

Mutations for Worse or Better: Low Fidelity DNA Synthesis by SOS DNA Polymerase V is a Tightly-Regulated Double-Edged Sword.

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ABBREVIATIONS

UV, ultraviolet light; ssDNA, single-stranded DNA; TLS, Translesion Synthesis; nt, nucleotide; DNA pol V, DNA polymerase V; pol V Mut, polymerase V Mutasome; pol III HE, polymerase III holoenzyme; ATP, Adenosine triphosphate; ATP γ S, Adenosine 5'-(3-thiotriphosphate) tetralithium salt; RecA*, RecA nucleoprotein filament; kDa, kilodalton; MALS, Multiangle Light Scattering; PAGE, polyacrylamide gel electrophoresis; sm, single-molecule; TIRF, Total internal reflection fluorescence; FRET, Förster resonance energy transfer.

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3 **ABSTRACT:** 1953, the year of Watson and Crick, bore witness to a less acclaimed, yet highly
4 influential discovery. Jean Weigle demonstrated that upon infection of *Escherichia coli*, λ phage
5 deactivated by UV radiation, and thus unable to form progeny, could be reactivated by
6 irradiation of the bacterial host. Evelyn Witkin and Miroslav Radman later revealed the presence
7 of the SOS regulon. The more than 40 regulon genes are repressed by LexA protein and induced
8 by the coproteolytic cleavage of LexA, catalyzed by RecA protein bound to single-stranded
9 (ss)DNA, the RecA* nucleoprotein filament. Several SOS-induced proteins are engaged in
10 repairing both cellular and extracellular damaged DNA. There's no "free lunch", however,
11 because error-free repair is accompanied by error-prone translesion DNA synthesis (TLS),
12 involving *E. coli* DNA polymerase V (UmuD'₂C) and RecA*. This review describes the
13 biochemical mechanisms of pol V-mediated TLS. Pol V is active only as a mutasomal complex,
14 pol V Mut = UmuD'₂C-RecA-ATP. RecA* donates a single RecA subunit to pol V. We highlight
15 three recent insights: 1) pol V Mut has an intrinsic DNA-dependent ATPase activity that governs
16 polymerase binding and dissociation from DNA; 2) active and inactive states of pol V Mut are
17 determined at least in part by the distinct interactions between RecA and UmuC; 3) pol V is
18 activated by RecA*, not at a blocked replisome, but at the inner cell membrane.
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3 Mutations for worse are responsible for death in microorganisms and disease in humans.
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5 Mutations for better are a central driving force for fitness and evolution in all forms of life.
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8 *Escherichia coli* DNA polymerase V (pol V) initiates the lion's share of mutations targeted at
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10 DNA template lesions, including those caused by UV radiation and a variety of chemicals that
11
12 can damage DNA.¹⁻⁴ The initial description of the polymerase function of the *E. coli* DinB (pol
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14 IV) and UmuDC (pol V) proteins in 1999⁵⁻⁷ was part of the broader discovery of a new and
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16 widespread family of specialized DNA polymerases.⁸ These are the Y family DNA polymerases,
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18 which promote a process called translesion DNA synthesis or TLS. These polymerases exhibit a
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20 relaxed specificity, increasing mutagenesis when they function. In *E. coli*, pol V is responsible
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22 for virtually all of the mutagenesis that accompanies the SOS response.^{1-4,9,10}
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28 The *umuD* and *umuC* genes encoding error prone pol V (UmuD'₂C) are induced in response
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30 to DNA damage as part of the SOS regulon.^{1,2} The promoter for the *umuDC* operon possesses a
31
32 near perfect match with the consensus LexA repressor binding motif resulting in its relatively
33
34 late SOS induction. The operon is not fully derepressed until ~15 min after DNA damage¹¹, and
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36 the UmuD'₂C proteins themselves do not accumulate until ~ 45 min after damage, due to rapid
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38 proteolysis of the Umu proteins.¹² Once formed, pol V is largely resistant to proteolysis, and the
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40 enzyme copies past DNA template lesions that normally arrest replication by the high fidelity pol
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42 III.¹³ Low-fidelity pol V lacks intrinsic 3'-5' exonucleolytic proofreading, so it cannot correct any
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44 mistakes it makes during TLS. However, if the pol V –dependent TLS tract is limited, then any
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46 misincorporated nucleotides introduced by pol V during TLS may be subject to extrinsic
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48 proofreading by the *dnaQ*-encoded epsilon subunit of pol III. The extent of extrinsic
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50 proofreading may be temporarily suppressed due to an increase in nucleotide pools that
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52 accompany the SOS response,¹⁴ and may allow pol III to elongate pol V-catalyzed
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3 misincorporations and thereby help sustain them as damage-induced mutations in the *E. coli*
4 genome.¹⁴
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9 RecA bound to single-stranded (ss) DNA in the presence of ATP as an active and extended
10 nucleoprotein filament is often referred to as RecA*.¹⁵ RecA has multiple roles in the SOS
11 response, and its effects are paramount to both SOS regulation and downstream implementation.
12 RecA* appears when RecA binds to the ssDNA byproduct of DNA damage events. Acting as a
13 coprotease, RecA* induces the SOS response by facilitating the autocatalytic cleavage of the
14 LexA repressor protein to upregulate transcription of the SOS regulon genes (Figure 1).¹⁶ The
15 RecA* coprotease activity is again needed to form pol V by facilitating the autocatalytic
16 cleavage of the UmuD protein to a shorter and mutagenically active UmuD'^{9,17,18} that dimerizes
17 and binds to UmuC¹⁹ (Figure 1). Finally, UmuD'₂C is activated by transfer of a RecA subunit
18 from RecA* to UmuD'₂C to form pol V Mut (UmuD'₂C-RecA-ATP), the active TLS
19 polymerase.²⁰
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36 Until 1998, UmuD'₂C was widely considered an auxiliary factor that, with RecA, somehow
37 modulated DNA polymerase III to effect mutagenic DNA synthesis across lesions in the DNA
38 template. However, when incubated in the presence of RecA*, the same UmuD'₂C promoted
39 TLS in the *absence* of any added DNA polymerase.¹³ Most notably, pol III holoenzyme (HE)
40 composed of pol III core ($\alpha\epsilon\theta$ subunits) + β sliding clamp + γ clamp loader complex, was
41 absent.¹³ Soon thereafter UmuD'₂C was identified as pol V,⁵ whose catalytic activity resides in
42 its UmuC subunit.^{5,6} The “auxillary factor” therefore evolved into a *bona fide* DNA polymerase,⁸
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60 many more have since been identified in prokaryotes and eukaryotes.^{8,21,22}

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Despite its demonstrable polymerase activity, pol V was largely catalytically inactive in the absence of RecA*.^{5,13} This review is centered on why and how RecA* is needed for pol V activity. Distinct from its role as a coprotease in converting UmuD → UmuD', RecA* plays a unique and (for RecA) uncharacteristically direct role in pol V function. RecA*, acting as a catalyst, transfers a RecA monomer from its 3'-proximal filament tip along with a molecule of ATP, to inactive pol V to form an activated pol V mutasome, pol V Mut (a complex of UmuD'₂C-RecA-ATP).²⁰ ATP may either be transferred in concert with RecA or it might perhaps bind separately.

The biochemical roles of RecA and ATP in regulating pol V Mut activity have come into focus since 2009, with surprises at every step. There are limited studies demonstrating the role of RecA outside nucleoprotein filaments, such as its ability to bind to DinB and modulate polymerase fidelity.^{23,24} However, pol V Mut is the only instance where a physical complex with a RecA monomer is absolutely essential for DNA polymerase activity.²⁰ Recent advances add to the dynamic. Pol V Mut has an intrinsic DNA-dependent ATPase activity that appears to act as internal clock to regulate polymerase processivity.²⁵ The RecA* activation of pol V is spatially regulated, most likely occurring at the cell membrane.²⁶ The last observation connects current research with the mid-'80s observation of Evelyn Witkin that RecA* is localized on the membrane in *E. coli* lysates.²⁷ More recently, it has become clear that initiation of the SOS response depends upon that same RecA membrane localization.²⁸ The robust field of SOS “error-prone” repair initiated by Witkin²⁹ and Miroslav Radman³⁰, just keeps on giving, thus providing impetus to reflect on the earlier genetic-based TLS models in light of recent biochemical and live cell imaging discoveries.

■ TLS BEFORE 1998

The Bridges-Woodgate two-step lesion bypass model³¹ involving the UmuDC proteins and RecA (Figure 2A) provides a mechanistic framework to trace the progress in dissecting the biochemical basis of pol V-catalyzed TLS. In the first step, nucleotide misincorporation at the site of DNA damage, pol III copies the lesion, requiring the action of RecA protein. In the second step, lesion bypass, UmuC and UmuD proteins are needed to facilitate continued DNA synthesis on the priming end opposite the lesion.³¹ The importance of the *umuDC* operon encoded damaged-induced genes for SOS mutagenesis^{1,2,32} was established before the Bridges-Woodgate model was proposed in 1985. Cells having defects in either *umuC* or *umuD* are UV non-mutable.¹ A direct role for RecA in pol III-catalyzed misincorporation was assumed in the model based on *recA* mutant strains that exhibited differences in their RecA* coprotease activity; those with good coprotease activity promoted higher levels of mutagenesis, while those with poor coprotease activity promoted low levels of mutagenesis.³³ Three years later, the discovery that UmuD' was the active form of UmuD, formed by the coproteolytic activity of RecA*, provided an alternative explanation for the role of RecA in SOS mutagenesis.^{17,18} The stage was set for another wrinkle.

Devoret and colleagues³⁴ soon demonstrated that RecA* had a separate and direct role in TLS. The RecA S117F mutant, named RecA1730, was able to catalyze homologous recombination, use its coproteolytic function to cleave the LexA repressor to turn on SOS and cleave UmuD to form UmuD'. However, SOS mutagenesis did not occur. This newly discovered direct role for RecA in TLS led to a new mutasome model proposed by Echols & Goodman³⁵ (Figure 2B). Relying on the known filament-forming properties of RecA, the model placed a RecA nucleoprotein filament in *cis*, at the lesion site on the template strand being copied. It was

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3 assumed that this filament would somehow facilitate replication past the lesion with reduced
4 fidelity. This might occur by inhibiting pol III proofreading,³⁶ or perhaps by reducing insertion
5 specificity by stretching the ssDNA at the stalled replication fork (Figure 2B). The mutagenically
6 active UmuD' was included in this "Echols mutasome" model.³⁵ Thus, although many parts of
7 the model proved incorrect, all of the main actors in the story were finally present. Quoting from
8 our contemporary 1990 review,³⁵ "What is the direct role of RecA in mutagenesis? At this point
9 we can make reasonable guesses, but the answer awaits a biochemical reconstitution of
10 translesion replication".

21 ■ BIOCHEMICAL RECONSTITUTION OF TLS

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24 In 1989 minuscule amounts of soluble UmuC were obtained by refolding the purified and
25 denatured protein.¹⁹ Soon after that detectable levels of TLS were generated in a reconstituted
26 system *in vitro* with UmuC, UmuD', pol III, and RecA.³⁷ However, it wasn't until 1998 when a
27 soluble UmuD'₂C complex was isolated¹³ and the biochemical reconstitution of TLS with
28 purified SOS proteins achieved.^{13,38} With the exception of UmuC, the individual components in
29 the sketch of the mutasome model (Figure 2B), RecA*, UmuD', pol III HE were available as
30 purified aqueous soluble proteins. Native UmuC, on the other hand was insoluble in aqueous
31 solution.¹⁹ Overexpression of UmuC and UmuD' in the absence of chromosomal UmuC and
32 most importantly UmuD allowed the purification of UmuC and UmuD' as a soluble
33 heterotrimeric complex, UmuD'₂C.³⁹ Parenthetically, although not appreciated at the time, it was
34 essential to delete the chromosomal *umuD* gene to prevent formation of a stable and interfering
35 UmuD'D complex that triggers the release and precipitation of UmuC.⁴⁰ Consistent with the
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3 biochemically recalcitrant nature of UmuC, we now know that, UmuC resides on the cell
4 membrane and only enters the cytosol upon binding to UmuD'₂C.²⁶
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9 Each of the required SOS mutagenesis protein components identified genetically were now
10 in-hand in a highly purified soluble form: UmuD'₂C, RecA*, presumably all acting to somehow
11 alter the activity of pol III HE. These proteins were used to copy primer-template (p/t) DNA
12 containing a site-directed template (abasic) lesion.^{13,38} Robust synthesis on the damaged DNA
13 template ensued. Unexpectedly, TLS occurred in the *absence* of pol III core.¹³ Indeed, pol III
14 acted as a competitive inhibitor to UmuD'₂C at the TLS step,¹³ and that was because UmuD'₂C is
15 a DNA polymerase.^{5,6} Examination of this TLS activity opened new questions about the role of
16 RecA. The pol V-catalyzed DNA synthesis was diminished markedly in the absence of
17 RecA*^{5,13}. What was RecA* doing, and where was it doing it?
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31 ■ TLS 1998 - 2006 RecA* CIS-ACTIVATION

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34 The elegantly simple 2-step model proposed in 1985 (Figure 2A) provided a focus for research
35 for over twenty years. It was flexible enough to incorporate each new biochemical advance in the
36 period. In the “mutasome” model,³⁵ a molecule of RecA was replaced by a RecA* nucleoprotein
37 filament, which was assumed to assemble at a DNA template lesion downstream of a stalled
38 replication fork (Figure 2B). The subsequent identification of UmuD'₂C as a TLS polymerase
39 resulted in a model in which pol III blocked at a lesion (Figure 3, left panel) was replaced on the
40 β-clamp by pol V (Figure 3, right panel). Although modified to include newly discovered
41 biochemical components, the common thread of each ensuing model remained the location of
42 RecA* on the template strand immediately downstream of, and perhaps even encompassing the
43 lesion (Figure 3). This has been defined as the *cis*-activation model for TLS, because RecA* is
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3 presumed to act in *cis* on the strand being copied to shepherd a stalled polymerase past the
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5 lesion.⁴¹⁻⁴³
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9 Figure 3 illustrates individual steps in TLS via *cis*-activation by RecA*. Initially, an
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11 advancing replication fork containing pol III HE becomes stalled at a lesion. Leading- and
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13 lagging-strand replication become uncoupled. Continued unwinding of dsDNA ahead of the
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15 blocked fork by the DnaB helicase results in a region of ssDNA downstream of the lesion on
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17 which RecA* can assemble (Figure 3, left panel). Pol V replaces pol III core on the β clamp and
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19 performs TLS accompanied presumably by progressive and concurrent 3' \rightarrow 5' disassembly of
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21 RecA*.⁴¹ Following TLS, pol III core would replace pol V to resume rapid DNA synthesis on
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23 undamaged DNA downstream from the lesion (Figure 3, right panel).
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30 Given the formation and requirement for RecA* nucleoprotein filaments, *cis*-activation
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32 models seemed intuitively obvious to many investigators, including us. If RecA* is required for
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34 TLS, its location should be in the single-strand DNA gap at the lesion, where it can interact with
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36 pol V. However, this construct had multiple problems. While one could visualize how the
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38 presence of RecA* might stimulate lesion bypass by interacting with pol III core, e.g., perhaps
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40 by inhibiting ϵ exonuclease proofreading, there was no obvious biochemical rationale for the
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42 workings of RecA* in conjunction with pol V. A RecA* filament on the DNA is a potentially
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44 formidable barrier to the progress of any DNA polymerase. The model envisioned displacement
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46 of RecA subunits at the RecA filament end where filament growth normally predominates.⁴⁴⁻⁵⁰
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48 Active RecA filament displacement from this filament end was proposed as a part of a model,
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50 where pol V was dubbed a “cowcatcher”.⁴¹ The DNA synthesis observed seemed to require such
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52 a displacement, but was not directly demonstrated as an activity of pol V. The subsequent
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54 demonstration of *trans*-activation of pol V⁵¹ provided an explanation of the pol V-mediated
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3 DNA synthesis results without invoking RecA displacement in *cis*. Currently, direct RecA
4 displacement function has been demonstrated only for specialized helicases such as UvrD⁵² and
5 PcrA.^{53,54}
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11 ■ TLS AFTER 2006 RecA* TRANS-ACTIVATION

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14 The *cis*-activation construct began to give way in 2006.⁵¹ When pol V was used to copy p/t DNA
15 in the form of a hairpin, containing a very short 3-nt template overhang (Figure 4), replication of
16 the short template by pol V required the presence of RecA* assembled on a separate ssDNA
17 molecule acting in *trans* (Figure 4). The new *trans* protocol also increased the overall efficiency
18 of primer utilization dramatically and consistently. Apparently, assembling the RecA* filament
19 in *cis* had constrained reaction efficiency in many experiments.^{55,56} The fraction of p/t DNA
20 extended by pol V was directly proportional to the concentration of *trans* RecA* in accord with
21 2nd order kinetics.⁵¹ The 3 nt template cannot support the assembly of a *cis*-RecA* filament (at
22 least one that is restricted to the ssDNA region) because the footprint of a single RecA molecule
23 is itself 3 nt. How might a transactivated pol V Mut behave were it to encounter RecA*
24 assembled downstream of a lesion? The presence of RecA* acts as a block to DNA synthesis and
25 would presumably need to be displaced, which could involve SSB, as proposed in our previous
26 “cowcatcher” model for TLS,⁴¹ or perhaps more likely by the UvrD⁵² or PcrA^{53,54} helicases.
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46 The pol V trans-activation method offered a straightforward approach to determine the
47 molecular basis for the enigmatic requirement for RecA* during TLS. By locating pol V and
48 RecA* on separate macromolecules, one had only to mix the pol V together with *trans*-RecA*
49 and ask: what happens? To answer this question it was necessary to completely separate the
50 *trans*-RecA* from pol V following incubation, to eliminate any possibility of continued
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3 transactivation of pol V. Quantitative separation was achieved by forming RecA* filaments on
4 ssDNA that was covalently linked to streptavidin-coated agarose beads, spinning the beads out of
5 solution to remove the *trans*-RecA*, and isolating a putative modified form of pol V from the
6 supernatant (Figure 5).²⁰
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13 **RecA* – Pol V Transactivation Mechanism.** When pol V (UmuD'2C) was exposed to
14 RecA* in *trans*, a molecule of RecA accompanied by an ATP molecule was transferred from the
15 3'-proximal tip of the RecA nucleoprotein filament to form an active mutasomal complex, now
16 referred to as pol V Mut. The final activated pol V Mut complex is UmuD'2C-RecA-ATP²⁰
17 (Figure 5). The transfer of RecA to pol V was revealed using MALS (Multi Angle Light
18 Scattering).²⁰ This powerful technique provides an absolute measurement of molecular mass.⁵⁷
19 UmuD'2C (72 kDa)-RecA (38 kDa) has a predicted molecular mass of 110 kDa, in excellent
20 agreement with 113 kDa obtained with MALS. Pol V in the absence of bound RecA has a
21 molecular mass of 73 kDa in close agreement with its predicted value. A PAGE analysis verified
22 that RecA was present in the light scattering peak corresponding to pol V Mut, but absent in the
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41 **Pol V Mut Copies DNA in the Absence of RecA*.** The identification of active pol V Mut as a
42 stand-alone polymerase meant that undamaged and damaged DNA could, for the first time, be
43 replicated efficiently in the absence of RecA*. In order to form pol V Mut, there are stringent
44 spatial restrictions on the RecA*-pol V interaction. The key amino acids involved in the
45 productive transfer of RecA to pol V include RecA residues 112 – 117; these are located at the
46 solvent exposed surface near the 3'-tip of RecA*, in close proximity to UmuC (e.g., N113 forms
47 a crosslink with UmuC).⁵⁸ A D112R mutation causes a 4-fold reduction in activity, and a
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3 D112R/N113R double mutant abolishes polymerase activity. Most importantly, from a historical
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5 perspective, pol V Mut assembled with Devoret's SOS non-mutable RecA mutant, UmuD'₂C-
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7 RecA(S117F),³⁴ is catalytically dead,²⁰ thus most likely accounting for the absence of damage
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9 induced mutagenesis in RecA1730 (S117F) mutant cells *in vivo*.³⁴ In addition, the RecA surface
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11 represented by residues 112-117 is the site of mutations that relieve inhibition of RecA-mediated
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13 recombination by overexpressed UmuC and UmuD proteins.⁵⁹
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19 The RecA subunit of activated pol V Mut must come from the 3'-proximal end of the RecA*
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21 filament. Although a molecule of RecA can also be transferred from the 5'-proximal filament tip
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23 to UmuD'₂C, that complex lacks detectable polymerase activity.²⁰ A stable complex can also be
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25 formed between RecA and UmuD'₂C in the absence of RecA*, but this form of UmuD'₂C-RecA
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27 has no detectable polymerase activity in the presence or absence of ATP.^{20,60} Thus, although
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29 RecA* is required to turn on the SOS regulon (Figure 1) and convert UmuD → UmuD' to form
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31 pol V = UmuD'₂C, the precursor form of pol V Mut (Figure 1), the most direct role of RecA*
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33 during SOS mutagenesis resides in the formation of a functional pol V Mut by transferring a
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35 RecA subunit to pol V.
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42 **Pol V Mut Requires Bound ATP_γS/ATP.** Pol V Mut assembles in 1:2:1:1 stoichiometry
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44 with UmuC, UmuD', RecA, and ATP/ATP_γS.²⁰ The transfer of one molecule of RecA from the
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46 3'-proximal tip of RecA* to UmuD'₂C occurred concomitantly with the binding of one molecule
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48 of ATP_γS.²⁰ This slowly hydrolyzing nucleotide cofactor ensures stable RecA* filament
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50 assembly, since filaments formed with ATP undergo a dynamic assembly-disassembly process
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52 resulting from ATP hydrolysis.^{46,61,62} However in the original transactivation assays, the
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54 stoichiometric binding of ATP_γS to UmuD'₂C had actually taken place adventitiously from the
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3 supernatant containing pol V Mut (Figure 5), and not directly during the transfer of RecA.²⁵ The
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5 role of the nucleotide cofactor in the pol V Mut complex, if any, was not understood at that time.
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9 A more efficient transactivation protocol was developed, utilizing cyanogen-bromide
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11 Sepharose beads with RecA* bound to covalently attached ssDNA in the presence of ATP γ S,
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13 along with the use of spin column to separate pol V Mut from the *trans*-RecA*, unbound RecA,
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15 as well as the free ATP γ S/ATP used to form RecA*.²⁵ We recovered a form of pol V Mut
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17 (UmuD'₂C-RecA) devoid of both ATP γ S/ATP and polymerase activity.²⁵ Restoration of pol V
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19 Mut activity occurred upon addition of new ATP γ S/ATP. Therefore, an adenosine nucleoside
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21 triphosphate cofactor ATP, dATP or ATP γ S was necessary as an integral component of pol V
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23 Mut to catalyze DNA synthesis on undamaged and damaged (TLS) DNA templates. Other
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25 nucleotide forms are unable to substitute for ATP γ S or ATP, e.g., pol V Mut is not activated by
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27 GTP, ADP or dTTP.²⁵
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33 34 ■ POL V MUT IS A DNA-DEPENDENT ATPASE

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37 The molecular basis for the A nucleotide cofactor requirement was soon revealed. Pol V Mut
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39 cannot bind to a DNA primer-3'-end, and hence has no polymerase activity, unless a molecule of
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41 ATP γ S/ATP is present in the complex.²⁵ More remarkable, pol V Mut has an intrinsic DNA-
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43 dependent ATPase activity.²⁵ This activity is distinct from the canonical DNA-dependent ATPase
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45 of RecA*. Pol V Mut retains its ATPase activity when assembled using a mutant RecA*
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47 (RecAK72R), which essentially eliminates RecA*-dependent ATPase activity.^{25,63,64}
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53 Playing the reciprocal role for ATP in binding to p/t DNA, the ATPase is required to release
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55 pol V Mut from a primer-3'-end. There is a very close correspondence of ATP hydrolysis rate
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3 (54 ± 9 × 10⁻³ s⁻¹) to the first order pol V Mut-p/t DNA release rate (53 ± 2.5 × 10⁻³ s⁻¹), strongly
4 suggesting that the hydrolysis of one ATP molecule triggers the release of pol V Mut from p/t
5 DNA.²⁵ In contrast, pol V Mut remains stably bound to DNA in the presence of ATPγS where
6 hydrolysis does not occur.²⁵ The DNA-dependent ATPase can be viewed as an internal clock that
7 limits pol V Mut processivity so as to minimize the occurrence of mutations on undamaged DNA
8 following TLS. The individual steps in the ATP-ATPase regulation of pol V Mut activity are
9 shown in Figure 6.
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20 ■ SPATIAL REGULATION OF POL V MUT ACTIVATION *IN VIVO*

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24 The activity of pol V Mut is clearly regulated on many levels. Formation of the enzyme requires
25 an elaborate activation process with multiple steps. The lifetime of the active complex on DNA
26 is regulated by an intrinsic ATPase activity.²⁵ But this is not the end of the regulation story. Yet
27 another level of regulation of pol V Mut, this one spatial, has recently been revealed in living
28 cells by fluorescence imaging at single-molecule (sm) resolution.²⁶ This study addressed the
29 cellular location of pol V Mut after induction of the SOS regulon. Replacement of wild-type
30 *umuC* with fluorescently labeled *umuC*-mKate2 showed that UmuC is synthesized and localized
31 at the inner cell membrane ~ 45 min after UV irradiation. Thereafter, UmuC remains sequestered
32 on the membrane and is released into the cytosol only after RecA*-mediated UmuD → UmuD'
33 cleavage occurs. The membrane association explains why UmuC is inherently insoluble in
34 aqueous solution and is solubilized by binding with UmuD'₂³⁹ to form pol V.^{5,13} In accord with
35 the biochemical data, live-cell imaging shows that UmuC stays bound to the membrane in a non-
36 cleavable *umuD*(K97A)⁹ mutant strain that cannot form UmuD'. The fluorescently labeled pol V
37 Mut (UmuC-mKate2) is partially functional in the cell, promoting increased DNA damage
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3 induced spontaneous mutagenesis.²⁶ The role of the membrane in the activation mechanism of
4 pol V, as now understood, is shown in Figure 7. Most of the current information centers on the
5 membrane sequestration of UmuC prior to its incorporation into active complexes. RecA protein
6 is also present in the membrane,^{27,28} but it is not clear if that RecA is in a form that can activate
7 pol V. It is possible that UmuC is removed from the membrane by association with UmuD'₂ and
8 later activated to functional pol V Mut after diffusion and interaction with RecA* bound to the
9 bacterial chromosome. It is also possible that additional steps occur at the membrane, such as
10 UmuD cleavage and activation to pol V Mut, involving currently unknown functions of
11 membrane-associated RecA protein.
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26 Once active pol V Mut is formed, where does it go? Instead of a complex activated at the site
27 of a lesion, we now see a freely diffusible activated pol V Mut complex. In principle, it could act
28 at a stalled replication fork, or in a single strand and lesion-containing gap left behind after
29 restart of that stalled fork.⁶⁵ Following the release of pol V into the cytosol, the co-localization of
30 pol V mutasomes with replication forks depends on the RecA variant that is present²⁶. When pol
31 V Mut contains wild type RecA, i.e., UmuD'₂C-RecA_{WT}-ATP, there is no co-localization
32 between pol V Mut and pol III HE above what would be predicted if the two species were
33 located randomly throughout the cell (~ 5%).²⁶ The absence of co-localization between pol V
34 Mut and pol III HE in replication foci suggests that either the polymerases form a very transient
35 ternary complex, with pol V Mut bound for < 30 ms (the imaging rate used was 30 Hz and there
36 were no indications of transient focus formation), or that the presumed exchange of the high
37 fidelity pol III HE blocked at a DNA damage site with the low fidelity pol V Mut to permit TLS
38 may in fact not be occurring. The result brings into question the widely held view that mass-
39 action binding of pol V occurs uniquely at stalled replication forks. Instead, when pol III skips
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3 over a replication-blocking lesion and initiates DNA synthesis downstream, a β -clamp may be
4 left at the lesion site to which pol V Mut can bind and catalyze TLS. Our *in vivo* observation
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8 comports with the pol III lesion-skipping model proposed by Yeeles and Marians⁶⁵ based on
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12 biochemical data showing that when impeded by DNA damage, pol III jumps past the damage
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15 site leaving behind a β -clamp bound to a 3'-primer end. The polymerase then reinitiates synthesis
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18 on the *same* template strand downstream from the lesion on a re-loaded β clamp using an RNA
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21 primer formed by DnaG primase.^{66,67}

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23 In contrast to the behavior observed in cells with wild type RecA, a significant proportion of
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26 pol V Mut foci ($27 \pm 3\%$) co-localize with pol III HE in cells containing the constitutively
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29 expressed RecA E38K mutant (previously referred to as RecA730).²⁷ These data suggest that pol
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32 V can replace pol III during normal DNA synthesis on undamaged templates. Constitutively
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35 expressed in undamaged cells, RecA E38K will activate pol V in an environment where lesion-
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38 containing gaps are less common. Its subsequent action at replication forks may help explain the
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41 100-fold increase in pol V Mut-catalyzed SOS mutagenesis in the absence of exogenous DNA
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44 damage observed in cells constitutively induced for RecA E38K.¹⁰ When copying undamaged
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47 DNA, the fidelity of proofreading-deficient pol V Mut, deoxynucleotide misincorporation, $\sim 10^{-2}$
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50 $- 10^{-3}$,⁴ is typically more than a thousand-fold lower than that of proofreading proficient pol III
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53 HE.⁶⁸

54 ■ SOS MUTAGENESIS MODEL 2015

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56 We suggest a unified model for the regulation of SOS mutagenesis that integrates the original *in*
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59 *vivo* genetic-based concepts to *in vitro* biochemical data obtained since 2009, with current live-
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cell imaging microscopy that furnishes essential physiological missing links coupling the old

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3 genetics and new biochemistry. The model is divided into three distinct mechanistic stages.
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5 Stage 1 embodies numerous genetic studies showing that *umuDC* is transcribed as a late SOS
6 function (Figure 1). Stage 2 portrays combined genetic and biochemical studies depicting the
7 temporally retarded RecA*-mediated cleavage of UmuD \rightarrow UmuD', required for SOS
8 mutagenesis (Figure 7), and the identification of the activated form of pol V (UmuD'₂C) as pol V
9 Mut (UmuD'₂C-RecA-ATP) (Figure 5). The individual biochemical steps to convert pol V to
10 activated pol V Mut are presented in Figure 6. Stage 3 illustrates how ATP and ATP hydrolysis
11 regulate pol V Mut activity. The steps for ATP activation and ATPase deactivation of pol V Mut
12 are shown in Figure 6. This 3rd stage describing a unique internal regulation mechanism
13 governing pol V Mut activity, presumably limits error-prone DNA synthesis to include TLS and
14 short regions of undamaged DNA in the vicinity of a damaged template base. The mutational
15 load associated with pol V is thus minimized.
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33 When it was discovered that pol V could be activated by RecA* acting in *trans* (Figure 4)
34 and didn't need to be located on ssDNA proximal to a DNA damage site being copied (see, e.g.,
35 Figure 3), the question of cellular location of these events had no obvious answer. Things
36 became a bit easier to explain when the molecular mechanisms of RecA* transactivation was
37 established (Figure 5). A stand-alone form of pol V was identified that, once formed, works in
38 the absence RecA* (Figure 6). Yet the salient question remained, if not activated by RecA* at a
39 blocked replication fork as originally proposed (Figures 2 and 3), then where in the cell did pol V
40 encounter a transactivating RecA* nuclear protein filament? At the cell membrane seems likely
41 to be the answer based on the recent live cell imaging studies,²⁶ thus providing a directly
42 visualized missing link between the old genetics,²⁷ new biochemistry,^{26,28} and cell physiology.
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■ A MYOPIC LOOK BEYOND 2016

Given the many unforeseeable twists encountered in the story of this system to date, any look ahead is a bit adventurous. Akin to a toggle switch, pol V Mut turns on and off repeatedly with RecA staying bound when in activated and deactivated states (Figure 6B).²⁰ Activated pol V Mut (Figure 6A) can perform limited numbers of DNA synthesis cycles before it deactivates (Figure 6B). If needed, deactivated pol V Mut can be reactivated by fresh RecA* that exchanges an "old" RecA monomer for a new one, but presumably in a different position in relation to UmuC.⁵⁸ When SOS is dialed down, a reduced availability of RecA* would presumably ensure that the deactivated form of pol V Mut is unlikely to be reactivated.

The location of RecA in relation to UmuC in activated and deactivated conformations is the next issue to resolve. The dynamics of RecA-UmuC switching could be visualized at single-molecule resolution, in real time, using TIRF-FRET microscopy, provided that a dual-labeled pol V Mut (e.g., Cy3-UmuC, Cy5-RecA) remains active. Upon a first stab at amino-acid crosslinking, tandem mass spec analysis found that contacts between RecA and UmuC differed significantly in active and inactive forms of pol V Mut.⁵⁸ A high-resolution crystal structure for even just one of the two pol V Muts, live or dead, would prove invaluable. Parenthetically, however, despite the rapid advances in cryo EM, pol V Mut (110 kDa) is currently too small to be resolved.

Pol V Mut's intrinsic ATPase appears unique, lacking Walker A or B motifs. RecA*'s ATPase seems not germane because pol V Mut assembled with an ATPase-deficient RecA K72R mutant, pol V Mut E38K/K72R, has an avid DNA-dependent ATPase.²⁵ Thus, a current challenge is to identify the interacting surfaces between RecA and UmuD'2C that form this potentially new type of hybrid ATPase active site. Recently, we have used desthobiotin-ATP

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3 crosslinking⁶⁹ and tandem mass spectrometry to identify a lysine residue in UmuC (K403) that
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5 contributes to ATP binding-initiated DNA synthesis and to ATPase activity. This is admittedly a
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7 small beginning.
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11 Apart from TLS, pol V Mut has an existential role in bacterial fitness and evolution, as do its
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13 two companion SOS-induced pols II and IV (Figure 1).^{70,71} Pols II and IV are present at high
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15 constitutive levels (50 and 400 molecules/cell, respectively) in the absence of external stressors,
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17 whereas pol V Mut is present at no more than about 10 molecules per cell.^{26,72} Yet each
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19 contributes to about the same extent in ensuring cell survival by generating life-sustaining
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21 mutations, with different spectral signatures.^{70,71} The cell survival data using all possible
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23 combinations of SOS polymerase genetic backgrounds suggests that pol II works most
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25 effectively during exponential growth, with pols V and IV taking over to maximize fitness in
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27 stationary and deep-stationary phases.^{70,71} Live-cell imaging is ideally suited to visualize when
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29 and under what conditions (e.g., \pm UV) any of the SOS polymerases co-localize with pol III, and
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31 further to determine whether the presence or absence of each SOS polymerase alters the
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33 competition for binding to DNA. The power of the live-cell imaging techniques comes into full
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35 play here because individual cells can be studied as part of an ensemble of cells. Here, as before,
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37 live cell imaging can provide a missing link between genetics, biochemistry and cell physiology.
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FIGURES

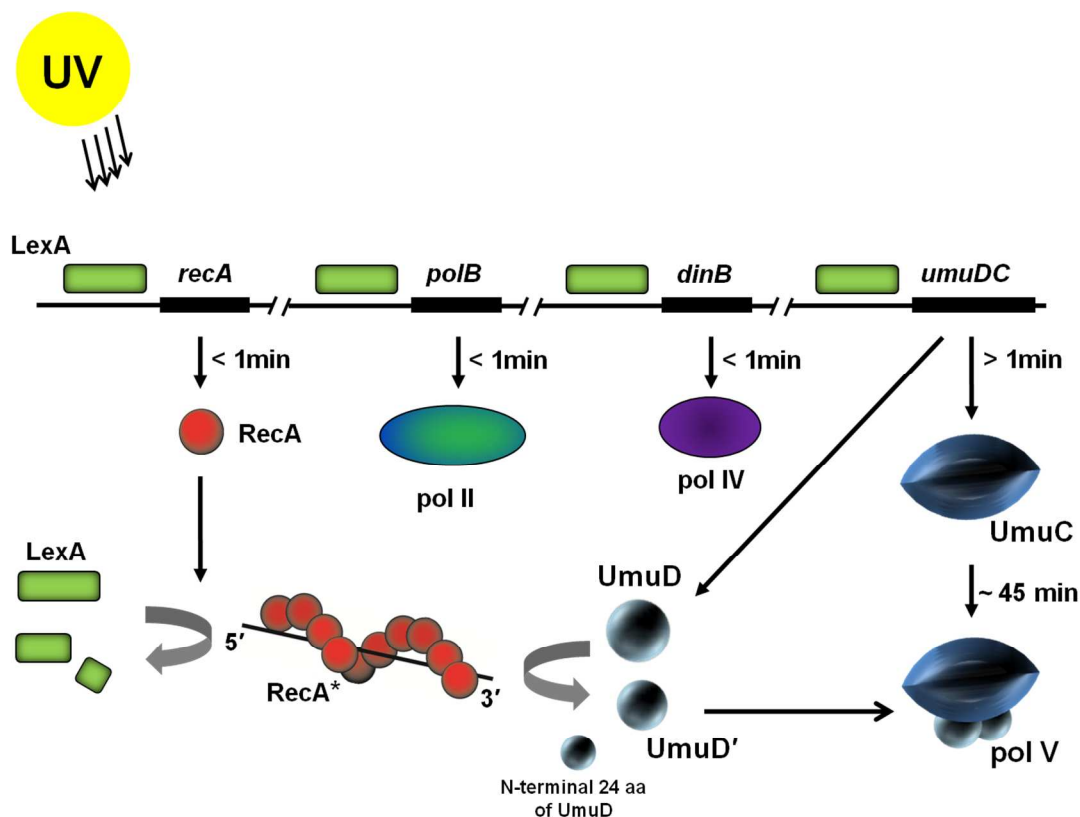
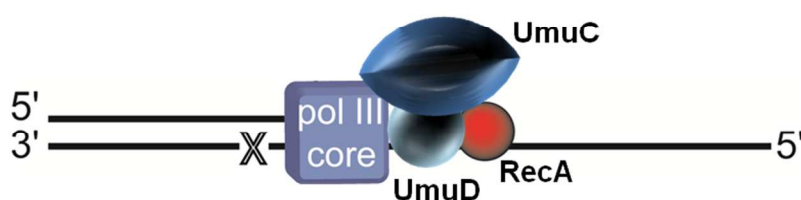


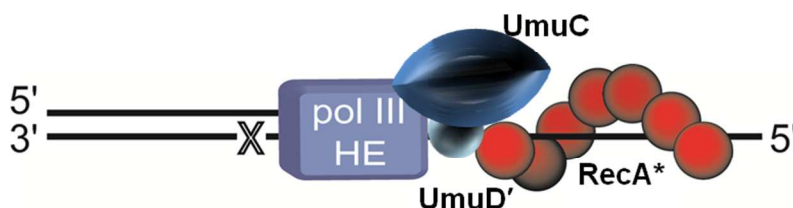
Figure 1. Induction of TLS DNA polymerases during the SOS response in *E. coli*. The LexA repressor (green box) binds to a 20 base pair consensus sequence in the operator region of the SOS genes suppressing their expression. Upon UV irradiation, expression of RecA (red spheres) is induced almost immediately after DNA damage occurs. RecA forms a nucleoprotein filament on ssDNA (RecA*) and functions as a coprotease to cleave and thereby inactivate the LexA repressor. Binding affinity of LexA to operon sequences of various SOS genes determine their induction time. Proteins with operators bound weakly by LexA, such as RecA, pol II and pol IV are induced soon after DNA damage occurs whereas proteins with strongly bound operators, such as the *umuDC* operon, are induced later (>1 min).¹¹ The *umuDC* operon is fully derepressed ~ 15 min after DNA damage,¹¹ however, due to rapid proteolysis of the Umu

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3 proteins¹², the UmuD'₂C complexes themselves do not accumulate until ~ 45 min after damage.
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6 RecA* facilitates cleavage of UmuD (15 kDa) to UmuD' (12 kDa) removing the first 24 aa from
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8 the N-terminus of UmuD. Two molecules of UmuD' (UmuD'₂) form a physical complex with
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10 UmuC (48kDa) forming pol V (72 kDa).
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A Bridges-Woodgate Two-Step Model

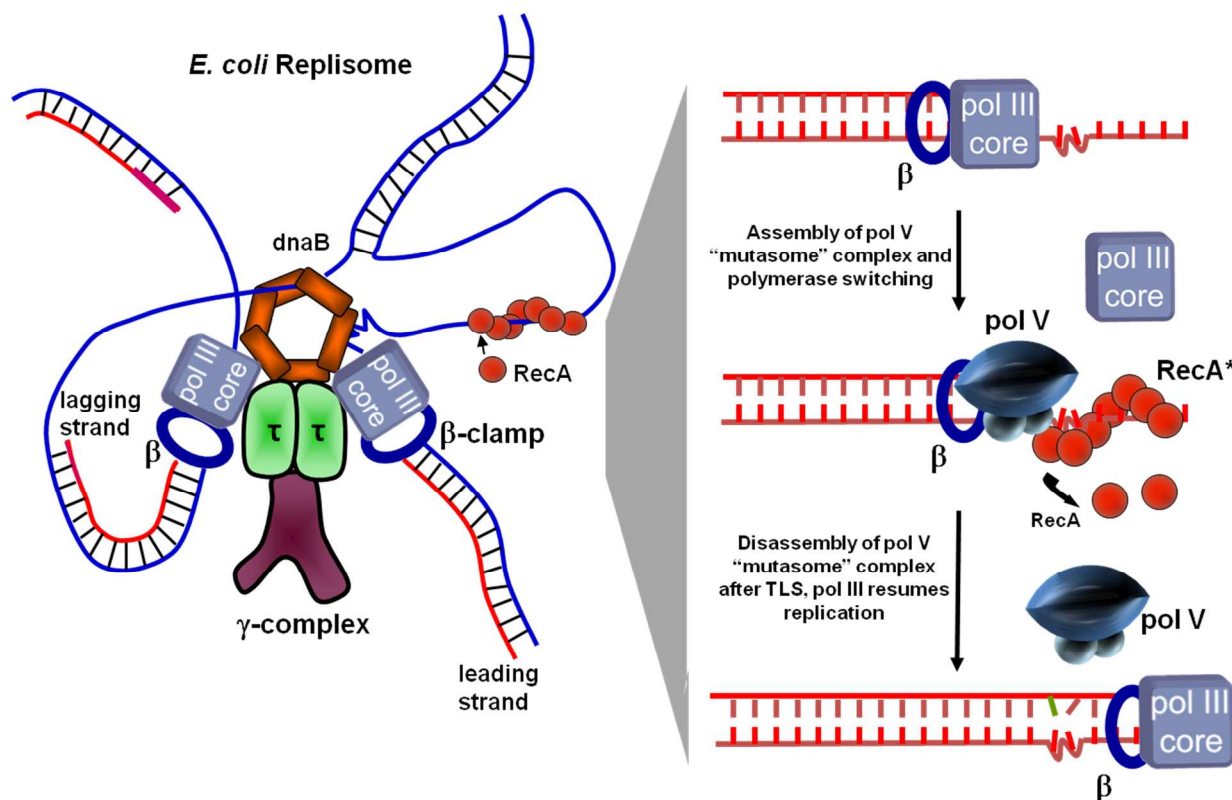


B Echols Mutasome



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43 **Figure 2.** Translesion synthesis (TLS) models before 1998. (A) The Bridges–Woodgate two-step
44 model.³¹ In this model translesion synthesis (TLS) is catalyzed by DNA polymerase (pol) III that
45 requires a RecA molecule for nucleotide insertion opposite a template lesion (X) and then
46 requires the UV mutagenesis gene products UmuDC to copy past the lesion. (B) The Echols
47 mutasome model.³⁵ RecA nucleoprotein filament (RecA*) assembles *in cis* at a lesion site (X) on
48 the template strand of DNA being copied. A multiprotein complex, including pol III
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3 holoenzyme, UmuC, and UmuD', is then recruited to the DNA allowing TLS resulting in
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5 mutations targeted opposite the lesion.
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Figure 3. TLS *cis*-activation model. A model of an *E. coli* replisome stalled at a lesion site is depicted in the *left panel* and individual TLS steps are shown in the *right panel*. DNA pol III stalls at the lesion site, DnaB helicase unwinds dsDNA ahead of the blocked fork resulting in regions of ssDNA where RecA* assembles in *cis*. The *cis*-activation model requires polymerase switching. DNA pol V replaces pol III core on the β clamp and bypasses lesions with concurrent 3' \rightarrow 5' displacement of RecA* formed ahead of the lesion by pol V. Following TLS, pol III core replaces pol V to continue rapid replication of undamaged DNA downstream of the lesion.

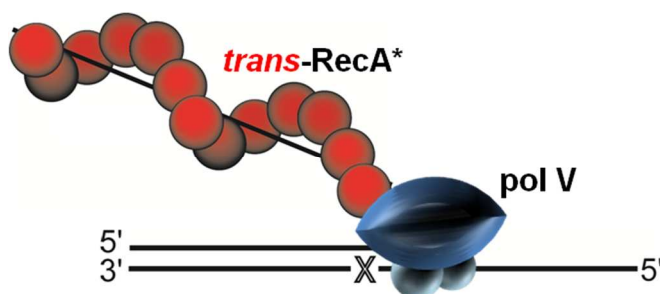


Figure 4. DNA pol V transactivation by RecA*. RecA* is assembled in *trans* on ssDNA separate from p/t DNA. DNA pol V is then activated by trans-RecA* resulting in TLS.

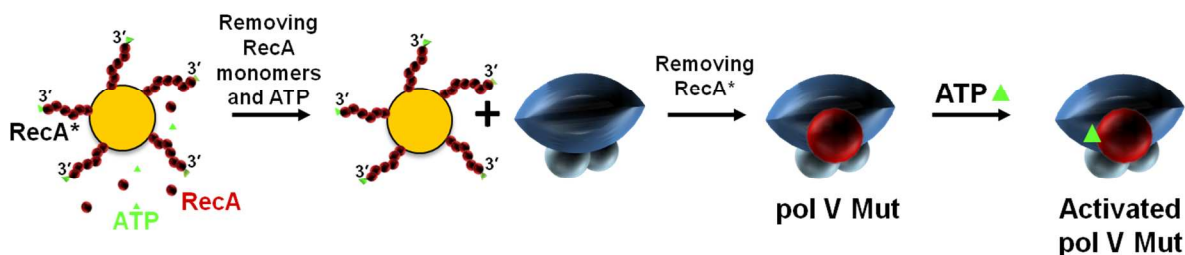


Figure 5. Pol V Mutasome (Mut). Pol V Mut isolation is a multi-step procedure requiring direct contact between pol V and RecA*. First, RecA* is formed on ssDNA covalently attached to agarose beads, and unbound RecA monomers (red spheres) and ATP (green triangle) are removed by extensive washing. Pol V is then added. Interaction of pol V with RecA* leads to transfer of a RecA subunit from the 3'-proximal tip of RecA* to form pol V Mut. Activated pol V Mut is composed of UmuC-UmuD₂-RecA-ATP.

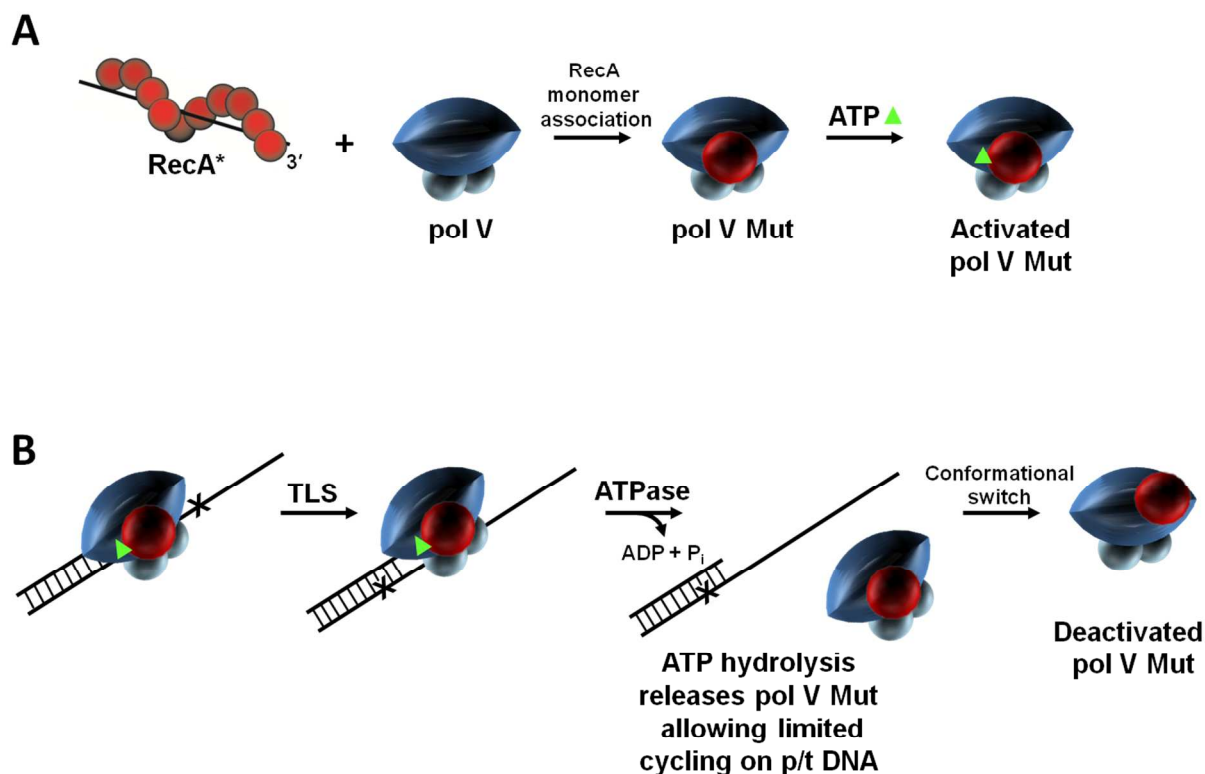


Figure 6. RecA and ATP regulation of pol V Mut activity. Pol V activity is regulated in multiple steps. (A) First, a RecA subunit is transferred to pol V from the 3'-proximal tip of RecA* to form pol V Mut. Pol V Mut is activated by binding a molecule of ATP (green triangle). (B) Activated pol V Mut associates with p/t DNA and DNA synthesis proceeds until ATP is hydrolyzed. Pol V Mut is a unique DNA-dependent ATPase, where a single ATP hydrolytic event leads to enzyme dissociation from p/t DNA, followed by limited cycling on p/t DNA. After DNA synthesis, pol V Mut becomes deactivated but can be reactivated by exposure to a new RecA*. We propose that active and deactivated pol V Mut are determined by the location of RecA on pol V.

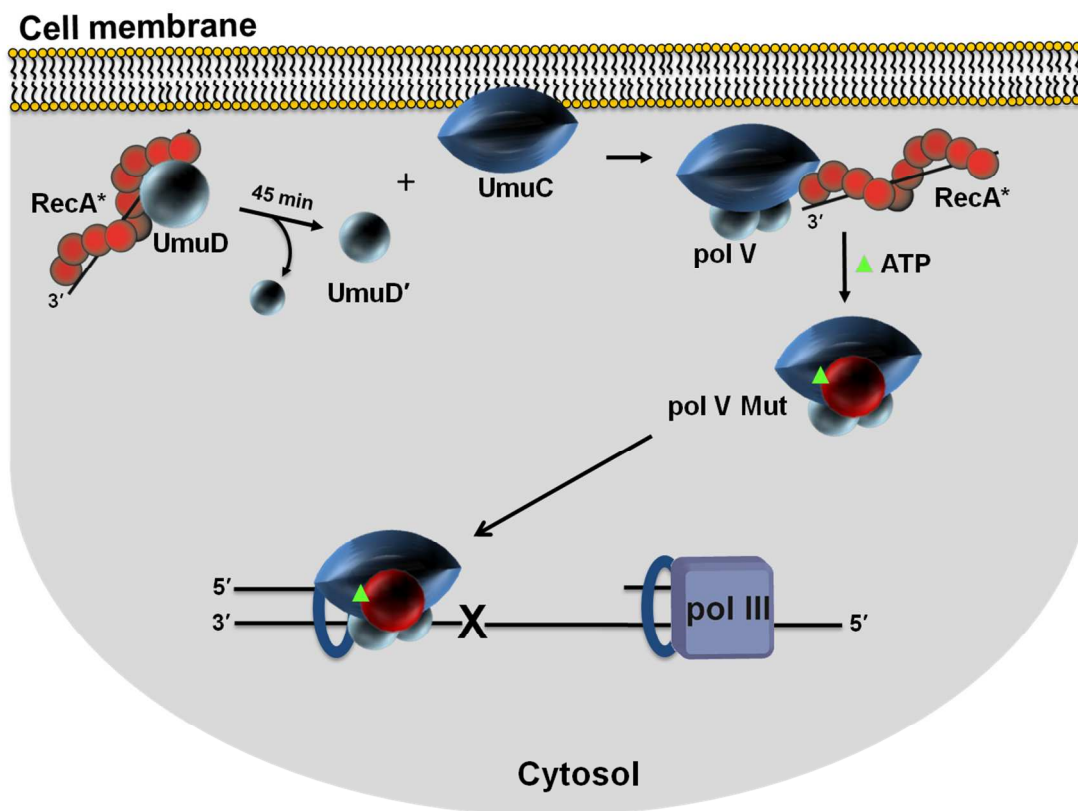


Figure 7. Spatial regulation of pol V Mut activity *in vivo*. Sketch depicting cell membrane localization of UmuC. Single molecule live cell fluorescence imaging data indicate that after an initial delay post UV irradiation, UmuC is synthesized and localized on the inner cell membrane. UmuC is released into the cytosol only after it interacts with UmuD'₂. As RecA and UmuD are found in or near the membrane, additional steps in the formation of pol V Mut (UmuD'₂C-RecA-ATP) may occur in proximity to the membrane. It is not clear what functionality may be associated with the RecA protein found in the membrane. Active RecA*, as studied to date, consists of RecA protein filaments bound to DNA. Sequestration of UmuC at the membrane provides additional levels of pol V Mut regulation. A delay between the production of UmuC and the formation of cytosolic pol V Mut provides time for error-free DNA repair to occur before mutagenic pol V Mut lesion bypass is necessary.

AUTHOR INFORMATION

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Notes

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58
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1
2
3 **For Table of Contents Use Only**
4
5
6
7
8

9
10 **Mutations for Worse or Better: Low Fidelity DNA**
11
12
13
14 **Synthesis by SOS DNA Polymerase V is a Tightly-**
15
16
17
18 **Regulated Double-Edged Sword**
19
20
21
22

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25
26 *Woodgate,[#] Michael M. Cox,¹ and Myron F. Goodman^{*§†}*
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