

TABLE 2 | Summary of small molecules targeting Mcms.

Name	Classification	Targeting mechanism	References
Genistein	Isoflavone	Downregulation of Mcm2	Majid et al., 2010
Trichostatin A	HDAC inhibitor	Downregulation of Mcm2 (through the activation of JNK signaling pathway)	Majid et al., 2010; Liu et al., 2013
Widdrol	Aromatic compund	Downregulation of Mcm2-7 (through the activation of RB by DNA damage)	Hong et al., 2009; Kwon et al., 2010; Yun et al., 2012
Lovastatin	HMG-CoA reductase inhibitor	Downreguation of Mcm2 (through the activation of JNK signaling pathway)	Zhang X. et al., 2015
Metformin	Biguanide	Downregulation of Mcm2-7	Kim et al., 2017
BETi	Molecular mimicker of acetylated histone	Downregulation of Mcm5	Mio et al., 2016
Breviscapine	Flavonoid	Downregulation of Mcm7	Guan et al., 2017
Heliquinomycin	Antibiotic compound	Inhibition of Mcm4/6/7 helicase	Ishimi et al., 2009; Toyokawa et al., 2011
Ciprofloxacin	Fluoroquinolone	Inhibition of Mcm2-7 and Mcm4/6/7 helicase	Simon et al., 2013

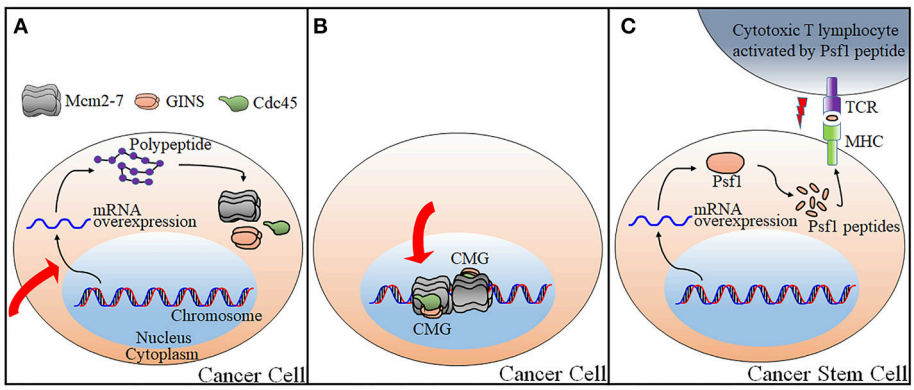

FIGURE 2 | Current strategies applicable for targeting the CMG for anti-cancer therapy. Three possible strategies to control CMG are as illustrated. **(A)** Control of expression levels of the CMG subunits either by transcriptional or post-transcriptional regulations. **(B)** Control of CMG helicase by targeting the catalytic center of the CMG helicase or by disrupting the proein-protein interactions required for the CMG complex formation and activation. **(C)** CTL-mediated growth inhibition of cancer stem cells that overexpresses Psf1 by vaccination with antigenic Psf1 peptides. The red arrows in panels **A** and **B** indicate a process which is blocked by a small molecule. The red "lightening" symbol in panel **C** indicates death signal from CTL. TCR, T-cell receptor; MHC, Major histocompatibility complex.

some of them were shown to inhibit proliferation of cancer cells. In leukemic cells, repression of the c-MYC oncogene appears to be responsible for the antiproliferative activity of BETis (Ott et al., 2012). In one study, it was shown that treatment of BETis in anaplastic thyroid carcinoma (ATC) cell lines induced S-phase arrest and cell death (Mio et al., 2016). Significant increases in Mcm5 levels were detected both in human and mouse ATC cells compared to normal thyroids. Transcriptome analysis revealed that Mcm5 belonged to the top 20 downregulated genes in ATC cells treated with BETis.

Breviscapine (BVP) is a natural flavonoid, extracted from the Chinese herb *Erigerin breviscapus*. It has been demonstrated that BVP suppressed growth of human prostate cancer cell lines and tumors in mouse xenograft experiments (Guan et al., 2017). BVP reduced Mcm7 expression and authors claimed that this was associated with DNA damage in prostate cancer cells and tumor tissues, although detailed mechanism still remains unclear. BVP also induced apoptosis of cancer cells through the caspase-3 pathway.

The second theoretic mechanism involved in targeting Mcms for anti-cancer therapy described above could be used for combinatorial therapy, that is, knockdown of a constituent of the CMG in the presence of a drug that gives replicative stress to cancer cells. Gemcitabine and 5-FU have been widely used to treat several cancers (Longley et al., 2003; Brown et al., 2014). Gemcitabine is a nucleoside analog in which two hydrogen atoms at the C-2 position of deoxycytidine are replaced by fluorine atoms and 5-FU is ~~a~~n uracil analog which has a fluorine atom at the C-5 position in place of hydrogen. Both drugs induce DNA replication fork stalling by terminating DNA strand elongation by polymerases or by negatively affecting deoxyribonucleotide (dNTP) pool required for DNA synthesis (Longley et al., 2003; de Sousa Cavalvante and Monteiro, 2014). In one study, a combination therapy using siRNA against Mcms and the chemotherapeutic drug was attempted to increase treatment efficacy in pancreatic ductal adenocarcinoma (PDAC) (Bryant et al., 2015). Reduction of Mcm4 or Mcm7 sensitized PDAC cells to gemcitabine and 5-FU

by preventing the formation of Mcm complex reservoir required to activate backup origins.

Screening and modification of small molecules modulating CMG expression and development of delivery materials of small molecules and siRNAs against components of the CMG including nanoparticles and conjugate delivery systems will improve targeting the CMG for cancer therapy (Kanasty et al., 2013).

Strategy II. Inhibition of Enzymatic Activity of the CMG Complex

The second strategy for targeting the CMG is to directly inhibit enzymatic activity of the CMG complex (**Figure 2B**). There are a few small molecules identified that are able to inhibit helicase activity of the Mcm complexes including the Mcm2-7 and the Mcm4/6/7.

Heliquinomycin was originally discovered from *Streptomyces* species using helicase assays carried out with fractionated nuclear extracts from HeLa cells for the purpose of isolating DNA helicase inhibitors (Chino et al., 1996). Heliquinomycin was demonstrated to have antibiotic effects on microorganisms and inhibited DNA replication and RNA synthesis in cultured cancer cell lines (Chino et al., 1996, 1998). Ishimi and his colleagues performed DNA unwinding assays using several candidate helicases in the presence of heliquinomycin to identify target helicases of heliquinomycin and found that inhibitory effect was most dramatic with human Mcm4/6/7 compared to other helicases including SV40 large T antigen and Werner helicase (Ishimi et al., 2009). The IC_{50} value of heliquinomycin in the inhibition of Mcm4/6/7 was 2.4 μM and it was similar to that of heliquinomycin in inhibiting cellular DNA replication. The inhibitory effect of heliquinomycin on ATPase activity of the Mcm4/6/7 was observed only in the presence of ssDNA. Heliquinomycin also effectively suppressed the growth of cancer cells including lung adenocarcinoma, lung large cell carcinoma, and bladder cancer cells (Toyokawa et al., 2011).

Fluoroquinolones are a group of antibiotics targeting bacterial gyrase and topoisomerase IV enzymes, and it was reported that they possess inhibitory effect on the helicase activity of SV40 large T antigen (Ali et al., 2007). Among fluoroquinolones, ciprofloxacin displayed selective inhibition toward yeast Mcm2-7 (Simon et al., 2013). While ofloxacin inhibited both the Mcm2-7 and the Mcm4/6/7 with similar IC_{50} values, 4.17 and 5.29 mM, respectively, ciprofloxacin inhibited the Mcm2-7 helicase activity more efficiently (Mcm2-7, $IC_{50} = 0.63$ mM; Mcm4/6/7, $IC_{50} = 1.89$ mM). The IC_{50} of ciprofloxacin to SV40 large T antigen was higher (4 mM) that those with the Mcm helicases. Thus, ciprofloxacin appears selective toward the Mcm2-7 from human and yeast cells and its selectivity was further verified by examining its cytotoxicity. This holds promises in designing drugs that target a specific helicase only. In this case, the CMG complex could be such a specific target.

It has been pointed out that the CMG complexes are difficult to purify in sufficient amounts for extensive high-throughput screening application and only a few groups succeeded to purify the CMG (Moyer et al., 2006; Kang et al., 2012; Georgescu et al., 2014; Simon and Schwacha, 2014; Abid Ali et al., 2016; Zhou et al., 2017). The yield of human CMG was ∼40 pmol (0.03 mg) from 2-liter Sf9 insect cells, and 0.3 mg of yeast CMG was purified from 12-liter culture of overexpressing haploid yeast strain (Kang et al., 2013; Georgescu et al., 2014). Recently, significant large amounts of yeast CMG have been successfully purified from a diploid strain (Zhou et al., 2017). Therefore, initial screenings of small molecules that inhibit helicase or ATPase activities could be performed with yeast CMG, followed by subsequent validation of the hit molecules with human CMG. On the other hand, it would be worthwhile to construct a yeast strain co-expressing 11 subunits of human CMG. This would allow to increase the purification scale to obtain CMG in large amounts enough for high-throughput screenings.

Strategy III. Cancer Vaccine

The third strategy is not intended to regulate either expression levels of the CMG constituent or to inhibit enzymatic activity of the CMG complex. Rather, it targets a CMG constituent with somewhat different ways and it is irrelevant to the regulation of the CMG action on DNA replication. Cancer immunotherapy triggers the immune system by recognizing molecular entities expressed specifically on the surface of cancer cells to eliminate these cells (Yoshida et al., 2017). Cytotoxic T lymphocytes (CTLs) attack cancer cells by recognizing cancer-specific antigenic peptides presented with human leukocyte antigen (HLA) on the cell surface. Recently, Yoshida and his colleagues has identified a Psf1-derived peptide presented by HLA through bioinformatics approach and mass spectrometric analyses. The HLA-Psf1 peptide complex was purified from breast cancer cell lines by overexpressing both HLA and Psf1 and this attempt resulted in the identification of $Psf1_{79-87}$ peptide. Splenocytes obtained from HLA expressing transgenic mice vaccinated with $Psf1_{79-87}$ peptide secreted interferon-γ when incubated with T2 cells pre-incubated with $Psf1_{79-87}$. In addition, CTLs generated by peptide stimulation of peripheral blood mononuclear cells from human blood were purified, followed by co-culture with monocyte-derived dendritic cells pulsed with $Psf1_{79-87}$. The peptide-specific CTLs killed $Psf1_{79-87}$-pulsed T2 cells more effectively than non-pulsed cells (2.4 fold), indicating that vaccination is effective in inducing immune response (**Figure 2C**). This result is promising because the same group also reported a close association of Psf1 overexpression with cancer stem cells (Nagahama et al., 2010b). Previously, the same strategy had also been applied to Cdc45 (Tomita et al., 2011). Highly immunogenic Cdc45-derived peptides induced CTLs to be reactive to lung cancer cells. Further studies are required to evaluate this technique in clinical trial for targeting cancer stem cells.

PROSPECTS FOR NEW STRATEGIES TARGETING THE CMG

Development of novel strategies targeting the CMG will shed light on the field of cancer therapy. Currently, most strategies are based on the regulation of protein expression and control of enzymatic activities by small molecules. However,

peptides and antibodies, could be used as an alternative to small molecule-based therapeutics in downregulating CMG activity.

Depletion strategy has been tested not only with Mcms, but also with other subunits of CMG. It has been proposed that 60% of total genes in humans are regulated by miRNA, and it was recently discovered that a miRNA governs Sld5 expression and Sld5 was robustly expressed in human bladder cancer due to the reduced level of a candidate miRNA that interacts with the 3′-UTR of the SLD5 gene (Yamane et al., 2016). The candidate miRNA turned out to be miRNA-370 and was downregulated in the bladder cancer cells. Overexpression of IL-6 (Interleukin 6) was also observed and enhanced expression of DNA-methyltransferase1 (DNMT1), leading to suppression of miR370, which in turn resulted in overexpression of Sld5. Thus, downregulation of IL-6 by siRNA could suppress Sld5 expression in T24 bladder cancer cell line and injection of miR-370 could inhibit tumor growth in the mouse xenograft experiment. IL-6 is a valuable target for treatment of dysimmune diseases and cancers (Rossi D. et al., 2015; Rossi J. F. et al., 2015). Many antagonistic monoclonal antibodies against IL-6 and the IL-6 receptor are under clinical trials and several of them were shown to be effective in certain diseases including rheumatoid arthritis. Therefore, bladder cancer could be tested for anti-IL-6 therapies to find whether tumor growth could be suppressed by preventing the autocrine loop of cancer cells which leads to Sld5 downregulation. Alternatively, sgp130, a natural inhibitor for IL-6 signaling could also be used for the inhibition of cancer growth (Hong et al., 2016).

The inhibition of the CMG helicase activity could take place in a variety of ways. For example, ciprofloxacin is likely to negatively interact with the ATPase active sites of the Mcm2-7 helicase to inhibit its helicase activity, although how it does is not clear at present (Simon et al., 2013). Small molecules could inhibit the CMG helicase activity via their competitive binding with the ATP binding pocket, interfering with the CMG-DNA interaction or via disruption of subunit interactions. PCNA is similar to the CMG in terms of its role in cell proliferation and acts as a marker for cancer. Therefore, studies on the PCNA inhibitor could give insight into development of the CMG inhibitors. Several compounds and peptides have been reported to inhibit the PCNA functions (Wang, 2014). PCNA forms a ring-shaped homotrimeric complex that encircles DNA double-helix and functions as a processivity factor for DNA polymerases, and it coordinates DNA synthesis by direct interactions with other replication proteins including ligases and topoisomerases. PCNA also has diverse roles in many cellular pathways including DNA damage repair, DNA damage avoidance pathway, cell cycle regulation, chromatin assembly, and transcription. Cellular reactions mediated by the PCNA usually occur through recruitment of the PCNA binding proteins on each subunit of the PCNA trimer. The interdomain-connecting loop (IDCL) is the major constituent participating in the PCNA-protein interaction by binding to the PCNA-interaction protein (PIP) box in the binding proteins. Several small molecules and peptides were identified that could inhibit the IDCL-PIP box interaction. For example, the PIP-box containing peptide derived from p21 inhibited DNA replication

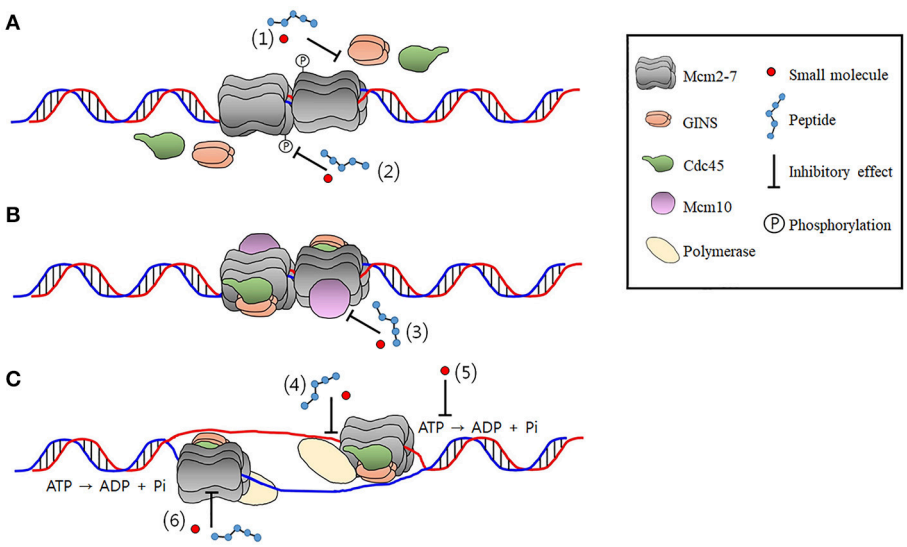

FIGURE 3 | The potential CMG-targeting sites of small molecules or peptides. The findings that multiple essential steps are required to form a functional CMG complex make the CMG a target well suited to develop drugs with a number of different inhibition mechanisms. These include inhibition of **(A)** formation, **(B)** activation, and **(C)** prevention of the catalytic activity of the CMG complex. The number in paranthesis denotes a site of inhibition. (1) Inhibition of the CMG formation by preventing interactions among the Mcm2-7, Cdc45, and the GINS. Inhibition of phosphrylation of Sld2/RecQL4 and Dpb11/TopBP1 by CDK and protein-protein interactions that occur in the precess of the CMG formation is not shown for simplicity. (2) Inhibition of the Mcm2-7 phosphorylation by DDK required for the CMG formation. (3) Inhibition of Mcm10 required for the activation of the CMG. (4) Uncoupling of the CMG-polymerase interactions during initiation and elongation stage of DNA replication. (5) Inhibition of enzymatic activity of the CMG required for duplex unwinding by targetting catalytic center of Mcms or residues required for translocation on DNA. (6) Disruption of intermoleculr interactions in the CMG.

and cell growth (Pan et al., 1995; Warbrick et al., 1995; Chen et al., 1996). In addition, compounds that inhibit formation of the PCNA trimer by docking at the monomer interface of the PCNA and a peptide that prevents phosphorylation of the PCNA Y211 inhibited cancer growth (Zhao et al., 2011; Tan et al., 2012).

The CMG is an 11-subunit complex and complex formation is essential for its helicase activation and cell proliferation. Furthermore, the CMG formation and activation is a multistep process that requires a variety of protein-protein interactions. Therefore, designing of small molecules and peptide inhibitors targeting inter-subunit interfaces within the CMG complex and protein-protein interactions between the CMG and other replication factors has immense importance in development novel anti-cancer drugs. It has been shown that the N-terminal peptides of the RB protein suppressed CMG helicase in *Xenopus* egg extract experiment, although it needs to be verified with the purified CMG complex (Borysov et al., 2015). Other Mcm-interacting proteins that have potentials to block replication or checkpoint activation, reviewed elsewhere, could be candidates for designing peptide drugs inhibiting the CMG activity (Simon and Schwacha, 2014). For example, prohibitin protein, which suppressed E2F-mediated transcription to inhibit cell proliferation, was shown to physically interact with Mcms and inhibit DNA replication *in vivo* (Rizwani et al., 2009). Posttranslational modifications of proteins also could be targeted as in the case of PCNA, because phosphorylation of Sld3/Treslin and Sld2/RecQL4 by CDK and phosphorylation of Mcms by DDK are essential steps for the CMG formation and stimulation (Tanaka and Araki, 2013). In addition, phosphorylation of Mcm7 increases complex formation with other Mcms and enhances chromatin binding, resulting in cell proliferation. Potentiation of Mcm7 phosphorylation has been observed in several cancers (Huang et al., 2013; Fei et al., 2017). Sites that could be targeted by small molecules and peptides during the formation and activation of CMG as well as replication initiation are illustrated in **Figure 3**.

Helicases are emerging targets that have great potential for the treatment of serious human proliferative diseases such as cancers. Identification of novel-type drugs targeting CMG, the core of DNA replication and cell proliferation, is of immense importance in developing new therapeutics against cancers. For this purpose, we may need more understanding of diverse features of the CMG complex that includes structural aspects, complex assembly and activation, mechanism of action, involvement with checkpoint, and intracellular and extracellular factors affecting transcriptional regulation of the CMG. The more information in these regards we obtain, the more possibility we will have for better drug design. Development of specific assays for assessing each step of CMG assembly, testing protein-protein interactions, and the success of high-yield purification of the CMG combined with high-throughput drug screenings will provide innovative means to identify small molecules targeting CMG assembly and helicase activity, ultimately contributing to development of novel and effective cure for human cancers.

AUTHOR CONTRIBUTIONS

Both Y-SS and Y-HK conceived and outlined the manuscript. Y-HK wrote the main text and produced the tables and figures. Y-SS critically reviewed and revised the manuscript.

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REVIEW
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Chromatin-Bound Cullin-Ring Ligases: Regulatory Roles in DNA Replication and Potential Targeting for Cancer Therapy

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Cullin-RING (Really Interesting New Gene) E3 ubiquitin ligases (CRLs), the largest family of E3 ubiquitin ligases, are functional multi-subunit complexes including substrate receptors, adaptors, cullin scaffolds, and RING-box proteins. CRLs are responsible for ubiquitination of ∼20% of cellular proteins and are involved in diverse biological processes including cell cycle progression, genome stability, and oncogenesis. Not surprisingly, cullins are deregulated in many diseases and instances of cancer. Recent studies have highlighted the importance of CRL-mediated ubiquitination in the regulation of DNA replication/repair, including specific roles in chromatin assembly and disassembly of the replication machinery. The development of novel therapeutics targeting the CRLs that regulate the replication machinery and chromatin in cancer is now an attractive therapeutic strategy. In this review, we summarize the structure and assembly of CRLs and outline their cellular functions and their diverse roles in cancer, emphasizing the regulatory functions of nuclear CRLs in modulating the DNA replication machinery. Finally, we discuss the current strategies for targeting CRLs against cancer in the clinic.

Keywords: DNA replication, chromatin, ubiquitin ligases, cancer, therapy

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STRUCTURE AND REGULATION OF CRLs

CRLs are composed of four components (**Figure 1**): cullins as molecular scaffolds, adaptor proteins, substrate receptors at the N-termini of cullins, and RING proteins at the C-termini of cullins, recruiting ubiquitin-loaded E2 enzymes (Bulatov and Ciulli, 2015). The evolutionarily conserved cullin family encompasses eight key members (CUL1, 2, 3, 4A, 4B, 5, 7, and 9) that exhibit similar structural architectures and contain cullin homology domains (Sarikas et al., 2011). Activation of CRLs is commonly regulated by NEDD8 modifications at lysine residues located at the C-termini of cullins (Soucy et al., 2010). Otherwise, individual CRLs include specific components, employing substrate receptors as critical determinants of substrate specificity.

CRL1, also known as SCF (SKP1-Cullin 1-F box protein), utilizes S-phase kinase-associated protein 1 (SKP1) as an adaptor protein and recognizes its substrates through substrate recognition proteins known as F-box proteins, which contain 40-amino-acid F-box domains (Zheng et al., 2002). Sixty nine F-box proteins are known to be encoded by the human genome to date and are classified into sub-groups based on their different substrate binding domains, including FBXW (F-box and WD40 domains) FBXL (F-box and leucine-rich repeats) and FBXO (F-box only) (Skaar et al., 2013). CRL2 and CRL5 share an identical adaptor, Elongin C (EloC), known to enhance

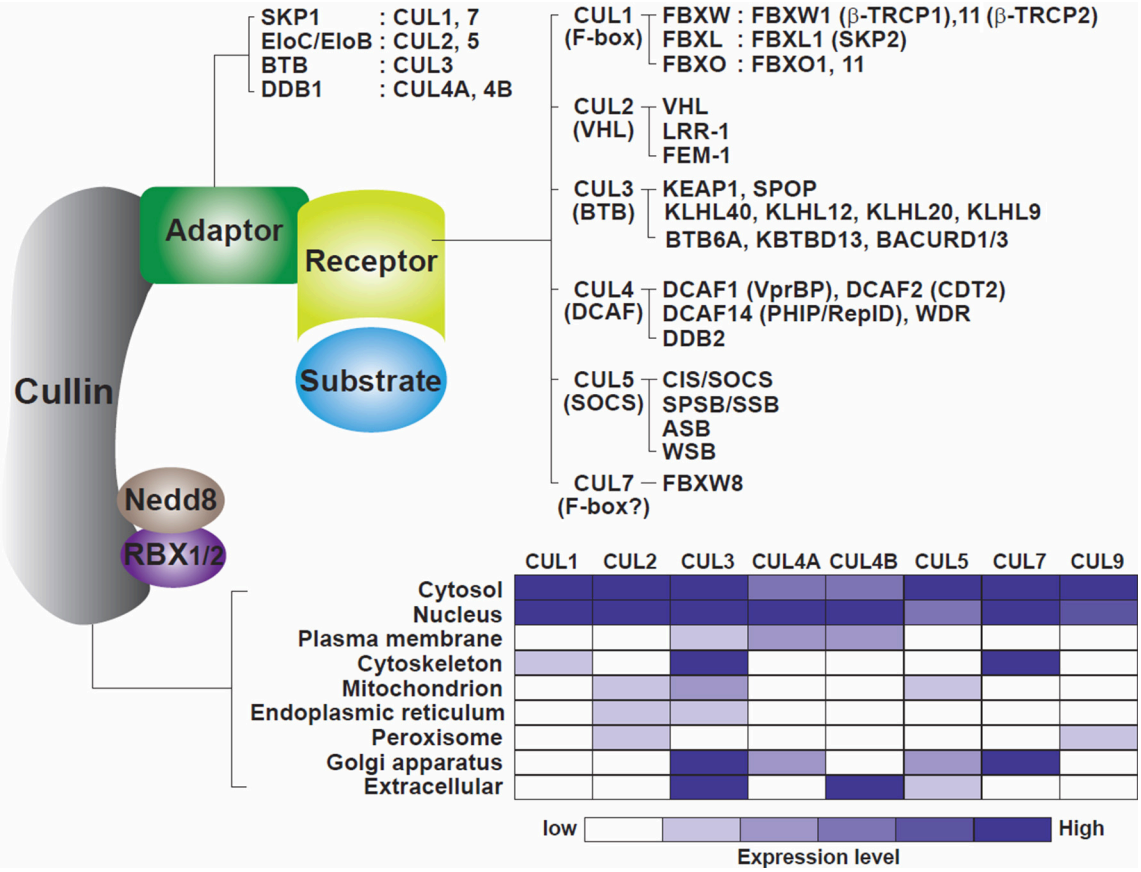

FIGURE 1 | Model of the cullin-RING ligase complexes. Cullins 1, 2, 3, 4A, 4B, 5, 7, and 9 are scaffold proteins that assemble with RING finger proteins (RBX1/2), adaptor proteins (SKP1, EloC/EloB, BTB, DDB1) and receptor or substrate recognition proteins (F-box family, VHL family, BTB, DCAF family, SOCS family among others). A non-exhaustive but known list of the CRLs, adaptors, receptors, and RING proteins is shown. The bottom part of the figure illustrates the cellular localization of the cullins. Cullins are broadly distributed in the different compartments of the cells with CUL4A and CUL4B mostly located in nuclei (table constructed from data gathered from Genecards.org).

the rate of RNA polymerase II elongation (Bradsher et al., 1993), and utilize either von Hippel-Lindau (VHL) or suppressors of cytokine signaling (SOCS)-box proteins as distinct substrate receptors (Muniz et al., 2013; Cardote et al., 2017). CRL3 interacts with several BTB (Bric-a-brac, Tramtrack, Broad-complex) domain-containing proteins that implement dual functions as adaptor and receptor subunits (Pintard et al., 2004). The BTB domains of these subunits act as adaptors by associating with CUL3 and RBX1, whereas their MATH (meprin and TRAF homology) motifs and Kelch beta-propeller repeat and zinc finger motifs recognize the substrates (Stogios et al., 2005). CRL4 is anchored by two highly similar scaffold proteins, CUL4A and CUL4B, and an adapter, DDB1 (damage-specific DNA binding protein 1). DDB1 contains three WD40 propeller domains (BPA, BPB, and BPC) and links the CUL4 scaffold with multiple substrate receptors termed DCAFs (DDB1-CUL4-associated factors). Over 100 DCAFs have been identified to date (Zimmerman et al., 2010; Harper and Tan, 2012). CRL7 and CRL9 contain the two largest cullin scaffold proteins, CUL7 (1698 amino acids) and CUL9 (2517 amino acids). As these two cullins are much larger than the cullins anchoring the other CRLs (745–913 amino acids), CRL7/9 may have additional specific, unique functions and/or protein partners. CRL7 is similar to CRL1 in that it includes SKP1 as an adaptor and FBXW8 as a substrate receptor (Dias et al., 2002; Sarikas et al., 2008), but unlike CUL1, it does not interact with the adaptor/receptor complexes SKP1/βTRCP2 or SKP1/SKP2 (Dias et al., 2002). These variations delineate distinct ubiquitin-dependent proteolysis pathways that may be involved in the degradation of specific substrates involved in specific cellular processes and /or in specific cell compartments. Cellular activities of the different CRLs can be co-regulated. For example, CUL7 (with OBSL1 and CCDC8) regulates CUL9 and its substrates to maintain genome stability (reviewed in Jackson, 2014) while degradation of the CRL4 component CDT2 can be orchestrated by the CRL1 (CUL1/FBXO11) complex (Abbas et al., 2013; Abbas and Dutta, 2017).

CRLs are involved in diverse biological processes including cell cycle control, DNA replication, DNA-damage repair, and chromatin remodeling through the selective degradation of various protein substrates, mediated by specific interactions with various substrate receptors. As an example, the SCF (CRL1)

complex plays a vital regulatory role by ubiquitinating a series of cell cycle regulators including EMI1 (Margottin-Goguet et al., 2003), CDC25A and B (Busino et al., 2003; Kanemori et al., 2005), WEE1A (Watanabe et al., 2004), Cyclin D1 (Wei et al., 2008), and PTTG1/Securin (Limón-Mortés et al., 2008). These substrates are recognized by F-box proteins that bind consensus sequences such as D-pS/pT-G-X-X-pS and/or **D**/E/S-**S**/E/D-**G**/A-$\mathbf{x_{2-4}}$-**S**/E/D for β-TrCP (Limón-Mortés et al., 2008) (Hansen et al., 2004) or 0X000S/TPXXS/T/E for FBW7 (Limón-Mortés et al., 2008; Wertz et al., 2011) (X, 0 = random or hydrophobic amino acids, respectively). F-box proteins exhibit a high affinity for serine or threonine residues phosphorylated by specific kinases such as JNK, p38, and CKII (Limón-Mortés et al., 2008; Wertz et al., 2011). While the assembly of cullins, adaptors, and substrate receptors into multiple combinations is necessary for specific arrays of cellular biological responses through time and space, such combinatorial complexity is a major challenge for understanding CRLs' roles in cell signaling and diseases. Some substrates of CRL targeted ubiquitination and their roles are listed in **Table 1**.

CRLs associate with the small protein NEDD8 (Neural precursor cell expressed developmentally down-regulated protein 8) and this interaction is essential for their ubiquitin ligase activities. NEDDylation is accomplished by the sequential action of an NAE (Nedd8-activating enzyme) and a Nedd8-conjugating enzyme, UBC12 (Haas, 2007). CRLs can be deNEDDylated by the zinc-dependent metalloenzyme CSN5, a component of the COP9 signalosome (CSN) complex, which cleaves the isopeptidic bond between cullin and NEDD8 (Cope and Deshaies, 2003). NEDDylation is also regulated by CAND1 (Cullin-associated Nedd8-dissociated protein 1), which binds to unneddylated cullins, inhibiting NEDD8 conjugation and consequently resulting in inhibition of both cullin NEDDylation and CRLs activities (Duda et al., 2011).

FUNCTIONS OF CRLs IN DNA REPLICATION AND CELL CYCLE PROGRESSION

CRLs serve key functions in the regulation of chromosome duplication, modulating crucial steps in the assembly and disassembly of the DNA replication machinery during normal growth and in response to perturbed replication. Roles for cullin-based ring E3 ligases in DNA replication and cell cycle progress ion have been recently extensively discussed elsewhere (Abbas and Dutta, 2017), and the involvement of CRLs in the early stages of DNA replication in various organisms are briefly summarized below.

The first step in the DNA replication process in all eukaryotes is the loading of the origin recognition complex (ORC) and recruitment of the MCM2-7 helicase complex by the licensing factors CDC6 and CDT1. This complex assembly occurs during late mitosis and the early G1 phase, to form an inactive pre-replication complex (pre-RC). Pre-RCs are subsequently activated by the recruitment of additional factors and by cyclin-dependent kinases (CDKs) and DBF4-dependent kinases (DDKs) (Parker et al., 2017). CRL-controlled levels and/or activities of proteins involved in pre-RCs assembly and activation are crucial for the orderly initiation of DNA replication and the prevention of re-replication.

In yeast, RTT101, the human CUL4 homolog, modulates MRC1 (human claspin homolog) interaction with the CMG (Cdc45-MCM-GINS) helicase (Buser et al., 2016). RTT101 deletion leads to reduced association of both the replicative helicase MCM and FACT, a complex that assemble or partially disassemble nucleosomes, to replication origins (Han et al., 2010). Cells lacking RTT101 are defective in DNA replication through DNA damaged sites (Zaidi et al., 2008). The yeast CDC6, crucial for pre-RC licensing is degraded in a CRL-dependent pathway (Drury et al., 1997). CRL-induced CDC6 degradation is required to prevent DNA rereplication (Ikui et al., 2012). The yeast CMG is ubiquitinated and disassembled by DIA2 (a F-box protein related to the human CUL1/F-box complex) that binds replication origins (Koepp et al., 2006; Maculins et al., 2015).

In vertebrates, CRLs demonstrate similar functions. In *Xenopus*, CUL2 is a key player during the termination of DNA replication, disassembling the CMG helicase complex (Sonneville et al., 2017). In mammalian cells, MCM3, an essential subunit of the replicative DNA helicase, is a CRL3 substrate (Mulvaney et al., 2016). Both CRL1 and CRL4 can play important roles in the regulation of pre-RC assembly by modulating the chromatin association of two essential licensing factors, CDC6 and CDT1. CDC6 is targeted for degradation by the CRL4-CDT2 and the CRL1-CyclinF complex in S phase (Clijsters and Wolthuis, 2014) and G2-M (Walter et al., 2016) respectively. Targeted degradation of CDT1 in S-phase is shared between CRL1/SKP2 (at the G1/S transition) and CRL4/CDT2 (during S-phase) (Kim and Kipreos, 2007; Pozo and Cook, 2016; Abbas and Dutta, 2017). Thus, deregulation of CRLs in the nucleus leads to CDT1 accumulation and, in turn, to DNA re-replication and genomic instability (Kim and Kipreos, 2007; Pozo and Cook, 2016). CRL4-CDT2 also mediates the degradation of the histone H4 methyltransferase SET8, an enzyme catalyzing the monomethylation on lysine 20 of histone H4 that allows the loading of the pre-RC component ORC1 and the ORC-associated protein ORCA (Beck et al., 2012). SET8 degradation is essential to prevent DNA re-replication (Abbas et al., 2010). Another protein involved in DNA replication initiation include MMSET, a histone methyltransferase degraded during S phase in a CRL4 dependent manner and necessary for the optimum association of pre-replication factors (Evans et al., 2016). The CRL4-interacting DCAF, RepID (DCAF14/PHIP) binds a subset of replication origins and is essential for initiation from those origins (Zhang Y. et al., 2016). The mechanism by which a DCAF can facilitate initiation is unclear, however recent evidence suggests that the CRL4/CUL4B complex facilitates replication licensing through a CUL4B-CDK2-CDC6 cascade, leading to the upregulation of CDK2 and protecting CDC6 from degradation (Zou Y. et al., 2013).

CRLs also control DNA replication via indirect mechanisms. Increased CDK1/2 activities, necessary for origin firing, occur in late G1 and at the G1/S transition through the CRLs-controlled degradation of CDK inhibitors such as p27, p21, and p57 (reviewed in Abbas and Dutta, 2017). Following DNA replication

TABLE 1 | Non-exhaustive list of CRLs substrates.

CRLs	Substrates	Receptors	Substrate roles	References
CRL1	EMI1/Cyclin A	β-TrCP1	Regulates mitosis entry	Guardavaccaro et al., 2003
CRL1	CDC25A	β-TrCP1/2	Required for progression from G1 to the S phase of the cell cycle	Busino et al., 2003
CRL1	CDC25B	β-TrCP1/2	Required for entry into mitosis	Kanemori et al., 2005; Uchida et al., 2011
CRL1	WEE1	β-TrCP1/2	Cell cycle progression, G2/M transition	Watanabe et al., 2004
CRL1	Cyclin D1	β-TrCP1/2	Progression through the G1 phase of the cell cycle	Wei et al., 2008
CRL1	Claspin	β-TrCP1/2	Checkpoint mediated cell cycle arrest in response to replication stress and DNA damage	Peschiaroli et al., 2006
CRL1	PR-SET7/SET8	β-TrCP1/2	Epigenetic regulation/Histone modification	Wang et al., 2015
CRL1	Securin	β-TrCP	Prevent sister chromatin separation	Limón-Mortés et al., 2008
CRL1	SAK/PLK4	β-TrCP1	Prevents centrosome amplification	Cunha-Ferreira et al., 2009
CRL1	MCL1	FBXW7	Involved in apoptosis regulation	Wertz et al., 2011
CRL1	$P27^{KIP1}$	FBXL1/SKP2	Involved in cell cycle progression	Nakayama et al., 2001
CRL1	$P21^{Cip1}$	FBXL1/SKP2	Cell cycle progression	Bornstein et al., 2003
CRL1	$P57^{Kip2}$	FBXL1/SKP2	Inhibitor of several G1 cyclins	Pateras et al., 2006
CRL1	P130	FBXL1/SKP2	Heterochromatin formation	Bhattacharya et al., 2003
CRL1	CDT1	FBXL1/SKP2	DNA replication licensing factor	Li et al., 2003
CRL1	Cyclin D	FBX4/FBXL1/SKP2	G1/S transition	Yu et al., 1998; Gong et al., 2014
CRL1	Cyclin G2	FBXL1/SKP2	Regulation of cell cycle progression	Xu et al., 2008
CRL1	Cyclin D2	FBXL2	Progression through the G1 phase of the cell cycle	Chen et al., 2012b
CRL1	Cyclin D3	FBXL2	G1/S transition	Chen et al., 2011
CRL1	Cyclin E	FBXW7	G1/S transition	Gong et al., 2014
CRL1	P85beta	FBXL2	Control PI3K signaling cascade	Kuchay et al., 2013
CRL1	VPS34	FBXL20	Catalytic subunit of the PI3K complex kinase	Xiao et al., 2015
CRL1	JMJD2A	FBXL4	Epigenetic regulation/Histone modification	Das et al., 2014
CRL1	CITED2	FBXL5	Transcription regulation	Machado-Oliveira et al., 2015
CRL1	Aurora A	FBXL7	Regulates mitosis	Coon et al., 2012
CRL1	Aurora B	FBXL2	Regulates mitosis	Chen B. B. et al., 2013
CRL1	CaMK1	FBXL12	Calcium/calmodulin-dependent protein kinase	Mallampalli et al., 2013
CRL1	CDC6	FBXO1/Cyclin F	DNA replication licensing factor	Walter et al., 2016
CRL1	DCAF2/CDT2	FBXO11	Efficient progression through S and G2/M phases	Abbas et al., 2013
CRL1	UHRF1	β-TrCP/FBW1A	Maintenance of DNA methylation patterns during DNA replication	Chen H. et al., 2013
CRL2	HIF1alpha	VHL	Response to hypoxia	Ohh et al., 2000
CRL2	SPRY2	VHL	May function as an antagonist to several growth factors	Anderson et al., 2011
CRL2	RNA polII subunit	VHL	Transcription	Kuznetsova et al., 2003
CRL2	CKI1	LRR1	Casein kinase involved in several cellular functions	Merlet et al., 2010
CRL2	$P21^{Cip1}$	LRR1	Cell cycle progression	Starostina et al., 2010
CRL2	TRA1	FEM1	Epigenetic regulation/Histone modification	Shi et al., 2011
CRL2	TOPBP1	–	DNA replication	Blackford et al., 2010
CRL2	H2B	–	Core component of the nucleosome	Li et al., 2010
CRL3	NRF2	KEAP1	Negative regulation of antioxidant response	McMahon et al., 2003
CRL3	WNK4	KEAP1	Blood pressure regulation	Andérica-Romero et al., 2014
CRL3	DAXX	SPOP	Transcription repressor	Sakaue et al., 2017
CRL3	MCM3	KEAP1	DNA replication	Mulvaney et al., 2016
CRL3	PP2A	–	Resistance of cancer cells to death receptor-induced apoptosis	Xu et al., 2014
CRL3/CRL4	TOP1	–	DNA replication, transcription	Zhang et al., 2004; Kerzendorfer et al., 2010
CRL4	CDT1	DCAF2/CDT2	DNA replication licensing factor	Zhong et al., 2003; Higa et al., 2006
CRL4	$P21^{Cip1}$	DCAF2/CDT2	Cell cycle progression	Abbas et al., 2008; Nishitani et al., 2008
CRL4	PR-SET7/SET8	DCAF2/CDT2	Epigenetic regulation	Jørgensen et al., 2011

(Continued)

TABLE 1 | Continued

CRLs	Substrates	Receptors	Substrate roles	References
CRL4	$P27^{Xic1}$	DCAF2/CDT2	Cell cycle arrest	Chuang and Yew, 2001
CRL4	CKI1	DCAF2/CDT2	Casein kinase involved in several cellular functions	Kim et al., 2008
CRL4	E2F	DCAF2/CDT2	Cell cycle regulation	Shibutani et al., 2008
CRL4	TDG	DCAF2/CDT2	DNA glycosylase	Slenn et al., 2014
CRL4	CHK1	DCAF2/CDT2	Checkpoint mediated cell cycle arrest in response to DNA damage	Huh and Piwnica-Worms, 2013
CRL4	Histone H2A, H3, H4, DDB2	DDB2	Core components of the nucleosome	Kapetanaki et al., 2006
CRL4	SLBP	DCAF11	Histone biosynthesis regulation	Djakbarova et al., 2016
CRL4	CK1alpha	CRBN	Casein kinase involved in several cellular functions	Krönke et al., 2015; Petzold et al., 2016
CRL4	ZFP91	CRBN	E3 ubiquitin protein ligase	An et al., 2017b
CRL4	APP	CRBN	Cell surface receptor	Del Prete et al., 2016
CRL4	IKZF1, 3	CRBN	Transcription	Krönke et al., 2014
CRL4	Merlin	DCAF1/VprBP	Probable regulator of the Salvador/Warts/Hippo (SWH) signaling pathway	Huang and Chen, 2008
CRL4	FOXM1	DCAF1/VprBP	Transcription	Wang et al., 2017
CRL4	MCM10	DCAF1/VprBP	Replication initiation factor	Kaur et al., 2012
CRL4	TSC2	FBXW5	Regulator of several GTPases	Hu et al., 2008
CRL4	MMSET	DCAF2/CDT2	Epigenetic regulation	Evans et al., 2016
CRL4	LIG I	DCAF7	DNA replication	Peng et al., 2016
CRL4	p12 subunit of DNA polymerase δ	DCAF2/CDT2	DNA replication	Zhang et al., 2013
CRL4/CRL1	CHK1	?	Checkpoint mediated cell cycle arrest in response to DNA damage	Lampert et al., 2017; Tu et al., 2017
CRL4	SLBP	WDR23/DCAF11	Stem-loop binding protein	Lampert et al., 2017
CRL4	FBH1	DCAF2/CDT2	Helicase with a role in response to stalled/damaged replication fork	Bacquin et al., 2013
CRL4	ORCA/LRWD1	?	G1/S transition. Recruits and stabilizes replication origin complexes	Shen and Prasanth, 2012
CRL4	PCNA	?	DNA replication	Lo et al., 2012
CRL4	p53	DCAF2/CDT2	Transcription/apoptosis	Banks et al., 2006
CRL5	iNOS	SOCS	Nitric oxide production	Kuang et al., 2010; Nishiya et al., 2011
CRL5	TRII	SOCS	Enhanced migration and invasion of tumor cells by SOCS silencing	Liu et al., 2015
CRL5	GHR	SOCS	Regulation of growth hormone signaling	Bullock et al., 2006
CRL5	TRAF6	SOCS	Regulation of lipopolysaccharide signaling	Zhu et al., 2016
CRL7	Cyclin D1	FBXW8	Cell cycle arrest	Okabe et al., 2006
CRL7	IRS1	FBXW8	Regulation of insulin signaling	Xu et al., 2008
CRL7	GRASP65	FBXW8	Maintenance of the Golgi apparatus integrity	Litterman et al., 2011
CRL7	EAG1	FBXW8	Potassium channel modulation	Hsu et al., 2017
CRL9	Cytochrome C	?	Promotes cell survival	Gama et al., 2014
CRL9	Survivin	?	Genome integrity maintenance	Li et al., 2014

initiation, cell cycle progression is also controlled, in part, by the CRL1-timely degradations of the CDK positive regulators cyclin E (for S phase progression), cyclin A, cyclin D1, and WEE1 (for G2 progression) (Watanabe et al., 2004; Abbas and Dutta, 2017). Similarly, progression over mitosis is ensured through EMI degradation by the CRL1- β-TrCP1 complex, leading to increased activity of the anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase utilizing the cullin-like scaffold protein APC2. Other CRL-targeted proteins associated with cell cycle progression include Claspin, PCNA, MCM10, the DNA polymerase alpha, and histones H2A, H2B, H3, H4 among others (**Table 1**).

CULLIN-BASED RING E3 LIGASES AND CANCER

Since CRLs play critical roles in a myriad of biological processes, it is reasonable to think that the deregulation of cullins and/or other CRLs components can play a major role in cancer

progression. While cullin deregulation (mostly upregulation) have been observed in cancer, downregulation or suppression of some of the CRL components can lead to tumor suppression. Deregulated expression of cullins and cullin-associated factors may occur through CpGs methylation, gene coding region mutations, or promoter deletion and/or micro-RNA-induced silencing among others. Below are several examples linking deregulation of CRLs to cancer.

CUL1-Based Ubiquitin Ligase Complexes

CRL1 is the most studied cullin-associated complex in the context of cancer (**Figure 2**). Deregulated CUL1 expression was reported in lung and gastric cancers (Le Gallo et al., 2012) and during the early stages of melanoma development (Lee et al., 2010). A myriad of CRL1-associated F-box proteins and their substrates are involved in cancer (for a review, see Kitagawa and Kitagawa, 2016).

Altered expression and mutations in several F-box proteins, including SKP2, are hallmarks of several cancers. SKP2 (FBXL11) is part of the FBXL subfamily that comprises 22 members (FBXL1 to FBXL22) containing an F-box motif and a C-terminal Leu-rich repeat (LRR) domain. SKP2, which has been characterized as an oncoprotein, is by far the most studied F-box protein of the FBXL subfamily. SKP2 associates with the SCF (SKP1-Cullin1-F-box) complex and targets p27 for degradation, with major developmental consequences in mice (Nakayama et al., 2004). The absence of SKP2 in mice results in the accumulation of p27, nuclear enlargement, cell polyploidies, and centrosome overduplication (Nakayama et al., 2000), phenotypes that disappeared in $SKP2^{-/-}/p27^{-/-}$ double-mutant mice (Nakayama et al., 2004). Chemical-induced skin tumorigenesis is inhibited in SKP2(−/−) mice (Sistrunk et al., 2013) whereas overexpression of *Skp2* in mice led to tumor development in the prostate (Shim et al., 2003), suggesting that a SKP2 deregulation-induced oncogenesis may be tissue specific. SKP2 is also a crucial mediator of BCR-ABL-induced leukemogenesis (Agarwal et al., 2008). SKP2 is deregulated and correlated with poor prognosis in a wide array of human cancers including breast, prostate, colon, lung, brain, gastric, and blood (Frescas and Pagano, 2008; Zheng et al., 2016). Therefore, the inhibition of SKP2 could be a novel strategy for the treatment of some human cancers.

Other FBXL proteins, also mediate the degradation of substrates involved in cell-cycle progression (**Table 1**). Ectopic expression of FBXL2 in transformed lung epithelia facilitates polyubiquitination and degradation of cyclin D3, leading to G2/M-phase arrest, increased frequency of apoptotic cells, and chromosomal anomalies (Chen et al., 2011). FBXL2 recognizes a canonical calmodulin-binding motif within cyclin D3 and compete with calmodulin for cyclin D3 binding (Chen et al., 2012a). It is thought that FBXL2 targets cyclin D2 for degradation to inhibit cancer cell proliferation. Several patient samples show suppressed expression of FBXL2 together with robust cyclin D2 levels in acute myelogenous leukemia and acute lymphoblastic leukemia (Chen et al., 2012b). Ectopically expressed FBXL2 significantly inhibited the growth and migration of tumorigenic cells and tumor formation in athymic nude mice (Chen et al., 2012a). FBXL2 was also shown to ubiquitinate Aurora B, an integral regulator of cytokinesis that inhibits tumorigenesis (Chen B. B. et al., 2013). FBXL3 was described as a regulator of the circadian rhythm by targeting Cryptochrome (Cry1/Cry2) proteins (Siepka et al., 2007). Since, growing evidence are pointing out that deregulation of the circadian clock plays an important role in carcinogenesis (Savvidis and Koutsilieris, 2012), one putative role for FBXL in cancer could be through the disruptions of normal circadian rhythms.

FBXL proteins are also involved in the epithelial to mesenchymal transition, which often accompanies tumor progression. For example, FBXL5 and FBXL14 inhibit cell invasiveness by targeting SNAIL1 in gastric cancer cells (Vinas-Castells et al., 2010; Wu et al., 2015; Cen et al., 2017). FBXL10 (also known as KDM2B), which contains a JmiC

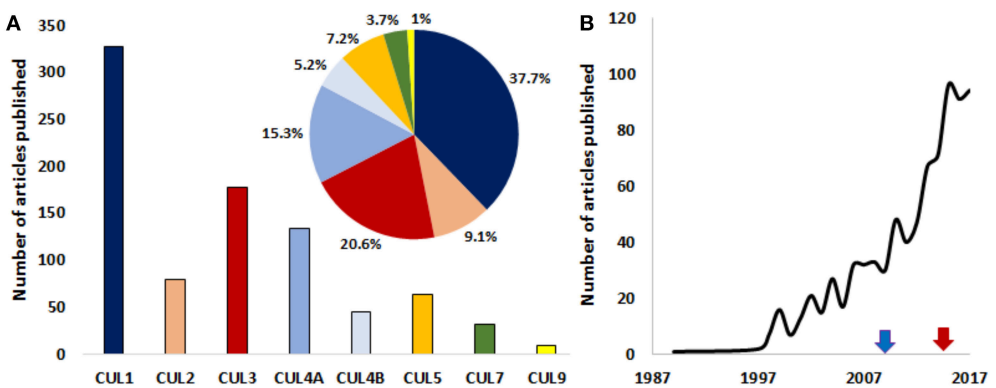

FIGURE 2 | A growing interest for the roles of cullins in cancer. **(A)** Graph depicting the number of published articles studying the role of cullin1, 2, 3, 4A, 4B, 5, 7, and 9 in cancer. The pie chart shows the relative distribution (%) of published articles among the different cullins (the color code is the same as in the bar graph). **(B)** The number of published studies looking at the role of cullins and/or cullin-interacting proteins in the pathology of cancer are increasing exponentially. Blue arrow and red arrows denote the first studies targeting cullins in mice and human respectively. The number of publications for the year 2017 is an estimation made from the number of articles that were published in the first 10 months of the same year. (*Source: PubMed*).

domain (Tsukada et al., 2006) and primarily regulates metabolic and developmental genes (Zheng et al., 2016), is involved in H2AK119 ubiquitination and histone H3K36 demethylation (Wu et al., 2013) and affects TRAIL-induced apoptosis (Ge et al., 2011). The latter observation implies that targeting FBXL10 could overcome resistance to TRAIL treatment in human cancer. Like other CRL1 interacting proteins, FBXL10 role in cancer seems to be tissue specific in humans with higher levels of FBXL10 observed in several types of cancers while it is downregulated in brain tumors (Frescas et al., 2007; Tzatsos et al., 2013). A tissue-specific relationship between FBXL10 and cancer is also observed in transgenic mice. Mice with hematopoietic stem cells overexpressing FBXL10 were shown to develop myeloid or B-lymphoid leukemia (Ueda et al., 2015) whereas FBXL10 depletion was shown to abrogate tumorigenicity in the pancreas (Tzatsos et al., 2013).

Deregulation of the F-box and WD containing protein β-TrCP is associated with several cancers, including breast, colon, pancreatic, liver, gastric, and prostate (Frescas and Pagano, 2008), and both overexpression and mutations in β-TrCPs have been reported in gastric, prostate, breast, pancreas, colon liver, and skin cancer (reviewed in Zheng et al., 2016). In line with these observations, a study using transgenic mice expressing human β-TrCP 1 targeted to epithelial cells under the control of the mouse mammary tumor virus (MMTV) showed that 38% of these mice developed mammary, ovarian, and uterine carcinomas (Kudo et al., 2004). Beyond altering cell cycle progression, β-TrCP1 and β-TrCP2 are involved in the degradation of the transcription factors SNAIL and TWIST and the extracellular matrix fibronectin, all involved in metastasis (Ray et al., 2006; Kitagawa and Kitagawa, 2016). In addition, invasion of human melanoma cells is suppressed by silymarin, a plant flavonoid, in part through β-TrCP-mediated degradation of β-catenin (Vaid et al., 2011). Further evidence linking β-TrCPs to skin cancer is the observation that degradation of IκBα and PDCD4 by β-TrCPs can contribute to the development of skin squamous carcinoma (Dorrello et al., 2006; Gu et al., 2007) while expression of a dominant negative β-TrCP in mouse epidermis confers skin proliferation and apoptosis resistance in response to UVB irradiation (Bhatia et al., 2011). The role for β-TrCPs in carcinogenesis is complex since these proteins also promotes anti-cancer activities by controlling the degradation of several pro-apoptotic proteins such as MCL-1 (Ding et al., 2007), BimEL (Dehan et al., 2009), PDCD4 (Dorrello et al., 2006), pro-caspase 3 (Tan et al., 2006).

Other F-box proteins, including FBXW7, FBXW8, and FBXW9 play roles in carcinogenesis, mainly through regulating the levels of factors involved in cell cycle progression (**Table 1**). FBXW7, a major tumor suppressor, negatively regulates more than a dozen of oncogenic proteins with pivotal roles in cell cycle progression, proliferation, and cell division. FBXW7 also regulates protein degradation involved in DNA damage repair, cell apoptosis and metastasis (for review, see Cheng and Li, 2012) (**Table 1**). The *FBXW7* 4q31.3 locus is deleted in ∼30% of cancers (Knuutila et al., 1999) with a *FBXW7* mutation rate of ∼6% in primary tumors (Akhoondi et al., 2007). FBXW7 mutations and deletions have been described in various type of tumor types including T-cell leukemia, stomach, pancreas, breast, colon, bladder, prostate cancer, gastric, and cholangiocarcinoma with T-cell leukemia and cholangiocarcima harboring the highest mutations rates of 31 and 35% respectively (reviewed in Cheng and Li, 2012; Zheng et al., 2016). However, mutation in *FBXW7* alone may not be sufficient for carcinogenesis since a recent study showed that both *FBXW7*and *NOTCH1* deregulation may be needed for the induction of human T-ALL (Takeishi and Nakayama, 2014). The *FBXW12* gene coding regions or promoter were found to be deleted in several epithelial ovarian cancers (De la Chesnaye et al., 2015). FBXW8 modulates cancer cell proliferation through cell-type specific cyclin D1 degradation (for review, see Zheng et al., 2016). Thus, FBXW8 is involved in the proliferation of human choriocarcinoma cells via G2/M phase transition with the regulation of CDK1, CDK2, cyclin A, cyclin B1, and p27 expression (Lin et al., 2011). FBXW8 also promotes the degradation of the hematopoietic progenitor kinase 1 (HPK1), a member of mammalian STE20-like serine/threonine kinases that is lost in >95% pancreatic cancer via proteasome-mediated degradation (Wang et al., 2014). The mouse FBXW12 homolog (FBXW15) interacts with histone acetyltransferase binding to the origin recognition complex (HBO1) to mediate its CUL1-regulated ubiquitination (Zou C. B. et al., 2013). Because HBO1 plays a crucial role in DNA replication licensing and cell proliferation, FBXW15 could control DNA replication licensing and cell proliferation.

CUL2-Based Ubiquitin Ligase Complexes

CUL2 is the scaffold protein of the CRL2 complex, recruiting the substrate receptor von Hippel-Lindau protein (pVHL) through the dimer complex EloB and EloC (Pause et al., 1997). pVHL can also be associated to CUL5 (Okumura et al., 2016). A germline mutation in VHL is the basis of familial inheritance of von Hippel-Lindau syndrome, which is characterized by the development of cysts and tumors in multiple organ systems (reviewed in Johnson et al., 2007). Mutations in pVHL or loss of heterogeneity result in high levels of HIF proteins and VHL tumorigenesis (Cassol and Mete, 2015). Deregulation of these two protein is involved in the development of VHL-associated clear-cell renal cell carcinoma (Maynard and Ohh, 2004) with pVHL ectopic expression in $VHL^{-/-}$ renal cell carcinoma leading to suppression of tumor formation in mice (Maynard and Ohh, 2004). HIF-1α is also often overexpressed in several other cancers (Zhong et al., 1999).

In addition to the extensively studied HIF-1α, many other CRL2 substrates have been identified (**Table 1**). HIF-1α triggers a transcriptional response to hypoxia, a key process critical to promote tumor progression and an important determinant of resistance to therapy (Vaupel and Mayer, 2007). Disruption of CRLs components (mutation, gene loss) associates with enrichments of HIF-target genes in several tumor types (Rowbotham et al., 2014). Still, deregulation of CRLs may not solely account for deregulated HIF-1α in cancer since HIF-1α levels are controlled by different signaling mechanisms, including regulation by the HSP90 pathway, the HIF-1 pathway and the MDM2-p53 mediated ubiquitination pathway (Rowbotham et al., 2014; Cassol and Mete, 2015; Masoud and Li, 2015). Since

no CUL2 mutation was found to play a critical role in HIF-1α activation in several cancers (Park et al., 2009), it is likely that HIF-1α loss of homeostasis in cancer is mediated primarily by deregulation of cullin expression rather than cullin point mutations (Zhong et al., 1999). The silencing of another CUL2 substrate, RhoB, is a crucial step driving carcinogenesis (Huang and Prendergast, 2006). In liver cancer, RhoB is targeted for degradation via the CUL2-RBX1 complex, an important effector that drives liver carcinogenesis (Xu et al., 2015). Similarly, CUL2 silencing in HPV16 positive cervical cancer cells resulted in slow growth of xenograft tumors retarding G1-S transition of the cell cycle and favoring apoptosis (Xu et al., 2016). As observed with other cullins, CUL2 deregulation may be driven by microRNAs. For example, CUL2 overexpression in gastric cancer tissues may be driven, in part, by aberrant levels of miR-574-3p (Su et al., 2012), suggesting a role for the CUL2/miR-424 pathway in promoting growth in cancer cells.

CUL3-Based Ubiquitin Ligase Complexes

Alterations of signaling pathways caused by the deregulation of CUL3 and/or CUL3-associated factors can give rise to cancer. For example, CUL3, in complex with the substrate adaptor Ketch-like family member 20 (KLHL20), is thought to promote cancer progression through increased ubiquitination and degradation of the Promyelocytic leukemia (PML) protein (Yuan et al., 2011). Hypoxia may exacerbate PML-KLHL20-driven carcinogenesis, since the promoter of *KLHL20* contains several hypoxia-response elements (Yuan et al., 2011) and PML is a negative regulator of HIF-1 (Bernardi et al., 2006). Thus, degradation of PML by KLHL20 would potentiate a strong induction of several hypoxia pathways. Indeed, KLHL20 expression is elevated in prostate cancer and correlates with HIF-1α upregulation, and PML downregulation (Yuan et al., 2011). It should be noted however that a study with HeLa cells suggests that HIF-2α, not HIF-1α, interacts with KLHL20 and that knockdown of KLHL20 decreased HIF-2α but not HIF-1α protein levels (Higashimura et al., 2011). Although this study did not show directly that KLHL20-mediated protection of HIF-2α from degradation involves CRL3, it suggested that both HIF-1α and HIF-2α may be controlled by CRLs in cancer.

Another Kelch-like family member, KLHL39, is down regulated in cancer and correlates with both low PML expression and cancer progression (Chen et al., 2015). Unlike KLHL20, KLHL39 does not bind CUL3 but acts as an inhibitor by blocking KLHL20-mediated ubiquitination of PML by inhibiting KLHL20 binding to both CUL3 and its putative substrates (Chen et al., 2015). Thus, KLHL39 may act as a tumor suppressor by blocking KLHL20-dependent ubiquitination of PML and other substrates (Yuan et al., 2011).

The CRL3 substrate Kelch-like ECH-associated protein (KEAP1) is a key inhibitor of the transcription factor NRF2 that regulates genes involved in the antioxidant response and drugs detoxification (Chen and Chen, 2016). The CUL3-KEAP1-NRF2 pathway prevents oxidative stress-induced DNA damage and carcinogenesis in normal cells and mediates the response to oxidative stress, cell growth, and survival in cancer cells. The CRL3-KEAP1-NRF2 pathway contributions to cancer development are reinforced by the observed deregulation of KEAP1 and the presence of CUL3 mutations that could lead to NRF2 overexpression in many cancers (Chen and Chen, 2016 and references therein).

Other dual adaptor/receptor BTB proteins may have a crucial role in maintaining specific metabolic pathways controlled by hormone receptors (Zhuang et al., 2009). Speckle type BTB/POZ protein (SPOP) is one of the highest loci to exhibit loss of heterozygosity in breast cancers (Li et al., 2011) and has high mutation rates in prostate (Kan et al., 2010) and endometrial cancers (Le Gallo et al., 2012). Frequent mutations in SPOP occur in domains that interfere with E3 substrate binding and may affect SPOP's ability to degrade androgen receptors that contribute to cancer development in prostate cancer (An et al., 2014) and progesterone receptors in breast cancer cells (Gao et al., 2015).

CUL4-Based Ubiquitin Ligase Complexes

Deregulation of CUL4A leads to tumorigenesis in transgenic mice (Jia et al., 2017) and the CUL4A locus is often amplified in many human cancers, including hepatocellular carcinomas, pleural mesotheliomas, breast and prostate cancers, squamous cell carcinoma, adrenocortical carcinoma, medulloblastoma, and ovarian invasive carcinoma (Sharma and Nag, 2014 and references therein). CUL4A overexpression in cancer is associated with tumor size, cell proliferation, migration, invasion, and cancer aggressiveness (Song et al., 2015; Deng et al., 2016; Ren et al., 2016; Jia et al., 2017; Nagel et al., 2017). In addition, CUL4A silencing can inhibit cell proliferation and invasion, and induce cell apoptosis. These processes are concomitant with increased expression of p53 and p27 and decreased expression of the metastasis—associated matrix metalloproteinase MMP-2 (Song et al., 2015). CUL4A involvement in tumorigenesis may be directly linked to its pivotal roles in the degradation of tumor suppressors or proto-oncogenic proteins associated with growth regulation, including p21, p73, p150/Sal2 and RASSF1A, N-and c-Myc and c-Jun (Sharma and Nag, 2014; Song et al., 2015). CUL4A may also play a crucial role in the regulation of PAQR3 (progestin and adipoQ receptor family member III), a newly discovered tumor suppressor that exerts its biological function through negative regulation of the oncogenic Raf/MEK/ERK signaling (Qiao et al., 2015).

Overexpression of CUL4B in several cancers such as lung, colon, pancreatic, esophageal, liver, kidney, bladder, and cervical cancer, generally associated with poor patient prognosis, has been reviewed elsewhere recently (Li and Wang, 2017). The critical role for CUL4B in tumorigenesis can be explained by its pleiotropic roles in cellular mechanisms such as cell cycle progression, DNA damage repair and apoptosis (see Li and Wang, 2017 for more details). In cervical carcinoma, CUL4B expression has been shown to be linked to histological grades with high expression related to tumor size, invasion, and metastasis (Yang et al., 2015; Jia et al., 2017; Li and Wang, 2017).

CUL5-Based Ubiquitin Ligase Complexes

Like the CUL3-BTB complex, CUL5 may be involved in hormone receptor homeostasis. CUL5 overexpression led to decreased cell

proliferation of T47D breast cancer cells (Burnatowska-Hledin et al., 2004) and attenuates estrogen receptor alpha and estrogen-dependent growth in a MAPK-dependent manner (Johnson et al., 2007). Similarly, CUL5 is significantly decreased in endometrial cancers with the most aggressive type of cancer displaying the highest CUL5 reduction (Devor et al., 2016). In concordance, CUL5 overexpression led to significantly slower growth in some endometrial cancer cells. CUL5 expression is negatively regulated by miR-19a and miR-19b (Xu et al., 2012), which are highly expressed in cervical cancer cells and are important determinants of the malignant phenotype in those cells, a phenotype that was suppressed when CUL5 3′UTR was deleted. CUL5 is also a direct target of miR-7 in liver cancer through direct miR-7 binding to the CUL5 3′UTR (Ma et al., 2013). CUL5 silencing by miR-7 led to cell cycle arrest and suppression of colony formation, suggesting that the role of CUL5 downregulation in carcinogenesis could be tissue specific.

CUL7-Based Ubiquitin Ligase Complexes

CUL7 was first identified as a novel antiapoptotic oncogene associated with the regulation of p53 levels (Kim et al., 2007). Breast cancers can overexpress CUL7, leading to p53 downregulation (Guo et al., 2014; Men et al., 2014). CUL7 was also identified as a gene involved in liver carcinogenesis through cirrhosis associated with non-alcoholic fatty liver disease, a disease connected with metabolic syndrome. CUL7 maps to the 6p21.1 amplicon characteristic of this type of liver cancer, suggesting that this particular cancer is driven by the anti-apoptotic effect of increased CUL7 through p53 downregulation (Paradis et al., 2013). CUL7 also promotes epithelial-mesenchymal transformation of liver cancer and its high expression in liver tumors is associated with poor prognosis (Zhang D. H. et al., 2016; An et al., 2017a).

CUL9-Based Ubiquitin Ligase Complexes

CUL9 (formerly known as PARC) is a cytoplasmic, p53-binding protein, and a p53-dependent tumor suppressor in mice (Pei et al., 2011) as well as in murine and human leukemic cells (Seipel et al., 2016; Li and Xiong, 2017). CUL9 deletion-induced tumorigenesis tends to be organ specific since mice lacking CUL9 were shown to develop tumors in sarcoma, lung, liver, and ovary only. CUL9's role in protecting genome integrity and tumor suppression is facilitated by mediating the degradation of survivin and cytochrome C in normal and cancer cells (Gama et al., 2014; Li et al., 2014).

TARGETING CRL COMPLEXES IN CANCER THERAPIES

Since cullins are overexpressed in many cancer types, many novel cancer therapy strategies aim to inhibit cullin-ring ligase activity. MLN4924 (pevonedistat), a selective inhibitor of NEDD8-activating enzyme (NAE) structurally related to adenosine 5′ monophosphate that inhibits cullin NEDDylation and CRLs activity, was first shown to inhibit the growth of human colon tumor xenografts in nude mice (Soucy et al., 2009). In promising experiments, the drug was able to induce rereplication and permanent growth arrest in melanoma cells but not in immortalized non-transformed melanocytes (Benamar et al., 2016). Another NEDDylation inhibitor, TAS4464, is also tested in clinical trials (**Table 2**). NEDDylation inhibitors inactivate CRL E3 ubiquitin ligases and causes the cellular buildup of many substrates involves in different cellular functions (see Oladghaffari et al., 2016 for a review). Most clinical studies involving pevonedistat/MLN4924 or TAS4464 (**Table 2**) are still restricted to phase I and II trials.

As shown in **Table 2**, the first phase I study involving a NEDDylation inhibitor (pevonedistat) investigated both pharmacokinetics and pharmacodynamics in patients with acute myeloid leukemia and myelodysplastic syndromes and demonstrated a modest clinical activity (Swords et al., 2015). Subsequent phase I studies evaluated the use of pevonedistat against relapsed/refractory multiple myeloma or lymphoma (Shah et al., 2016), advanced nonhematologic malignancies (Sarantopoulos et al., 2016), and metastatic melanoma (Bhatia et al., 2016). At the time of writing, there are 11 ongoing clinical trials using MLN4294/pevonedistat targeting both solid tumors (4) and blood cancers (7) (**Table 1** and clinicaltrials.gov). Since MLN4924 sensitizes cancer cells to several chemotherapeutic drugs (reviewed in Oladghaffari et al., 2016), the majority of ongoing trials (10/11) are evaluating pevonedistat in combination with other anti-tumor drugs such as DNA damaging agents such as carboplatin, nucleoside analogs (azacitidine, gemcitabine, decitabine), and tubulin-binding drugs (paclitaxel, vincristine). In many studies, NAE inhibition by pevonedistat was confirmed *in vivo* by the accumulation of cullin-ring ligases substrates, including CDT1 and NRF2 in solid tumors and upregulation of NRF2 gene in blood. In the metastatic melanoma study (Bhatia et al., 2016), an additional panel of NAE-regulated substrates (ATF3, GCLM, GSR, MAG1, NQO1, SLC7A11, SRXN1, TXNRD1) was used to confirm inhibition of NAE in blood and increases in pevonedistat–NEDD8 adducts. CDT1 and NRF2 protein levels were measured in tumor biopsies. In the study related to advanced nonhematologic malignancies, stable disease was observed in 80% of the patients receiving both dexamethasone and pevonedistat, and in 69% of patients receiving pevonedistat alone (Sarantopoulos et al., 2016). In patients with metastatic melanoma (Bhatia et al., 2016), one patient (3%) achieved partial response while 15 patients (48%) showed stable disease.

Several studies have shown the proof of concept by using MLN4924 for increased cancer cell killing by radiation. MLN4924 sensitized head and neck squamous carcinoma cells to ionizing radiation and enhances radiation-induced suppression of xenografts in mice (Vanderdys et al., 2017). MLN4924 also enhanced the susceptibility of nasopharyngeal carcinoma, colorectal, lung, pancreatic, and breast cancer cells to radiation (Oladghaffari et al., 2016, 2017; Wan et al., 2016; Xie et al., 2017). Importantly, MLN4924 was shown to sensitize several types of cancer cells to ionizing radiation with a minimal effect on non-cancerous cells (Wei et al., 2012). Mechanistically, MLN4924-increased radiosensitization may be due to induced G2 cell arrest, apoptosis, delayed DNA repair, and loss of

TABLE 2 | A non-exhaustive list of clinical studies targeting cullin-RING ubiquitin E3 ligases.

Condition	Drug(s)	Measurements	Phase	References or ClinicalTrials.gov identifier
Advanced solid tumors, neoplasms	(14C)-Pevonedistat	1. cumulative excretion of radioactive Pevonedistat in urine and feces/Circulatory and excretory pevonedistat metabolites 2. Report of TEAEs and SAEs	I	NCT03057366
Recurrent AML, therapy-induced AML, untreated or recurrent AML	Pevonedistat plus Decitabine	1. Safety and tolerability of Pevonedistat added to Decitabine 2. MTD of pevonedistat in combination to Decitabine 3. mIR-155 expression, promoter methylation, and mIR-155 target gene expression (SHIP1/PU.1) 4. NF-kappaB expression and enrichment on mIR-155 promoter	I	NCT03009240
Metastatic melanoma	Pevonedistat	1. MTD of 209 mg/m^2 2. Clinical activity: 3% PR, 48% *SD* 3. Pevonedistat plasma concentration increased approximately proportionally with dose from 50 to 278 mg/m^2 after Day 1 intravenous infusion	I	NCT01011530 (*) (Bhatia et al., 2016)
Solid tumors	1. MLN4924 plus Docetaxel 2. MLN4924 plus Docetaxel plus Carboplatin 3. MLN4924 plus Gemcitabine	1. Number of adverse events 2. Time course MLN4924 plasma concentration	I	NCT01862328
Advanced solid tumors	MLN4924 (schedules A and C) MLN4924 + Dexamethasone (Schedule B)	1. MTD of 50 mg/m^2 (schedule A) 50 and 67 mg/m^2 (schedule B and C, respectively) 2. 11/13 patients with > 20% increase in CDT1 and NRF2 CRLs substrates 3. 13/14 patients show NEDD8 adducts in tumor biopsies 4. Clinical activity: 74% SD for schedules B and C	I	NCT00677170 (*) (Sarantopoulos et al., 2016)
AML	MLN4924 plus Azacitidine	1. Safety and tolerability of MLN4924 in combination with Azacitidine 2. Disease response rate 3. 30-day and 60-day mortality rate	I	NCT01814826
Advanced solid tumors	MLN4924 Fluconazole Itraconazole Docetaxel Carboplatin Paclitaxel	1. TEAEs and disease response 2. MLN4924 plasma concentration, blood to plasma ratio. MLN4924 clearance 3. Clinical response	I	NCT02122770
AML, ALL, MDS	MLN4924 Intravenous infusion on days 1, 3, and 5 (schedule A) and 1, 4, 8, and 11 (schedule B)	1. MTD of 59 (Schedule A) and 83 mg/m^2 (Schedule B) 2. Clinical activity: 17% CR/PR (schedule A); 10% CR/PR (schedule B) 3. 32/35 patients with NEDD8 adduct in tumor biopsies 4. Pevonedistat increased within 4–8 h after infusion and returned to baseline within 24 h	I	NCT00911066 (*) (Swords et al., 2015)
Leukemia, MDS, Myeloid, Acute	1. Pevonedistat 2. Pevonedistat plus Azacitidine	1. TEAEs and dose limiting toxicities 2. Overall and complete responses 3. Pevonedistat plasma concentration and clearance	I	NCT02782468

(Continued)

TABLE 2 | Continued

Condition	Drug(s)	Measurements	Phase	References or ClinicalTrials.gov identifier
Relapsed/refractory multiple Myeloma or lymphoma	MLN4924 Intravenous infusion on Week 1, 2, 8, and 9 (schedule A) and 1, 4, 8, and 11 (schedule B)	1. MTD of 110 mg/m^2 (schedule A) and 196 mg/m^2 (schedule B) 2. 11/13 patients with NEDD8 adducts in bone marrow aspirates 3. CDT1 and NRF2 skin and NRF2 mRNA in blood increased in treated patients 4. Clinical activity: 1 patient with PR and 71% SD	I	NCT00722488 (*) (Shah et al., 2016)
Multiple myeloma Non-Hodgkin lymphoma	TAS4464	1. Investigate the safety and tolerability of TAS4464; identify TAS4464 MTD 2. Efficacy of TAS4464, defined as Objective Response Rate (ORR) per IWG criteria (NHL) and IMWG criteria (MM).	I II	NCT02978235
MDS leukemia, CML	1. Azacitidine 2. Azacitidine plus Pevonedistat	1. EVF 2. OS	II	NCT02610777
Non-small cell lung cancer	Pevonedistat plus Docetaxel	1. Response to treatment 2. Median progression free survival time, OS time, and patients who achieve stable disease 3. Toxicities by system organ class	II	NCT03228186
MDS leukemia, CML, AML	1. Azacytidine 2. Azacytidine plus Pevonedistat	1. EVF, OS, partial remission 2. overall response. 6 months and 1 year survival rate	II	NCT02610777
MDS leukemia, CML	1. Azacitidine 2. Azacitidine plus Pevonedistat	1. Overall response and EVF 3. OS 3. Pevonedistat plasma concentration 4. EVF and OS in participants with TP53 mutations or any adverse cytogenetic risk group	III	NCT03268954

EVF, Event-Free Survival; OS, Overall Survival; AML, Acute Myeloid Leukemia; CML, Chronic Myelomonocytic Leukemia. TEAEs, Treatment Emergent Adverse Events; SAEs, Serious Adverse Events; MDS, Myelodysplastic Syndrome; MTD, maximum tolerated dose. CR, complete response; PR, partial response; SD, Stable diseases. (*) Study completed.

radical oxygen species homeostasis (Oladghaffari et al., 2016; Wang et al., 2016). For all these reasons, future clinical trials may expand the use of NAE inhibitors to radiotherapy to treat cancer.

FUTURE DIRECTIONS

Developing a better understanding of the contributions of each Cullin-Ring Ligase complex in cellular homeostasis remains a challenging task. A large portion of the 100,000 different proteins that are present per cell needs to be recycled or eliminated in a timely manner during development or cell cycle progression. The complexity of such a task explains the extreme intricacies of protein degradation, where substrate recognition (or protein modification recognition) is crucial. It also suggests that most of the CRL substrates are yet to be discovered. Future studies are expected to reveal new CRL-interacting factors and new regulatory pathways, and provide further insights into the existence of regulatory crosstalk among the different CRLs. New roles for cullins in carcinogenesis will assuredly emerge in the near future since the relationship between cancer and some cullins (i.e., CUL7 and CUL9) is still a relatively new concept (**Figure 2**).

While drugs that inhibit all CRLs are currently being validated, it is plausible that future drug development will also target individual CRLs or specific CRLs-interacting factors. For example, a specific SKP2 inhibitor that selectively suppresses the CRL1 E3 ligase activity was reported to exhibit anticancer activity against human tumor xenografts in mice (Chan et al., 2013). In another approach, homo-bivalent molecules aiming to target CRL2-VHL induced preferential dimerization and isoform-selective degradation of VHL (Maniaci et al., 2017). Further development of agents that modulate specific interactions with substrate receptors is expected in the future. Of particular interest are CRLs that play regulatory roles in molecular pathways altered in cancer cells, such as components

of chromatin-associated CRLs that modulate DNA replication. Such a development will benefit from further understanding of the interactions that fine-tune DNA replication, such as selective interactions of groups of replication origins with distinct regulatory proteins, including DCAF members of CRL4 (Zhang Y. et al., 2016; Aladjem and Redon, 2017). Future advances will be likely target CRL-mediated pathways that maintain genomic stability by preventing DNA rereplication and modulate the S-phase DNA damage response via protein degradation.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Targeting DNA Replication and Repair for the Development of Novel Therapeutics against Tuberculosis

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Mycobacterium tuberculosis is the etiological agent of tuberculosis (TB), an infectious disease which results in approximately 10 million incident cases and 1.4 million deaths globally each year, making it the leading cause of mortality from infection. An effective frontline combination chemotherapy exists for TB; however, this regimen requires the administration of four drugs in a 2 month long intensive phase followed by a continuation phase of a further 4 months with two of the original drugs, and is only effective for the treatment of drug-sensitive TB. The emergence and global spread of multidrug-resistant (MDR) as well as extensively drug-resistant (XDR) strains of *M. tuberculosis*, and the complications posed by co-infection with the human immunodeficiency virus (HIV) and other co-morbidities such as diabetes, have prompted urgent efforts to develop shorter regimens comprising new compounds with novel mechanisms of action. This demands that researchers re-visit cellular pathways and functions that are essential to *M. tuberculosis* survival and replication in the host but which are inadequately represented amongst the targets of current anti-mycobacterial agents. Here, we consider the DNA replication and repair machinery as a source of new targets for anti-TB drug development. Like most bacteria, *M. tuberculosis* encodes a complex array of proteins which ensure faithful and accurate replication and repair of the chromosomal DNA. Many of these are essential; so, too, are enzymes in the ancillary pathways of nucleotide biosynthesis, salvage, and re-cycling, suggesting the potential to inhibit replication and repair functions at multiple stages. To this end, we provide an update on the state of chemotherapeutic inhibition of DNA synthesis and related pathways in *M. tuberculosis*. Given the established links between genotoxicity and mutagenesis, we also consider the potential implications of targeting DNA metabolic pathways implicated in the development of drug resistance in *M. tuberculosis*, an organism which is unusual in relying exclusively on *de novo* mutations and chromosomal rearrangements for evolution, including the acquisition of drug resistance. In that context, we conclude by discussing the feasibility of targeting mutagenic pathways in an ancillary, "anti-evolution" strategy aimed at protecting existing and future TB drugs.

Keywords: DNA replication, Tuberculosis, bacteria, drug resistance, drug targets

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INTRODUCTION

The Need for New TB Drugs

According to the most recent WHO report, 10.4 million people developed tuberculosis (TB) and 1.8 million died from this disease in 2015 (WHO, 2016), making TB the leading cause of death from an infectious disease. The threat that TB presents to global health has been significantly heightened by the evolution and spread of drug-resistant TB: in 2015, a staggering 480,000 people across the world developed multi-drug resistant (MDR)-TB, defined as TB that is resistant to isoniazid (INH) and rifampicin (RIF), with or without resistance to other first-line anti-tubercular drugs. Of these, 9.5% had extensively drug-resistant (XDR)-TB, which is resistant to INH and RIF (i.e., MDR-TB) in addition to any fluoroquinolone and at least one of the injectable second-line drugs, kanamycin, amikacin, or capreomycin. Unfortunately, this alarming situation has continued to worsen with the ongoing evolution of XDR-TB to forms of the disease that are functionally untreatable with existing antibiotics (Dheda et al., 2014).

Drug-sensitive TB is treated with a standard "short-course" regimen comprising a 2-month intensive phase of treatment with four drugs—INH, RIF, pyrazinamide (PZA), and ethambutol (EMB)—followed by four additional months of treatment with INH and RIF in a continuation phase. Under optimal conditions, this regimen is highly effective at achieving durable cure of drug-sensitive TB. However, non-adherence to this protracted therapeutic regimen is common among TB patients and may result in the emergence of drug resistance through the acquisition of chromosomal mutations in the aetiological agent, *Mycobacterium tuberculosis* (*M. tuberculosis*), leading to prolonged infectiousness and poor treatment outcomes (Dheda et al., 2014). Drug-resistant TB is far more challenging to treat, requiring the administration of combinations of second- and third-line drugs that are more toxic, more expensive, and less efficacious. As a result, this form of the disease is associated with substantial morbidity and mortality, while consuming a disproportionate share of national budgets for TB control in disease-endemic countries—thus compromising TB control programmes (Dheda et al., 2014, 2017).

The need for new TB drugs for the treatment of drug-susceptible as well as drug-resistant TB is therefore clear and urgent. After decades of neglect, a renewed interest in TB drug development in the late 1990s, which coincided with major scientific advances including the completion of the first genome sequence of *M. tuberculosis* (Cole et al., 1998), has resulted in a pipeline populated with new as well as repurposed drugs and drug combinations at various stages of development (http://www.newtbdrugs.org). A number of criteria are being used to guide this process: for example, all new TB drugs should: (i) have novel mechanisms of action to permit their use in the treatment of drug-resistant forms of the disease; (ii) have significant treatment-shortening potential when combined with other agents; (iii) be safe and tolerable; (iv) simplify treatment by reducing the pill burden and dosing frequency; and, (v) be compatible with antiretroviral drugs to enable treatment of patients co-infected with HIV (Zumla et al., 2013, 2014). The ability to meet these criteria is dependent upon the quality of compounds that enter the pipeline at the lead optimization stage. The identification of high-quality leads has, in turn, been critically reliant on harnessing biological insight from studies on *M. tuberculosis* pathogenesis in various models of infection. A major theme emerging from this work is the biological complexity of TB at the level of both host and pathogen, with the genotypic and phenotypic heterogeneity of *M. tuberculosis* posing particularly onerous challenges for new TB drug discovery, as discussed below.

Approaches to TB Drug Discovery

Genome-wide mutagenesis studies in *M. tuberculosis* (Long et al., 2015) have identified genes that are (conditionally) essential for growth and survival of the bacillus *in vitro* (Sassetti et al., 2003; DeJesus et al., 2017), in macrophages (Rengarajan et al., 2005), and in animal models of infection (Sassetti and Rubin, 2003). This information has underpinned target-based drug discovery efforts aimed at crippling essential cellular functions in *M. tuberculosis*. However, as in other areas of antimicrobial drug discovery (Payne et al., 2007), the approach has met with very limited success in the TB field, and has been confounded by a general lack of information about target vulnerability as well as the impact of compound metabolism, permeability, and efflux on efficacy. For this reason, small molecules that potently inhibit *M. tuberculosis* enzymes in biochemical assays have failed to translate into leads with activity against the bacillus *in vitro* and/or *in vivo*. In contrast, phenotypic screening, in which compound libraries are screened for activity against *M. tuberculosis* to identify molecules with whole-cell activity, has been far more successful, and has delivered the clinically approved drugs, bedaquiline (Sirturo) and delamanid (Deltyba), a number of drug candidates that are currently in development (Mdluli et al., 2015; Singh and Mizrahi, 2017)—including griselimycin (Kling et al., 2015), PA-824 (pretomanid) (Stover et al., 2000), PBTZ169 (Makarov et al., 2014), and Q203 (Pethe et al., 2013)—and other promising leads such as the Pks13 inhibitor, TAM16 (Aggarwal et al., 2017). It is worth noting, however, that this approach, too, has its challenges as mechanisms of action (MOA) of potent molecules with whole-cell activity can be difficult to elucidate, thereby complicating the progression of individual compounds or compound series through the pipeline. Importantly, though, there are signs indicating greater integration of the two approaches: on the one hand, target-based whole-cell screening, in which hit identification from phenotypic screening is biased toward prioritized targets and pathways, has begun to gain traction (Abrahams et al., 2012) while, on the other hand, screening collections of whole-cell actives identified by phenotypic approaches against high-value *M. tuberculosis* targets offers the prospect of discovering new drug-target pairs as starting points for hit-to-lead (H2L) programs (Esposito et al., 2017).

Managing Biological Complexity in TB Drug Discovery

Genotypic and phenotypic heterogeneity of *M. tuberculosis* must be taken into account from the earliest stage of TB drug

discovery. Genotypic heterogeneity is managed by screening promising hits for activity against representatives from the major strain lineages of *M. tuberculosis* (Coscolla and Gagneux, 2014) and against panels of drug-resistant strains (e.g., Aggarwal et al., 2017; Blondiaux et al., 2017). The other major mechanism underlying differential drug susceptibility in *M. tuberculosis* is phenotypic antibiotic tolerance (Aldridge et al., 2014; Brauner et al., 2016), which is thought to be the main reason why prolonged TB therapy is required in order to achieve relapse-free cure (Kester and Fortune, 2014; Gold and Nathan, 2017). Antibiotic efficacy can be influenced profoundly by the physiology, metabolic state, and growth rate of the organism, with most TB drugs showing significantly reduced efficacy against *M. tuberculosis* in slow- or non-growing states (Baer et al., 2015). Thus, drugs that target cellular processes required to support bacterial growth tend to have reduced efficacy against slow- or non-growing organisms (Gold and Nathan, 2017). *Mycobacterium tuberculosis* encounters complex, hostile environments during transmission, infection, and disease (Pai et al., 2016). As an exquisitely adapted human pathogen endowed with a rich and highly flexible metabolic repertoire (Baughn and Rhee, 2014; Warner, 2014), the bacillus is able to adapt its physiology and metabolism in response to the conditions encountered during each of these stages. These conditions include intracellular residence in macrophages and other phagocytic cells, exposure to nitrosative and oxidative stress, hypoxia, nutrient deprivation, alterations in carbon source availabilities, and low pH (Baer et al., 2015). In a single patient, therefore, *M. tuberculosis* infection can be characterized by mixed populations of intracellular and extracellular bacilli in a variety of metabolic states and with variable growth rates. This complicates treatment (Dartois and Barry, 2013) and has led to the suggestion that TB should be treated as a polybacterial infection (Evangelopoulos and McHugh, 2015). The problem is further complicated by the impact of lesion heterogeneity on drug pharmacokinetic/pharmacodynamic (PK/PD) parameters (Dartois, 2014). To address this complexity, assays designed to recapitulate at least some of the conditions encountered during infection have been incorporated into drug screening cascades with the aim of identifying "pan-active" compounds with the ability to kill *M. tuberculosis* in as wide a range of metabolic states as possible.

Major Mechanistic Classes of TB Drugs

TB drugs fall into a relatively small number of mechanistic classes. A defining characteristic of the tubercle bacillus is its unusual and highly complex cell envelope, which has a number of distinguishing features including the mycolyl-arabinogalactan-peptidoglycan complex that links the peptidoglycan to the mycobacterial outer membrane. Not surprisingly, a disproportionate number of TB drugs act on biogenesis of the cell envelope; these include INH and ethambutol (EMB), the second-line agent, D-cycloserine, and those that act on the new targets, DprE1 (e.g., PBTZ169) (Makarov et al., 2014), MmpL3 (e.g., BM212 and other chemotypes) (Xu et al., 2017), and Pks13 (TAM16) (Aggarwal et al., 2017). Other drugs target transcription (RIF), protein synthesis (e.g., linezolid), and energy metabolism (bedaquiline, Q203). Furthermore, and consistent with the formidable capacity of *M. tuberculosis* to metabolize xenobiotics (Awasthi and Freundlich, 2017), prodrugs are common in the TB drug arsenal and, for compounds such as PZA, delamanid, and pretomanid, the respective active metabolites have pleiotropic effects on mycobacterial metabolism (Matsumoto et al., 2006; Singh et al., 2008; Anthony et al., 2016).

An important, albeit small, category of TB drugs includes those that target DNA replication. Until recently, these have been limited exclusively to the fluoroquinolones, in particular, moxifloxacin and gatifloxacin, which inhibit DNA gyrase, and are widely used for the treatment of MDR-TB. However, another component of the DNA replication machinery has emerged as an exciting new target for TB drug development through the discovery that griselimycins target the β-clamp protein, DnaN (Kling et al., 2015). In the following sections, we consider the DNA replication and repair pathways of *M. tuberculosis* as potential sources of new targets for TB drug development. This terrain has been extensively reviewed recently, perhaps signaling the increasing appreciation of DNA metabolism as underrepresented among common antibiotic targets. The interested reader is encouraged to consult a number of excellent articles, both specific to *M. tuberculosis* (Plocinska et al., 2017) and of more general interest (Robinson et al., 2012; Sanyal and Doig, 2012; van Eijk et al., 2017).

THE MYCOBACTERIAL DNA REPLICATION MACHINERY

Chromosomal replication in bacteria is performed by a large, multiprotein replisome that ensures coordinated synthesis of the leading and lagging DNA strands with high efficiency and accuracy (Beattie and Reyes-Lamothe, 2015; Yao and O'Donnell, 2016a,b). Broadly, this is accomplished through the concerted action of three catalytic centers: the helicase-primase complex, the core complex, and the clamp loader complex [for comprehensive recent reviews, please refer to Ditse et al. (2017) and Plocinska et al. (2017). The helicase-primase complex comprises the DnaB helicase, which unwinds the two DNA strands, and the DnaG primase, which synthesizes short RNA primers on the lagging strand to initiate replication by the replicative DNA polymerase, Pol IIIα. Two core complexes containing Pol IIIα, the exonuclease subunit, ε, and the small subunit, θ, synthesize the new DNA strand on both leading and lagging strand templates. In elegant *in vivo* studies that were directed by earlier *in vitro* studies by Yao and O'Donnell (Yao and O'Donnell, 2016a,b) and the identification of the β-clamp (O'Donnell and Kuriyan, 2006), Reyes-Lamothe and colleagues demonstrated that these core complexes bind to the torroidal β-clamp proteins that encircle the DNA, providing a tether that enables processive synthesis and dynamic exchange of replisome components (Reyes-Lamothe et al., 2012). A $\tau_3\delta_1\delta'_1\chi_1\psi_1$ clamp-loader complex loads the β-clamp proteins onto newly synthesized RNA primers, with the τ subunits also binding to the Pol IIIα subunits to couple leading and lagging strand biosynthesis, and the χ/ψ subunits guiding single-stranded DNA binding (SSB) proteins onto the DNA lagging strand.

The composition of the replisome is dynamic (Beattie et al., 2017; Lewis et al., 2017) and, as evident from the brief description above, the majority of the constituent proteins perform specialist functions ranging across DNA unwinding, RNA primer synthesis, clamp loading, and DNA synthesis. It is not surprising, therefore, that most of the replisome components are conserved across bacteria (Robinson et al., 2012), including *M. tuberculosis* (Ditse et al., 2017). So, while replisome function has been most thoroughly investigated in organisms such as *E. coli* and *B. subtilis* (Beattie and Reyes-Lamothe, 2015), the resulting models of the bacterial replication machinery are considered readily applicable to less studied systems, such as *M. tuberculosis*, with some notable exceptions (Ditse et al., 2017). For example, there are no clear homologs of several initiation proteins (DnaC, DnaT, PriB, and PriC) in *M. tuberculosis*, neither is there a *holE*-encoded θ subunit, nor *holC*- and *holD*-encoded χ and ψ clamp-loader subunits, respectively. Moreover, recent studies have revealed additional departures of the mycobacterial system from the classic replication models, most notably in demonstrating a dominant role for the PHP domain of the essential Pol IIIα subunit, DnaE1, in proofreading in *M. tuberculosis* (Rock et al., 2015; Gu et al., 2016), as discussed elsewhere (Ditse et al., 2017).

Targeting DNA Replication in *M. tuberculosis*

The *M. tuberculosis* genome comprises approximately 3950 genes (Cole et al., 1998; Wang and Chen, 2013), of which ∼10% (461 genes) are absolutely required for growth and survival of the bacillus under standard aerobic growth conditions *in vitro* (DeJesus et al., 2017). Among the "essential" genes, 15 encode components of the DNA replication machinery; these include the DnaA replication initiator, PriA helicase loader, DnaB helicase, DnaG primase, SSB, clamp loader subunits (τ/γ, δ, δ′), DNA polymerases I and III, DnaN β-clamp, DNA ligase I, and type I and II topoisomerases (Ditse et al., 2017). It is notable that effective inhibitory agents are available for only a small number of these essential mycobacterial proteins (**Table 1**), with DNA gyrase representing the only clinically validated target—of the fluoroquinolones, which are used in treatment of MDR-TB. This implies considerable scope for developing new compounds targeting the other essential DNA replication components, as has been proposed recently for *M. tuberculosis* as well as other bacterial pathogens (Robinson et al., 2012; Sanyal and Doig, 2012; Plocinska et al., 2017; van Eijk et al., 2017). In turn, it also suggests the possible utility in investigating the potential antimycobacterial efficacies of compounds developed for use against homologous DNA replication and repair proteins in other bacteria (**Table 2**).

As applies to antibiotic development in general, overcoming the natural defenses of the target organism—in particular, the permeability barrier presented by the (myco)bacterial cell wall, and the capacity for xenobiotic extrusion via multiple efflux pumps—is often a key challenge, particularly in converting hits from biochemical assays into whole-cell actives. Avoiding compound metabolism (degradation or modification) by the target bacillus or its human host can present an additional obstacle. For DNA replication and repair specifically, the non-availability to date of purified forms of many of the mycobacterial proteins and/or reconstituted complexes has further restricted the number of *in vitro* screens against purified proteins, and has required that researchers rely on homology models developed using template structures from other bacteria. Importantly, this can also complicate any assessment of the druggability and ligandability of the target protein—both key additional factors in determining the success of the antibiotic development process, and which render gene essentiality alone insufficient for target validation (Hopkins and Groom, 2002; Edfeldt et al., 2011). It is pleasing to note, therefore, that several recent successes in expressing different components of the mycobacterial DNA metabolic machinery (Gong et al., 2004; Rock et al., 2015; Gu et al., 2016; Banos-Mateos et al., 2017) suggest this critical roadblock will be overcome shortly.

Targeting DNA and the array of proteins which ensure its replication and maintenance within the cell presents an additional challenge, namely ensuring specificity of the applied drug for its target organism. This can be onerous given that the proteins which interact with and modify this macromolecule have retained many key features and commonalities as they have evolved in different species. For TB, which requires lengthy treatment, the need to avoid toxicity in the human host presents an additional major challenge, and one which is likely to exclude drugs which target DNA directly, such as DNA intercalating agents (Zhang et al., 2017), and inducers of replication stress in mammalian cells (i.e., anticancer compounds). Instead, antitubercular chemotherapies need to be designed to exploit specific nuances of, and vulnerabilities within, the complement of mycobacterial DNA replication and repair proteins (Mizrahi and Huberts, 1996; Rock et al., 2015).

Despite all these challenges, there have been some exciting recent discoveries—for example, the novel DnaN-targeting natural product antibiotic, griselimycin (Kling et al., 2015), and the DnaE inhibitor, nargenicin (Young et al., 2016)—which support the potential for new drug discovery in this area, and also suggest that natural product sources are likely to offer the most promising new agents (Wright, 2017). In the ensuing sections, we discuss the very limited number of validated and experimental anti-TB drugs targeting DNA replication, and provide brief updates on recent progress suggesting the potential to develop additional experimental compounds to inhibit other components of the mycobacterial replication machinery.

Targeting the Mycobacterial Pol III Holoenzyme

Although the MOA of antifolate drugs such as sulfamethoxazole, trimethoprim, and *para*-aminosalicylic acid includes depletion of dNTP pools, preventing DNA replication, the impact of these agents on *M. tuberculosis* is polypharmacologic as it also involves inhibition of RNA and protein synthesis (Minato et al., 2015). Therefore, in its strictest sense, there are no anti-TB drugs in clinical use which directly target the DNA biosynthetic machinery in mycobacteria. That said, a handful of very exciting

TABLE 1 | Essential proteins involved in DNA replication targeted by anti-tubercular compounds.

Name[a]	*In vitro* essentiality	Inhibitor or compound series	Target IC_{50} (μM)	MIC (μM)	References
DnaN/β (Rv0002)	Essential[1,3]	Griselimycins		0.05–0.84	Kling et al., 2015
GyrB (Rv0005)	Essential[1,2]	Novobiocin	1	6.5	Chopra et al., 2012
		Pyrrolamides	<0.5	0.026–1.7	Hameed et al., 2014
		Thiazolopyridine		0.0005	Kale et al., 2013
		Aminopyrazinamides	<0.002–>50	<1.0–>81	Shirude et al., 2013
		Thiazole-aminopiperidine hybrid analogs	50	28.44	Jeankumar et al., 2013
		Methoxyquinolone carboxylic acids	>102.96	0.16–6.43	Senthilkumar et al., 2008
		Benzothiazinone-piperazine derivatives	0.51-26	1.82–52	Chandran et al., 2015
		N-linked aminopiperidines	>3.6	6.2–132	Jeankumar et al., 2014
		Benzofurans	0.81		Renuka et al., 2014
			0.42		Reddy et al., 2014
		Quinoxalines and quinoxaline analogs	12–50		Sipos et al., 2015
		Phenylthiophene carboxamides	>0.76	4.84–78.5	Saxena et al., 2015
		Quinoline–aminopiperidine hybrid analogs	0.62–34.5	1.72–67.94	Medapi et al., 2015a
		7-Methyljuglone	30	2.6	Karkare et al., 2013
		Diospyrin	15	21.4	Karkare et al., 2013
		Indoline-dione Schiff bases	>40		Aboul-Fadl et al., 2011
		4-Aminoquinoline derivatives	0.63–23.92	1.47–49.75	Medapi et al., 2015b
		Thiazolopyridone ureas		0.2–19	Kale et al., 2013, 2014
		7-chloroquinolinyl-piperazinyl-pyridinylmethyl acetamide derivatives	1.82–28.3	7.26–76.55	Jeankumar et al., 2016a
		Benzo-imidazolyl acid derivatives	0.5–25	7.2–64.14	Jeankumar et al., 2016b
		VXc-486		0.28–0.58	Locher et al., 2015
		7-substituted-naphthyridinone derivatives[#]		0.02–0.65	Blanco et al., 2015
GyrA (Rv0006)	Essential[1,2]	Moxifloxacin[#]	11.2	0.31–2.49	Aubry et al., 2004; Sulochana et al., 2005
		Gatifloxacin[#]	7.99	82.58–319.7	Alvirez-Freites et al., 2002; Aubry et al., 2004
		Ofloxacin derivatives[#]	>10	0.47–10	Dinakaran et al., 2008
		Gatifloxacin derivatives[#]	8–26.6	0.033–2.1	Sriram et al., 2006
		Fluoroquinolone DC-159a[#]		0.143	Disratthakit and Doi, 2010
		Acridine derivatives	5.21–33.9	6.46–57.80	Medapi et al., 2016
DnaE1/α (Rv1547)	Essential[1,2]	251D			Butler et al., 2007; Chhabra et al., 2011
DnaG (Rv2343c)	Essential[1,2]	Doxyrubicin (anthracyclines)	100		Kuron et al., 2014; Gajadeera et al., 2015
		Aloe-emodin			Gajadeera et al., 2015
LigA (Rv3014c)	Essential[1,2]	Bis-xylofuranosylated diamines	11.4–260		Srivastava et al., 2005a,b
		N-substituted tetracyclic indoles	13.5		Srivastava et al., 2007
		Pinafide and Mitonafid	>50	>25	Korycka-Machala et al., 2017
		Pyridochromanone	0.6		Gong et al., 2004
TopA/Top I (Rv3646c)	Essential[1,2]	Polyamine scaffolds	5-15		Sandhaus et al., 2016
		Hydroxycamptothecin derivatives	>2.9	5.46–48.36	Sridevi et al., 2015
		Amsacrine and Tryptanthrin	15-42		Sridevi et al., 2015
		m-AMSA		125	Godbole et al., 2014
		Norclomipramine and Imipramin		60–250	Godbole et al., 2015
		Dihydrobenzofuranyl urea	60		Ravishankar et al., 2015

[a]Cole et al. (1998), [1]DeJesus et al. (2017), [2]Griffin et al. (2011), [3]Xu et al. (2014), [#]Elucidation of the targets of DNA gyrase inhibitors is often complex and involves both GyrA and GyrB subunits.

TABLE 2 | Compounds worth investigating that inhibit homologs of essential *Mtb* proteins validated in other bacterial species[a].

Mtb homologue[b]	Annotated function[b]	*In vitro* essentiality	Inhibitor or compound series	Organism	References
DnaA (Rv0001)	Initiation of DNA replication	Essential[1,2]	3-acetoxy-bi-indols	*E. coli*	Mizushima et al., 1996
			Sporulation protein SirA	*B. subtilis*	Rahn-Lee et al., 2011
DnaN (Rv0002)	β subunit of DNA polymerase III	Essential[1,3]	Small-molecule RU7	*S. pyogenes*; *E. coli*	Georgescu et al., 2008
			Toxin-antitoxin SocB	*C. crescentus*	Aakre et al., 2013
GyrB (Rv0005)	DNA gyrase, subunit B	Essential[1,2]	Spiropyrimidinetriones	Various Gram-negative and Gram-positive bacteria	Basarab et al., 2014, 2015
			Quinoline pyrimidine triones	Various Gram-negative and Gram-positive bacteria	Miller et al., 2008
			Isothiazolopyridones	*E. coli*; *S. aureus*	Wiles et al., 2006a
			Isothiazoloquinolones	*E. coli*; *S. aureus*	Wiles et al., 2006b
			3-amino quinazolinediones	Various Gram-negative and Gram-positive bacteria	Tran et al., 2007; Hutchings et al., 2008
			Cyclothialidines	Various Gram-positive bacteria	Angehrn et al., 2004, 2011; Lubbers et al., 2007
			Benzothiazole ethyl urea inhibitors	Various Gram-negative and Gram-positive bacteria	Stokes et al., 2013
			Tricyclic pyrrolopyramidine derivatives	Various Gram-negative and Gram-positive bacteria	Tari et al., 2013a,b
			Indazole derivatives	*S. aureus*; *S. pneumoniae*; *E. faecium*; *E. faecalis*	Zhang et al., 2015
			Benzimidazole ureas	*S. aureus*; *E. faecium*; *S. pneumoniae*; *E. faecalis*	Grillot et al., 2014
GyrA (Rv0006)	DNA gyrase, subunit A	Essential[1,2]	Simocyclinone D8	*E. coli*	Flatman et al., 2005
			Novel bacterial topoisomerase inhibitors	*S. aureus*; *E. coli*	Bax et al., 2010
			NXL101	Gram-positive bacteria	Black et al., 2008
SSB (Rv0054)	Helix-destabilizing protein	Essential[1,3]	Small-molecule inhibitors	*K. pneumonia*	Voter et al., 2017
			SSBA inhibitors	*E. coli; S. aureus; B. anthracis; F. tularensis*	Glanzer et al., 2016
DnaB (Rv0058)	DNA helicase	Essential[1,2]	Coumarin scaffolds	Gram-positive bacteria	Aiello et al., 2009; Li et al., 2012, 2013
			Flavonols	*E. coli*	Griep et al., 2007
				K. pneumoniae	Chen and Huang, 2011; Lin and Huang, 2012
			Triaminotriazines	*S. aureus*	McKay et al., 2006
PriA (Rv1402)	Primosomal helicase	Essential[1]	Kaempferol	*S. aureus*	Huang et al., 2015
			Small-molecule inhibitors	*K. pneumonia*	Voter et al., 2017
DnaE1 (Rv1547)	DNA polymerase III α	Essential[1,2]	Nargenicin	*S. aureus*; *E. coli*	Painter et al., 2015
			6-anilino-pyrimidine-diones	*B. subtilis*	Tarantino et al., 1999a
			Substituted deazaguanines	*B. subtilis*; *S. aureus*	Xu et al., 2011

(Continued)

TABLE 2 | Continued

Mtb homologue[b]	Annotated function[b]	*In vitro* essentiality	Inhibitor or compound series	Organism	References
DnaG (Rv2343c)	Primase	Essential[1,2]	Phenolic monosaccharides	*E. coli*	Hegde et al., 2004
			(p)ppGpp	*E. coli*; *B. subtilis*	Maciąg et al., 2010
			Bicyclic macrolide	*E. coli*	Chu et al., 2003
			Pyrido-thieno-pyrimidines	*E. coli*	Agarwal et al., 2007
			Benzo-pyrimido-furans	*E. coli*	Agarwal et al., 2007
LigA (Rv3014c)	DNA ligase	Essential[1,2]	6-azaindazoles	Gram-positive bacteria	Howard et al., 2013
			Pyridochromanones	*S. aureus*; *E. coli; S. pneumoniae; B. subtilis*	Brotz-Oesterhelt et al., 2003
			Arylamino compounds	*E. coli*; *S. typhimurium*	Ciarrocchi et al., 1999
			Adenosine analogs	Variety of Gram-negative and positive bacteria	Mills et al., 2011; Stokes et al., 2011
			Diamino-dimethylamino-pyrimido-pyrimidine	*S. pneumoniae*; *S. aureus*; *H. influenzae*	Meier et al., 2008
			Aminoalkoxypyrimidine carboxamides	*S. aureus*	Gu et al., 2012
			2-amino-naphthyridine-carboxamides	*S. aureus*; *S. pneumoniae*; *H. influenzae*	Surivet et al., 2012
			4-aminopyrido-pyrimidin-ones	*S. aureus*; *S. pneumoniae*; *H. influenzae*	Wang et al., 2012
			Adenine-based inhibitors	*S. pneumoniae; H. influenzae*	Buurman et al., 2012

B. anthracis, Bacillus anthracis; B. subtilis, Bacillus subtilis; C. crescentus, Caulobacter crescentus; E. faecium, Enterococcus faecium; E. faecalis, Enterococcus faecalis; E. coli, Escherichia coli; F. tularensis, Francisella tularensis; H. influenzae, Haemophilus influenzae; H. pylori, Helicobacter pylori; K. pneumoniae, Klebsiella pneumoniae; S. aureus, Staphylococcus aureus; S. pneumonia, Streptococcus pneumoniae; S. pyogenes, Streptococcus pyogenes; S. typhimurium, Salmonella typhimurium.
[a]Inhibition of either purified protein or bacterial growth; [b]Cole et al. (1998); [1]DeJesus et al. (2017), [2]Griffin et al. (2011), and [3]Xu et al. (2014).

recent studies have established the utility of a number of compounds that prevent DNA synthesis by targeting novel Pol III holoenzyme components in *M. tuberculosis*.

Targeting the β Clamp, DnaN

Together with the *dnaN*-encoded β clamp, the Pol III* core complex (comprising α and ε subunits only, as *M. tuberculosis* lacks θ) and the clamp loader complex (τ/γ, δ, δ′) form the Pol III holoenzyme (Ditse et al., 2017). Griselimycin, a cyclic peptide antibiotic produced by *Streptomyces* spp., was originally discovered 50 years ago, but was abandoned owing to its unfavorable pharmacologic profile and the availability of other drugs such as RIF (Herrmann et al., 2017). Resurgent interest in neglected antibiotics led a team of investigators from Sanofi and Helmholtz Institute for Pharmaceutical Research Saarland to revisit this compound (Kling et al., 2015) as part of a so-called "rekindling" strategy (Herrmann et al., 2017) to identify potential anti-TB agents. In MOA studies, it was discovered that griselimycin and its metabolically more stable derivative, cyclohexylgriselimycin, bound with very high affinity (equilibrium dissociation constants of 1.0×10^{-10} and 2.0×10^{-10}, respectively) to the *dnaN*-encoded β sliding clamp of *M. tuberculosis*. Importantly, the contrastingly poor binding of these compounds to the human DNA clamp protein, PNCA, results in a very high selectivity index, eliminating any concerns of general cytotoxicity.

X-ray crystallography revealed that griselimycin preferentially binds within a hydrophobic pocket located between domains II and III of DnaN—a target site known to be involved in protein-protein interactions between the β_2 sliding clamp and other DNA replication and repair proteins such as the Pol IIIα replicative polymerase subunit. As such, griselimycin functions as a protein-protein interaction inhibitor and, notably, is bactericidal against mycobacteria. Moreover, resistance is rare (resistant mutants are identified at a frequency of $\sim 5 \times 10^{-10}$) and incurs a very severe fitness cost: in the non-pathogenic *M. smegmatis* as well as *M. tuberculosis*, griselimycin resistance was shown to depend on sequential amplification of the genomic region containing *dnaN* and the mycobacterial origin of replication (*ori*) site. Perhaps unsurprisingly, this resulted in a severe (slow) growth defect *in vitro*, and did not confer cross-resistance to other antibiotics.

From a drug development perspective, the addition of a cyclohexyl group to Proline-8 in the griselimycin backbone resulted in greater metabolic stability as well as increased lipophilicity, in turn increasing the antimycobacterial potency significantly from an initial minimum inhibitory concentration (MIC) of 1.0 μg/ml for the parental compound to 0.06 μg/ml in the derivative, all under aerobic conditions *in vitro*. The compound was also highly active against intracellular *M. tuberculosis* within macrophages, and in a mouse model—both as a single drug and in combination with the first-line drugs, RIF and PZA. These observations support the potential utility

of griselimycin derivatives as anti-TB compounds, possibly to shorten therapeutic duration—though it should be noted that, under anaerobic conditions, the compound exhibited a 100-fold increase in MIC, a result which may have implications for its efficacy as a sterilizing drug. Nevertheless, griselimycin remains an exciting prospect, and is undergoing lead optimization by Sanofi and the TB Alliance (https://www.tballiance.org/portfolio/compound/cyclopeptides). Of further interest, very elegant recent work elucidating the pathway for griselimycin biosynthesis in the producer organism, *Streptomyces* DSM 40835, suggests the feasibility of rational modifications to the core pharmacophore (Lukat et al., 2017), thereby overcoming a common stumbling block in natural product drug development.

Targeting the Clamp Loader Complex

Mycobacterium tuberculosis possesses a restricted set of four clamp loader subunits: τ/γ, encoded by *dnaX* (though it must be noted that the alternative gene product, γ, has not been observed in mycobacteria), and the δ and δ' ATPases, encoded by *holA* and *holB*, respectively (Ditse et al., 2017). Consistent with their role in loading the β clamp and co-ordinating leading and lagging strand synthesis, all four subunits are essential in *M. tuberculosis* (DeJesus et al., 2017); however, aside from a number of studies which have identified these components as potentially attractive targets for novel antimycobacterial agents (Anishetty et al., 2005; Kinnings et al., 2010; Xu et al., 2014), there are no reports of any experimental approaches to this effect (Plocinska et al., 2017). For this reason, these proteins are included in the small set of "non-validated, essential targets" identified as worthy of future investigation (**Table 3**).

Targeting the Pol IIIα Subunit, DnaE1

Mycobacterium tuberculosis encodes a single DNA Pol IIIα subunit, DnaE1, which is essential for chromosomal replication (Boshoff et al., 2003) and, therefore, a potentially attractive target for TB drug discovery (Banos-Mateos et al., 2017). Despite the fact that RNA polymerase represents a very successful therapeutic target in *M. tuberculosis* (Koch et al., 2014) and other pathogens (Ma et al., 2016), and that DNA polymerases have been exploited as therapeutic targets for both anti-viral and anti-cancer drugs (Lange et al., 2011), the number of compounds with demonstrated activity against bacterial replicative polymerases is very low and reduces even further when demonstrated whole-cell activity is applied as a filter (Robinson et al., 2012; van Eijk et al., 2017). There are several classes of compound known to inhibit the PolC-type polymerases: the 6-anilinouracils, which are competitive inhibitors of dGTP binding (Tarantino et al., 1999b; Wright et al., 2005); the guanine inhibitors, which are similar to the 6-anilinouracils in functioning as competitive inhibitors, but which target both PolC and DnaE (Wright et al., 2005; Xu et al., 2011); the non-nucleobase inhibitors, which include the anilinopyrimidinediones (such as 6-anilinouracils, competitive inhibitors of dGTP) (Rose et al., 2006) and the quinazolin-2-ylamino-quinazolin-4-ols (or BisQuinols), whose precise MOA remains to be elucidated but appears to involve competitive binding with the DNA template (Guiles et al., 2009); and, finally, the very recently described dicoumarin, 3,3′-(4-Nitrobenzylidene)-bis-(4-hydroxycoumarin) (Hou et al., 2015). In contrast, finding DnaE1-specific inhibitors has proved much more challenging, with some encouraging exceptions.

Very recent work has identified another natural product, nargenicin A1, as a putative DnaE1 inhibitor (Painter et al., 2015). This compound, a macrolide produced by *Nocardia* sp. ACC18, was shown to be active against both *E. coli* and *S. aureus in vitro* and, importantly, was effective against *S. aureus* in two separate mouse infection models. In *S. aureus*, spontaneous resistance was observed at a very low frequency ($\sim 10^{-9}$), and mapped to *dnaE*. This observation—in combination with *in Vitro* data which confirmed that nargenicin binds to the *S. aureus* DnaE protein in the presence of DNA, thereby inhibiting DNA replication—identified the replicative polymerase as the likely molecular target (Painter et al., 2015). However, the MOA remains to be elucidated definitively: the sole SNP in *dnaE* was not located in the DnaE active site, moreover nargenicin-resistant mutants displayed only low-level resistance (∼4-fold over MIC). Although limited literature are available to support the potential antimycobacterial utility of nargenicin, a patent lodged by Merck claims that the compound is bactericidal against *M. tuberculosis* and, on that basis, under development as potential anti-TB agent (Young et al., 2016). It is assumed, therefore, that ongoing work aims to determine whether DnaE1 is the molecular target in *M. tuberculosis* and, furthermore, whether the bacillus is able to develop resistance—and at what cost to replicative fitness.

Compound 251D, a hybrid molecule comprising 6-(3-ethyl-4-methylanilino)uracil and fluoroquinolone moieties is another bacterial Pol IIIα inhibitor that has been identified as worthy of investigation as a potential anti-mycobacterial agent. Whether it will prove efficacious though is unclear: the target of 251D is the PolC-type replicative polymerase (Butler et al., 2007), most commonly found in low-GC Gram-positive bacteria (Timinskas et al., 2014). As noted above, *M. tuberculosis* encodes only the *dnaE*-type, which is found in both Gram-positive and Gram-negatives. Therefore, while bioinformatic analyses have predicted that the compound might be capable of docking with DnaE1, these studies utilized a model based on the replicative subunit from the Gram-negative *Thermus aquaticus* (Chhabra et al., 2011); inhibition of the mycobacterial DnaE1 *in vitro* is still to be demonstrated, so too is the activity in whole-cell assays. As noted elsewhere (Plocinska et al., 2017), an attraction of this type of hybrid compound is the potential to target both DNA gyrase and Pol IIIα with a single molecule, thereby limiting the potential for resistance development in drug-susceptible cases and retaining activity against fluoroquinolone-resistant isolates in drug-resistant TB.

Targeting the Mycobacterial Primosome

Together with nine other proteins (namely, Pol IIIα, the β_2 sliding clamp, ε proofreading subunit, τ, δ, and δ', DnaA, DNA ligase, and Pol I), the DnaB helicase, DnaG primase, and SSB constitute the basic replication module that is found across almost all sequenced bacterial genomes (McHenry, 2011; Robinson et al., 2012). DnaB and DnaG form the helicase-primase complex which, in combination with the PriA helicase

TABLE 3 | Potential, non-validated, essential *Mtb* targets involved in DNA replication.

Name	Encoding gene[a]	*In vitro* essentiality	Comment	References
PolA/Pol I	*Rv1629*	ED[1]/E[2]	Only the 5′-3′ exonuclease domain is essential; the polymerase domain is dispensable in *Mycobacterium smegmatis* and yields a phenotype of DNA damage hypersensitivity. The exonuclease domain is unable to discriminate against dideoxynucleotide 5′-triphosphates and can be inhibited by chain-terminating nucleotide analogs during DNA synthesis.	Gordhan et al., 1996; Mizrahi and Huberts, 1996
RecO	*Rv2362c*	ED[1]	Involved in DNA repair and RecF-dependent recombination; functions to assemble and disassemble RecA filaments at single-stranded gaps	Mizrahi and Andersen, 1998; Singh et al., 2016
HolA	*Rv2413c*	E[1,3]	Putative DNA polymerase III δ subunit	
UvrD2	*Rv3198c*	ED[1]/E[2]	Component of nucleotide excision repair and methyl-directed mismatch repair; possesses an essential DNA-dependent ATPase activity linked to DNA translocation and protein displacement, as well as a dispensable helicase activity	Kazarian et al., 2010; Williams et al., 2011
DnaZX	*Rv3721c*	E[1,2]	Putative DNA polymerase III τ and γ subunits	

E, Essential; ED, Essential domain.
[a]Cole et al. (1998); [1]DeJesus et al. (2017); [2]Griffin et al. (2011); [3]Xu et al. (2014).

loader, functions as the mycobacterial primosome: there are no identifiable mycobacterial homologs of DnaC, DnaT, PriB, or PriC. As core proteins, these represent compelling drug targets and, while much further work is required, some recent progress (summarized below) suggests that a validated clinical candidate targeting different components of the primosome is a genuine possibility.

Within any cell, ssDNA generated during DNA replication (as well as other processes, including exposure to genotoxic stress) is vulnerable to damage and prone to form secondary structures that can restrict DNA metabolic processes with potentially lethal consequences. SSB proteins have evolved to protect ssDNA, and so are essential to bacillary viability during normal replication as well as under DNA damaging conditions. Several recent high-throughput screens have been successful in identifying small-molecule inhibitors of SSB-protein interactions (Lu et al., 2010; Marceau et al., 2013; Glanzer et al., 2016). These include an attempt to identify inhibitors of SSB that might disrupt both DNA replication and SOS-mediated resistance pathways within Gram-positive and Gram-negative bacteria (Glanzer et al., 2016): following *in vitro* screening, six molecules were identified which successfully inhibited a broad range of bacterial SSBs, with a further four exhibiting species-specific activity—thereby establishing the potential for both broad-spectrum and species-targeted use. Notably, five of the six compounds were found to have whole-cell activity against a variety of the tested species, of which a single compound, 9-hydroxyphenylfluoron, was associated with minimal activity against the human SSB homolog. While the potential utility of this approach remains to be determined for *M. tuberculosis*, these results suggest the value of investigating SSB as novel anti-mycobacterial target.

An analogous approach sought to identify compounds that specifically target the eight highly-conserved residues at the C-terminus of *Klebsiella pneumonia* SSB with the objective of inhibiting interactions between SSB and other proteins (Voter et al., 2017). Using the interaction between SSB and the essential protein helicase, PriA, as basis for a high-throughput screen of more than 72,000 compounds, this study aimed to identify small molecules capable of inhibiting SSB interactions. Seven SSB-PriA interaction inhibitors were found to bind to SSB, with a further two binding PriA, all with IC_{50} values below 40 μM. No data were presented on the activity (or lack thereof) of these compounds in whole-cell assays; however, this work reinforces a common theme which suggests that protein-protein interaction inhibitors may be of specific value in inhibiting the large complex of proteins which enables DNA replication. In a similar vein, two potential PriA inhibitors, kaempferol and myricetin, were shown to inhibit the ATP hydrolysis activity of *S. aureus* PriA *in vitro* (Huang et al., 2015). While these compounds were also not validated in whole-cell assays, they too represent encouraging steps in the effort to identify antibiotics that target primosome proteins and, importantly, provide useful insight into the isolation of tractable pharmacophores for optimization against the mycobacterial homologs as part of rational structure-activity relationship (SAR) efforts.

The DnaG primase synthesizes primers for lagging strand Okazaki fragments. An early study investigating plant-derived natural products discovered two phenolic monosaccharides from *Polygonum cuspidatum* with low micromolar IC_{50} values against *E. coli* DnaG (Hegde et al., 2004). Similarly, another molecule from *Penicillium verrucosum* was shown to inhibit *E. coli* primase activity in biochemical assays (Chu et al., 2003). However, whole-cell activity was attainable only in a mutant *E. coli* strain deficient in the lipopolysaccharide layer of the cell wall as well as the AcrAB efflux system, reinforcing the potential obstacles associated with permeation and efflux as part of antibiotic discovery. This is echoed in another study which identified two classes of compounds with efficacy against *E. coli* DnaG *in vitro* and in efflux pump-deficient whole-cell assays (Agarwal et al., 2007). In that case, *in vitro* and whole-cell activity analyses of related pyrido-thieno-pyrimidines and benzo-pyrimido-furans identified numerous hits with attractive IC_{50} and MIC values, again suggesting the potential to identify novel primase inhibitors as possible anti-mycobacterial agents. In this context, it is worth noting the "natural validation" of DnaG as a suitable target for inhibiting replication: in many

bacteria including *M. tuberculosis*, endogenous production of guanosine tetra- and penta-phosphate, (p)ppGpp, as part of the stringent response prevents the function of the replicative primase, curtailing bacterial growth (Maciąg et al., 2010).

The interaction of primase with the ssDNA template is facilitated by the replicative DNA helicase encoded by DnaB, another essential protein in *M. tuberculosis* (Sassetti et al., 2003; DeJesus et al., 2017). A number of flavonols have been shown to inhibit DnaB function in other bacteria (Griep et al., 2007; Lin and Huang, 2012), though no reports exist regarding the activity of these (or other) compounds in *M. tuberculosis*. Unusually, DnaB is among five mycobacterial proteins that contain inteins, two others of which are also involved in DNA replication: GyrA and RecA. This observation recently prompted the interesting proposal from Dziadek and colleagues (Plocinska et al., 2017) to block the protein splicing machinery as part of a polypharmacologic approach that would prevent activation of these intein-containing proteins, potentially disrupting multiple pathways simultaneously.

Targeting DNA Unwinding: DNA Gyrase and DNA Topoisomerase

Replication of the chromosomal DNA requires controlled alterations of the DNA topology to ensure processive synthesis while limiting the stresses imposed by negative supercoiling and concatenation of the double-stranded DNA molecule. The type II topoisomerase, DNA gyrase, functions to relieve torsional strain by introducing transient double-strand DNA (dsDNA) breaks which generate negative supercoils in the bacterial chromosome. Unlike those bacteria which rely on two type II topoisomerase enzymes—DNA gyrase and TopoIV—to accomplish these tasks, *M. tuberculosis* employs only a $GyrA_2B_2$ gyrase comprising *gyrA*-encoded supercoiling subunits and *gyrB*-encoded ATPase proteins. As a drug target, DNA gyrase represents one of the most successful in antibiotic history, primarily of the fluoroquinolones which have been used to treat both Gram-negative and Gram-positive bacterial pathogens. A series of chemical scaffolds has been employed in developing successive generations of fluoroquinolones, all of which function as topoisomerase II poisons, stabilizing the cleaved DNA-topoisomerase II complex and so resulting in a large number of double-stranded DNA breaks within the replicating bacillus which are thought to overwhelm the repair machinery, triggering a cascade of events that results in bacterial death (Dwyer et al., 2015). Fluoroquinolones are currently used as second-line anti-TB agents; however, the imperative to reduce the duration of therapy has seen several large clinical trials of novel combination regimens comprising a fluoroquinolone as frontline agent (Gillespie et al., 2014; Jindani et al., 2014; Merle et al., 2014). Although unsuccessful, these trials yielded valuable lessons about the types of preclinical data which might better inform the design of new therapies (Warner and Mizrahi, 2014), as well as the potentially critical role of drug distribution and lesion penetration in ensuring efficacy (Prideaux et al., 2015).

Other classes of gyrase inhibitors include the aminocoumarins, such as novobiocin, which were the first of many natural products found to act as gyrase inhibitors (Barreiro and Ullán, 2016). Since these compounds preferentially inhibit ATPase (GyrB) function (Lewis et al., 1996), they are less vulnerable to pre-existing resistance against the fluoroquinolones, which generally maps to mutations in *gyrA* (Chopra et al., 2012). Moreover, in contrast to the fluoroquinolones which result in dsDNA breaks and so upregulate the mycobacterial DNA damage response (Gillespie et al., 2005), the risk of aminocoumarin-induced mutagenesis is likely to be lower, especially in *M. tuberculosis* in which exposure to novobiocin does not trigger expression of the SOS regulon (Boshoff et al., 2004). However, the relatively poor penetration of aminocoumarins across cell membranes, their limited solubility, and the development of the synthetic fluoroquinolones have limited the clinical utility of this compound class (Barreiro and Ullán, 2016). In addition, issues with cytotoxicity remain a major hurdle, particularly for TB which requires extended therapeutic duration. Recent progress in the development of novel bacterial topoisomerase inhibitors (NBTIs) targeting DNA gyrase (Grillot et al., 2014; Blanco et al., 2015; Jeankumar et al., 2015a,b; Locher et al., 2015) nevertheless suggests that alternatives to the fluoroquinolones might become available in the future.

In contrast to the Type II enzymes, Type I topoisomerases have been very sparsely explored for antibiotic drug discovery. These enzymes, which cause single-stranded nicks in relaxing the DNA, perform an essential function in remodeling the chromosome for various processes including DNA replication and recombination, RNA transcription, and condensation and therefore represent an attractive target (Tse-Dinh, 2016). For this reason, Sridevi et al. conducted virtual screens of two chemical libraries for the capacity to dock with *M. tuberculosis* TopA (Sridevi et al., 2015). Subsequent *in vitro* verification of the putative hit compounds identified three with activity against purified TopA: amasacrine, tryptanthrin, and hydroxycamptothecin, a derivative of the anticancer topoisomerase inhibitor camptothecin (Wall et al., 1966). The latter hit compound was subsequently modified with terminal hydrophobic moieties to yield a library of fifteen 7-ethyl-10-hydroxycomptothecin derivatives which exhibited activity against both drug-susceptible and XDR *M. tuberculosis*, with MICs as low as 5.92 and 2.95 μM, respectively—a significant improvement over previous TopA inhibitors (Godbole et al., 2014, 2015). Moreover, the XDR isolates exhibited enhanced susceptibility to five of the hydroxycamptothecin derivatives relative to the drug-susceptible strains, suggesting that this might offer an attractive target in these otherwise highly resistant forms. Furthermore, four hydroxycamptothecin derivatives were identified to be more effective at inhibiting the resuscitation of non-replicating persisters in both nutrient starvation as well as oxidative and nitrosative stress models. These results, together with compounds identified in other studies and which still require validation in whole-cell assays (Ravishankar et al., 2015; Sandhaus et al., 2016), highlight the possibility of successfully and specifically inhibiting TopA as a novel therapeutic target for drug-susceptible and drug-resistant TB.

TARGETING OTHER FUNCTIONS IN CHROMOSOMAL REPLICATION

During replication, Okazaki fragments are generated which must be joined together by the bacterial NAD^+-dependent DNA ligase. The enzyme is therefore essential, making it a highly attractive target for drug development. Inhibition of purified *M. tuberculosis* LigA has been reported numerous times (Gong et al., 2004; Srivastava et al., 2005a,b); however, very few compounds have been shown to exhibit whole-cell, micromolar-range activity against *M. tuberculosis*. Following the high-throughput, *in silico* screening of potential LigA inhibitors, Korycka-Machala et al. identified pinafide and mitonafide as attractive inhibitors of *Mtb* growth *in vitro* (Korycka-Machala et al., 2017). Both compounds exhibited an MIC of 25 μM in 7H9 liquid media and half maximal inhibitory concentration (IC_{50}) of 50 μM. In addition, the *in vitro* analysis of LigA inhibition suggested that the two compounds failed to inhibit T_4 ATP-dependent DNA ligase effectively and, therefore, had specificity for NAD^+-dependent DNA ligase, which is not utilized by eukaryotes. Although preliminary, these results hold great promise for the development of similar compounds or analogs capable of inhibiting *Mtb* growth at low-micromolar concentrations *in vivo* through the inhibition of LigA.

Proof-of-concept Targets from Other Bacterial Systems

Sporulation Protein SirA and the SocB Toxin

Further insight into the inhibition of essential DNA replicative pathways can be obtained from natural phenomena which characterize normal bacterial physiology. Both DnaA and DnaN have been shown to be inhibited by bacterial-derived molecules, including a sporulation protein in *B. subtilis* and a toxin-antitoxin (TA) system found in *Caulobacter crescentus*. In the first example, the interaction of the sporulation protein SirA with domain I of DnaA prevents the replication initiator protein from binding to the origin of replication during the start of sporulation of *B. subtilis*, effectively inhibiting DNA replication initiation (Rahn-Lee et al., 2011). As noted above, DnaA is essential for initiation of DNA replication, with domain I of DnaA being required for interactions between DnaA monomers and other proteins, such as the essential *dnaB*-encoded helicase (Seitz et al., 2000; Abe et al., 2007). This domain of DnaA is thus an attractive target for therapeutic intervention.

In a further example, the toxin component of the atypical SocAB TA system in *C. crescentus* was found to inhibit DNA elongation through an interaction with the *dnaN*-encoded β sliding clamp (Aakre et al., 2013). Notably, mutations conferring resistance to SocB mapped to the hydrophobic Pol III-binding domain of DnaN, indicating a similar binding site to the previously mentioned antibiotic, griselimycin. The mechanism of resistance is different, though, and so cross-resistance is unlikely. In summary, these examples further validate the inhibition of novel DNA replication components and can potentially be used as a basis for the rational design of synthetic inhibitors against *M. tuberculosis* DnaA and DnaN in the future.

OTHER MYCOBACTERIAL DNA REPLICATION AND REPAIR FUNCTIONS

In addition to the specialist DNA replication proteins detailed above (and see **Figure 1**), *M. tuberculosis* encodes a number of other DNA metabolic functions which are essential for cellular viability. For some of these, the potential to yield novel drugs and drug targets is compelling, and includes pathways and enzymes required for *de novo* synthesis, salvage, and recycling of dNTPs for incorporation in newly synthesized DNA, as well as during repair. A detailed discussion is beyond the scope of this review, however some examples include the mycobacterial ribonucleotide reductase (Nurbo et al., 2013; Bueno et al., 2014; Karlsson et al., 2015), thymidylate synthase (Kogler et al., 2011; Fivian-Hughes et al., 2012; Singh et al., 2015), and inosine monophosphate dehydrogenase (Park et al., 2017; Singh et al., 2017) enzymes. Notably, the roles of these and other related proteins in maintaining nucleotide homeostasis within the mycobacterial cell suggests the potential to inhibit replication and repair functions at multiple stages and, moreover, raises the possibility of disrupting indirectly other macromolecular pathways such as RNA transcription and cell wall biosynthesis owing to their convergence on many common metabolic precursors and intermediates (Singh et al., 2015, 2017).

A deeper analysis of other specialist DNA replication and repair functions reveals several more candidates such as Pol I, RecO, and UvrD2, all of which either are essential or contain essential domains (**Table 3**; DeJesus et al., 2017). For some of these, their perceived potential as novel drug targets requires further validation. PolA/Pol I is a DNA-dependent polymerase that possesses bi-directional exonuclease activity. Previous work identified the 5′-3′ exonuclease domain of PolA as essential to the growth of *M. tuberculosis*; furthermore, while the polymerase domain was shown to be dispensable in *M. smegmatis*, deficiency in this function was associated with DNA damage hypersensitivity (Gordhan et al., 1996;

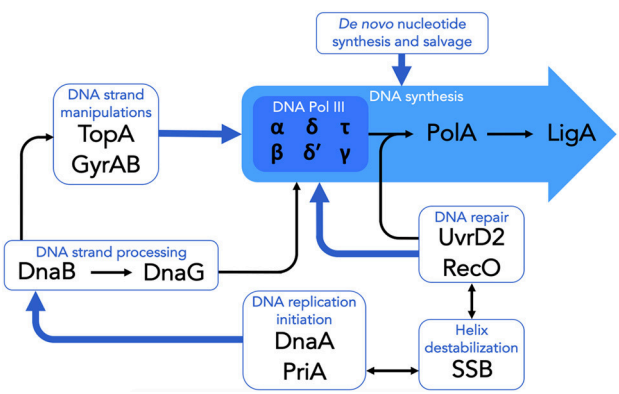

FIGURE 1 | Essential components of DNA replication and repair in *M. tuberculosis*. The schematic highlights the essential DNA replication and repair functions which are targeted by existing clinical or experimental drugs, as well as those which have been identified as potential targets for the development of novel antimycobacterial compounds. See text for details.

Mizrahi and Huberts, 1996). Importantly, the exonuclease domain was determined incapable of discriminating against dideoxynucleotide 5′-triphosphates, and could be inhibited by chain-terminating nucleotide analogs during DNA synthesis, suggesting the druggability of the target (Mizrahi and Huberts, 1996). Moreover, the involvement of Pol I in DNA damage tolerance has identified this protein as potential target for antimutagenesis agents (Plocinska et al., 2017), as explored further below.

Mycobacterium tuberculosis Rv2362c exhibits 28% identity with *S. typhimurium* RecO (Mizrahi and Andersen, 1998), a protein required in DNA repair and RecF-dependent recombination and which functions to assemble and disassemble RecA filaments at single-stranded gaps. Recently, it was reported that *Rv2362c* contains a domain that is essential for *M. tuberculosis* growth (DeJesus et al., 2017), indicating potential of targeting the under-investigated protein with therapeutic compounds in the future. Similarly, UvrD2—a component of nucleotide excision repair and methyl-directed mismatch repair pathways—is another mycobacterial protein containing an essential DNA-dependent ATPase activity implicated in DNA translocation and protein displacement, as well as a dispensable helicase activity (Kazarian et al., 2010; Williams et al., 2011). Although no compounds have been reported to inhibit either of these proteins, their implication in essential replication functions appears to warrant further investigation.

Targeting Mutagenesis

The notion of developing "anti-evolution" drugs to prevent the function of mutagenic repair pathways in *M. tuberculosis* has been discussed previously (Warner, 2010). This strategy seems likely to be especially appropriate for *M. tuberculosis* as adaptive evolution of this organism depends solely on chromosomal rearrangements and point mutations, and all drug resistance arises through spontaneous mutations in target or complementary genes (Galagan, 2014). These factors suggest that inducible mutagenic mechanisms—such as the *imuA'-imuB/dnaE2* mycobacterial mutasome (Warner et al., 2010)—might drive the evolution of *M. tuberculosis* within its host. The limited distribution of ImuA′ and ImuB among sequenced bacterial genomes therefore identifies the mutasome as a compelling target for limiting drug resistance. In some ways, this strategy is analogous to targeting virulence factors (Liu et al., 2008) and assumes that the selective pressure to mutate to antibiotic resistance is not as great where the pathway is essential for pathogenesis but not survival (Clatworthy et al., 2007). Moreover, inhibiting mutagenesis should be effective in immune compromised individuals, and might facilitate clinical trials by identifying compounds that could supplement existing regimens without compromising efficacy.

To this end, several approaches appear worth pursuing: in the first, recent evidence suggests that selective inhibition of DnaE2 by anilinouracils might be possible (Jadaun et al., 2015), provided structural data are available to enable rational identification of compounds which target this alternative α subunit, and not the replicative DnaE1, the structure of which was recently elucidated (Banos-Mateos et al., 2017). For this reason, nargenicin may not be appropriate, though dual targeting of both DnaE proteins might nevertheless represent a profitable strategy. Secondly, targeting the PHP domain exonuclease of DnaE1 provides another attractive option as inactivation of this domain was found to render *M. smegmatis* hypersensitive to the chain-terminating adenosine analog, ara-A (Rock et al., 2015). The recent determination of the structure of the PHP domain, which lacks a human homolog, has created an opportunity for structure-guided design of inhibitors against this exonuclease (Banos-Mateos et al., 2017). In the third approach, the identification of griselimycin supports the potential for developing novel protein-protein interaction inhibitors designed to disrupt mutasome function. Further work is underway in our laboratory to elucidate the molecular interactions which are essential to DnaE2-dependent mutagenesis, with genetic evidence indicating that preventing ImuB from functioning as "hub" protein might collapse this pathway (Warner et al., 2010). In conclusion, the possibility of targeting replication and repair mechanisms implicated in the evolution of drug resistance seems a challenge worth tackling: if successful, it is proposed that these compounds might be co-administered with other agents in novel combination therapies designed to protect existing antibiotics.

AUTHOR CONTRIBTIONS

MAR produced the tables and figure, contributed to the main text, and edited the manuscript; DFW co-developed the outline, wrote the main text, and edited the manuscript; and VM developed the outline, wrote the introduction and edited the manuscript.

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