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Recognition and repair of chemically heterogeneous structures at DNA ends

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Abstract

Exposure to environmental toxicants and stressors, radiation, pharmaceutical drugs, inflammation, cellular respiration, and routine DNA metabolism all lead to the production of cytotoxic DNA strand breaks. Akin to splintered wood, DNA breaks are not “clean”. Rather, DNA breaks typically lack DNA 5'-phosphate and 3'-hydroxyl moieties required for DNA synthesis and DNA ligation. Failure to resolve damage at DNA ends can lead to abnormal DNA replication and repair, and is associated with genomic instability, mutagenesis, neurological disease, ageing and carcinogenesis. An array of chemically heterogeneous DNA termini arises from spontaneously generated DNA single-strand and double-strand breaks (SSBs and DSBs), and also from normal and/or inappropriate DNA metabolism by DNA polymerases, DNA ligases and topoisomerases. As a front line of defense to these genotoxic insults, eukaryotic cells have accrued an arsenal of enzymatic first responders that bind and protect damaged DNA termini, and enzymatically tailor DNA ends for DNA repair synthesis and ligation. These nucleic acid transactions employ direct damage reversal enzymes including Aprataxin (APTX), Polynucleotide kinase phosphatase (PNK), the tyrosyl DNA phosphodiesterases (TDP1 and TDP2), the Ku70/80 complex and DNA polymerase β (POL β). Nucleolytic processing enzymes such as the MRE11/RAD50/NBS1/CtIP complex, Flap endonuclease (FEN1) and the apurinic endonucleases (APE1 and APE2) also act in the chemical “cleansing” of DNA breaks to prevent genomic instability and disease, and promote progression of DNA- and RNA-DNA damage response (DDR and RDDR) pathways. Here, we provide an overview of cellular first responders dedicated to the detection and repair of abnormal DNA termini.

INTRODUCTION

Spontaneous chemical processes generating DNA strand breaks create damaged DNA termini. Modified non-canonical DNA termini are also generated during base excision repair reactions, from aberrant DNA ligation [Tumbale et al. 2014], and from failed topoisomerase–DNA transactions [Deweese and Osheroff 2009; Nitiss 2009; Pommier 2013]. Emerging evidence also indicates that the contamination of RNA in DNA created by

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polymerase misincorporation of ribonucleotides (rNMPs) into genomic DNA is also an important, poorly understood source of non-canonical DNA termini, whose efficient repair is required to prevent replication stress and genomic instability [Nick McElhinny et al. 2010; Reijns et al. 2012; Williams et al. 2013; Tumbale et al. 2014]. To exacerbate these primary challenges to genomic integrity, the enzymatic conversion of existing damage by DNA metabolic processes can generate additional forms of compound DNA-end damage. Furthermore, collisions of damaged DNA strand breaks with the DNA and RNA metabolic enzymes can collapse DNA replication forks, impact the cellular transcription apparatus, and create recombinogenic and carcinogenic DNA double strand breaks (DSBs).

Eukarya are equipped with an equally diverse set of enzymatic processing activities for detecting and processing damaged DNA termini. These enzymes include direct chemical reversal mechanisms yielding “canonical” 5′-phosphate (5′-P) and 3′-hydroxyl (3′-OH) termini, as well as multi-purpose genome maintenance nucleolytic enzymes. These enzymatic activities are integral to major DNA strand break repair pathways including DNA base excision repair (BER), single strand break repair (SSBR) [Caldecott 2007; Caldecott 2008] and DNA double strand break repair (DSBR) [Williams et al. 2007; Ciccia and Elledge 2010]. We divide this overview broadly into two parts: first we summarize sources and the chemical nature of DNA termini at strand breaks (see Figures 1 and 2) and second, we survey molecular mechanisms for recognition and enzymatic “healing” of damage at DNA ends, with a focus on factors for which molecular structure information is available.

SOURCES OF MODIFIED DNA TERMINI

IR and ROS induced DNA end damage

DNA damage is unavoidable. Radiation from various natural and unnatural sources along with the generation of reactive oxygen species (ROS) during normal cellular metabolism or during inflammation mount a constant assault on our genetic material, and can produce DNA strand breaks. ROS modification of DNA is estimated to generate DNA base damage and strand break lesions on the order of 10,000–100,000 events per mammalian cell per day [Lindahl and Nyberg 1972; Drinkwater et al. 1980; Nakamura and Swenberg 1999; Roberts et al. 2006]. Ionizing radiation (IR) triggers production of SSBs and DSBs bearing “dirty” DNA ends by inducing free radical attack on the deoxyribose sugar backbone [Dedon 2008; Weinfeld et al. 2011; Povirk 2012]. The stable end products on 3′ termini of IR-induced breaks and those created by endogenous ROS or oxidants such as hydrogen peroxide (H₂O₂) include 3′-phosphate (3′-P) and 3′-phosphosphoglycolate (3′-PG) moieties (Figure 1). In oxic conditions, IR generated strand breaks harbor 3′-PG and 3′-P in an approximate 1.6:1 3′-PG:3′-P ratio [Buchko and Weinfeld 1993]. At the 5′ terminus of breaks, abundant stable products of IR insult and oxidation are 5′ phosphate (5′-P) groups, however 5′ -aldehyde and 5′ hydroxyl (5′-OH) moieties also constitute significant measurable fractions of damaged [Dedon 2008; Weinfeld et al. 2011; Povirk 2012].

DNA glycosylase sources of modified termini

DNA base excision repair (BER) acts in the removal of damaged DNA base lesions in mammalian cells [Friedberg et al. 2006; Liu and Wilson 2012; Sykora et al. 2013]. The

DNA processing reactions carried out by BER monofunctional and bifunctional DNA glycosylases create variable modified DNA ends. In removing damaged bases via hydrolysis of the N-glycosidic bond, monofunctional DNA glycosylases generate abasic or apurinic (AP) sites. Spontaneous depurination of DNA also creates AP sites in large numbers in cells. Following glycosylase action, endonucleolytic incision at the 5' side of the AP site by AP endonuclease 1 (APE1) during BER yields DNA strand breaks bearing 5'-deoxyribose-phosphate (5'-dRP) groups and 3'-OH ends [Wilson and Barsky 2001; Demple and Sung 2005]. By comparison, following damaged base removal, β,δ -elimination or β -elimination reactions of the bifunctional DNA glycosylase-lyases yield DNA breaks bearing 3'-phosphate or 3'-phospho- α , β -unsaturated aldehyde groups [Wallace 2013].

Topoisomerase-DNA crosslinks

Key cellular processes including transcription, DNA replication, and cell division are impossible without a mechanism to relieve DNA topological strain and DNA entanglement generated by nuclear DNA compaction and translocation of DNA and RNA polymerases. To achieve this, DNA topoisomerases generate transient and reversible SSBs (type I topoisomerases) or DSBs (type II topoisomerases), [Nitiss 2009a; Nitiss 2009b; Deweese and Osherooff 2009; Pommier 2013]. During these transactions, the formation of enzyme-phosphotyrosyl-DNA linkages with DNA 5' and 3' termini by topoisomerases can lead to protein-DNA adducts in cases where the topoisomerase reactions are perturbed (Figures 1 and 2).

Type I topoisomerases (eg. TOP1) relieve topological strain by a mechanism that uses an active-site tyrosine residue to introduce a single-strand break, thereby linking the enzyme covalently to the 3'-phosphate side of the nick (TOP1-cleavage complex) [Pommier 2013]. This bond ensures that the enzyme remains bound to the nick while the DNA is allowed to rotate and relieve topological strain before the nick is re-sealed. However, this reaction is sensitive to a variety of compounds and pre-existing DNA damage that can "poison" the topoisomerase by preventing re-ligation and release of TOP1 from DNA. Camptothecin and its derivatives are TOP1 poisons and are potent chemotherapeutics [Pommier 2009]. TOP1 incision adjacent to a DNA mismatch, AP site, or a nick also leads to irreversible TOP1-DNA cleavage complex formation [Pourquier et al. 1997; Lebedeva et al. 2006; Pourquier et al. 1997]. The presence of oxidative damage (e.g. 8-oxoG) near the TOP1-cleavage site can also impair re-ligation, and extend the lifetime of the TOP1-cleavage complex [Pourquier et al. 1997; Pourquier et al. 1999]. TOP1 and TOP2 adducts lead to detrimental effects such as blocking transcription and creating replication fork collapse [Nitiss 2009a; Nitiss 2009b].

TOP2 alters supercoiling of DNA and relaxes tangles or knots by creating paired DNA strand cleavages spaced 4 base-pairs apart [Liu et al. 1983], gating passage of duplex DNA through the break, then resealing the break to restore genome integrity [Roca and Wang 1994]. This reaction is driven by a trans-esterification of a DNA backbone phosphate to an active-site tyrosine in TOP2, creating a covalently linked TOP2-DNA cleavage complex [Rowe et al. 1984]. TOP2-DNA intermediates pose unique challenges to genomic integrity. TOP2 reactions are sensitive to both genetic and environmental perturbations that can accelerate TOP2 DNA cleavage or stall topoisomerase re-ligation. As is the case for TOP1,

these changes poison the reaction cycle by altering the cleavage/ligation equilibrium towards production of excessive DSBs in the form of TOP2 cleavage complexes and protein linked strand breaks. Anticancer chemotherapeutic TOP2 poisons such as etoposide target this vulnerability by creating genomic instability leading to cell death [Hande 1998; Baldwin and Osheroff 2005; Pommier 2013]. Additional TOP2 poisons include dietary bioflavonoids like genistein [Okura et al. 1988], which is prominent in soy, or EGCG [Bandelet and Osheroff 2008] from green tea, as well as metabolic products of the environmental toxicants polychloro biphenyls (PCBs) [Bender et al. 2006], benzene (1,4 benzoquinone) [Bender et al. 2007], and drug metabolites of acetaminophen (NAPQI) [Bender et al. 2004]. Significantly, DNA base damage repair intermediates including AP-sites and DNA SSBs can be converted to DSBs when TOP2 engages these structures and processes them to protein-linked strand breaks [Wilstermann and Osheroff 2001].

Damage generated by DNA ligases

The final critical step of DNA replication, recombination and repair is DNA ligation, during which the chemical joining of broken DNA strands occurs. The eukaryotic DNA ligases (DNA Ligases I, III and IV) catalyze ligation in a 3-step, ATP-dependent DNA nick sealing reaction. In the first step, an active-site ligase lysine is adenylated. In step two, the AMP moiety is transferred to the 5' phosphate of the DNA substrate to facilitate step 3, phosphodiester bond formation and sealing of the DNA backbone by nucleophilic attack of the 3'-OH on the 5'-adenylate [Pascal et al. 2004; Tomkinson et al. 2006]. Like many key biological processes, ligation can fail. Environmental and metabolic sources of DNA damage trigger this failure causing DNA ligase-dependent production of 5'-adenylated DNA adducts (5'-AMP) (Figures 1 and 2).

Structural studies of ligase-DNA complexes revealed how the nick sealing reactions are orchestrated by the complete encirclement of the DNA nick by the conserved 3-domain architecture in DNA Ligase I and Ligase III [Cotner-Gohara et al. 2010; Pascal et al. 2004]. Like topoisomerase reactions, the essential genome maintenance functions of DNA ligases are also prone to DNA damage-induced side reactions. During a process termed "abortive ligation", 5'-AMP strand breaks can arise if DNA ligases engage damaged DNA structures including direct oxidative single-strand breaks [Ahel et al. 2006], DNA nicks bearing 3' AP sites, oxidative DNA base damage (e.g 8-oxo-dG) [Harris et al. 2009], 5'-dRP groups generated in BER [Rass et al. 2007], or RNA-DNA junctions arising during ribonucleotide excision repair (RER) [Tumbale et al. 2014] (see below).

Premature detachment of DNA ligases from the 5'-AMP reaction intermediate is problematic because under physiological ATP concentrations eukaryotic DNA ligases are predicted to exist predominantly in the enzyme-AMP state [Tomkinson et al. 2006]. Ligase-AMP cannot perform the nick sealing reactions, or deadenylate 5'-termini. Thus, while typically viewed as the ultimate step in DNA repair pathways, DNA ligases paradoxically can also generate unwanted toxic intermediates in the form of adenylated DNA. While the molecular mechanisms underlying abortive ligation are currently ill defined, presumably substrate-induced distortions of damaged substrates in the ligase active site permit 5'

adenylation, but reduce the ligase nick-sealing reaction efficiency such that completion of this step is not ensured before ligase dissociates from its substrate.

Genomic RNA-derived intermediates

During DNA replication in eukaryotic cells, replicative DNA polymerases Pol ϵ and Pol δ incorporate ribonucleotides (ribonucleotide monophosphates, rNMPs) into genomic DNA. It is estimated that greater than 10,000 rNMPs are incorporated by replicative DNA polymerases each cell cycle in budding yeast [Nick McElhinny et al. 2010, Williams et al. 2013; Nick McElhinny et al. 2010], and greater than ~1,000,000 rNMPs are incorporated each cell division in murine cells [Reijns et al. 2012; Hiller et al. 2012]. These numbers overshadow classically viewed common forms of DNA damage such as AP sites, which are produced ~10,000 times per mammalian cell per day [Lindahl, 2000]. Overall, the transient contamination of nuclear genomic DNA by RNA is estimated to outnumber all other known types of DNA damage combined [Caldecott 2014]. Further, Pol α Primase synthesizes ~5% of the nascent lagging strand using rNTPs. Ribonucleotides are also incorporated into mitochondrial DNA [Kasiviswanathan and Copeland 2011; Yang et al. 2002], and rNTPs are utilized by DNA repair Polymerases μ and λ [Nick McElhinny and Ramsden 2003]. In non-cycling cells where dNTP concentrations are decreased relative to rNTPs [Chabes et al. 2003; Ferraro et al. 2010], elevated rNTP:dNTP ratios [Ferraro et al. 2010] may favor increased ribonucleotide incorporation by DNA repair polymerases [Nick McElhinny and Ramsden 2003]. The presence of ribonucleotides in genomic DNA poses unique challenges to genomic stability. Ribonucleotides, unlike deoxyribonucleotides, bear a reactive 2'-OH on the ribose sugar that renders the sugar-phosphate backbone prone to hydrolysis in physiological conditions, creating strand breaks with 2'-3'-cyclic phosphate and 5'-OH groups (Figure 1). A major enzymatic removal pathway for ribonucleotides is RER [Sparks et al. 2012]. RER is initiated when RNase H2, a heterotrimeric enzyme, cleaves on the 5' side of a DNA embedded ribonucleotide. These RER strand break intermediates bear a 5'-P ribonucleotide and 3'-OH end.

The fate of ribonucleotides, and their impact on genomic integrity is highly relevant to the current topic of damaged DNA end repair, and is the least explored in the field. Significantly, ribonucleotides introduce local geometric distortions into DNA [Egli et al. 1993; Ban et al. 1994; DeRose et al. 2012] that can influence enzymatic transactions of DNA metabolic enzymes engaging ribonucleotide containing RNA-DNA [Kim et al. 2011] or nicked RER intermediates generated by RNase H2 [Tumbale et al. 2014]. In the case of ribonucleotides embedded in DNA, TOP1 incision on the 3' side of a ribonucleotide, like hydrolysis, can generate the end-blocking 2'-3'-cyclic phosphate group when the ribonucleotide 2'-OH attacks the RNA 3'-phosphotyrosyl-linked TOP1-cleavage complex [Sekiguchi and Shuman 1997; Shuman 1998; Kim et al. 2011]. These events are also associated with mutagenic TOP1 processing of rNMPs in genomic DNA, and the generation of 2–5 bp deletions in repetitive sequences [Kim et al. 2011; Cho et al. 2013; Williams et al. 2013]. In a second example of metabolism of rNMPs leading to non-canonical DNA termini, it has been demonstrated recently that the incised RNA-DNA junctions from RER are potent triggers for abortive ligation by DNA ligases. In this context, the misincorporation of a ribonucleotide by a DNA polymerase, followed by abortive DNA ligase processing of the

RER intermediate generates adenylated RNA-DNA, or so called “RNA-DNA damage” [Tumbale et al. 2014].

DNA END PROCESSING AND REPAIR MACHINERIES

Just as there are multiple forms of damaged DNA termini, a number of direct reversal DNA end processing enzymes exist to convert damaged DNA ends to ligatable substrates. These proteins factors are described below.

Reversal of 5'-adenylation by Aprataxin (APTX)

Analogous to the correction of nucleotide misincorporation errors made by DNA polymerases, DNA ligation errors in the form of adenylation DNA damage are “proofread” [Ahel et al. 2006]. Eukaryotic Aprataxin (APTX) is the only known enzyme to catalyze direct reversal of DNA or RNA-DNA 5'-adenylation to generate a 5'-phosphorylated terminus, [Ahel et al. 2006; Reynolds et al. 2009; Harris et al. 2009; Tumbale et al. 2014]. DNA damage repair roles for APTX in BER, SSBR, and DSBR are underlined by the facts that APTX deficient AOA1 cells show elevated levels of oxidative DNA damage [Harris et al. 2009; Hirano et al. 2007] and have increased DNA damage following treatment with the anticancer topoisomerase inhibitor camptothecin [Mosesso et al. 2005]. Although direct quantitative measures of genomic DNA adenylation have not been reported, genetic studies link the budding yeast APTX homolog Hnt3p to RNA-triggered abortive ligation during RER [Tumbale et al. 2014], as well as pathways for repair of alkylation and oxidative DNA damage [Daley et al. 2010]. Identification and characterization of a mitochondrial isoform of mammalian APTX suggests these activities are also critical for maintenance of mitochondrial DNA [Sykora et al. 2011].

X-ray structures of *Schizosaccharomyces pombe* and human APTX deadenylases bound to DNA and RNA-DNA targets revealed a conserved two-domain architecture that constructs APTX deadenylases [Tumbale et al. 2011; Tumbale et al. 2013] (Figure 3). APTX belongs to the functionally diverse histidine triad (HIT) family of nucleotide hydrolases and transferases. APTX is unlike other HIT family members, such as the fragile histidine triad (FHIT) protein [Lima et al. 1997a; Lima et al. 1997b], which self-associate via an extended HIT domain β -sheet interaction, and function as dimeric molecules. Rather, APTX is monomeric and the HIT domains mixed α - β fold catalytic core is fused to an APTX-specific C₂H₂ (C₂HE in *S. pombe* Aptx) zinc-finger (ZnF) domain that confers DNA damage recognition and substrate specificity for adenylated polynucleotide substrates [Rass et al. 2008; Tumbale et al. 2011; Tumbale et al. 2013]. Vertebrate APTX orthologs also contain a forkhead associated domain (FHA) that mediates APTX interactions with multi-protein DNA repair complexes via phosphorylation regulated protein-protein interactions with XRCC1 [Clements et al. 2004; Luo et al. 2004], XRCC4 [Clements et al. 2004] and MDC1 [Becherel et al. 2010]. Additional APTX interactions with PCNA suggest roles for APTX in DNA replication and/or long patch BER [Hirano et al. 2007].

DNA damage sensing by APTX is achieved through two-point nucleic acid binding by both the HIT and Znf domains. Sensing of DNA ends or nicks is regulated by the HIT domain amino terminal α -helix. This helix forms a wedge that binds and interrogates the exposed

damaged DNA base stack of DSB end substrates [Tumbale et al. 2011; Tumbale et al. 2013]. The binding mode of blunt-ended nucleic acid substrates in the APTX active site [Tumbale et al. 2011] suggests the α 1 helical wedge would clash with the upstream region of nicked or gapped adenylated substrates, and that APTX must significantly distort nicked or gapped DNA to facilitate catalysis. Intriguingly, the APTX active site histidine triad “HIT” (H ϕ H ϕ H, where ϕ is a hydrophobic residue) loop and α -helical wedge undergo conformational changes that may gate assembly of the active site, thereby ensuring non-specific hydrolysis of adenine nucleotides is prevented.

Conserved elements from both the HIT domain and Znf converge on the adenylated DNA 5' terminus, unwind the terminal base pair, and direct the AMP lesion into a deep binding groove. As APTX engages the DNA 5'-end in an A-form conformation, APTX appears engineered to process adenylated 5'-termini of both DNA and RNA-DNA substrates, consistent with functions of APTX in RER [Tumbale et al. 2014]. Structural and biochemical data support a two-step metal-independent catalytic mechanism for DNA-adenylate reversal by APTX. In the first step, an active site nucleophile, His260 of hAPT_X, directly attacks the 5'-adenylate phosphoanhydride resulting in the formation of a transient enzyme-AMP intermediate [Rass et al. 2008] and liberation of the DNA 5'-P product. A structure containing a vanadate transition state mimic of RNA-DNA bound hAPT_X shows that the histidine triad HIT substrate binding loop completely encircles the AMP lesion to stabilize the transition state during the first step. The second step involves hydrolysis of the ensuing His260-AMP intermediate, and is likely facilitated by activation of a solvent by a His251-Ser281 catalytic dyad [Tumbale et al. 2014]. Whilst the APTX deadenylation reaction appears metal independent, binding of a Na⁺ ion suggests roles for solvent in stabilization of the charged 5'-phosphate reaction product.

Polynucleotide kinase phosphatase (PNK)

Mammalian Polynucleotide kinase/phosphatase (PNK or PNKP) is a critical DNA end-processing factor participating in BER, SSBR and DSB (Non-homologous end-joining or NHEJ) repair [Chappell et al. 2002; Wiederhold et al. 2004; Weinfeld et al. 2011]. PNK has two enzymatic activities [Jilani et al. 1999]. It is both a DNA 5'-kinase that phosphorylates 5'-OH termini, and 3' phosphatase that resolves 3'-phosphorylated termini produced by ROS and DNA glycosylases [Pheiffer et al. 1982]. Clearance of 3'-phosphate moieties generated with TDP1 resolution of TOP1 protein-DNA adducts (see below) is also an important function of PNK [Plo et al. 2003].

X-ray structures of full-length mouse PNK [Bernstein et al. 2005] showed the enzyme adopts a modular architecture characterized by three globular domains; an N-terminal phosphoprotein binding FHA domain, and a C-terminal paired kinase-phosphatase domain. The catalytic domains are fused, and the flexible kinase-phosphatase interface builds a composite DNA interaction interface for substrate binding and catalysis [Garces et al. 2011]. The PNK phosphatase is structurally related to the haloacid dehalogenase (HAD) family of enzymes that includes Phosphoserine phosphatase [Bernstein et al. 2005]. In phosphatase-DNA complex structures [Coquelle 2011; Garces et al. 2011], basic residues lining the phosphatase active site interact with the phosphate backbone. Together with aromatic side

chains that stack against the substrate nucleobases, these backbone contacts orient the substrate in a highly extended ssDNA conformation for catalysis. The 3'-P is found in a deep active site pocket evidently well suited for dephosphorylation of 3'-P, but not for accomodating larger modifications such as 3'-phosphoglycolate [Inamdar et al. 2002]. An active site Asp (Asp170 in mPNK) is proposed to catalyze the Mg^{2+} assisted nucleophilic attack of the 3'-P to form a phospho-aspartate intermediate that is subsequently hydrolyzed.

A probable mode of substrate binding by the kinase domain has been established from a structure of PNK bound to a self-annealing 5mer ssDNA that assembles into a pseudo-nicked DNA structure occupying the kinase active site. In this work, the 5' end of the duplex is bound in the kinase active site as a phosphorylated product of the ATP-dependent kinase reaction [Garces et al. 2011]. To recognize the nicked DNA junction, a helical element from the kinase domain wedges into the pseudo-nick duplex, directing the 5' and 3' strands to their respective active sites. Such coupling of the phosphatase and kinase substrate interaction sites may provide a mechanism for kinase-phosphatase crosstalk [Garces et al. 2011, Schellenberg and Williams 2011].

Tyrosyl DNA phosphodiesterase 1 (TDP1)

Failure to complete a TOP1 reaction cycle creates a DNA SSB with a 3' covalently linked protein adduct. In the case of stalled TOP1, an initial processing step using the ubiquitin-proteasome system degrades TOP1, yielding a peptide linked to a DNA 3'-P end by a phosphotyrosine linkage [Desai et al. 1997; Lin et al. 2008]. A direct reversal pathway of 3'-TOP1 adducts is catalyzed by TDP1 [Yang et al. 1996].

A series of structures of human TDP1 complexes have been described [Davies et al. 2002; Davies et al. 2003; Davies et al. 2004]. TDP1 is a member of the phospholipase D (PLD) enzyme superfamily. PLD enzymes often function as dimers, but TDP1 contains tandem PLD repeats (Figure 4), with each half of the TDP1 catalytic domain recognizing one side of the substrate phosphodiester. PLD proteins catalyze phosphotransfer reactions with an active site His-Lys-Asp (HKD) motif; in the case of TDP1 the C-terminal aspartate of the motif is not conserved.

Structures of TDP1 bound to transition-state mimics containing vanadate show that ssDNA binds in an extended conformation in an active site trench, which positions the scissile 3'-P in the active site (Figure 4), where the two conserved HK motifs catalyze a 2-step phosphotyrosine adduct hydrolysis [Davies et al. 2003; Davies et al. 2004; Raymond et al. 2004]. The first step involves metal independent nucleophilic attack by His263 on the scissile phosphate, while His493 protonates the leaving group. Two TDP1 lysine residues provide charge-neutralization of a negatively charged transition state in this reaction. The ensuing DNA phosphohistidyl intermediate is resolved by hydrolysis. The 3'-P product of the TDP1 reaction requires subsequent processing by PNK to yield a 3'-OH end (Figure 1, Figure 3). In a transition-state mimic structure containing a TOP1-derived peptide [Davies et al. 2003], there are few specific interactions from TDP1 with the TOP1 active-site tyrosine. TDP1 also processes other small 3' adducts such as 3'-phosphoglycolates which

although chemically distinct from phosphotyrosine, are also accommodated in the TDP1 active site [Inamdar et al. 2002; Interthal et al. 2005; Akopiants et al. 2013].

A deficiency in TDP1 activity has recently been shown to cause a neurodegenerative disease called spinocerebellar ataxia with axonal neuropathy (SCAN1), caused by mutation of an active-site histidine (H493R) [Interthal et al. 2005]. This mutation has the effect of extending the lifetime of the phosphohistidyl intermediate, thereby linking TDP1 to the 3'-end of DNA in place of the TOP1-derived peptide.

Tyrosyl DNA phosphodiesterase 2, TDP2

Stalled TOP2 cleavage complexes are processed by the proteasome [Alchanati et al. 2009; Ban et al. 2013; Fan et al. 2008], presumably leaving a TOP2 derived peptide linked through a tyrosine to the 5'-end of a DNA break. Although the DSB structure generated in this way has four-nucleotide "sticky" ends, they cannot be directly re-ligated without removal of the 5'-peptide adduct. A vertebrate tyrosyl-DNA phosphodiesterase (TDP2^{TTRAP/EapII}) was identified, which efficiently processes TOP2-adducts to 5'-phosphorylated termini through a direct reversal mechanism of DSBs [Cortes Ledesma et al. 2009]. TDP2 processing generates a 5'-phosphate, which can be used directly by DNA Ligase IV from NHEJ to seal the break. Consistent with a role in TOP2 processing, targeted RNAi knockdown of TDP2 or knockout sensitizes cells to etoposide, and increases formation of nuclear γ H2AX foci, a marker of DSBs [Cortes Ledesma et al. 2009; Gomez-Herreros et al. 2013].

The catalytic domain of TDP2 belongs to the endonuclease/exonuclease/phosphatase (EEP) family of metalloenzymes (Figure 3). High resolution X-ray structures of a series of TDP2-DNA complexes shows TDP2 binds the 5'-end of tyrosylated ssDNA and 5'-overhanging DNA substrates in a narrow trench, similar to TDP1, with direct protein-DNA contacts with the sugar-phosphate backbone and hydrophobic faces of the terminal nucleobases [Schellenberg et al. 2012; Shi et al. 2012] (Figure 3). Although structures of TDP2 containing a TOP2 derived peptide have not yet been reported, a structure containing a non-hydrolysable 5'-6-aminohexanol adduct identified a hydrophobic 5'-adduct binding patch that likely participates in recognition of the peptide half DNA-protein phosphotyrosine substrate [Schellenberg et al. 2012]. The recent discovery that TDP2 can remove the 5'-VPg peptide adduct from picornaviral mRNA [Virgen-Slane et al. 2012], a peptide unrelated to TOP2, suggests that specific TDP2 interactions with the peptide portion of the adduct may be limited to the phosphotyrosine and peptide mainchain.

As with TDP1, the size of the tyrosine-binding pocket is too small to accommodate a deoxyribose-nucleotide, providing a mechanism of excluding DNA from endonucleolytic incision. X-ray structures support a model where TDP2 hydrolyses the 5'-phosphotyrosine bond with a single Mg^{2+} ion mediated catalytic mechanism, similar to structure-based models for APE1 catalysis [Mol et al. 2000]. In this scheme, a conserved aspartate residue (Asp262, human TDP2 numbering) functions as a catalytic base to activate a water molecule for nucleophilic attack in an S_N2 displacement reaction. Bound Mg^{2+} along with three additional conserved residues (His226, His351 and Ser229) bind the 5'- PO_4 and are positioned to interact with the substrate phosphate moiety to stabilize a pentacovalent reaction transition state in this reaction [Schellenberg et al. 2012].

DNA polymerase β AP lyase

The incision of AP sites during base excision repair generates abundant DNA nicks bearing 5'-dRP groups. To resolve these intermediates, mammalian cells employ DNA polymerase β (POL β) in the single nucleotide BER (SN-BER) as well as long patch BER (LP-BER, also see section below on FEN1) pathways [Liu and Wilson 2012]. The multifunctional 39 kDa POL β is the smallest of the cellular eukaryotic polymerases, and acts as both a 5'-dRP lyase, and DNA polymerase. In SN-BER, POL β lyase activity excises the dRP, leaving a gap that is filled by the polymerase domain, resulting in a nick that can be sealed by DNA Ligase III. Polymerase activity resides in the POL β 31-kDa C-terminal domain, consisting of palm, finger and thumb subdomains, which utilizes a 3-metal ion mechanism to catalyze nucleotidyl transfer [Freudenthal et al. 2013]. The 8-kDa, N-terminal domain harbors the rate-limiting 5'-dRP lyase activity [Matsumoto and Kim 1995; Piersen et al. 1996; Prasad et al. 1998; Bennett et al. 1997] (Figure 3).

POL β uses a metal-independent 5'-dRP lyase mechanism, involving a lysine mediated nucleophilic attack on C1' of the sugar to form a Schiff base intermediate, followed by a β -elimination and release of dRP by hydrolysis [Prasad et al. 2005; Matsumoto and Kim 1995; Matsumoto, 1995]. Structures of POL β bound to nicked and gapped DNA show these interactions bend the DNA 90° across the surface of the protein, positioning the DNA for optimal enzymatic activity [Pelletier et al. 1996; Piersen et al. 1996; Sawaya et al. 1997]. The 5 α -helices of the lyase domain form a lysine rich pocket, that engages the 5'-end, as observed in structures of POL β bound to both nicked and gapped DNA [Prasad et al. 1994; Pelletier et al. 1994]. Site-directed mutagenesis and mass spectrometry both indicate that Lys72 within the pocket acts as a potential nucleophile in the lyase reaction [Matsumoto et al. 1998]. However, a crystal structure of POL β bound to nicked DNA bearing the dRP analog tetrahydrofuran, found Lys72 or other potential nucleophiles lie distal from the 5'-dRP. Evidence from the crystal structure suggests that a 120° rotation of dRP about its 3'-phosphate would be required to position the lesion for catalysis.

Ku70/80 AP lyase

While abasic sites generated during BER are processed by POL β , clustered ROS damage creating DNA strand breaks can also lead to production of AP sites near DSB termini, which are processed by Ku70/80 in NHEJ. Ku70/80 is a heterodimeric, high affinity DSB DNA end binding protein, and is amongst the first proteins to be recruited to DSBs, encircling the DNA ends as a ring structure (Figure 4) [Walker et al. 2001]. The Ku80 C-terminus forms a small helical domain that is required for binding and DNA-dependent activation of the DNA-protein kinase catalytic subunit (DNA-PKcs), a member of the phosphoinositide-3 kinase-related protein kinase family [Hartley et al. 1995; Hammel et al. 2010; Gell and Jackson, 1999; Harris et al., 2004]. In addition to structural DNA end sensing and protein-protein interactions, recent evidence indicates that similar to POL β , Ku70/80 is a dRP/AP lyase. Ku70/80 removes abasic sites found within 2 nucleotides of a 5'-overhang most efficiently, restricting lyase activity to abasic sites that could potentially directly impact DNA ligation [Roberts et al., 2010; Strande et al. 2012]. Lys31 and Lys160 of the Ku70 monomer are located in the N-terminal α/β domain near the central dimeric pore, and are

required for this activity, as K31A/K160A mutants decrease lyase activity 4-fold [Strande et al., 2012].

NUCLEOLYTIC PROCESSING MECHANISMS

Genome maintenance endonucleases and exonucleases are also well suited for clearance of blocked termini, by providing redundant means for DNA end processing of damaged termini. The 5' nucleases FEN1 and the MRE11/RAD50/NBS1/CtIP complex are the best characterized of these, and have been implicated in alternate pathways for direct reversal of 5'-end blocking lesions. Acting at the 3'-terminus, the AP endonucleases possess activities appropriate for resolving a variety of blocked termini structures. Other DNA damage response nucleases for which we lack structural information include Artemis, Metnase, and Slx1/4, and have been recently reviewed elsewhere [Povirk 2012].

Flap endonuclease (FEN1)

Varying chemistries on DNA 5' termini can impair normal processing and clearance of blocked ends by direct reversal processes. For example, during BER if a 5'-dRP group is oxidized [Wong et al. 2010; Jacobs et al. 2011] POL β lyase activity is impaired. In this case, the polymerase can replace the damaged nucleotide at the 5' terminus by strand-displacement synthesis (LP-BER). Polymerase displacement then creates a flap structure that is a substrate for FEN1, which recognizes and cleaves the 5'-damaged strand. FEN1 cleavage creates a ligatable nick that is then sealed by ligase. *In vitro* this general 5'-adduct processing mechanism is also capable of resolving 5'-aldehyde blocked ends, for which there are no known direct reversal pathways [Jung et al. 2011], as well as 5'-ribonucleotides [Sparks et al. 2012] generated by RNaseH2 incision at embedded RNA in DNA during RER. Genetic evidence in budding yeast also implicates the FEN1 homolog Rad27 in resolution of 5' adenylated termini *in vivo*, a function that is redundant with Hnt3p, the yeast APTX homolog [Daley et al., 2010]. Thus FEN1, in addition to its critical role in resolving ~50 million flap structures generated during Okazaki fragment maturation [Balakrishnan and Bambara, 2013] is an important damage response enzyme participating in nucleolytic resolution of several 5' lesions.

FEN1 is the founding member of the FEN family of structure-specific nucleolytic processing enzymes, and is related in structure to EXO1 [Orans et al. 2011], XPG and GEN1 [Williams and Kunkel 2011]. X-ray structures of human FEN1 bound to flap DNA structures revealed that FEN1 overall resembles a left-handed glove, and shows that the enzyme recognizes both the upstream and downstream regions of the flap [Tsutakawa et al. 2011] (Figure 5). At the flap junction, α -helical wedges separate the DNA duplex to segregate 5' downstream and 3' upstream DNA. These interactions induce a 100° DNA bend proximal to the cleavage site [Tsutakawa et al. 2011; Chapados et al. 2004], and unpairing of DNA 5' end that provides access to the scissile phosphodiester bond.

The FEN1 structure explains efficiency and specificity of the enzyme on double-stranded flap structures. Significantly, extensive protein-nucleic acid contacts are made to the 3' flap region. These protein-DNA contacts set the endonuclease register for cleavage of the displaced 5' strand, and ensure generation of a ligatable nick following cleavage. The DNA

5' strand of the flap is bound using a helix-two turn helix containing (H2TH) potassium-binding fold and two nucleotides are unpaired at the 5' end, where the flap is "threaded" through a helical arch structure that engulfs the 5' terminus. Protein disorder-to-order transitions within the helical arch, and a disorder-thread-order mechanism of substrate engagement helps to explain how unique mode of FEN1 substrate binding accommodates diverse 5' end structures [Patel et al. 2012].

AP Endonucleases

APE1 is a multifunctional protein acting in BER, transcription [Okazaki et al. 1994; Kuninger et al. 2002], and granzyme A-mediated cell death [Fan et al. 2003; Tell et al. 2009]. The catalytic core of APE1 the endonuclease/exonuclease/phosphatase (EEP) fold is related in sequence and structure to vertebrate TDP2, and E. coli Exonuclease III [Mol et al. 2000] (Figure 5). The EEP nucleases adopt a globular α - β fold with a central β -sandwich surrounded by helical elements and surface loops dictating unique substrate interaction specificities [Schellenberg et al. 2012]. In the human APE1 DNA complex structures [Mol et al. 2000] APE1 kinks and completely surrounds the abasic site containing DNA. A substrate recognition arginine interrogates the abasic site for positioning of the scissile phosphate in a metal ion bound active site. In a single metal ion mechanism that is supported by structural studies of human and N. meningitidis APE1 [Lu et al. 2012], and similar to a mechanism proposed for TDP2, Asp210 (human APE1 numbering) deprotonates water, which performs nucleophilic attack on scissile phosphate 5' of the AP site [Mol et al. 2000].

In addition to roles in endonucleolytic processing of abasic sites, APE1 harbors 3'-5' exonuclease, 3'-phosphodiesterase, 3'-phosphatase, and endoribonuclease activities [Wilson 2003; Kim et al. 2011; Barnes et al. 2009; Barzilay et al. 1995; Chou et al. 2002]. DNA end-processing reactions of APE1 are important during end-healing processes in BER. For example, APE1 phosphodiesterase activity processes 3'-phospho- α,β -unsaturated aldehyde (PUA) generated by β -elimination reactions of glycosylase-lyases, yielding a 3'-OH for repair synthesis and ligation. Presumably APE1 engages damaged 3'-termini in a manner analogous to that observed for the AP-endonuclease reactions (Figure 5), however a detailed structural basis for these transactions is currently undefined.

APE2 (Apn2 in yeast), a related enzyme with sequence homology to APE1, was later identified as an alternate AP endonuclease [Johnson et al. 1998]. However, APE2 exhibits distinct substrate preferences [Hadi et al. 2002; Burkovics et al. 2006; Unk et al. 2000; Unk et al. 2001]. APE2 displays 3'-5' exonuclease and 3' phosphodiesterase activity, and weak AP-endonuclease when compared to APE1. Divergence in APE1 and APE2 substrate interaction pocket, DNA binding loops, and active sites suggest substitution of the hydrophobic AP-site interacting groove of APE1 (formed by F266, W280, and L282, human APE1) may underlie differing substrate specificities between the two AP endonucleases [Hadi et al. 2002]. APE2 may play more significant roles in processing of mismatched 3' nucleotides and damaged 3' termini. Consistent with roles in clearance of blocked termini, *Xenopus* APE2 participates in "single strand break end resection" and is required for checkpoint activation in response to oxidative stress in *Xenopus* extracts [Willis et al. 2013].

MRE11/RAD50/NBS1^{Xrs2}/CtIP^{Sae2/Ctp1} processing of blocked termini

The MRE11/RAD50/NBS1 (MRN) complex, in conjunction with CtIP^{Sae2/Ctp1} (CtIP in humans, Ctp1 in *S. pombe* and Sae2 in *S. cerevisiae*), generates 3' ssDNA overhangs required for the initiation of DNA DSB repair by homologous recombination (HR) [reviewed in Williams et al. 2007; Williams and Tainer 2005; Mimitou and Symington 2011]. CtIP is also involved in alternative-NHEJ [Bennardo et al., 2008; Zhang et al., 2011; Lee-Theilen et al., 2011]. This multifunctional complex directly tethers DSB DNA termini [Moreno-Herrero et al. 2005; Williams et al. 2008] and initiates 5'-3' end resection at DNA breaks [Mimitou and Symington 2008; Zhu et al. 2008; Niu et al. 2010]. Resection, as characterized in *S. cerevisiae*, is coincident at both ends of a DSB [Westmoreland et al. 2013]. MRN also signals DSB damage and regulates cell cycle checkpoints via interactions with ATM [Lee and Paull 2005]. The nucleolytic core of the MRN complex is MRE11. MRE11 directly binds RAD50, NBS1 and DNA. *In vitro*, MRN possesses 3'-5' Mn²⁺-dependent exonuclease activity and ATP-dependent endonuclease activities [Paull and Gellert 1998; Trujillo et al. 1998] that are also modulated by CtIP^{Sae2/Ctp1} [Nicolette et al. 2010; Sartori et al. 2007]. Evidence in fission yeast [Hartsuiker et al. 2009] and mammalian cells [Quennet et al. 2011] indicates MRN/CtIP is required for nucleolytic processing of etoposide generated TOP2-DNA damage adducts, representing an alternative repair pathway to direct reversal by TDP2. MRN/Ctp1^{sae2} dependent clearance of protein-blocked 5'-termini is also key during meiosis where the complex functions in removal of Rec12-protein-DNA complex in fission yeast [Rothenberg et al. 2009] or Spo11 in budding yeast [Garcia et al. 2011] to facilitate meiotic strand break processing.

Important insights into MRN/CtIP nucleolytic processing mechanisms have come from X-ray structures of *Pyrococcus furiosus* (Pf) MRE11-DNA [Williams et al. 2008] and MRE11/RAD50 complexes [Mockel et al. 2012; Williams et al. 2011; Lim et al. 2011] (Figure 5). The PfMRE11 enzyme is a homodimer that associates via a structurally conserved four-helix bundle at the nexus of two N-terminal Calcineurin protein phosphatase related catalytic domains. A mixed α - β fold nuclease "capping domain", emanates from the nucleolytic cores forming a U-shaped DNA processing cradle, with dsDNA bound in the cleft between the two MRE11 monomers via minor groove sequence-independent interactions in the DNA sugar-phosphate backbone [Williams et al. 2008]. MRE11 splays the duplex apart to access the sugar phosphate backbone for nucleolytic activity. The MRE11 active site consists of five conserved phosphoesterase motifs coordinating two Mn²⁺ ions [Hopfner et al. 2001; Williams et al. 2008]. Two of these motifs contain histidine residues (His52, His85 of PfMRE11) critical for exo- and endonuclease activity. In the crystal structure bound to duplex DNA, His52 is proposed to participate in positioning the DNA backbone phosphate upstream of the cleavage site, and guides the 3' terminus into the active site for a 3'-5' exonucleolytic reaction. His85 likely stabilizes the transition state and Mn²⁺ acts to align the scissile bond [Hopfner et al. 2001; Williams et al. 2008].

Neale and colleagues [Garcia et al. 2011] proposed an attractive bidirectional resection model in which the combined action of *S. cerevisiae* MRN/Sae2 endonucleolytic nicking creates an entry point for the MRE11 3'-5' exonuclease activity that tracks towards the 5' end. This model provides a reasonable explanation for how a 3'-5' exonuclease

paradoxically dictates the resection reaction that is in the opposite polarity (5'-3') as well as a probable mechanism for MRN dependent clearance of 5'-topoisomerase or Spo11 blocked termini [Garcia et al. 2011]. The nucleolytic transactions in the MRE11 binding groove are also intimately regulated by RAD50 [Deshpande et al. 2014] and NBS1 [Schiller et al., 2012]. NBS1 homologs also bind CtIP/Ctp1 [Sartori et al. 2007; Williams et al. 2009] which influences MRE11 nuclease activity *in vitro* [Sartori et al. 2007], however, the precise role for these factors in regulating nucleolytic activities awaits definitive co-complex X-ray structure determination of intact MRN/CtIP-DNA complexes.

COORDINATION OF DNA STRAND BREAK PROCESSING REACTIONS

The DNA damage first-responders discussed herein act in coordinated, multistep DNA repair events. The linking together of individual activities via multi-protein interaction hubs or nodes can achieve high specificity, as well as facilitate efficient substrate transfer of the damaged DNA substrates. The employment of hub proteins, generally non-enzymatic interaction polypeptides, enables integrated orchestration of DNA end recognition events with activation of cellular DNA damage signaling, and coordination of multi-step enzymatic processing events to ensure efficient damage recognition, DNA break processing and nick sealing transactions. Hub protein interactions involve: 1) formation of proteinaceous molecular filaments, 2) regulated (eg. phosphorylation-dependent) binding events, 3) multivalent interactions to several proteins and 4) dynamic and flexible scaffold-to-effector linkages. Two examples of scaffolds that link end-processing activities are XRCC1, which acts in SSBR, and XRCC4, which acts in DSB repair (Figures 6, 7).

The XRCC1 scaffold in SSBR

SSBR generally requires four coordinated reaction steps to complete the repair: 1) DNA damage detection by XRCC1/Parp1, 2) DNA end processing by PNK, APTX, TDP1 and others, 3) gap filling by POL β , and 4) final nick sealing by DNA Ligase III. The XRCC1 protein couples these steps by coordinating recruitment and targeting repair factors that catalyze these reactions, thereby accelerating overall rates of repair by acting as a DNA processing assembly line [Whitehouse et al. 2001; Caldecott 2008; Kubota et al. 1996] (Figure 6).

XRCC1 is a multi-domain protein containing three structurally defined regions, the XRCC1 N-terminal domain (NTD) and two BRCA1 Carboxyl Terminal (BRCT) domains. The NTD directly interacts with POL β near the POL β thumb domain [Cuneo and London 2010] (Figure 6). Intriguingly, the NTD is capable of adopting dramatically different redox-dependent protein folds that may alter POL β interactions in response to oxidative stress. X-ray structures show the C-terminal BRCT adopts a canonical BRCT fold characterized by a central 4-stranded β -strand, surrounded by 3 helical elements (Zhang et al. 1998; Williams et al. 2001; Williams et al. 2004). The XRCC1 C-terminal BRCT directly interacts with the DNA Ligase III BRCT region [Caldecott et al. 1994] in a heterodimeric BRCT-BRCT interaction [Cuneo et al. 2011] (Figure 6).

Casein kinase 2 (CK2) phosphorylates XRCC1 between the two BRCT domains [Loizou et al. 2004] at multiple sites [Ali et al. 2009], and these modifications promote efficient SSBR

[Parsons et al. 2010]. These phosphorylation sites create a docking platform for the FHA domains of APTX and PNK [Clements et al. 2004]. Flexibility is likely a common feature of the FHA-dependent interactions with XRCC1 (and XRCC4, see below). Small angle X-ray scattering (SAXS) [Bernstein et al. 2009] and limited proteolysis experiments [Bernstein et al. 2005] show a flexible region of low structural complexity bridges the PNK FHA domain and phosphatase-kinase C-terminus. Similarly, the linkage between the N-terminal APTX FHA domain, and the catalytic deadenylase HIT-Znf domains is proteolytically labile [Tumbale et al. 2014], suggesting APTX architecturally parallels PNK in having a phosphoprotein targeting FHA domain linked flexibly to its DNA processing catalytic domain. For PNK, the interaction with XRCC1 also regulates substrate interactions, by promoting PNK dissociation from DNA products following either 5'-phosphorylation or 3'-dephosphorylation [Mani et al. 2007].

Overall an emerging view of the XRCC1 complex is that it enhances transitions between DNA repair enzymes through multivalent protein-protein interactions, both in SSBR and alternative-NHEJ [Della-Maria et al. 2011]. Connecting functionally intertwined activities provides a means for enhancing local concentration of repair factors, and for coordinating sequentially acting end processing activities that are needed in a variety of contexts that may arise with variable damage at DNA ends.

The XRCC4 scaffold in NHEJ

XRCC4 acts as a central hub in non-homologous end joining (NHEJ), and interacts with XLF, DNA Ligase IV, APTX, and PNK [Mahaney et al. 2013]. XRCC4 is comprised of an N-terminal head domain, made up of a 7-stranded anti-parallel beta sandwich, and a helix-turn-helix motif [Junop et al. 2000; Dore et al. 2006; Sibanda et al. 2001]. It also has an elongated, flexible α -helical coiled-coil region, and carboxyl termini tails which are phosphorylated by CK2 [Koch et al. 2004]. In addition to protein-protein interactions, XRCC4 efficiently binds long DNA substrates (>100bp), preferring nicks or broken ends *in vitro* [Modesti et al. 1999].

The XRCC4 head domain binds to the structurally related protein XLF [Hammel et al. 2011; Ropars et al. 2011; Wu et al. 2011; Andres et al. 2012]. X-ray structures, transmission electron microscopy (TEM), and SAXS indicate that alternating XRCC4 and XLF interact to form helical filaments. Crystal lattice packing and atomic force microscopy (AFM) imaging of XRCC4/XLF revealed large superhelical filaments (Figure 7A). DNA binding by the superhelical filament may occur in grooves outside the filament or through a central pore that has been calculated to be large enough to enclose dsDNA (Figure 7A), or even an entire nucleosome. The filament has the potential to align mismatched broken DNA ends, providing a molecular basis for XRCC4/XLF mediated enhancement of ligation efficiency in NHEJ [Andres et al 2012; Ropars et al. 2011; Wu et al. 2011; Hammel et al. 2011].

C-terminal to the head domain, the XRCC4 homodimeric coil binds, stimulates, and controls nuclear import of DNA Ligase IV [Junop et al. 2000; Critchlow et al. 1997; Grawunder et al. 1997; Berg et al. 2011]. The XRCC4 coiled-coil region is encircled and bent by the Ligase IV helix-loop-helix clamp region between the Ligase IV tandem BRCT domains [Wu et al. 2009] (Figure 7B). At the XRCC4 unstructured C-terminus, CK2-phosphorylation sites

regulate protein-protein interactions and binding of the FHA domains of PNK, APTX and the Aprataxin and PNK-like factor (APLF) [Clements et al. 2004; Koch et al. 2004; Iles et al. 2007; Macrae et al. 2008]. These interactions are hypothesized to coordinate requisite end processing reactions with the DNA ligation step in NHEJ, which is catalyzed by DNA Ligase IV [Koch et al. 2004].

Structural scaffolding by XRCC1 and XRCC4 may act in limiting release of mutagenic DNA-strand break intermediates, and provide a physical mechanism to efficiently orchestrate molecular hand-offs in DNA damage repair to avoid disrupting transcription and replication processes, as originally proposed for BER proteins [Mol et al. 2000; Wilson and Kunkel 2000]. Aside from logical inferences from structural characterizations, experimental evidence for substrate channeling between enzymatic activities remains limited [Prasad et al. 2010]. A significant challenge in the future requiring multi-pronged biochemical, structural and cell biological approaches will be to establish detailed mechanistic understanding of how these protein scaffolds and flexible multi-enzyme processing assemblies achieve high specificity, efficiency, and coordination in DNA strand break repair.

SUMMARY AND PERSPECTIVES

Ultimately, the mechanistic study of the structural chemistry of DNA damage response pathways has broad ranging implications for understanding cellular mechanisms of DNA strand break repair. This work is providing insights into individual genetic vulnerabilities to DNA damage, and an intimate understanding of the molecular principles of susceptibility and resistance to commonly employed cancer chemotherapeutics.

The importance of DNA end-processing factors in preserving genomic integrity is underscored by the fact that deficiencies in protein factors discussed here contribute to a variety of neurological diseases and cancer susceptibility [Rulten and Caldecott 2013]. For example, heritable mutations in the human *APTX* gene are linked to the autosomal recessive neurological disorders Ataxia with Oculomotor Apraxia 1 (AOA1) [Date et al. 2001; Moreira et al. 2001], a Parkinsons-like multiple system atrophy (MSA) [Baba et al. 2007], and Ataxia with coenzyme Q10 (coQ10) deficiency [Quinzii et al. 2005]. Germline hypomorphic *NBS1* and *MRE11* mutations cause radiosensitivity and chromosome instability syndromes, Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia-like disorder (ATLD), respectively [Carney et al. 1998; Stewart et al. 1999]. Human CtIP mutations are linked to the microcephaly and dwarfism Seckel syndrome [Qvist et al. 2011]. Mutations in *PNK* are associated with microcephaly with early onset seizures (MCSZ) [Shen et al. 2010; Reynolds et al. 2012], and *TDPI* mutation is associated with SCAN1 [El-Khamisy et al. 2005; Takashima et al. 2002]. The emerging detailed understanding of DNA end processing mechanisms derived from structural biology is shedding light onto precisely how heritable DNA repair defects in these factors contributes to neurodegeneration, as well as heritable and somatic carcinogenesis.

Tumor cells are oftentimes deficient in repair and/or signaling of DNA damage, such that therapeutic targeting of backup DNA repair pathways that compensate for such deficits can be a winning strategy to selectively kill cancer cells [Martin et al. 2008; Curtin 2012]. Thus,

the systematic definition of the molecular mechanisms of genome repair by DNA end processing factors may open new doors to these “synthetic lethal” treatment regimens, and also facilitate discovery and development of novel targeted DNA repair inhibitors for treatment of cancers that have acquired resistance to DNA crosslinkers, alkylating agents, topoisomerase poisons, and other DNA-targeted chemotherapeutics.

Finally, we note the recent advances in the field that indicate the abundance of RNA contamination in the genome outnumbers all other known potential threats to genomic integrity combined [Nick McElhinny et al. 2010; Reijns et al. 2012; Williams et al. 2013]. This raises important questions: 1) How does ribose chemistry influence DNA transactions carried out by known repair factors, and 2) what additional factors operate in the RNA-DNA realm to preserve integrity of the genome? Further, the identification of an RNA ligase that can function to join 3'-phosphorylated or 2'-3' cyclic phosphate ends to 5'-OH termini in the context of DNA [Das et al. 2013] suggests that our *de facto* views of DNA end joining may require revision in the context of RNA-DNA (Huang and Pommier 2013). Given the abundance of RNA incorporation in the genome it seems likely that other known and yet to be discovered factors may play roles in detection and resolution of RNA-derived lesions and act in RNA-DNA damage responses (RDDR) similar to that recently documented for APTX [Tumbale et al. 2014].

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5' end damage

Damage	5' -hydroxyl	5'-deoxyribose phosphate (dRP)	5'-Ribonucleotide	5'-aldehyde	5'-Top 2 adduct	5'-adenylate (AMP)
Source	ROS	Depurination, BER	RER	ROS	Stalled Top2	Abortive DNA ligase reactions
Repair Enzyme (s)	PNK	Polβ	Fen1	Polβ, Fen1	Tdp2, Mre11/Rad50/Nbs1/CtIP	Aptx, Fen1

3' end damage

Damage	3'-phosphate	3'-phosphoglycolate (PG)	3'-phospho-α, β-unsaturated aldehyde (PUA)	3'-Top1 adduct	2'-3' cyclic phosphate
Source	ROS, Tdp1	ROS, AP-lyase	AP-lyase	Stalled Top1	Top1 incision at ribonucleotides
Repair Enzyme (s)	PNK/Ape1	Ape1/Tdp1	Ape1/Tdp1	Tdp1	?

Figure 1. Chemically heterogeneous, “dirty”, DNA end structures

Repair of strand breaks by DNA ligases requires 5'-phosphate and 3'-hydroxyl groups.

Dirty ends have chemical modifications (red) at either the 5'- or 3'-ends that will interfere with ligation. Sources of specific types of damage and enzymes that can repair them are indicated.

Figure 2.

Map of DNA end cleansing pathways. Networks of end-cleansing proteins repair specific types of end damage before DNA ligase can complete the final break–sealing reaction. Unrepaired DNA damage can interfere with the function of enzymes such as DNA topoisomerases or DNA ligase to create compound metabolized DNA damage (see blue brackets and arrows).

5' direct reversal mechanisms

Figure 3. Structural basis for repair of 5'-DNA ends by end-cleaving enzymes

(Left, gray box) Specific examples of 5'-end damage are removed or corrected to leave ligatable ends (5'-phosphate termini). Right: cartoon representations of repair enzymes with α -helices (blue) and β -strands (green) highlighting their catalytic domain bound to DNA (yellow). Surface representation of repair enzymes showing damaged DNA ends (red) engaged in the enzyme active site. Figures were generated from PDB entry 4NDH for APTX, 3ZVN for PNK kinase domain, 4GZ0 for TDP2, 1JEY for Ku70/80, 2P66 for POL β ,

Figure 4. Structural basis for repair of 3'-DNA ends by end-cleaving enzymes
 (Left, gray box) Specific examples of 3'-end damage are removed. Right: cartoon representations of repair enzymes with α -helices (blue) and β -strands (green) highlighting their catalytic domain bound to DNA (yellow). (right) Surface representation of repair enzymes showing damaged DNA ends (red) engaged in the enzyme active site. Figures were generated from PDB 1RFF for TDP1, and 3U7F for the PNK phosphatase-DNA complex.

Nucleolytic processing enzymes

Mre11

Monomer A

Fen1

Ape1/Ape2

Figure 5. Structural basis for repair of DNA ends by nucleolytic processing enzymes (Left, gray box) DNA structures (red) recognized and cleaved by nucleolytic enzymes. (Center) Cartoon representations of repair enzymes with α -helices (blue) and β -strands (green) highlighting their catalytic domain bound to DNA (yellow). (Right) Surface representation of repair enzymes showing substrate DNA engaged in the enzyme active site. Figures were generated from PDB entry 3DSD for MRE11, 3Q8L for FEN1, and 1DE9 for APE1.

Figure 6. XRCC1 acts as a strand break repair assembly line

XRCC1 orchestrates SSBR proteins by serving as an extended protein-protein interaction platform. An example form of complex DNA damage generated by reactive oxygen is displayed (red rectangle). Multistep processing of DNA damage requires. Variable outcomes, including abortive ligation reactions are resolved by FHA domains of DNA end-processing enzymes APTX and PNK (PDB ID 3KT9, 1YJM) associate with phosphorylated XRCC1, localizing the catalytic APTX deadenylase (PDB ID 4NDH) and PNK phosphatase and kinase domains (PDB ID 1YJ5) to damaged DNA ends. Efficient repair also requires binding of POLβ through the XRCC1 NTD (PDB ID 3K75). XRCC1's BRCT2 and Ligase III's BRCT modules (PDB ID 3QVG) link DNA Ligase III's catalytic domain (PDB ID 3L2P) to the DNA break site for final sealing of the nicked DNA backbone. XRCC1 BRCT1 (PDB ID 2D8M).

Figure 7. XRCC4-XLF coordinates DNA double strand break repair

A.) XLF occupies binding sites at XRCC4's NTD (PDB ID 3RWR), forming an elongated, helical filament of alternating XRCC4 and XLF proteins. Both proteins lack enzymatic activity but are proposed to coat DNA strand breaks and align DNA breaks for efficient end processing and ligation. B.) XRCC4-NHEJ repair protein interactions drive DNA end processing and ligation of double-strand breaks. Like XRCC1, XRCC4's flexible, phosphorylated C-terminal tails mediate binding for both APTX (3KT9) and PNK FHA domains (1YJM). DNA Ligase IV stability is dependent on its association with XRCC4, encasing XRCC4's helical tails with tandem BRCT domains (PDB ID 3II6) that bind Ligase IV's catalytic domain (PDB ID 3W50).