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# A Comprehensive View of Translesion Synthesis in *Escherichia coli*

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1 **A Comprehensive View of Translesion Synthesis in *Escherichia coli* and beyond**

2

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12 Running Head: Comprehensive View of TLS

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**SUMMARY**

The lesion bypass pathway, translesion synthesis (TLS), exists in essentially all organisms and is considered a post-replicative gap repair pathway. Likewise to “a trip is not over until you get back home”, studying TLS only at the site of the lesion is not enough to understand the whole process of TLS. Recently, a genetic study uncovered that Pol V, a poorly expressed *E. coli* TLS polymerase, is not only involved in the TLS step *per se* but participates in the gap filling reaction over several hundred nucleotides. In contrast, the same study revealed that Pol IV, another highly expressed TLS polymerase, does essentially not take part in the gap filling reaction. These observations imply the existence of fundamentally different ways these two polymerases are recruited to DNA. While access of Pol IV appears to be governed by mass action, efficient recruitment of Pol V involves a chaperon-like action of the RecA filament. We present a model of Pol V activation: 3'-tip of RecA filament initially stabilizes Pol V to allow stable complex formation with a sliding  $\beta$ -clamp, followed by the capture of the terminal RecA monomer by Pol V thus forming a functional Pol V complex. This activation process mediated by RecA likely determines higher accessibility of Pol V compared to Pol IV onto normal DNA. Finally, we discuss the biological significance for the participation of TLS polymerases during gap filling reactions: error-prone gap filling synthesis may contribute as a driving force for genetic diversity, adaptive mutation and evolution.

**KEYWORDS**

1 Translesion synthesis, gap filling synthesis, post-replicative gap repair, untargeted  
2 mutagenesis, replicative polymerase, Pol IV, Pol V, RecA,  $\beta$ -clamp, adaptive mutation,  
3 evolution

4

## 5 **INTRODUCTION**

6 Cells have evolved robust and versatile defense mechanisms to cope with various  
7 types of DNA damages derived from endogenous and exogenous sources. Endogenous  
8 DNA damage stems from normal metabolic processes (e.g., reactive oxygen species  
9 (ROS) via respiration of mitochondria (1)) or intrinsic instability of DNA *per se* (e.g., abasic  
10 lesion (apurinic/apyrimidinic (AP) site) (2)). The steady-state number of DNA damages  
11 caused by endogenous sources is estimated to be over 40,000 in human DNA per cell  
12 (AP site is the most prevalent damage and is estimated to be  $\approx 30,000$ ) (3). With respect  
13 to exogeneous sources, they are derived from numerous chemicals (e.g., polycyclic  
14 aromatic hydrocarbons (PAHs) formed during incomplete combustion in cigarette smoke  
15 or car exhausts (4); alkylating agents during chemotherapy (5)) or environmental sources  
16 (e.g., UV light via sunshine (6)). Most DNA damage is effectively repaired through  
17 canonical DNA repair pathways such as nucleotide excision repair (NER) and base  
18 excision repair (BER) (7). If impaired in such repair pathways, cells become highly  
19 sensitive to DNA damages (8). However, even in the presence of efficient DNA repair  
20 systems, a subfraction of DNA damage escapes repair and has the opportunity to  
21 encounter the replicative DNA polymerase (Pol) at the fork (step a in Fig. 1). In case of  
22 replication blocking lesions, replication forks skip over the DNA damage and re-initiate  
23 downstream leaving a single-stranded (ss) DNA gap (9). As a consequence, the  
24 unrepaired lesion is now located in the context of ssDNA (step b in Fig. 1) where it

1 becomes irreparable by regular DNA repair systems that only function on double-stranded  
2 (ds) DNA. In order to cope with DNA damage located in ssDNA, cells have evolved two  
3 so-called DNA damage tolerance strategies that take place in the context of post-  
4 replicative gap repair (Fig. 1). The most straightforward damage tolerance strategy  
5 involves specialized DNA Pols that bypass directly DNA lesions, a process called  
6 translesion synthesis (TLS) (step c in Fig. 1) (10-12). Whereas some types of DNA lesions  
7 are bypassed by the replicative Pols themselves (e.g., 8-oxo-dG) (13), in this review we  
8 define TLS as an event requiring the action of a specialized Pol (i.e., TLS Pol). TLS is  
9 potentially highly mutagenic when DNA lesions are non-instructive (e.g., AP site; TT  
10 pyrimidine-pyrimidone (6-4) photoproduct (TT (6-4))). Therefore, the resulting nascent  
11 strand often contains a mutation opposite the template lesion site (a process referred to  
12 as “targeted mutagenesis”).

13         The second DNA damage tolerance strategy, referred to as homology-dependent  
14 gap repair (HDGR) or homology-directed repair (HDR) operated through template  
15 switching (for reviews see (11, 14)), aims at relocating the lesion into a double-stranded  
16 context by means of a homologous recombination intermediate with the complementary  
17 sister chromatid (step f in Fig. 1) (8, 11, 15-17). Irrespective of the precise model, the main  
18 purpose of HDGR (that is also the case for TLS) is to prevent deleterious discontinuities  
19 in DNA strands and to reinstate the DNA damage from its irreparable ssDNA context to a  
20 repairable dsDNA context (steps c-e or f-h in Fig. 1). Let us note that a similar template  
21 switching mechanism, usually referred to as fork regression or fork reversal, has been  
22 proposed to allow a replication fork, arrested in the vicinity of a lesion or a secondary  
23 structure in the leading strand template, to restart (11, 18).

1 Owing to the intrinsic low-fidelity of TLS Pols, the process of TLS is highly  
2 mutagenic both at the lesion bypass step *per se* (targeted mutagenesis) and downstream  
3 from the lesion on normal template DNA (untargeted mutagenesis) (steps b to c in Fig. 1).  
4 As discussed later in this review, despite their high mutagenic load, TLS pathways are  
5 present in most species (19, 20) strongly suggesting that TLS pathways are likely to be  
6 beneficial for evolution in the long term and for prevention of persistent ssDNA gaps in the  
7 short term, rather than being an extra back-up pathway.

8 The bypass of many different DNA lesions by different TLS Pols has been  
9 extensively studied *in vitro* (10, 21). However, the overall process of TLS *in vivo* has  
10 received little attention until recently (22, 23). The present article aims at discussing how  
11 the various Pols that are encoded and expressed at different levels in *E. coli* manage the  
12 overall process of post-replicative gap repair *in vivo*. We will propose a TLS model  
13 involving an interplay between the error-prone TLS Pols and the high-fidelity Pols in the  
14 context of physiologically relevant conditions, and discuss its biological consequences.

15

## 16 **WHAT HAPPENS WHEN A REPLICATION FORK ENCOUNTERS A LESION?**

17 When an ongoing replication fork encounters a replication blocking lesion in one of  
18 the template strands, it was initially expected that the fate of the fork would depend on  
19 whether the lesion is located in the leading or the lagging strand template. For a lesion in  
20 the lagging strand, the fork would go ahead leaving a gap (i.e., an unfinished Okazaki  
21 fragment). With a leading strand lesion, the fork may stall due to the concept of continuous  
22 leading strand replication as observed with reconstituted replication forks *in vitro* (24, 25).  
23 However, initial data from Okazaki indicated that, in *E. coli*, normal replication is  
24 discontinuous in both leading and lagging strands (26). Discontinuous strand synthesis

1 was also demonstrated when *E. coli* is damaged by UV irradiation (27) suggesting that  
2 the fork can skip over UV-induced lesions during both leading and lagging strand synthesis.

3 The reason for which such observations had not been taken into account came  
4 from the lack of biochemical evidence for leading strand repriming. Nearly 40 years after  
5 the classic *in vivo* observations, it was shown that repriming events relying on DnaG  
6 primase can occur during leading strand synthesis *in vitro*, either in the presence of  
7 accessory factors related to *de novo* priming activities (PriA- or PriC-dependent pathway)  
8 (28) or in the absence of such factors (29). In addition, an alternative pathway for skipping  
9 over a lesion on leading strand was also proposed based on *in vitro* observation that a  
10 leading strand polymerase can utilize a mRNA formed downstream of the leading strand  
11 template DNA (30). These observations led to the suggestion that repriming of leading  
12 strand synthesis can occur downstream from a replication-blocking lesion leaving a gap  
13 behind the fork. As a consequence, the process of TLS became regarded as part of post-  
14 replicative gap repair for both leading and lagging strand lesions, rather than a continuous  
15 process occurring at the fork (31), sometimes referred to as “TLS on the fly” (32).  
16 Discontinuous DNA replication of both leading and lagging strands was also supported *in*  
17 *vivo* using a ligase mutant (33, 34) and as well as by fluorescence-based imaging in live  
18 *E. coli* cells as discussed below (35). Indeed, the scenario of TLS being implemented  
19 behind the replication fork, was supported via single molecule fluorescence assays in  
20 which the localization of a TLS Pol, Pol V, was shown to be distinct from the localization  
21 of the Pol III replisome following UV irradiation *in vivo* (36). Similarly, single molecule  
22 fluorescence assays *in vivo* also indicated that Pol IV, another TLS Pol, principally  
23 colocalizes outside of the replisome following exposure of DNA damaging agents (e.g., 4-

1 nitroquinoline N-oxide (4-NQO)), implying that Pol IV-mediated TLS also predominantly  
2 occurs behind rather than at the replication fork (37).

3

#### 4 **CHARACTERISTIC FEATURES OF DNA POLYMERASES IN *E. COLI***

5 *E. coli* possesses five DNA polymerases (Pol I to V). Whereas classical Pols (i.e.,  
6 Pol I to III) were shown to exhibit bypass properties for some lesions under certain  
7 experimental conditions (38-40), their contribution to TLS under physiological conditions,  
8 is limited (largely unknown) except for a few examples (10). In contrast, the two remaining  
9 Pols (i.e., Pol IV and V) have characteristic features of TLS Pols, namely their lack of  
10 proofreading function and their capacity to accommodate bulky lesions given their  
11 spacious catalytic domain (19, 41). Interestingly, Pol IV and Pol V appear to be adapted  
12 for the bypass of minor and major groove lesions (i.e., division of labor), respectively (10).  
13 It may appear awkward to refer to minor versus major groove location during TLS as the  
14 lesion is located at the ss to dsDNA junction (step b in Fig. 1). However, in the Pol active  
15 site, the conformation of the damaged nucleotide (at the ss to dsDNA junction) may adopt,  
16 during the nucleotide insertion step, the same conformation as in dsDNA, allowing thus  
17 for Pol IV versus Pol V selectivity. Of note, TLS polymerase discrimination at the insertion  
18 step is undoubtedly performed through stochastic accesses of Pols (trial and error) (10),  
19 rather than during a scenario where a lesion would specifically recruit a Pol that is best  
20 suited for its bypass.

21 When a replicative Pol stalls at a replication blocking lesion and then dissociates,  
22 a 3'-end of the nascent strand is typically located one nucleotide before the lesion (42);  
23 upon dissociation, the stalled Pol leaves its processivity factor (the  $\beta$ -clamp) bound to the  
24 template DNA (42, 43). Downstream of the lesion, an exposed ssDNA gap becomes first

1 coated by ssDNA binding (SSB) proteins that are subsequently replaced by a RecA  
2 filament (10, 17, 44, 45) forming the so-called SOS signal, the bacterial stress response  
3 (46-48). Any Pols (or other types of DNA binding proteins like exonucleases and helicases  
4 (49)) can stochastically access the vacant 3'-end of the nascent strand. If a classical Pol  
5 (i.e., Pol I to III) accesses the 3'-end in the vicinity of a lesion (e.g., TT (6-4)), it either  
6 quickly detaches as a consequence of non-productive association/dissociation cycles (Fig.  
7 2A) or causes degradation/resynthesis cycles upstream from the lesion site (42, 43).

8

### 9 **What Is the Mechanism Underlying Functional Access of Pol IV on Template DNA?**

10 If a lesion is located in the minor groove (e.g., [-ta]-benzo[a]pyrene-N<sup>2</sup>-dG ([-ta]-  
11 BaP-dG)), Pol IV bypasses the lesion *in vivo* (50). Pol IV first accesses a 3'-end of nascent  
12 strand in the vicinity of the lesion and subsequently interacts with a freely sliding  $\beta$ -clamp,  
13 leading to the formation of a functional complex (Pol IV-fc) (Fig. 2B). It should be stressed  
14 that the opposite scenario (i.e., the  $\beta$ -clamp recruits Pols) is unlikely since the  $\beta$ -clamp is  
15 freely sliding on DNA (51). Interaction with the  $\beta$ -clamp is genetically essential for Pol IV-  
16 mediated TLS (52); moreover, it was shown that, under multiple-hit conditions *in vitro*, the  
17 processivity of Pol IV is only one nucleotide in the absence of the  $\beta$ -clamp (53, 54),  
18 indicating high instability of Pol IV on T/P. These observations imply that only a minor  
19 fraction of transiently bound Pol IV will succeed to interact with a freely sliding  $\beta$ -clamp. In  
20 conclusion, the rate limiting factor for successful TLS by Pol IV is the formation of Pol IV-  
21 fc (Fig. 2B).

22 Intriguingly, under normal growth conditions, the basal expression level of Pol IV  
23 ( $\approx$ 250 molecules per cell), is the second most abundant among all Pols and is further  
24 induced to  $\approx$ 2,500 molecules per cell under SOS induction (55). One may conclude that

1 the high abundance of Pol IV counteracts its intrinsically low T/P binding affinity by  
2 increasing the probability of stochastic access thus allowing the formation of Pol IV-fc  
3 required for the minor groove adduct bypass. This notion is supported by the observation  
4 that Pol IV-mediated TLS across [-ta]-BaP-dG adducts *in vivo* indeed occurs under non-  
5 SOS induced condition and is only moderately stimulated by SOS induction (S. Fujii, A.  
6 Isogawa, R. Fuchs, unpublished data). In contrast, on normal T/P, it is known that Pol IV-  
7 induced untargeted mutagenesis *in vivo* was below detection limits even under SOS  
8 induced conditions, while being only detected upon artificially high overexpression of Pol  
9 IV (56, 57). These observations suggest that the number of Pol IV molecules required for  
10 stochastic access during TLS is below the number required for access on normal T/P.  
11 Under physiological levels of expression, Pol IV cannot efficiently compete with classical  
12 Pols on normal T/P given its instability in the absence of interaction with the  $\beta$ -clamp (54).  
13 In contrast, when a post-replicative ssDNA gap contains a minor groove lesion, owing to  
14 the lack of elongation capacity by classical Pols, multiple accesses of Pol IV can lead to  
15 formation of a functional complex (Pol IV-fc) by interaction with a freely sliding  $\beta$ -clamp.  
16 Formation of this functional complex is thus restricted to Pol IV specific substrates such  
17 as minor groove lesions.

18

### 19 **The Pivotal Role of RecA for Assembly of Functional Pol V**

20 If a lesion is located in the major groove (e.g., N-2-acetylaminofluorene-dG (AAF-  
21 dG) (58)), Pol V bypasses the lesion *in vivo* (59). For Pol V-mediated TLS, interaction with  
22 RecA is essential as demonstrated both genetically (60-62) and biochemically (63, 64).  
23 Interaction of Pol V with the  $\beta$ -clamp is also genetically essential for Pol V-mediated TLS  
24 (52), as previously mentioned for Pol IV.

1           Subsequently, both genetic requirements (i.e., interaction with both the  $\beta$ -clamp  
2 and RecA) were also demonstrated to be essential factors *in vitro* (65). When Pol V  
3 accesses a lesion-containing T/P in the vicinity of a RecA-coated ssDNA gap, the following  
4 scenario is likely to occur (Fig. 2C). The interaction of Pol V with T/P is, as already noted  
5 for Pol IV, highly unstable (66); the RecA molecule located at a 3'-tip of the RecA  
6 nucleoprotein filament (RecA\*) stabilizes a Pol V molecule transiently bound to the T/P,  
7 leading to the formation of a complex referred to as pre-Pol V-fa (pre-Pol V functional  
8 assembly: Pol V with RecA\*). The resulting increase in residency time allows Pol V to  
9 encounter and to associate with a freely sliding  $\beta$ -clamp. Consequently, a functional  
10 assembly of Pol V with RecA\* and the  $\beta$ -clamp (Pol V-fa) is reached, allowing TLS to  
11 ensue (Fig. 2C) (65, 66).

12           A single RecA molecule in RecA\* typically occupies 3 nt (17); the length of the  
13 ssDNA gap between a 3'-end of primer and a 3'-tip of RecA\* is therefore 0, 1 or 2 (modulo  
14 3) nt long. Potentially, only one of the three gaps has the proper size to form a pre-Pol V-  
15 fa. To allow formation of a correct pre-Pol V-fa complex, the adjustment of the gap size  
16 may occur owing to the intrinsic dynamic features of RecA\* (67), or as a result of the  
17 dynamic state of 3'-end of primer that is subject to degradation/resynthesis cycles by  
18 classical Pols as mentioned above (42).

19

## 20 **Characteristic Features of Pol IV- and Pol V-Mediated TLS Pathways**

21           The major difference between Pol IV and Pol V in terms of their respective  
22 activation procedure for TLS is the unique requirement of RecA for Pol V. Under SOS-  
23 induced conditions, the estimated number of Pol V (15 ~ 60 molecules per cell) (68) is  
24  $\approx 40 \sim 170$ -fold lower than that of Pol IV ( $\sim 2,500$  molecules per cell) (55). Pol IV and Pol V

1 implement different strategies to compensate for their low T/P affinity. For Pol IV, its high  
2 abundance compensates for its low affinity to T/P, while Pol V qualitatively benefits from  
3 additional stabilization through a specific interaction with RecA. During SOS induction, in  
4 contrast to *dinB* encoding Pol IV that is induced early (69), the *umuDC* genes are induced  
5 late (70) and the UmuD<sub>2</sub>C (i.e., Pol V) appears only ≈50 minutes after SOS induction (71,  
6 72). In case of a major groove lesion like AAF-dG, given that Pol V induction is delayed to  
7 a late time point during SOS induction, one can assume that all other Pols have randomly  
8 accessed the 3'-end of primer in a non-productive way (Fig. 2A). Thus, depending upon  
9 the type of lesion to be bypassed (i.e., minor or major groove lesion), the timing of TLS  
10 occurrence largely differs between Pol IV- and Pol V-mediated pathways. Since bypass  
11 across [-ta]-BaP-dG *in vivo* does not require SOS induction, Pol IV-mediated TLS can  
12 occur at an earlier time compared to TLS mediated by Pol V, reducing thus the time  
13 harmful ssDNA is exposed. This notion is also supported by single molecule fluorescence  
14 assays *in vivo* (37).

15

16 **UNTARGETED MUTAGENESIS DURING COMPLETION OF THE WHOLE PROCESS**  
17 **OF POL V-MEDIATED TLS**

18 In case cells would aim to minimize mutagenesis during lesion bypass (Fig. 3A),  
19 one would assume that the error-prone TLS Pols would dissociate from the template DNA  
20 as soon as a TLS patch reaches a minimal length (pink line in Fig. 1c) sufficient for  
21 extension by high-fidelity Pols (e.g., Pol III) (42).

22 We have previously shown that a 5 nt long TLS patch is efficiently extended by Pol  
23 III (42). In such a case, the occurrence of untargeted mutations would be exclusively  
24 limited to a short TLS patch (Fig. 3B). However, for Pol V-mediated TLS *in vivo*,

1 unexpectedly, untargeted mutagenesis is spread over essentially the whole post-  
2 replicative gap and even in the upstream region (Fig. 3C) (23). Our work revealed that the  
3 error frequency of Pol V-induced untargeted mutagenesis during the gap filling synthesis  
4 lies in the  $10^{-5}$  range per base. If a gap size is 1 kb, then cumulative untargeted mutations  
5 reach a  $10^{-2}$  frequency for every TLS event. The data clearly indicate that, *in vivo*, access  
6 of Pol V to undamaged template DNA is not strictly limited to a minimal TLS patch but  
7 extends over several hundred nt. In addition, in contrast to replication errors (73),  
8 untargeted mutations assigned to Pol V are refractory to mismatch repair (MMR)  
9 correction (23). Indeed, efficient MMR requires association with the replication apparatus  
10 (74, 75). Thus, the observed lack of MMR correction is likely due to the absence of the  
11 replication fork context. Such a phenomenon was also observed in yeast where  
12 untargeted mutations induced by Pol  $\zeta$  during gap filling synthesis, are refractory to MMR  
13 (22, 76). Taken together, in addition to the intrinsic low fidelity of TLS Pols, ineffectiveness  
14 of MMR during post-replicative gap repair exacerbates the genetic instability caused by  
15 untargeted mutagenesis.

## 16 17 **ALTERNATE ACCESSES OF LOW- AND HIGH-FIDELITY POLS DURING POST-** 18 **REPLICATIVE GAP REPAIR**

19 During the overall process of TLS *in vivo*, Pol V-mediated untargeted mutagenesis  
20 was revealed to extend over several hundred nt around a lesion site (Fig. 3C) (23). The  
21 intensity of untargeted mutations downstream from the lesion site globally decreases with  
22 the distance from the lesion. Surprisingly the decrease is not uniform but exhibits an  
23 oscillating (wavy) pattern of valleys and hills suggesting alternate accesses of high- and  
24 low-fidelity Pols, respectively (23).

1 In principle, any Pol can stochastically access vacant 3'-ends in nascent strands  
2 irrespective of whether or not the binding event will be productive, i.e. lead to DNA  
3 synthesis. The probability for stochastic access of a given Pol to a vacant T/P will depend  
4 upon the relative number of Pol molecules per cell. When Pol V functions, cells are under  
5 SOS-induced conditions. Under such conditions, the estimated number of molecules per  
6 cell, from Pol I to Pol V, are  $\approx 400$ ,  $\sim 350$ ,  $\sim 20$ ,  $\sim 2,500$ , and  $\approx 40$ , respectively (25, 55, 68,  
7 77, 78). The total number of high-fidelity Pols (i.e., the classical Pol I to III) is  $\sim 770$ ; the  
8 relative proportion of "classical Pols", Pol IV and Pol V is thus approximately 23%, 76%  
9 and 1.2%, respectively. Despite the high relative abundance of Pol IV (76%), its  
10 contribution to untargeted mutagenesis during gap filling synthesis is essentially negligible  
11 (23). This observation reflects the above mentioned feature of Pol IV, namely its inefficient  
12 functional access to normal T/P. Thereby, it was concluded that Pol V is the main  
13 contributor to untargeted mutagenesis (23)

14 Within the oscillating pattern of untargeted mutations, the apparent length of valleys  
15 (i.e., error-free stretches) is relatively short (e.g., a few tens nt) (Fig. 3C) (23), a size that  
16 is compatible with the processivity of classical high-fidelity Pols in the absence of the  $\beta$ -  
17 clamp (43, 79, 80). This observation suggests that, when classical Pols access 3'-ends of  
18 primers, most Pols begin to elongate and dissociate before spontaneously encountering  
19 a diffusing sliding  $\beta$ -clamp (Fig. 4A).

20 The apparent length of the hills (i.e., error-prone stretches) in the oscillating pattern  
21 is around 50 nt (Fig. 3C) (23). In contrast to Pol IV on normal T/P (i.e., quick dissociation  
22 or one nt elongation) (Fig. 4B), Pol V becomes stabilized by interacting with a 3'-tip of  
23 RecA\* (i.e., pre-Pol V-fa) thus providing a long enough residency time to interact with a  
24 freely sliding  $\beta$ -clamp (i.e., pre-Pol V-fa is converted to Pol V-fa) on normal T/P (Fig. 4C)

1 (66), as discussed before in the context of TLS (Fig. 2C) (65, 66). Pol V-fa begins to  
2 elongate on normal T/P with an average processivity of  $\approx 25$  nt as determined *in vitro* (66),  
3 a size that is compatible with the apparent length of the observed hills (23). Thereafter,  
4 Pol V likely dissociates from the T/P as a sub-complex composed of Pol V and a single  
5 RecA monomer (Pol V-dc: Pol V-dissociation complex) (23, 81). Upon dissociation of Pol  
6 V and its associated RecA monomer (Pol V-dc), a short ssDNA gap is generated between  
7 the vacant 3'-end of the primer and the new 3'-tip of RecA\* (Fig. 4C).

8  
9 **What is the Underlying Mechanism of the Alternate Access of Pol V and classical**  
10 **Pols?**

11 In addition to the untargeted mutation pattern indicating alternate access of low-  
12 and high-fidelity Pols described above, intriguingly the overall decline of untargeted  
13 mutations with distance from the lesion site is conspicuously moderate (23) given the  
14 numerical superiority of classical Pols over Pol V (the relative ratio,  $\approx 19 : 1$ ). As a minor  
15 pathway, a small fraction of the classical Pols encounter a freely sliding  $\beta$ -clamp and  
16 synthesize a long stretch in an error-free manner, thus contributing to the overall decline  
17 of mutations. (step b in Fig. 5).

18 What may be the mechanism that allows Pol V to dominate over the classical Pols  
19 in spite of its numerical inferiority? If the vacant T/P is formed upon dissociation of Pol V,  
20 the short ssDNA gap that is formed may be geometrically unsuitable for functional access  
21 of a free Pol V molecule which requires concomitant interaction with T/P and RecA\* (step  
22 a in Fig. 5). Under such circumstances classical Pols will preferentially access the vacant  
23 3'-end and synthesize, without  $\beta$ -clamp, a relatively short error-free patch (step b in Fig.  
24 5). During synthesis of such short error-free patches by the classical Pols, RecA molecules

1 are dislodged from the 3'-tip of RecA\* (42, 44) forming new 3'-tips of RecA\* among which  
2 some may exhibit the proper configuration to allow Pol V to simultaneously bind the T/P  
3 and the 3'-tip of RecA\*. When a classical Pol dissociates, we suggest that its immediate  
4 reloading may be sterically hampered by the presence of the RecA filament, which in turn  
5 favors loading of Pol V despite its numerical inferiority. Subsequently, Pol V locked onto  
6 the T/P (i.e., pre-Pol V-fa) will associate with a freely sliding  $\beta$ -clamp and the resulting  
7 functional assembly of Pol V (i.e., Pol V-fa) synthesizes an error-prone patch (step c in  
8 Fig. 5), restarting a new cycle (step d in Fig. 5).

9

### 10 **The Region Upstream of the Lesion Displays a Dynamic State**

11 Unexpectedly, during the process of Pol V-mediated TLS *in vivo*, untargeted  
12 mutagenesis was also observed in the region upstream from the lesion site albeit with  
13 somewhat reduced intensity compared with the downstream region (Fig. 3C) (23). These  
14 events likely result from a scenario where exonucleases (with or without helicases) resect  
15 the 3'-end of nascent strand in the vicinity of a lesion or of a non-extendable short TLS  
16 patch produced by Pol V, thus expanding the single-stranded region upstream from the  
17 lesion site, followed by RecA\* formation. Subsequently, during gap filling of the region  
18 upstream of the lesion, Pol V-fa is formed and induces untargeted mutations. This  
19 observation implies that, until late SOS response, the region upstream from the lesion is  
20 in a dynamic dsDNA  $\rightleftharpoons$  ssDNA equilibrium state. During that period, classical Pols are  
21 essentially responsible for the fill-in reaction while accuracy of the fill-in reaction will be  
22 lower than that of nascent strands at a replication fork due to lack of efficient MMR  
23 correction. When Pol V becomes available in late SOS response, the subfraction of these

1 3'-ends located upstream from the lesion, will be extended in an error-prone way by Pol  
2 V. Thus, even before Pol V bypasses a lesion, genome integrity is already compromised.

3

#### 4 **HOW DOES POL V IMPLEMENT ELONGATION ON RECA-COATED ssDNA?**

##### 5 **Pol V Interacts with a Dynamically Formed RecA Filament**

6 When Pol V is expressed, all exposed (or SSB-coated) ssDNA regions are  
7 converted into RecA-coated ssDNA (RecA\*). Globally, the apparent growth of the RecA  
8 filament was shown to occur in the 5' → 3' direction on ssDNA (82). However, RecA  
9 filament formation is a highly dynamic process, and individual RecA monomers associate  
10 at and dissociate from both ends of the filament at different rates, leading to a net apparent  
11 growth in the 5' → 3' direction (Fig. 6) (67).

12 Genetic data suggested that a 3'-tip of RecA\* is essential for UmuD'<sub>2</sub>C-induced  
13 mutagenesis (i.e., targeted mutagenesis via Pol V), and this RecA function was referred  
14 to as third role of RecA (60-62). Thereafter, it was also shown that Pol V requires direct  
15 contact at the 3'-tip of RecA\* for activation of Pol V *in vitro* (83) and that the ultimate RecA  
16 monomer becomes stably associated with Pol V forming a complex composed of Pol V  
17 and a RecA monomer (81). It should be stressed that the stability of RecA bound to Pol V  
18 is much higher than the stability of the 3'-terminal RecA monomer in RecA\*; indeed, 50%  
19 of RecA monomer bound to Pol V dissociates in ≈30 min (81), while at the 3'-end of RecA\*,  
20 RecA monomer dissociates with a half-life ( $t_{1/2}$ ) of ≈4.3 s ( $= \ln 2 / k_{\text{off}}$  (≈0.16 s<sup>-1</sup>)).

21

##### 22 **Biochemical Features of RecA\* Filament Formation**

23 The first step in RecA\* formation is a nucleation step whereby ≈5 RecA monomers  
24 associate on a short stretch of ssDNA (≈20 nt), followed by an extension phase to form

1 RecA\* (67, 84, 85). In other words, the requirement of the nucleation step indicates that  
2 individual RecA monomers bound to ssDNA are intrinsically very unstable (e.g., very high  
3  $k_{\text{off}}$  of RecA) (67, 84-87). *In vitro*, the efficiency of RecA\* formation largely depends upon  
4 the nature of the nucleotide analogue that is used as a cofactor (88, 89). The affinity of  
5 RecA for ssDNA decreases in the order, ATP > no cofactor > ADP, and only ATP efficiently  
6 supports both co-protease and strand exchange reactions in the context of RecA\*. Thus,  
7 ATP is seemingly the most physiologically relevant cofactor during RecA\* formation *in vivo*  
8 (86, 88, 90). During RecA\* formation *in vitro*, the different cofactors induce distinct RecA\*  
9 filaments. Indeed, RecA-ATP (or RecA-ATP- $\gamma$ S: a poorly hydrolysable ATP analog) and  
10 RecA-ADP (or RecA with no cofactor) induce filaments on ssDNA referred to as extended  
11 and compressed, respectively (91-93). During *de novo* filament formation, RecA occupies  
12 3 nt in the extended filament (helical pitch:  $\approx 95$  Å) (17) while it occupies 5 nt in the  
13 compressed filament (helical pitch:  $\approx 75$  Å) (94).

14 Conversion from extended to compressed filament was reversibly observed on the  
15 same ssDNA molecule in response to changing the nucleotide cofactor, implying that  
16 conversion can elastically occur without varying nt occupation of RecA (92, 95). We thus  
17 suggest that, in the context of physiologically relevant *de novo* extended RecA-ATP  
18 filaments, the event observed in RecA-ADP (or RecA with no cofactor) occupying 5 nt (94)  
19 would be spatially compatible at RecA\* extremities (i.e., 3'- and 5'-tips) in a certain  
20 situation such as a free RecA-ADP molecule access RecA\* extremities.

21 RecA possesses a DNA-dependent ATPase activity and its turnover number ( $k_{\text{cat}}$ )  
22 for ATP hydrolysis was estimated at  $\approx 0.3 \sim 0.5$  s<sup>-1</sup> in the presence of ssDNA (96). Based  
23 on relatively similar range of  $k_{\text{off}}$  (5'-end,  $\approx 0.12$  s<sup>-1</sup>; 3'-end,  $\approx 0.16$  s<sup>-1</sup>) (67) and  $k_{\text{cat}}$  of RecA,  
24 dissociation of RecA monomers from ends of RecA\* could be linked to ATP hydrolysis,

1 accompanied with a conformational change of RecA (i.e., RecA-ATP → RecA-ADP) (67,  
2 87). On the other hand, as individual RecA monomers (i.e., not in RecA\*) on ssDNA are  
3 unstable as described above, dissociation events of such individual RecA monomers on  
4 ssDNA are likely not linked to ATP hydrolysis.

5

## 6 **The RecA-Fluttering Model**

7 **How is Pol V activated on RecA-coated ssDNA?** At the outset, Pol V accesses RecA-  
8 coated ssDNA leading to form pre-Pol V-fa, a prerequisite for subsequent conversion into  
9 Pol V-fa via interaction with the  $\beta$ -clamp (Fig. 7A). The RecA monomer at a 3'-tip of RecA\*  
10 has greater affinity towards Pol V than towards the next RecA monomer in the RecA\* as  
11 discussed above. As a consequence, this particular RecA monomer bound to Pol V  
12 detaches from RecA\* via ATP hydrolysis. The transition of the RecA monomer, from the  
13 3'-tip of RecA\* to its stable association with Pol V, is referred to as "RecA-capture". Under  
14 single-hit conditions *in vitro*, Pol V with RecA\* (i.e., pre-Pol V-fa) lacks measurable  
15 processivity on normal T/P (66). Thus, in the event where RecA-capture occurs in the pre-  
16 Pol V-fa state, Pol V with a RecA monomer would immediately dissociate from T/P (i.e., in  
17 the form of Pol V-dc), resulting in a non-productive event. On the other hand, in the context  
18 of Pol V-fa, owing to the additional stability conferred by the interaction with the  $\beta$ -clamp,  
19 the event of RecA-capture leads to a formation referred to as a Pol V functional complex  
20 (Pol V-fc: Pol V with a RecA monomer and the  $\beta$ -clamp) (Fig. 7A). The RecA-capture event  
21 generates a 3 nt ssDNA gap that cannot support Pol V-mediated DNA synthesis due to  
22 steric hinderance; indeed, as mentioned previously, given the change in status of RecA  
23 from its ATP to ADP form when it dissociates from ssDNA, re-access of the RecA-ADP  
24 onto ssDNA likely requires a 5 nt gap instead of 3 nt. We refer to the sterically blocked

1 form of Pol V as its “immovable state”. The transition between Pol V-fa and Pol V-fc would,  
2 thus, basically be irreversible (Fig. 7A). Consequently, DNA synthesis by Pol V-fc can only  
3 occur upon dissociation of the next 3'-tip of RecA\* ( $k_{\text{off}}: \approx 0.16 \text{ s}^{-1}$ ), thus generating a 6 nt  
4 gap and turning Pol V from an immovable to a movable state (Fig. 7B).

5  
6 **What is RecA-Fluttering?** During elongation, the RecA monomer that is part of Pol V-fc  
7 likely behaves as either a 5'-tip of RecA\* or a free RecA monomer. In either case, given  
8 the low binding affinity of a single RecA monomer to ssDNA, we suggest that the RecA  
9 monomer in Pol V-fc performs rapid association and dissociation cycles on ssDNA,  
10 referred to as “RecA-Fluttering” (Figs. 7C and 7D). We suggest that RecA-Fluttering  
11 serves to properly position Pol V with the  $\beta$ -clamp on T/P at the nucleotide insertion step  
12 during elongation. Indeed, in the absence of RecA under multiple-hit conditions *in vitro*,  
13 Pol V alone has the capacity to produce much longer elongation products than Pol V with  
14 the  $\beta$ -clamp at certain natural DNA sequence contexts (66). It implies that the  $\beta$ -clamp  
15 impairs proper positioning of Pol V on T/P such as a difficult-to-replicate DNA sequence  
16 or a lesion-containing template DNA (66). Immediately after Pol V-fc formation, since most  
17 captured RecA monomer would be associated with ADP, this form may be advantageous  
18 for Pol V-fc elongation owing to its lower ssDNA binding affinity. Otherwise, due to lack of  
19 sliding capacity of RecA on ssDNA (45, 97), a RecA monomer with higher ssDNA binding  
20 affinity such as RecA-ATP may modestly behave as an obstacle during Pol V-fc elongation.

21 While first and second roles of RecA correspond to up-regulation of the mRNA  
22 encoding UmuDC and post-translational cleavage of UmuD to UmuD', respectively (10),  
23 we previously suggested that the so-called third role of RecA (60-62), corresponds to the  
24 stabilization of Pol V on T/P via pre-Pol V-fa (i.e., Pol V with RecA\*) formation (10). As

1 discussed above, the pre-Pol V-fa complex facilitates the interaction of Pol V with a freely  
2 sliding  $\beta$ -clamp, leading to Pol V-fa formation (Fig. 4C). Additionally, another role of RecA  
3 allows smooth elongation of Pol V on difficult-to-replicate sequences (e.g., secondary  
4 structure) by stretching such sequences that otherwise would lead to strong pause sites  
5 (fourth role of RecA) (10, 66). In contrast to both third and fourth roles that involve RecA  
6 in the context of RecA\*, its role in Pol V-fc implicates RecA as a monomer. Here, we  
7 suggest that in the RecA-Fluttering mode, where the RecA monomer actively functions for  
8 proper positioning of Pol V bound to the  $\beta$ -clamp on T/P during the elongation, represents  
9 the fifth role of RecA.

10  
11 **Two elongation modes of Pol V-fc.** Within the context of a movable Pol V-fc complex,  
12 the RecA-Fluttering mode allows to sense whether DNA synthesis is possible when the  
13 gap is 6 or more nt long (movable state) or impossible if the gap is only 3 nt (immovable  
14 state) (Fig. 7B). Subsequently, elongation on RecA-coated ssDNA is operated via two,  
15 non-exclusive elongation modes (discontinuous and continuous modes) depending upon  
16 the position of a 3'-tip of RecA\* with respect to Pol V-fc. In the discontinuous mode, as a  
17 RecA-ADP monomer requires 5 nt for binding to ssDNA, elongation of Pol V-fc on a 6 nt  
18 gap would be sterically blocked after only 1 nt incorporation leaving a 5 nt gap (Fig. 7C).  
19 In the subsequent cycle, relying on dissociation of a RecA monomer from the 3'-tip of  
20 RecA\*, Pol V-fc encounters an 8 nt gap and succeeds to incorporate 3 nt leaving a 5 nt  
21 gap in each cycle (Fig. 7C). During elongation, when the RecA monomer in Pol V-fc is  
22 converted to RecA-ATP, Pol V-fc could be converted into Pol V-fa via contact of the RecA-  
23 ATP with a 3'-tip of RecA\*; in such a case, Pol V-fa can be reactivated through the process  
24 of RecA-capture as previously mentioned (Fig. 7A). In the continuous mode, when not

1 encountering a blockade (i.e., a 3'-tip of RecA\*), Pol V-fc can uninterruptedly elongate (Fig  
2 7D). Accordingly, a DNA patch synthesized by Pol V-fc could be produced by either mode,  
3 or a combination of both modes. When the Pol V-fc consumes its residency time on T/P,  
4 it dissociates as Pol V-dc (Pol V with a RecA monomer) being a non-productive by-product  
5 in the physiological context (Fig. 7E). Based on the average processivity of Pol V-fc (i.e.,  
6  $\approx 25$  nt), in the case of discontinuous mode, one can estimate that a total of 9 cycles has  
7 been performed (steps d to f in Fig. 8).

8         The apparent  $k_{\text{off}}$  of Pol V-dc is estimated to be  $\approx 0.012 \text{ s}^{-1}$  as derived from the  
9 residency time of Pol V-fc,  $\approx 86 \text{ s}$  (= processivity / velocity:  $\approx 25 \text{ nt} / \approx 0.29 \text{ nt s}^{-1}$ ) (66). When  
10 Pol V-dc dissociates, it concomitantly creates a 5-8 nt gap depending on whether  
11 dissociation occurs at the immovable or the movable state (Fig. 7E). The kinetics of  
12 nucleotide cofactor exchange from RecA-ADP to RecA-ATP (e.g., ADP  $\rightarrow$  no cofactor  $\rightarrow$   
13 ATP) as indirectly monitored by ATPase activity seemingly reaches a plateau value at  $\sim 10$   
14 min (86). The residency time of Pol V-fc ( $\approx 86 \text{ s}$ ) may thus indicate that during elongation  
15 most RecA monomers in Pol V-fc are in the ADP (or no cofactor) state or even after  
16 dissociation (Pol V-dc). In addition, a nucleotide cofactor such as ATP- $\gamma$ S was observed  
17 to remain in a RecA monomer associated to Pol V over 1 hr (81).

18         The overall view of the RecA-Fluttering model is delineated in Fig. 8. It is  
19 noteworthy that, while the average processivity of Pol V-fc is  $\approx 25$  nt, the distribution of  
20 processive synthesis is relatively broad up to around 100 nt (66). This observation may  
21 indicate that processive synthesis entails a combination of discontinuous and continuous  
22 elongation modes.

23

1 **Extremely slow velocity of Pol V-fc.** Intriguingly, on normal T/P the velocity of Pol V-fc  
2 is over 10-fold slower than that of Pol IV-fc ( $\approx 0.29$  vs  $3-5$  nt  $s^{-1}$ ) (54, 66). This significant  
3 difference likely reflects the unique feature of Pol V during elongation, i.e. its functional  
4 association with RecA. Residency times of RecA monomers at the 5'- and 3'-ends of  
5 RecA\*, deduced from their  $(k_{\text{off}})^{-1}$  values, are estimated to be  $\approx 8.3$  s and  $\approx 6.3$  s,  
6 respectively (67). Thus, the residency time of Pol V-fc ( $\approx 86$  s) is one order of magnitude  
7 higher than those of RecA monomers at ends of RecA\*. This suggests that, within the  
8 residency time of Pol V-fc, multiple RecA monomers ( $\approx 14$ ) are committed to dissociate  
9 from the 3'-ends of RecA\*. Intriguingly, Pol V-fc requires  $\approx 10$  s to fill the 3 nt freed upon  
10 dissociation of a single RecA monomer, a duration similar to the residency times of a single  
11 RecA monomer at the ends of RecA\*. Taken together, there appears to be fine tuning  
12 between the velocity of Pol V-fc and the dynamic state of RecA at the 3'-end of RecA\*.

13

#### 14 **DISTINCT MODES OF ACTION DURING DNA SYNTHESIS FOR POL IV AND POL V**

15 In contrast to Pol IV-fc (velocity,  $3-5$  nt  $s^{-1}$ ; processivity,  $300-400$  nt) (54), Pol V-fc  
16 exhibits slower velocity ( $\approx 0.29$  nt  $s^{-1}$ ) and lower processivity ( $\approx 25$  nt) on normal T/P (66).  
17 Interestingly, the residency times of Pol IV-fc and Pol V-fc on T/P exhibit similar duration,  
18  $\approx 97$  s (average from  $60-133$  s ( $= 300-400$  nt /  $3-5$  nt  $s^{-1}$ )) and  $\approx 86$  s ( $= \approx 25$  nt /  $\approx 0.29$  nt  $s^{-1}$ ),  
19 respectively. Thus, despite significant differences between Pol IV and Pol V in terms of  
20 velocity and processivity on normal T/P, their affinities for T/P are similar, implying that the  
21 residency times of both Pol IV and Pol V may largely depend upon their interaction with  
22 the  $\beta$ -clamp (54, 66).

23 Pol IV and Pol V exhibit distinct lesion bypass features, not only in terms of their  
24 substrate specificities (minor versus major groove lesions, respectively), but also in the

1 way their processivities are affected by lesions. The processivity of Pol V-fc ( $\approx 25$  nt on  
2 normal T/P) is only moderately reduced by the presence of a lesion (e.g., reduced to  $\approx 18$   
3 nt in TLS across an AAF-dG) (66). In contrast, *in vitro* the processivity of Pol IV-fc during  
4 [-ta]-BaP-dG bypass is intriguingly short (around 5 nt) compared to its processivity on  
5 normal T/P (300-400 nt) (54) suggesting that most of Pol IV's residency time is consumed  
6 by the lesion bypass step (S. Fujii, A. Isogawa, R. Fuchs, unpublished data). A similar  
7 phenomenon also occurs for Pol II during the bypass of an AAF-dG lesion in the *Nar I*  
8 sequence context, namely its processivity during TLS via a slippage mechanism is only 3  
9 nt, while it is  $>1,600$  nt on normal T/P (43, 99). It, thus, indicates that Pol IV and Pol V  
10 largely differ in their modes of action during lesion bypass. Indeed, the fact that Pol V-fc  
11 can deal with a T/P almost as efficiently whether or not a lesion is present can be attributed  
12 to its fine positioning by virtue of a RecA monomer in Pol V-fc (i.e., fifth role of RecA). This  
13 notion is also supported by the observation that, under multiple-hit conditions *in vitro*, Pol  
14 IV (or Pol II) alone can carry out robust DNA synthesis on both normal and lesion-  
15 containing T/P (N-2-furfuryl-dG for Pol IV; AAF-dG for Pol II) (43, 98); in contrast, under  
16 multiple-hit conditions *in vitro*, Pol V alone can perform DNA synthesis on normal T/P but  
17 not on a lesion-containing T/P (65).

18

## 19 **IMPACT OF TLS AND PERSPECTIVES**

20 In *E. coli*, TLS Pols modestly contribute to survival, but are essential for induced  
21 mutagenesis (10) strongly inferring that, in addition to preventing persistent ssDNA gaps,  
22 they represent a major driving force for genetic diversity in both short (e.g., adaptive  
23 mutations) or long time range (e.g., evolution) (10, 99, 100). Biological significance of  
24 genetic diversity in short time range is illustrated by, for instance, mutations in the

1 immunoglobulin gene locus (i.e., somatic hypermutation) in higher eukaryote or  
2 appearance of antibiotic resistant bacteria in the clinics. The former is driven by TLS Pols-  
3 mediated targeted and/or untargeted mutagenesis restricted to a localized region in the  
4 genome (101). The latter is presumed to result from antibiotic-induced stress that leads to  
5 SOS induction causing genome-wide mutagenesis mediated by TLS Pols (102).

6

### 7 **Why Many Different Pol V Models have been Proposed over the Years?**

8         Since Pol V was identified, various, sometimes conflicting, models to explain its  
9 biochemical features *in vitro* have been proposed (10, 103, 104). We suggest that the  
10 subtlety of Pol V's biochemistry stems from two distinct roles that RecA imparts Pol V: i)  
11 the ATP-bound form of RecA in RecA\* facilitates the interaction of Pol V with a freely  
12 sliding  $\beta$ -clamp (third role of RecA) and ii) the ADP-bound form of RecA monomer (fifth  
13 role of RecA) allows proper positioning of Pol V with the  $\beta$ -clamp during DNA synthesis.  
14 Complete physiological reconstitution of Pol V's mode of action is thus made difficult by  
15 the stepwise involvement of RecA-ATP first followed by RecA-ADP, in addition to the  
16 influence of the  $\beta$ -clamp into the Pol V's mode of action. As a consequence, the  
17 biochemical properties of Pol V sharply depend upon the experimental conditions such as  
18 the nature of the nucleotide cofactor as well as the geometry of the T/P, leading to  
19 conflicting results and contributing to the enigmatic feature of Pol V *in vitro* (10, 103, 104).  
20 In contrast to previous models, the "RecA-Fluttering" model proposed herein is likely to  
21 become a paradigm that reconciles these perplexing longstanding issues (Figs. 7 and 8).

22

### 23 **Physiological Roles of TLS Pols Beyond the Canonical TLS Pathway**

1           Recent finding that Pol V participates in gap filling synthesis associated with the  
2 process of TLS (23) raises the possibility that TLS Pols such as Pol V participate in any  
3 gap filling synthesis irrespective of the origin of the ssDNA gap. For instance, at a late  
4 time point during SOS response, a RecA-dependent recombination-mediated repair  
5 pathway is activated (step f in Fig. 1) (8, 69, 105). During the process, an ssDNA gap, in  
6 all points similar to the gap formed following TLS patch formation, appears (steps c to d  
7 vs f to g in Fig. 1). Indeed, it was suggested that TLS Pols potentially participate in  
8 homologous recombination *in vivo* (106). In addition, it was reported that TLS Pols can  
9 participate in NER *in vivo* (107) and confer a competitive fitness advantage during  
10 stationary phase (108). Thus, the working place of TLS Pols appears not to be restricted  
11 to the sole context of lesion bypass. Consequently, the name of “specialized Pols” instead  
12 of TLS Pols may be more appropriate to reflect their diverse functions.

### 13 14 **Untargeted Mutagenesis during Post-Replicative Gap Repair as a Beneficial Source** 15 **of Diversity in Evolution**

16           Classically, genome-wide mutagenesis has been extensively studied through the  
17 analysis of mutator mutants (e.g., MMR defective strains) in order to unravel their relative  
18 contribution to genome integrity (109). Differently from genome-wide mutators, Pol V-  
19 induced untargeted mutagenesis is restricted to a local area (Fig. 3C) resembling to  
20 somatic hypermutation as in immunoglobulin gene loci. Such localized mutagenesis may  
21 be beneficial to evolution (110). Moreover, as Pol V-induced untargeted mutations are  
22 inefficiently repaired by MMR (23), one daughter cell will acquire novel genetic information  
23 while the another daughter cell retains its original content. It is noteworthy that Pol IV  
24 frequently induces –1 frameshift mutations at monotonous base runs (56) while Pol V

1 essentially induces base substitutions (23). Errors induced by Pol V during gap filling  
2 synthesis, in addition to the lack of proofreading function, are not efficiently corrected by  
3 MMR, leading to  $\geq 10^4$ -fold increase compared to regular DNA replication errors (109, 111).  
4 Such features may be physiologically advantageous for generating genetic diversity in  
5 short and long time ranges.

6

### 7 **SOS-dependent Untargeted Mutagenic Roads**

8 Last but certainly not least, Pol IV is essentially inefficient in gap filling synthesis on  
9 normal template DNA as described previously. Therefore, in contrast to post-replicative  
10 gap repair associated to Pol V-mediated TLS (Fig. 3C), the mutation spectrum associated  
11 to Pol IV-mediated TLS is expected to carry minimum mutagenic load as in Fig. 3B.

12 Below we describe two situations where, in addition to Pol IV, Pol V becomes  
13 involved in completion of the whole process of minor groove lesion bypass. First, some  
14 minor groove lesions may require both Pol IV and Pol V for their bypass. Following  
15 metabolic activation of BaP *in vivo*, the ultimate metabolite (diol-epoxide) primarily reacts  
16 at the N<sup>2</sup> position of dG (112) forming [+ta]-BaP-dG and its stereoisomer [-ta]-BaP-dG  
17 that represent the major and a minor adduct, respectively (113). As mentioned above, *in*  
18 *vivo*, the [-ta]-BaP-dG adduct is bypassed by Pol IV under normal growth conditions. In  
19 contrast, bypass of [+ta]-BaP-dG interestingly requires both Pol IV and Pol V *in vivo* (50,  
20 59). Thus, depending upon the precise chemistry and conformation of a minor groove  
21 adduct, successful bypass requires collaboration between Pol IV and Pol V. In this  
22 situation, when Pol V is required, TLS necessarily occurs in late SOS and post-replicative  
23 gap filling becomes error-prone.

1           Second, when the number of minor groove lesion is high, i.e. exhausting the  
2 capacity of rapid Pol IV-mediated bypass, cells enter into late SOS response where Pol V  
3 is expressed and can functionally access RecA-coated ssDNA gap. Thus, depending  
4 upon the timing of Pol IV-mediated lesion bypass completion, untargeted mutation pattern  
5 gradually shifts from largely error-free (Fig. 3B) to error-prone (Fig. 3C) owing to the  
6 participation of Pol V.

7           At the end, considering TLS *in vivo* from the sole prospect of lesion bypass  
8 accompanied by a targeted mutation event, melts down to the famous saying “you cannot  
9 see the wood for the trees”. As mentioned throughout this review, based upon the overall  
10 context of post-replicative gap repair, our vision of TLS pathways should be thoroughly  
11 revisited.

12

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19

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17

18

1

2 **Fig. legends**

3 **FIG 1** Schematic overview of post-replicative gap repair. (a) The fork moves from left to  
4 right. The red triangle represents a replication blocking lesion in the leading strand. (b)  
5 The fork skips over the lesion and downstream repriming leaves an ssDNA gap.  
6 Subsequently the gap is repaired either via TLS (c to e) or HDGR (f to h). (c) The pink line  
7 represents a short DNA patch, mediated by a TLS Pol, across and beyond the lesion. (d)  
8 A DNA Pol fills in the remaining gap, followed by ligation. (e) At steps (c and d) the lesion  
9 may be repaired by regular repair systems. (f) The lesion located in ssDNA is relocated to  
10 dsDNA via sister chromatid exchange. (g) A DNA Pol fills in the newly appeared gap. (h)  
11 Resolution of the Holliday junction, followed by ligation. Any time (f to h), when the lesion  
12 is relocated to dsDNA, it may be repaired by regular repair systems.

13

14 **FIG 2** Stochastic access of DNA Pols on lesion-containing template DNA. Functional TLS  
15 requires a proper lesion/Pol combination. (A) We will only discuss the case of lesion that  
16 cannot be bypassed by classical Pols (i.e., Pol I to III). However, these Pols get  
17 occasionally involved in non-productive degradation/resynthesis cycles at primer ends in  
18 the vicinity of lesions (42). (B) For a minor groove lesion, Pol IV following interaction with  
19 the  $\beta$ -clamp (i.e., Pol IV-fc: Pol IV functional complex) bypasses the lesion in any stage of  
20 SOS response. (C) For a major groove lesion, during late SOS response, Pol V is  
21 stabilized by RecA leading to the formation of a pre-Pol V-fa complex (Pol V with RecA\*),  
22 followed by interaction with the  $\beta$ -clamp (Pol V-fa: Pol V functional assembly),  
23 subsequently bypassing the lesion.

24

1 **FIG 3** TLS elicits untargeted mutagenesis during post-replicative gap repair. (A)  
2 Schematic representation of template DNA undergoing TLS-mediated post-replicative gap  
3 repair. (B) Expected pattern of untargeted mutations in case the error-prone TLS Pol is  
4 restricted to the lesion bypass event *per se*. (C) Schematic representation of the  
5 untargeted mutation pattern as observed *in vivo* during Pol V-mediated TLS (23).

6  
7 **FIG 4** Stochastic access of DNA Pols on normal template DNA. (A) Classical Pols can  
8 elongate primers independently of the status of the template DNA such as naked, SSB-  
9 or RecA-coated (42). When a classical Pol occasionally encounters a freely sliding  $\beta$ -  
10 clamp, the resulting complex exhibits increased residency time leading to long DNA patch  
11 synthesis. (B) Pol IV quickly dissociates from template DNA. The vast majority of these  
12 transiently bound Pol IV molecules will not encounter a freely sliding  $\beta$ -clamp. Therefore,  
13 Pol IV binding events are either abortive or lead to one nt incorporation. (C) Pol V  
14 physically interacts with RecA on template DNA increasing substantially its residency time  
15 leading to the formation of pre-Pol V-fa (i.e., Pol V with RecA\*) and, in turn, allowing Pol  
16 V to encounter a freely sliding  $\beta$ -clamp (chaperon-like action of Rec A). Pol V in interaction  
17 with both RecA and the  $\beta$ -clamp (i.e., Pol V-fa) synthesizes a short DNA patch in an error-  
18 prone manner. When Pol V together with a RecA monomer (Pol V-dc) dissociates, it leaves  
19 behind a short ssDNA gap between the 3'-end of the primer and the 3'-tip of RecA\*.

20  
21 **FIG 5** Pol V-mediated TLS: overall view of the complete post-replicative gap repair  
22 process. This scheme is likely to describe the mechanism involved *in vivo* as depicted in  
23 Fig. 3C. (a) When Pol V with a RecA monomer (i.e., Pol V-dc) dissociates following Pol V-  
24 mediated TLS, a short ssDNA gap between the 3'-end of the primer and the 3'-tip of RecA\*

1 appears. (b) This short gap prevents proper re-access of a Pol V molecule to both the 3'-  
2 end of the primer and the 3'-tip of RecA\*, simultaneously. Thereby, functional access of  
3 one of the classical Pols dominates, leading to synthesis of a short error-free patch. Our  
4 working model supposes that, when a classical Pol dissociates, it eventually yields a 3'-  
5 end of primer to which Pol V can bind and simultaneously interact with the 3'-tip of RecA\*.  
6 (c) Pol V and classical Pols stochastically compete at the 3'-end of the primer. When Pol  
7 V accesses, it synthesizes a short DNA patch in an error-prone manner. A short gap again  
8 appears following dissociation of Pol V-dc. (d) The remaining overall ssDNA gap is filled  
9 in through repeated cycles of steps b and c.

10  
11 **FIG 6** Dynamics of RecA\* formation. The directionality of RecA filament formation was  
12 deduced from the following biochemical parameters (67): Dissociation rates ( $k_{\text{off}}$ ) of RecA  
13 monomers in RecA\* at 5'- and 3'-ends are essentially the same (5'-end,  $\approx 0.12 \text{ s}^{-1}$ ; 3'-end,  
14  $\approx 0.16 \text{ s}^{-1}$ ), and their association rates ( $k_{\text{on}}$ ) are as follows: 5'-end,  $\approx 0.11 \text{ s}^{-1}$  at 100 nM  
15 RecA; 3'-end,  $\approx 0.18 \text{ s}^{-1}$  at 8 nM RecA, leading to the dissociation constants ( $K_{\text{D}}$ ) at 5'-end,  
16  $\approx 100 \text{ nM}$  and at 3'-end,  $\approx 8 \text{ nM}$  (67). Thus, whereas both ends of RecA\* are in a highly  
17 dynamic state, the significant difference of  $K_{\text{D}}$  (i.e., the large difference of  $k_{\text{on}}$  dependent  
18 upon concentration of RecA) leads to the apparent directionality of RecA\* formation.

19  
20 **FIG 7** The RecA-Fluttering model. RecA molecules are numbered starting at the 3'-end of  
21 RecA\*. ATP and ADP forms of RecA are shown in light blue and yellow, respectively. If Pol  
22 V can carry out elongation (i.e., there is enough free ssDNA space), we will refer to it as  
23 an “movable” complex. If not, we will call it “immovable”. (A) pre-Pol V-fa (i.e., Pol V with  
24 RecA\*) is converted into Pol V-fa following interaction with the  $\beta$ -clamp. The 3'-end of

1 RecA molecule detaches from the filament while remaining tightly bound to Pol V. During  
2 that process, referred to as “RecA-capture”, ATP bound RecA is converted into ADP and  
3 Pol V becomes a fully functional complex (Pol V-fc: Pol V with a RecA monomer and the  
4  $\beta$ -clamp). During the RecA-capture event, a 3 nt gap forms and RecA molecule #2  
5 becomes the new 3'-tip of RecA\*. The transition between Pol V-fa and Pol V-fc is  
6 irreversible due to ATP to ADP conversion. Moreover, due to steric hindrance both Pol V-  
7 fa and Pol V-fc complexes are immovable. It should be noted that, if RecA-capture occurs  
8 at the pre-Pol V-fa state, it leads to a non-productive event as Pol V with a RecA monomer  
9 (i.e., Pol V-dc) dissociates immediately. (B) Upon dissociation of RecA molecule #2, the  
10 ensuing 6 nt gap allows Pol V-fc to enter its movable state. Thus, in the presence of the  
11  $\beta$ -clamp, “activation” of Pol V occurs on the T/P substrate through dynamic cooperation  
12 with RecA\*. Elongation will proceed either via discontinuous or continuous mode. (C)  
13 Discontinuous elongation mode. RecA #1 in Pol V-fc repeatedly binds to and detaches  
14 from template DNA (“RecA-Fluttering”). Since access of RecA-ADP spatially requires 5 nt  
15 on ssDNA (94), Pol V-fc synthesizes the first nt of the 6 nt gap and then pauses due to  
16 steric hindrance until RecA #3 dissociates generating an 8 nt gap. Pol V-fc can synthesize  
17 3 nt until reaching a 5 nt gap that again blocks further synthesis. Elongation by Pol V-fc  
18 proceeds in an inchworm-like motion (i.e., multiple cycles of 3 nt except for 1 nt  
19 incorporation at the first cycle). It should be noted that, if ATP is incorporated into the RecA  
20 monomer in Pol V-fc, it can be converted into Pol V-fa during elongation, then Pol V-fa  
21 would need to be reactivated via the RecA-capture event. (D) Continuous elongation  
22 mode. When the 3'-tip of RecA\* dissociates before encountering the moving Pol V-fc  
23 complex, smooth elongation (without stalling) is maintained until Pol V dissociates. (E)  
24 Based on the processivity of Pol V-fc on normal T/P (i.e.,  $\approx$ 25 nt) (66), when Pol V-dc

1 dissociates from T/P,  $\approx 9$  RecA molecules have been released from the 3'-end of the  
2 filament. When Pol V-dc dissociates, Pol V-fc is either in a "immovable" state leaving a 5  
3 nt gap or in a "movable" state leaving a 5-8 nt gap.

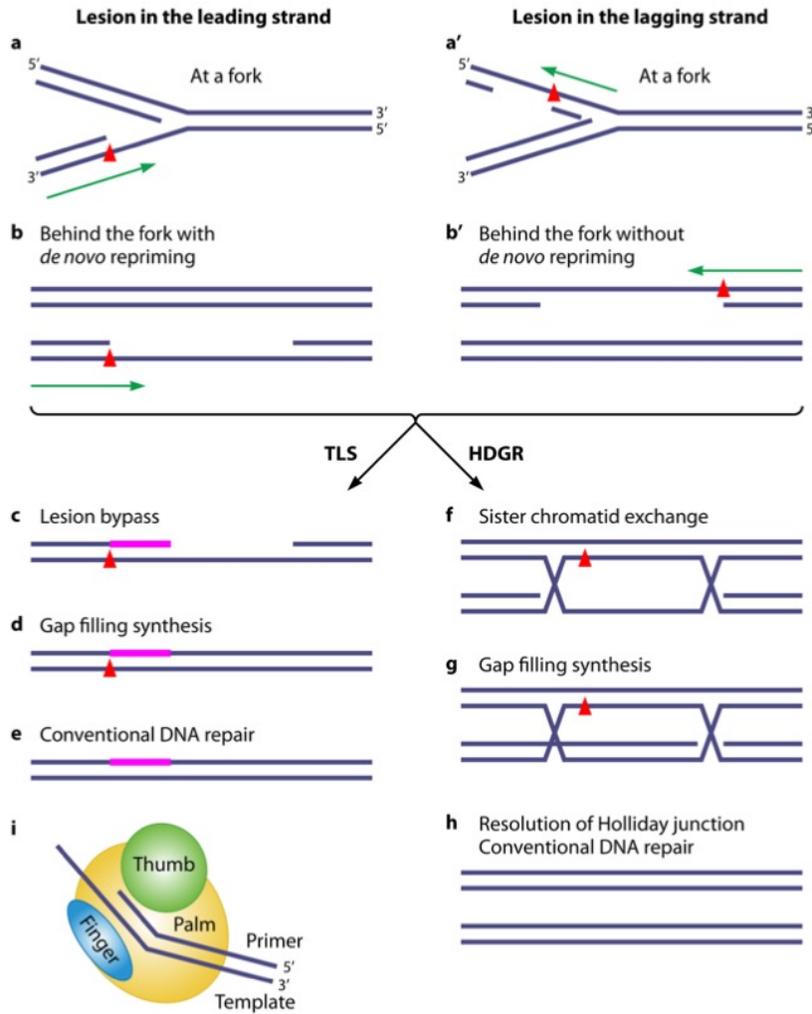
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5 **FIG 8** Overall view of the RecA-Fluttering model. (a) When Pol V accesses a vacant 3'-  
6 end of primer, it becomes stabilized by RecA\* leading to pre-Pol V-fa (Pol V with RecA\*)  
7 formation. (b to c) pre-Pol V-fa is first converted into Pol V-fa by interacting with the  $\beta$ -  
8 clamp and then to Pol V-fc by capturing the terminal RecA molecule from the filament.  
9 ATP hydrolysis is associated with the RecA-capture event. (d) When the new 3'-tip of  
10 RecA\* dissociates, it generates a 6 nt gap and Pol V-fc can start elongation. During  
11 elongation, we propose that Pol V-fc exhibits a dynamic state during which its associated  
12 RecA molecule alternately binds to and detaches from the DNA substrate, a motion we  
13 refer to as "RecA-Fluttering". RecA-Fluttering, to which we also refer to as fifth role of  
14 RecA, insures proper positioning of Pol V with the  $\beta$ -clamp during DNA synthesis. (e)  
15 Discontinuous elongation. Pol V-fc fills in 1 nt of the 6 nt gap. (f) In the next cycle, Pol V-  
16 fc fills in the first 3 nt of the 8 nt gap. Elongation of Pol V-fc is discontinuous until  
17 dissociation. (g) Continuous elongation. Pol V-fc continues elongation without stalling as  
18 long as it is not sterically hindered by a 3'-tip of RecA\*. (h) When Pol V-fc exhausts its  
19 residency time, Pol V-dc (Pol V with a RecA monomer) dissociates from T/P, leaving  
20 behind the  $\beta$ -clamp and a 5 to 8 nt gap on the T/P.

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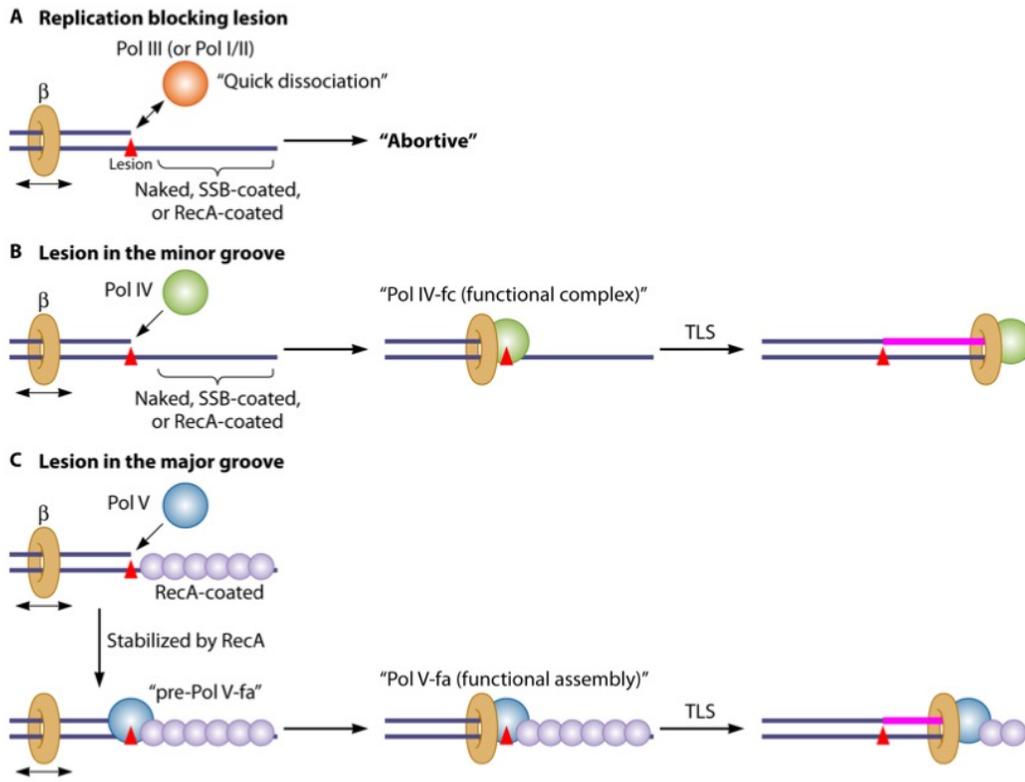
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4 **Fig. 1**

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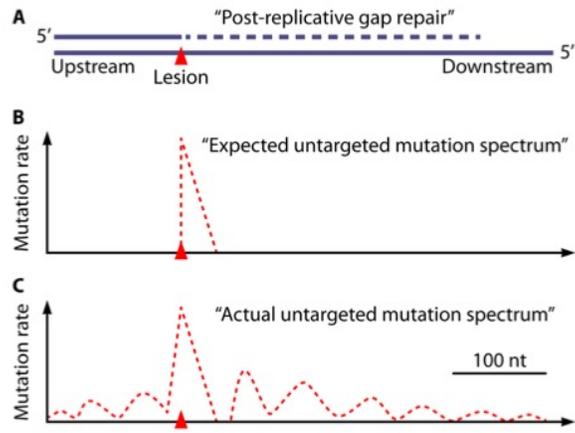
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4 **Fig. 2**

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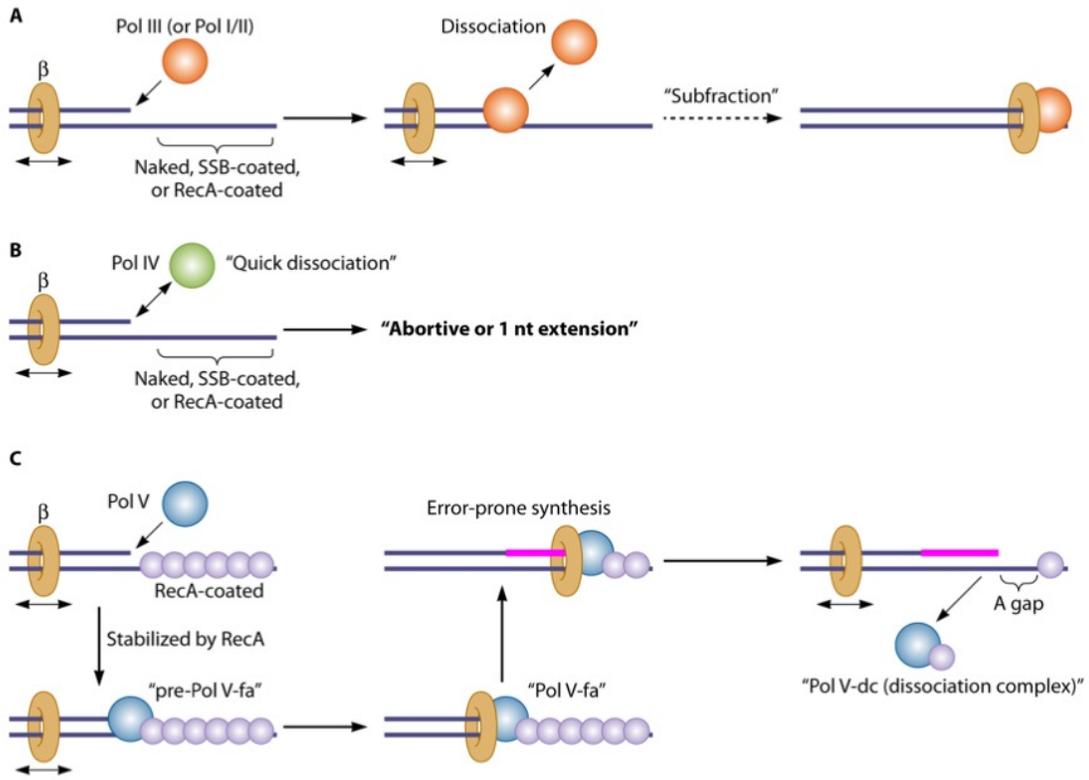
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4 **Fig. 3**

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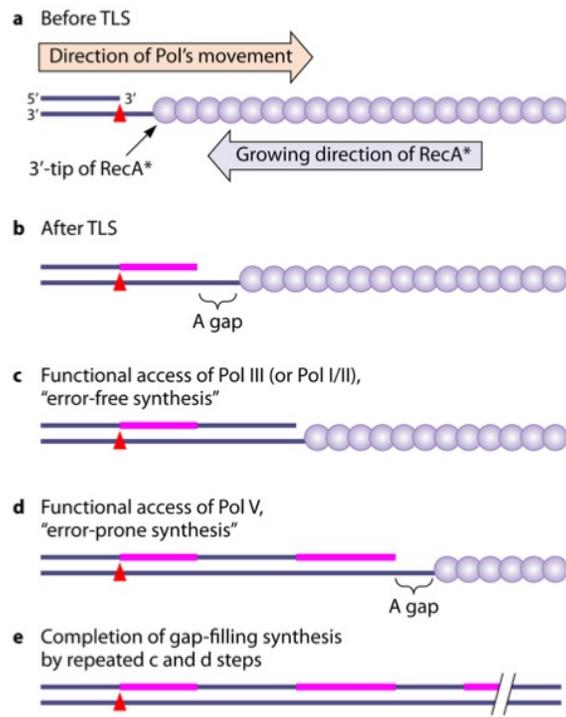
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4 **Fig. 4**

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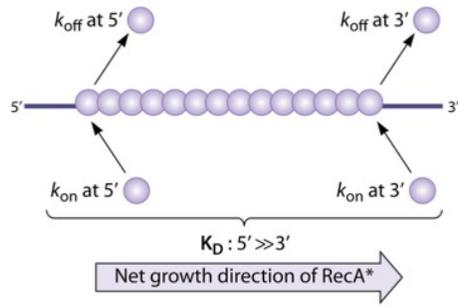
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4 **Fig. 5**

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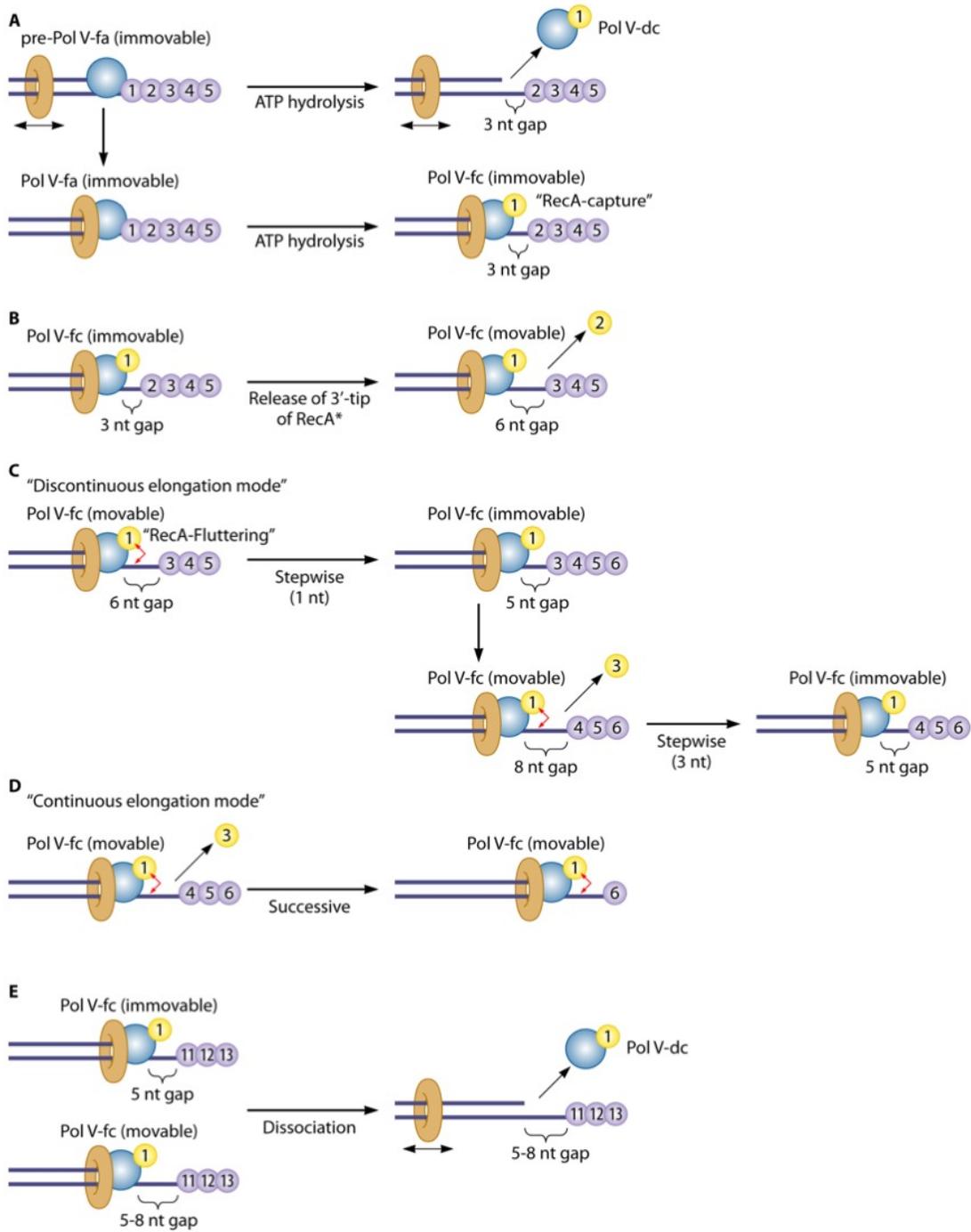
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4 **Fig. 6**

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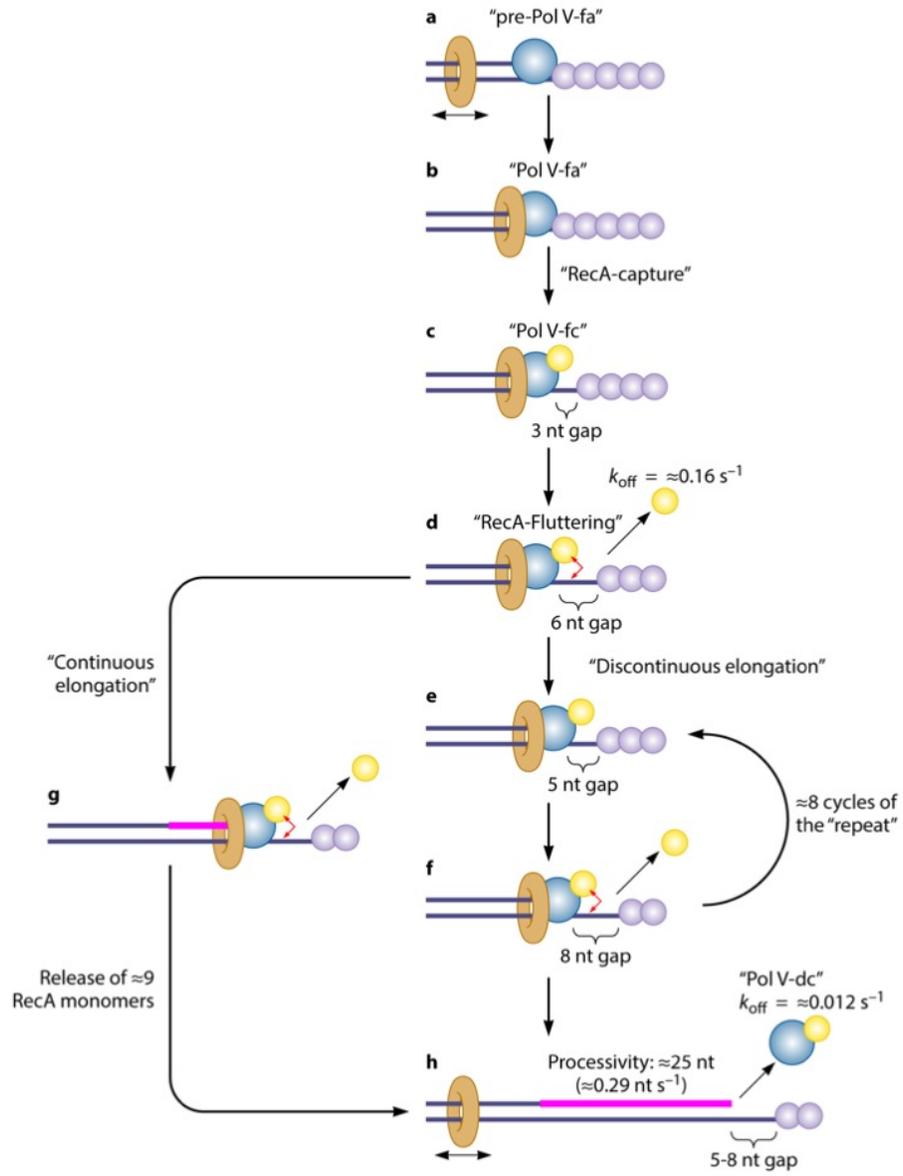
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4 **Fig. 7**

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4 **Fig. 8**

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