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Influence of DNA Lesions on Polymerase-Mediated DNA Replication at Single-Molecule Resolution

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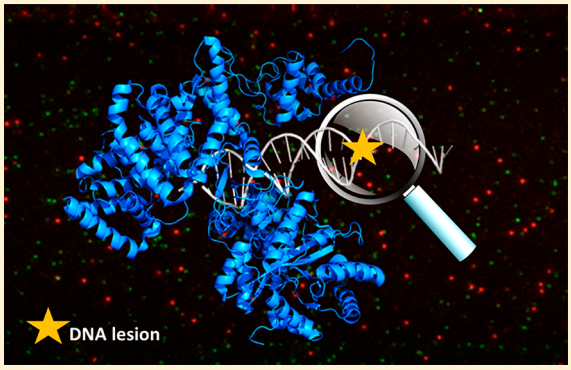
ABSTRACT: Faithful replication of DNA is a critical aspect in maintaining genome integrity. DNA polymerases are responsible for replicating DNA, and high-fidelity polymerases do this rapidly and at low error rates. Upon exposure to exogenous or endogenous substances, DNA can become damaged and this can alter the speed and fidelity of a DNA polymerase. In this instance, DNA polymerases are confronted with an obstacle that can result in genomic instability during replication, for example, by nucleotide misinsertion or replication fork collapse. It is important to know how DNA polymerases respond to damaged DNA substrates to understand the mechanism of mutagenesis and chemical carcinogenesis. Single-molecule techniques have helped to improve our current understanding of DNA polymerase-mediated DNA replication, as they enable the dissection of mechanistic details that can otherwise be lost in ensemble-averaged experiments. These techniques have also been used to gain a deeper understanding of how single DNA polymerases behave at the site of the damage in a DNA substrate. In this review, we evaluate single-molecule studies that have examined the interaction between DNA polymerases and damaged sites on a DNA template.



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1. INTRODUCTION

1.1. DNA Polymerases and DNA Replication. DNA polymerases (DNA Pols) catalyze the synthesis of DNA in a 5′ to 3′ direction by inserting a nucleotide at the 3′-end of a growing DNA primer hybridized to the template (parental) DNA strand. Catalysis occurs via the nucleophilic attack of the 3′ hydroxyl group of the primer strand on the α phosphate group of the enzyme-bound deoxynucleotide triphosphate. Replicative Pols perform synthesis at fast rates and high fidelity; for example, the holoenzyme of DNA polymerase III in *E. coli* can replicate at speeds of 600−1000 bases per second and an error rate of one mistake per million nucleotides inserted.[1,2] The nucleotidyl transfer reaction requires the presence of divalent ions, Mg^{2+} being the physiologically relevant ion used by most DNA Pols. However, other divalent metals such as Mn^{2+}, Ca^{2+}, and Co^{2+} can be substituted to activate certain polymerases.[3]

DNA replication must be extraordinarily accurate if there is to be the faithful duplication of the genomic information during cell division.[4] Although replicative DNA Pols catalyze DNA synthesis with very high fidelity (with replication error rates as low as 10^{-6} to 10^{-8}),[4,5] misinsertions and frameshifts can occur. In general, the fidelity of DNA replication depends on a variety of factors including canonical base paring with the template DNA, nucleotide selection, proofreading, and mismatch repair.[5] Canonical base pairing is the molecular pairing between A-T and G-C

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nucleotides and is dependent on the molecular shape complementarity as well as the hydrogen bonding capacity between the cognate base pairs. In cells, nucleotides are selected by DNA Pols that discriminate from a nucleotide pool consisting of four deoxynucleotide triphosphates (dNTPs) and four ribonucleotide triphosphates (NTPs), which are often in much higher concentration than dTNPs.[6] Nucleotides can become damaged prior to being incorporated during replication.[7,8] In the event of a misinsertion, high-fidelity DNA Pols have an exonuclease domain or an associated subunit that can perform proofreading where an incorrect nucleotide is removed from the 3′ end of DNA, thereby increasing replication fidelity.[4,9]

1.2. DNA Damage and Translesion DNA Synthesis. Exposure to either endogenous or exogenous substances can cause damage in DNA.[10−12] Exogenous substances such as environmental pollutants, UV light, or cigarette smoke can react with DNA by various mechanisms and change its molecular structure. Endogenous agents, such as reactive oxygen species (ROS) or byproducts from metabolic pathways of lipid peroxidation, can also react with DNA and cause damage.[13] The structural variety of DNA damage that can occur in our genome is very diverse, generating a myriad of potential structures that the polymerase must deal with to allow for faithful DNA replication. Common classes of lesions are UV-induced base damage, DNA alkylation, including bulky adducts, such as those formed by aromatic amines or polycyclic aromatic hydrocarbons, oxidative damage, and abasic sites (Figure 1). DNA lesions resulting from UV-exposure include cyclobutane pyrimidine dimers (CPD) and 6,4 photodimers (Figure 1),[14] which are the result of two monomeric nucleobases that react to form TT, CC, or TC dimers.[15−18] Alkylation or bulky DNA adducts result from the covalent attachment of a chemical agent to the DNA nucleobase such as methylating agents like methylmethanesulfonate (MMS)[19] or *N*-methyl-*N*-nitrosourea (MNU),[20] the environmental pollutant benzo[a]pyrene (B[a]P)[21−24] acetylaminofluorene,[25,26] or the fungal metabolite aflatoxin[27−29] that can contaminate food supplies. Oxidative DNA damage is generated by ROS reacting with nucleotides. Guanosine, with the lowest oxidation potential, is oxidized at the highest frequency compared to A, T, and C.[30] One of the most common forms of DNA damage in human cells is the abasic site, which can arise upon exposure to alkylating agents to produce positively charged bases that are hydrolytically cleaved at the *N*-glycosidic bond.[31] For example, exposure to substances that can react at the N7 position of guanine or N3 position of adenine can result in abasic site DNA damage.[32,33] Alternatively, abasic sites can be generated via the spontaneous loss of the nucleobase.[34] Collectively, it is thought that approximately 10^4 abasic sites occur per day per cell.[34−36] Given the constant attack of bioreactive agents on DNA, it is important to understand how each form of damage can contribute to the establishment of mutations during DNA replication.

An important repercussion of DNA damage is that these sites can slow or block the progression of the replication fork because high-fidelity polymerases are unable to accommodate these adducts in their active site.[37−41] To bypass the damaged site, the polymerase in the replisome exchanges with a lesion-specific Y-family polymerase, which can synthesize across from and past a specific class of lesion.[42] Structurally, Y-family DNA Pols have larger, more solvent exposed active sites that allow for the accommodation of bulky distortional DNA lesions, which otherwise stall more stringent replicative polymerases.[43,44] Their flexibility comes at a cost in fidelity with error rates ranging from 10^{-2}−10^{-4}.[45] Although translesion synthesis (TLS) is a more mutagenic pathway, it allows for DNA replication to continue past DNA lesions that could otherwise lead to replication fork collapse and double-strand breaks. After TLS, DNA repair pathways can survey and fix damaged DNA as well as correct genomic mutations. Seminal biochemical studies have been performed to elucidate how DNA lesions affect DNA Pol behavior and cause mutations.[46−49] However, these ensemble-averaged bulk experiments can miss some key transient mechanistic intermediates along the biochemical pathway. In recent years, numerous single-molecule experiments have helped overcome this issue by directly observing individual polymerases during replication.[50]

1.3. Single-Molecule Approaches To Study Lesion Bypass. In the last two decades, single-molecule biophysics

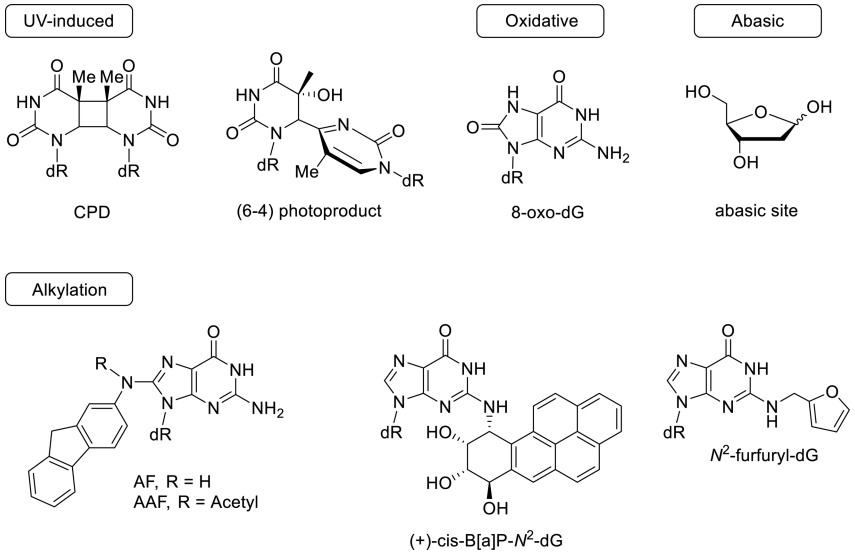

Figure 1. Common classes of DNA damage and their structural diversity. The four most common classes of lesions included are UV-induced photodamage (CPD and (6−4) photoproducts), oxidative damage (8-oxo-dG), abasic sites, and alkylation-derived DNA adducts (AF, AAF, (+)-*cis* B[*a*]P-N^2-dG, and N^2-furfuryl-dG).

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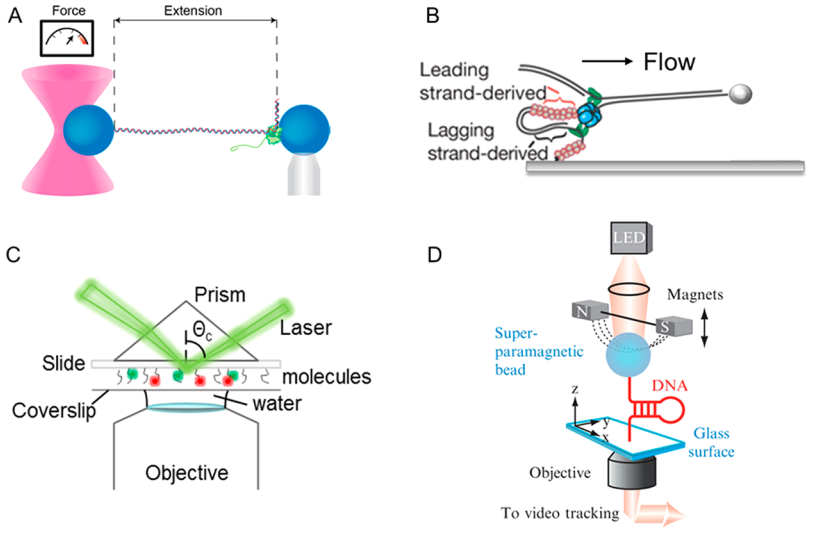

Figure 2. Typical single-molecule approaches to study lesion bypass in DNA replication. Schematic diagrams of fluorescence and force-based single-molecule techniques: (A) optical tweezers with a bead in an optical trapping laser (left) that is functionalized with DNA to monitor DNA replication (adapted from Heller, I.; Hoekstra, T. P.; King, G. A.; Peterman, E. J. G.; Wuite, G. J. L. Optical tweezers analysis of DNA−protein complexes, *Chemical Reviews* **2014**, *114* (6), 3087−3119. Copyright 2014 American Chemical Society[66]), (B) flow-based assay where a buffer flow is applied to stretch out DNA that is tethered to a bead to track DNA replication (adapted with permission from Hamdan, S. M., Loparo, J. J., Takahashi, M., Richardson, C. C., and van Oijen, A. M. Dynamics of DNA replication loops reveal temporal control of lagging-strand synthesis. *Nature* **2009**, *457* (7227), 336−339),[67] (C) total internal reflection single-molecule FRET where a laser is passed through a prism to generate an evanescent wave to excite immobilized molecules attached to a slide surface, and (D) magnetic tweezers that trap a magnetic bead functionalized with DNA and on the other side to a glass surface (adapted from Manosas, M.; Meglio, A.; Spiering, M. M.; Ding, F.; Benkovic, S. J.; Barre, F.-X.; Saleh, O. A.,; Allemand, J. F.; Bensimon, D.; Croquette, V. Magnentic tweezers for the study of DNA tracking motors. *Methods in Enzymology* **2010**, *475*, 297−320, with permission from the authors).[68]

has led to major advances in our understanding of nanoscopic biomolecular reactions like DNA replication. A variety of single-molecule techniques have been employed to interrogate mechanistic details of polymerase binding and catalysis at sites of DNA damage. Each technique has strengths and limitations; thus, it is advantageous to approach the complex biology of DNA replication with a variety of single-molecule strategies. Single-molecule imaging can generally be classified into fluorescence-based spectroscopy, for example, single-molecule Förster resonance energy transfer (smFRET) and force-based measurements, for example, optical and magnetic tweezers and flow-based assays (Figure 2). In addition to these *in vitro* techniques, super-resolution imaging has provided unprecedented insight into the mechanisms of DNA replication in the physiological environment of the cell.[51−53] Below, we briefly discuss these *in vitro* single-molecule techniques. For additional details, we direct the reader to more in-depth reviews on this topic[54−64] and a recent review discussing lesion bypass mechanisms by X-ray crystallography and smFRET.[65]

Two force-based methods that have been used to investigate DNA Pol mechanisms at a damaged site are optical and magnetic tweezers. In typical optical tweezers trapping experiments, a DNA molecule is tethered to polystyrene beads trapped by a highly focused laser beam (Figure 2A). Changes in DNA length and applied force are then measured during a replication reaction. DNA templates for optical trapping experiments should be ≥1 μm long (~3000 base pairs) to reduce photodamage caused by the trapping lasers at the DNA ends and to avoid the cross-talk between the traps.[69] With magnetic tweezers, instead of optically trapping polystyrene beads, magnetic beads are trapped in a magnetic field (Figure 2D). Here, the force is modulated by changing the relative position of the bead in the field. In force-based flow assays, flow is applied to stretch long DNA molecules tethered between the microscope slide surface and a bead (Figure 2B) while monitoring either the bead location or a fluorescence reporter. Owing to the different elastic properties of single and double-stranded DNA, replication and degradation can be monitored as changes in the total DNA length over time.

Fluorescence-based assays, like smFRET, can be performed with fluorescent dyes attached to either the DNA template and/or the DNA Pol to monitor the dynamics of protein-nucleic acid interactions.[70,71] Several strategies have been reported to assist in labeling challenging protein substrates with fluorescent dyes, which have been extensively reviewed elsewhere.[72−74] FRET relies on two appropriately positioned fluorescent dyes that are within a specific interaction distance of 10−100 Å. During FRET, a donor fluorophore is excited and transfers energy to an acceptor fluorophore through a nonradiative process.[75] The energy transfer efficiency is directly related to the intervening distance between the donor and acceptor dyes. Thus, FRET is often referred to as a molecular ruler as it can provide molecular-based distance information between two reporter dyes. For smFRET, total internal reflection (TIR) microscopy is used to create an evanescent field of excitation light, 100−200 nm from the surface (Figure 2C).[76] This reduces background fluorescence and allows for the visualization of single molecules.

One advantage of force-based techniques is that they can monitor reactions on long (1−15 μm) DNA substrates and are less constrained by distance limitations (<100 Å) in smFRET.[77] Moreover, force-based techniques often have a trade-off between spatial resolution and throughput. An advantage of smFRET is that detailed conformational changes of the protein can be monitored with higher resolution, a feature that is often lost in a force-based and flow-based assays with long DNA molecules. As there are both advantages and disadvantages for each single-molecule approach, it is beneficial to apply multiple techniques to obtain a broader understanding behind the biology of reactions *in singulo*. It is noteworthy that previous studies have combined both fluorescence and force- or flow-based techniques; however,

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these did not investigate polymerase behavior on damaged DNA strands and are, therefore, not reviewed here. These reports include observing DNA replication loops in real-time, understanding the influence of intercalators on DNA Pol dynamics,[78] watching fluorescently tagged DNA Pols replicate nascent DNA,[79] and assays involving rolling-circle DNA replication.[80−82]

In this review, we describe recent single-molecule reports that have studied the mechanism of Pol action on templates containing DNA damage. A range of studies have been performed with varying types of DNA lesions such as the oxidative DNA lesion 8-oxo-G, covalent adducts ranging from the N^2-furfuryl-dG adduct, and the AF and AAF bulky DNA adducts as well as UV-induced CPD lesions (Table 1).

Table 1. Summary of Single-Molecule Studies on DNA Pol-Mediated Replication and Respective DNA Lesions Reviewed Here

type of lesion	name of lesion[a]	DNA Pol	technique	ref
alkylation	N^2-furfuryl-dG	*E. coli* DNA Pol III/IV	flow assay	98
alkylation	AF/AAF	*E. coli* DNA Pol I	smFRET	97
alkylation	AF/AAF	Dpo4	smFRET	96
alkylation	(+)-*cis*-B[*a*]P-N^2-dG	Dpo4	smFRET	107
oxidative	8-oxo-dG	Dpo4	smFRET	108
UV	CPD	T7 DNA Pol	optical tweezers	88
UV	UV	*E. coli* DNA Pol V	PALM[b]	89

[a]AF, 2-aminofluorene; AAF, *N*-acetyl-2-aminofluorene; (+)-*cis*-B[*a*]P-N^2-dG; CPD, cyclobutane pyrimidine dimer. [b]Super-resolution microscopy, PALM images (for structures of lesions, see Figure 1).

2. SINGLE-MOLECULE STUDIES ON UV-INDUCED DNA LESIONS

While a variety of bulk biochemical studies have provided insight into the behavior and dynamics of DNA Pols at sites of UV-induced lesions,[49,83−87] fewer studies have been reported at single-molecule resolution. One study, performed by Sun and colleagues, investigated the ability of the T7 DNA Pol and helicase to bypass the *cis-syn* CPD lesion (Figure 1, CPD) using single-molecule optical tweezers.[88] In this study, a forked DNA substrate was held at each end under a constant low force (6−12 pN) to prevent mechanical unzipping of the DNA (see Figure 3A for an illustration of the DNA construct alone); thus, the DNA was only actively unwound by the action of the T7 helicase. The CPD lesion was placed on either the leading or lagging strand and no difference in T7 helicase-mediated DNA unwinding was observed, suggesting that the central channel of the helicase can accommodate the lesion and translocate without stalling or pausing. In contrast to this, T7 DNA Pol was unable to replicate past the CPD and stalled at the site of the lesion under forces in the 8−12 pN range (Figure 3A). Interestingly, T7 DNA Pol was found to replicate past the CPD lesion in the presence of T7 helicase, suggesting the presence of an interaction between the two enzymes (Figure 3B). The observation that T7 DNA Pol alone cannot replicate past a CPD and that a coupled DNA Pol-helicase complex is necessary to overcome lesion bypass demonstrates the importance of additional proteins to overcome DNA lesions that can act as roadblocks for DNA Pol-mediated TLS.

To probe whether a direct interaction between the DNA Pol and helicase was necessary for TLS, Sun and co-workers prepared a helicase deletion mutant lacking its T7 DNA Pol interaction domain (17 carboxy-terminal amino acid residues). In these conditions, T7 DNA Pol was not able to bypass the CPD, demonstrating that a direct interaction between the two proteins is necessary for TLS. Bulk assays were also carried out to confirm that helicase unwinding and DNA Pol replication were not a result of tension applied on the DNA. For this, primer extension reactions were carried out to show that T7 DNA Pol only bypassed the CPD lesion in the presence of the helicase (15% full-length extension) and that negligible TLS was observed in the presence of the helicase mutant (3% full-length extension).

Overall, this study provides insight into how the bacteriophage T7 replisome can tolerate DNA damage during replication.

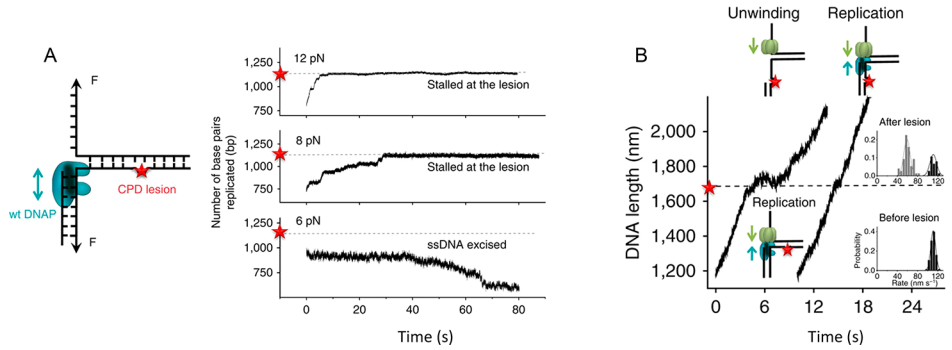

Figure 3. Schematic showing the single-molecule optical tweezer experiments on a CPD containing template (red star). (A) Three representative traces showing the number of base pairs replicated versus time at 1 mM each dNTP. T7 DNA Pol does not replicate past the CPD lesion and stalls at 8 pN and 12 pN, whereas at 6 pN the DNA Pol excises the DNA from the 3′ end. The dotted line indicates the position of the CPD lesion. (B) Speed of replication was determined by measuring the length of DNA over time in the presence of T7 DNA Pol and T7 helicase. The three cartoons show the varying protein complexes that can be present at the replication fork, which include helicase unwinding (top left), replication with the polymerase and helicase before the lesion (bottom) and after the lesion (top right). Before the lesion, the rate of DNA length increase was determined to be 109 nm s^{-1} (bottom inset). After replication of the lesion, two rates were observed a slower rate of 59 nm s^{-1} and faster rate of 109 nm s^{-1} (top inset). The slower rate is similar to helicase unwinding alone (63 nm s^{-1}), suggesting the helicase continues to unwind DNA while the polymerase stalls or dissociates (top left helicase alone unwinding scheme). The presence of the fast rate (109 nm s^{-1}) indicates that a fraction of DNA polymerases can bypass the lesion. Adapted from Sun, B. et al. T7 replisome directly overcomes DNA damage. *Nat. Commun.* **2015**, *6*, 10260.[88] This work is licensed under a Creative Commons Attribution 4.0 International License: http://creativecommons.org/licenses/by/4.0/.

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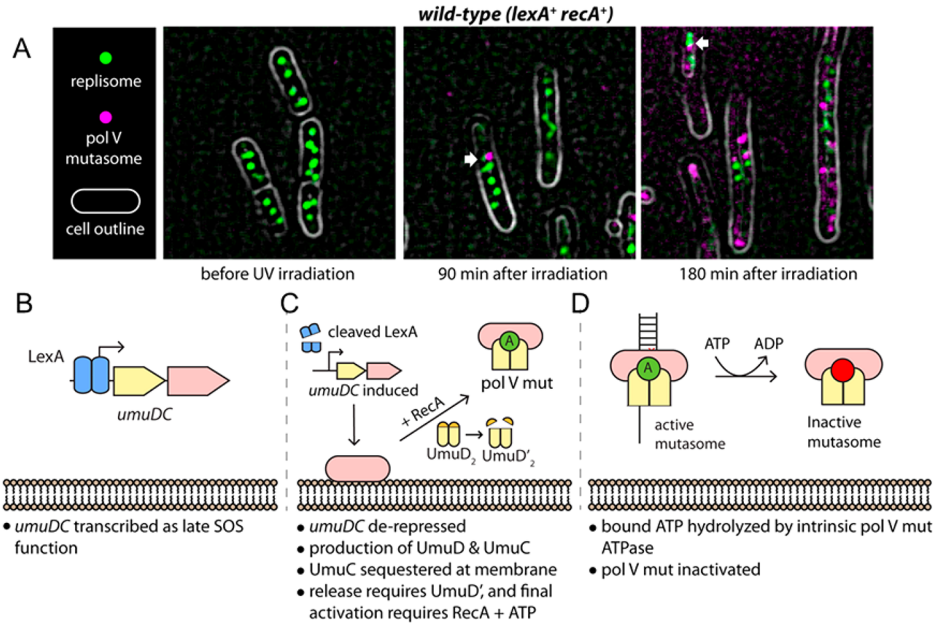

Figure 4. DNA Pol V activation scheme. (A) Average projection time-sampling movies in *E. coli* cells showing the colocalization of replisomes (green) and mutasomes (pink) before and after UV-irradiation at 90 and 180 min. Examples of colocalization are indicated with white arrows. (B–D) Schematic depiction of the mechanisms limiting Pol V activity on DNA (B) transcriptional level regulation for the expression of umuDC by LexA, (C) expression of UmuD and UmuC, subsequent localization of UmuC to the membrane and release of UmuC to the cytosol in the presence of $UmuD'_2$ to produce Pol V ($UmuD'_2C$), (D) the dependence on ATP for Pol V inactivation. Adapted from Robinson, A.; McDonald, J. P.; Caldas, V. E.; Patel, M.; Wood, E. A.; Punter, C. M.; Ghodke, H.; Cox, M. M.; Woodgate, R.; Goodman, M. F.; van Oijen, A. M. Regulation of mutagenic DNA polymerase V activation in space and time. *PLoS Genet.*, **2015**, *11* (8), e1005482.[89] This work is licensed under a Creative Commons Attribution 4.0 International License: http://creativecommons.org/licenses/by/4.0/.

It is interesting to note that bacteriophages lack TLS DNA Pols that are found in other organisms, so other pathways for lesion tolerance are important. Further, this work not only highlights important insights into replication past CPD lesions, but also illustrates that replication past DNA lesions can change with higher order protein complexes of the replisome in comparison to single enzyme conditions, for example, DNA Pol alone. To further elucidate lesion tolerance mechanisms in more complex biological assemblies, single-molecule studies containing all replisomal proteins are warranted.

A study performed in *E. coli* monitored the regulation of DNA Pol V, a TLS DNA Pol, upon irradiation with UV.[89] Using super-resolution single-molecule fluorescence microscopy, the spatial and temporal regulation of Pol V was measured in live cells (Figure 4A). Pol V is produced through a number of steps *in vivo* for which the nucleoprotein filament RecA is required. The last step involves the association of $UmuD'_2$ with UmuC proteins to form Pol V ($UmuD'_2C$). To achieve this, the operon of UmuC was engineered to incorporate the red fluorescent protein mKate2 at the C-terminus (UmuC-mKate2). Following UV irradiation, the number of molecules of UmuC-mKate2 was found to increase from ∼2 to ∼16 over 180 min. After UV treatment, the cellular location of UmuC-mKate2 was found to shift from the membrane periphery into the cytosol as a function of time. Further, with various mutants containing defects in the Pol V activation pathway it was found that the release of UmuC from the membrane is dependent on the cleavage of $UmuD_2$. The cleavage of $UmuD_2$ is facilitated by RecA to form $UmuD'_2$. The main findings provide support for a three-part mechanism in the regulation of DNA Pol V (Figure 4B–D).

The regulation of Pol V is tightly controlled because its replication is highly error-prone. Therefore, Pol V is upregulated when normal DNA replication is impeded, for example when there is an accumulation of DNA lesions. This study highlights the importance of examining the regulation of DNA Pols in response to DNA damage in the biological context of the cell. It is hoped that further studies can be performed that monitor the spatial and temporal regulation of DNA Pols upon exposure to oxidative and alkylative DNA damaging agents at a single-molecule level in live cells.

3. SINGLE-MOLECULE STUDIES ON ALKYLATED DNA ADDUCTS

DNA can react with endogenous and exogenous molecules leading to alkylation on DNA. This results in covalently modified DNA that can influence the dynamics and behavior of DNA polymerases at the site of the damaged DNA. Single-molecule FRET studies have been performed to investigate the effect of AF, AAF, (+)-*cis* B[*a*]P–N2-dG, and N^2-furfuryl-dG lesions (Figure 1). In these studies, the bulky DNA adducts impacted DNA Pol dynamics and the ability to perform DNA replication.

Humans are exposed to a vast array of carcinogenic aromatic amines present in cooked meats, tobacco smoke, and environmental pollutants.[90−92] Exposure to these chemicals can result in metabolically activated intermediates that react with DNA to form adducts. The model aromatic amine adducts, 2-aminofluorene and *N*-acetyl-2-aminofluorene linked to the C8 position on DNA, have been well studied by a variety of techniques.[93] Structurally, the acetyl group on an AAF adduct changes it conformational structure in DNA causing a greater distortion as compared to the analogous AF adduct. This is most likely due to the fact that AAF causes the modified guanine to adopt an anti-conformation, while AF-dG has a syn-conformation.[94] This conformational change in the adduct structure influences how DNA

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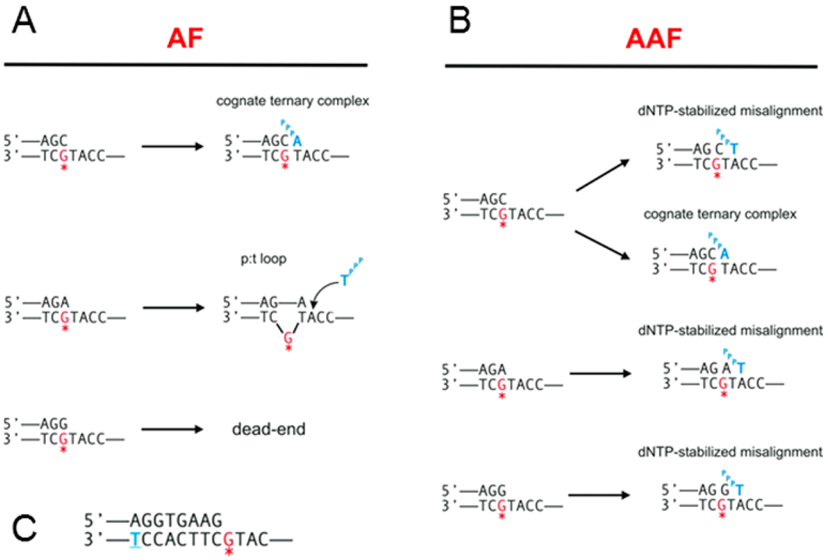

Figure 5. Models for the mutagenic bypass by Dpo4. (A) In the presence of an AF adduct, insertion of the correct dATP is preferred at a terminal C:G base pair (top). For an A:G mismatch, the misaligned DNA (middle) allows dTTP misinsertion by looping out the AF DNA adduct. The G:G mismatch is a dead end substrate for AF-containg DNA by Dpo4. (B) For the AAF DNA adduct, dTTP misinsertion is preferred in all cases suggesting a dNTP-stabilized misalignment mechanism. (C) Position of the DNA adduct is depicted as the red G and the position of the Cy3-donor dye is indicated as the blue T. Adapted from Brenlla, A.; Rueda, D.; Romano, L. J.Mechanism of aromatic amine carcinogen bypass by the Y-family polymerase, Dpo4. *Nucleic Acids Res.* **2015**, *43* (20), 9918−27.[96] This work is licensed under a Creative Commons Attribution 4.0 International License: http://creativecommons.org/licenses/by/4.0/.

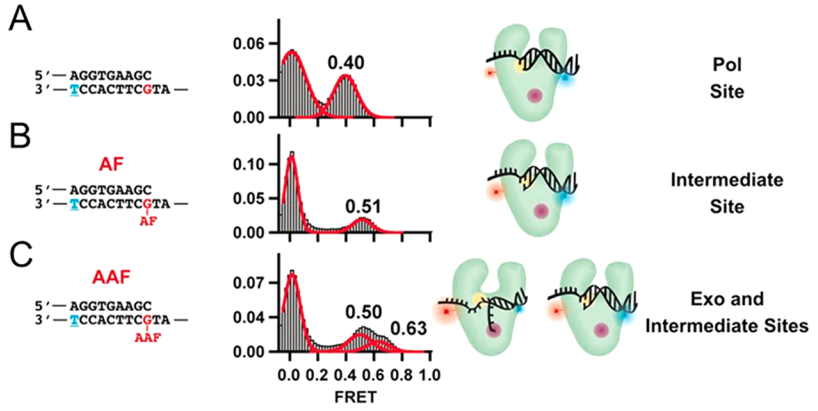

Figure 6. Distinct binding orientations for KF at (A) unmodified DNA where a single 0.4 FRET state is observed indicating Pol-binding mode and (B) AF DNA adduct where an intermediate 0.51 FRET state is observed and an (C) AAF DNA adduct where two FRET states, 0.5 and 0.63, are observed as the intermediate and exobinding mode, respectively. Adapted from Vrtis, K. B.; Markiewicz, R. P.; Romano, L. J.; Rueda, D. Carcinogenic adducts induce distinct DNA polymerase binding orientations. *Nucleic Acids Res.* **2013**, *41* (16), 7843−53.[97] This work is licensed under a Creative Commons Attribution 3.0 International License: https://creativecommons.org/licenses/by/3.0/.

Pols can perform TLS and AAF-dG adducts are stronger blocks to replication than AF-dG adducts.[95,96]

In a study by Brenlla and co-workers, the binding conformations of the *Sulfolobus sulfataricus* Y-family DNA Pol, Dpo4, on primer-templates containing either an AF or AAF DNA lesion were evaluated.[96] Here, Dpo4 was labeled with Cy5 at its sole native cysteine residue (C31) and the DNA constructs containing dG, AF-dG, or AAF-dG positioned on the template at the primer-template junction were labeled with a Cy3 donor. This study showed that both adducts caused significant structural distortion of the binary complex when the primer terminus was positioned before or across from the adduct site. The ternary complex, which forms upon the addition of the next correct dNTP, resulted in FRET efficiency histograms that resembled that measured with analogous unmodified templates. When similar experiments were carried out with mismatches across from the adduct positions, significant differences for the AF and AAF adducts were observed for both DNA extension reactions and the smFRET peak positions. Although the AF adduct induced a structure consistent with the adducted G looping out allowing the *n*+2 base to serve as the template (Figure 5A), the AAF adduct formed complexes that did not depend on the nucleotide across from the adduct. In the AAF case, the nucleotide incorporation results and smFRET analysis suggested that a nucleotide-stabilized misaligned structure had formed (Figure 5B).

Another study investigating the binding orientations at AF and AAF DNA adducts was reported with *E. coli* DNA Pol I (Klenow Fragment, KF).[97] Similar to the work with Dpo4 described above, distinct binding modes were observed for AF and AAF adducts. As with the Dpo4 experiments, the DNA templates containing AF and AAF were labeled with Cy3 and KF with Cy5.

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Primer extension analysis showed that KF could synthesize past the AF-dG adduct, while AAF-dG was a strong block, with synthesis completely inhibited once a nucleotide was incorporated across from the adduct. Single-molecule FRET studies on templates that terminate across from the adduct showed the formation of two structures that were different from that observed for unmodified primer-templates bound to the polymerase active site (Figure 6). In these studies, the AF adduct formed a structure similar to that observed when there was a single mismatched primer template terminus (intermediate site) (Figure 6B). In the AAF case, two structures formed, one similar to the intermediate site binding observed for AF and the other similar to that observed for unmodified DNA containing a double mismatch (exo site binding) (Figure 6C). It was suggested that the previously unreported intermediate binding orientation may represent a key intermediate in the polymerase proofreading mechanism, one that occurs as a step between active site binding and exo site binding.

Another study by Kath and co-workers investigated DNA polymerase exchange in the presence of a N^2-furfuryl-deoxyguanosine (N^2-furfuryl-dG, Figure 1) DNA adduct.[98] N^2-Furfuryl-dG is a carcinogenic adduct that originates from exposure to furfuryl alcohol, which is widespread in manufacturing and consumer goods like flavoring agents and food products.[99−101] Structurally, N^2-furfuryl-dG is a minor groove DNA adduct that does not appear to produce large distortions in B-form DNA as compared to bulky DNA adducts, such as AF and AAF.[102] In the study, Kath and co-workers investigated the mechanism of polymerase exchange between the Y-family DNA Pol in *E. coli*, Pol IV, and replicative DNA Pol III. It was found that the β clamp, a replisome processivity factor, can bind the DNA Pols simultaneously to allow for an exchange reaction to occur between the two Pols during TLS. In this single-molecule study, long DNA molecules containing a tethered bead on one end were immobilized at the opposite end onto a glass slide. Microfluidic flow cells were used to control flow in the chamber. With an applied force of approximately 3 pN, ssDNA is collapsed whereas dsDNA is extended, thus providing the ability to track DNA replication in real time (Figure 7A). The replication rate on nondamaged DNA was ∼222 bp/s for Pol III and ∼11 bp/s for Pol IV. In the presence of the N^2-furfuryl-dG lesion, DNA Pol III was blocked at the site of the lesion; however, replication past the lesion was observed when DNA Pol IV was added, demonstrating a role for Pol IV to bypass a N^2-furfuryl-dG lesion (Figure 7B). Additionally, it was found that a secondary contact between Pol IV and the β clamp restricted the ability for Pol IV-mediated synthesis and enabled Pol III displacement on DNA. This was shown by monitoring the processivity of Pol III in the presence of a mutant for Pol IV that lacked a clamp-binding motif and two β clamp mutants; one a single-cleft mutant ($\beta^{+/}\beta^{C}$) and another mutant with a weak Pol IV-interacting rim interface (β^{R}). Here, a slight increase in the processivity of DNA Pol III is observed in the presence of the β^{R} mutant as compared to conditions with wild type β clamp and $\beta^{+/}\beta^{C}$ (Figure 7C), indicating the presence of a secondary contact between Pol IV and the β clamp that influences the processivity of Pol III.

The polycylic aromatic hydrocarbon benzo[a]pyrene (B[a]P) is an environmental carcinogen that can be metabolically activated to a diol epoxide that reacts with DNA to form adducts, primarily at the N2 position of guanine. Various isomers of bioactivated B[a]P can be created leading to structural variation in the DNA adducts that can form.[103,104] Each of these adduct conformers have different mutagenic outcomes during replication.[105,106] A report by Liyanage and colleagues has investigated the mechanism of bypass at (+)-*cis*-B[*a*]P-N^2-dG (Figure 1), by the model Y-family polymerase Dpo4 at single-molecule resolution.[107] This study investigated the interaction between this bypass polymerase and templates containing the (+)-*cis* B[*a*]P-N^2-dG adduct in which the primer terminated just before or across from the adduct position. Similar to the prior Dpo4 studies, the polymerase was labeled with Cy5 and the template with Cy3. Primer extension analysis showed that this bypass polymerase could not synthesize past this adduct but that the addition of an organic solvent, such as DMSO, to the reaction mixture allowed for translesion synthesis to occur. Single-molecule FRET analysis showed that the binary complex adopted a different orientation in the presence of DMSO (Figure 8A), suggesting that this altered structure was what allowed for adduct bypass. MD simulations showed that the DMSO altered the stacking of the adduct in DNA and predicted structures were consistent with the adduct becoming solvent exposed on the exterior of the DNA helix in the presence of DMSO (Figure 8B,C). This study shows how the position of the adduct in the polymerase active site influences the ability of a polymerase to bypass the adduct position.

4. SINGLE-MOLECULE STUDIES ON OXIDATIVE DNA LESIONS

Oxidative stress in cells leads to ROS that can react with DNA causing oxidative DNA lesions that, if not repaired, become substrates for DNA Pols during replication. One of the best studied oxidative lesion is 8-oxo-dG (Figure 1) and this lesion has

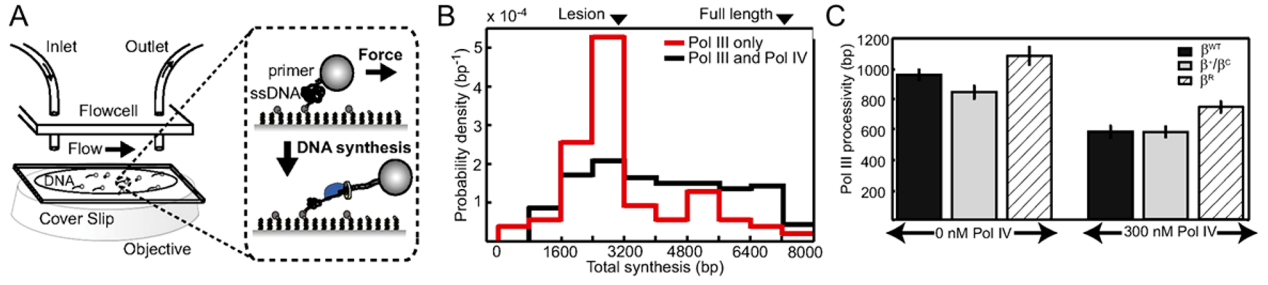

Figure 7. Single-molecule study with an N^2-furfuryl-dG DNA adduct. (A) Experimental setup for flow-based assay where DNA is immobilized on one end and stretched in the presence of flow. Here, ssDNA is entropically collapsed as compared to dsDNA that is more extended (inset). (B) More DNA synthesis is observed in the presence of the DNA lesion when both Pol III and Pol IV are present. (C) Processivity of DNA Pol III at 0 and 300 nM Pol IV and wild type β clamp (black) along with two β clamp mutants (gray = single-cleft mutant and dashed line = clamp with weakened Pol IV interacting rim interface). Figure adapted with permission from Kath, J. E.; Jergic, S.; Heltzel, J. M.; Jacob, D. T.; Dixon, N. E.; Sutton, M. D.; Walker, G. C.; Loparo, J. J. Polymerase exchange on single DNA molecules reveals processivity clamp control of translesion synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, (21), 7647−52.[98]

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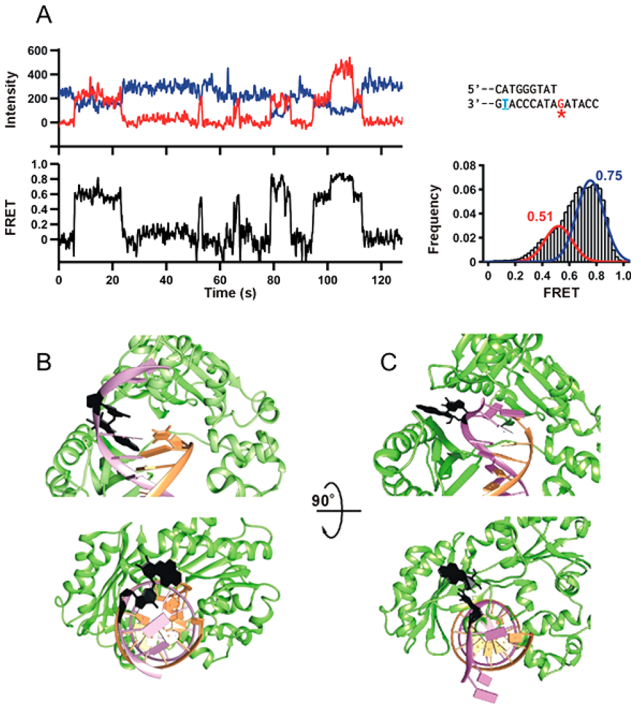

Figure 8. (A) smFRET analysis for Cy5-labeled Dpo4 binding to (+)-*cis* B[*a*]P-N^2-dG (here, blue T represents the site of Cy3 and red G represents adduct position); (B, C) MD simulations for binary Dpo4-DNA structure containing (+)-*cis* B[*a*]P-N^2-dG (B) in the presence of water, whereby the adduct is in the minor groove, and (C) in the presence of DMSO, whereby the adduct flips out of the DNA helix and is solvent exposed.

also been investigated with smFRET.[108] In this study, Raper and co-workers investigate the dynamics of a Cy5 labeled Dpo4 when bound to a Cy3 labeled primer-template containing an 8-oxo-dG adduct.[108] Upon the binding of Dpo4 to the DNA, dynamic transitions were observed by monitoring changes in FRET efficiencies. For the binary complexes of Dpo4 in the presence of damaged or undamaged DNA, three distinct FRET states were observed, suggesting that varying Pol conformations are adopted upon DNA binding (Figure 9). However, the distribution of the most populated FRET state differed. For Dpo4 bound to undamaged DNA, the mid-FRET (0.65) state was most frequent (51%), whereas for Dpo4 bound to 8-oxo-dG, the low-FRET (0.50) was most frequent (50%) (Figure 9A and B, respectively). Interestingly, in the presence of the next correct nucleotide, dCTP, only one FRET state was observed (0.50) for Dpo4 binding to 8-oxo-dG DNA, demonstrating the influence nucleotides have on the dynamics of DNA Pol binding DNA (Figure 9C). This behavior is similar to that observed in the presence of AAF and B[a]P,[96,107] raising the interesting possibility that Dpo4 can utilize a general mechanism to bypass a variety of damaged bases (such as 8-oxo-dG, AAF and B[a]P), but can also employ different mechanisms to bypass others (such as AF).

5. CONCLUSIONS

A variety of single-molecule studies with force and fluorescence-based imaging have been used to investigate the behavior and dynamics of DNA Pols on damaged DNA. These studies have revealed significant insights regarding the mechanism by which DNA adducts affect the interaction between a template and a DNA polymerase. Carefully controlled experiments with purified DNA Pols are critical to understand TLS and its role in mutagenesis and carcinogenesis. Single-molecule studies outlined in this review have shown two important mechanisms that affect polymerase-mediated lesion bypass: (1) higher order replisomal protein complexes can influence the ability of DNA Pol-mediated TLS and (2) that the structure and conformation of the DNA lesion influences polymerase dynamics.

Work by Sun and co-workers showed that both the T7 polymerase and the T7 helicase are required for replication past the UV-based CPD lesion, demonstrating a role for the helicase in lesion bypass in the bacteriophage T7. Also, work by Kath and colleagues showed that sliding clamp mutants with weakened

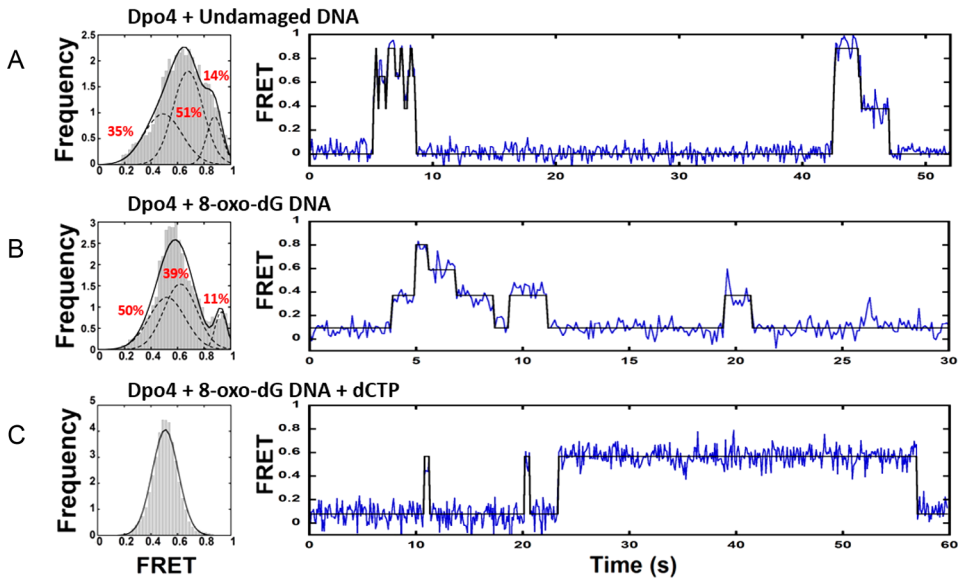

Figure 9. smFRET analysis for Dpo4 binding to damaged and undamaged DNA substrates. (A) Dpo4 in the presence of dG, (B) Dpo4 in the presence of 8-oxo-dG, and (C) the ternary complex of Dpo4 binding to 8-oxo-dG DNA and the next correct nucleotide, dCTP. Reproduced from Raper, A. T.; Gadkari, V. V.; Maxwell, B. A.; Suo, Z. Single-molecule investigation of response to oxidative DNA damage by a Y-family DNA polymerase. *Biochemistry* **2016**, *55* (14), 2187−96. Copyright 2016 American Chemical Society.[108]

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interactions with DNA Pol IV reduces the processivity of DNA Pol III in the presence of an N^2-furfuryl-dG lesion, demonstrating a role for the sliding clamp in TLS. These reports reveal the importance of testing the role for all proteins that are part of the replisome machinery, for example, helicases, single-stranded binding proteins, and processivity factors like the β clamp in *E. coli* or PCNA in humans. These types of studies are needed to expand on what is currently understood regarding lesion tolerance mechanisms in larger protein assemblies of the replisome.

The single-molecule studies on templates modified with AF, AAF, (+)-*cis* B[*a*]P–N^2-dG, and 8-oxo-dG adducts have shown that the structure of the lesion can influence polymerase dynamics and that binary and ternary structures can alter the overall dynamics of the system. Still, information is lacking to better understand polymerase dynamics with the expansive repertoire of DNA lesions that have been discovered. As these lesions pose a risk to human health and the fact that their presence is involved in the development of diseases like cancer, more studies are warranted. For example, studies with DNA lesions including oxidative hydantoins and glycol-derived DNA damage as well as bulky adducts including aflatoxin and the different conformers of BPDE-N^2-dG would benefit in this effort. Overall, the advancement of single-molecule imaging in the last two decades has strengthened our understanding in biological pathways by probing dynamics one enzyme at a time. Future studies investigating how single polymerases catalyze replication at damaged DNA substrates will deepen our understanding of lesion bypass and its role in carcinogenesis.

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Notes

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David Rueda is Professor and Chair of Molecular and Cellular Medicine at Imperial College London and Group Leader at the London Institute of Medical Sciences of the Medical Research Council (LMS-MRC). He was initially trained as a Chemical Engineer and received his DSc in Physical Chemistry from the EPFL (Lausanne, Switzerland). He switched to Single-Molecule Biophysics of RNA during his postdoc (University of Michigan) at the interface of Physics, Chemistry, and Biology. His research consists of developing and applying single-molecule approaches to study the mechanisms by which nucleic acid enzymes regulate cellular functions such as DNA replication and repair.

ABBREVIATIONS

DNA Pols, DNA polymerases; TLS, translesion DNA synthesis; smFRET, single-molecule Förester resonance energy; CPD, cyclobutane pyrimidine dimers; B[a]P, benzo[a]pyrene; AF, 2-aminofluorene; AAF, *N*-acetyl-2-aminofluorene

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