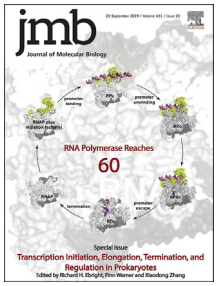
Review



Transcription-Coupled Repair: From Cells to Single Molecules and Back Again

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Abstract

Transcription-coupled repair is mediated by the Mfd protein. TCR is defined as the preferential repair of DNA lesions in the transcribed strand of actively transcribed genes, and is opposed to the strand-aspecific global genome repair. The Mfd protein mediates TCR by binding to and displacing RNA polymerase, which is stalled at a DNA lesion on the transcribed strand of DNA, then recruiting UvrA and UvrB. The repair cascade results in the recruitment of, and DNA excision by, UvrC; removal of the damage-bearing oligonucleotide by UvrD; "filling-in" of the DNA by DNA polymerase; and sealing of the strands by DNA ligase. The gene required for Mfd was originally identified as a gene needed for the "mutation frequency decline" phenotype in which the repair of certain UV-induced lesions in the transcribed strand of tRNA genes is increased when cells are forced to delay replication immediately following UV exposure. This review will focus on the genetics that led to the discovery of the Mfd gene; summarize the subsequent biochemical, structural and single-molecule interrogations of the Mfd protein; and explore the more recent findings of Mfd in mutagenesis.

Introduction

Transcription-coupled repair (TCR) refers to the preferential repair of bulky lesions or abasic sites located on the transcribed strand, as opposed to the coding strand, of actively transcribed genes [1–3]. In TCR, lesions are first identified by the fact that RNA polymerase (RNAP) has stalled atop them. Because RNAP sterically prevents DNA repair machineries from accessing the lesion, TCR appears as a specific pathway to handle a specific problem: the protection of a premutational lesion by a very stably bound molecular motor, which must be actively removed for repair to take place. Intriguingly, however, there is no observed structural feature to signal the outside world that elongating RNAP is in fact immobilized [4]. The features that allow for one protein molecule to know whether another protein molecule is moving or not within a diffusive environment are not simple to imagine *a priori*.

Identification of an *Escherichia coli* strain deficient for the transcription–repair coupling factor, Mfd, occurred over 60 years ago [5], and ultimately led to the identification of not just strand-specific TCR but also strand-aspecific GG-NER (or global genome nucleotide excision repair, or GGR, which, grossly speaking, targets the lesions which stall RNAP) [6]. Mfd interacts with both RNAP and GGR component UvrA [7] to enhance recruitment of UvrA to lesions on the transcribed strand of DNA [7–9]. GGR and TCR are complex molecular pathways that involve numerous protein partners acting through a succession of steps, making their biochemical analysis challenging. In addition, the two pathways share common protein components (e.g., UvrA) and compete with each other for substrate lesions, making it nontrivial to tease apart the two pathways' relative contributions to cell fitness and survival. Intriguingly, a number of genetic observations over the past few years have tended to indicate that Mfd

can not only repair premutational lesions in DNA but may also activate pro-mutagenic pathways to favor for instance the evolution of antibiotic resistance [10–12]. These counteracting effects indicate that we still have not fully understood all of the roles taken up *in vivo* by the Mfd protein and that new features still await discovery.

Although genetic analysis is unrivaled in its ability to identify biochemical pathways and then determine the necessary and sufficient components of those pathways, it must be complemented by biochemical and structural studies to provide a mechanistic basis for the action of components within those pathways. Importantly, in the past few decades, the field of single-molecule experimentation has contributed powerful new tools for the mechanistic characterization of biomolecular systems. Despite their name, single-molecule approaches have turned out to be particularly useful for the study *in vitro* of multi-component, multi-step reactions such as in DNA transcription [13,14], splicing [15,16], and DNA repair [17–20], allowing researchers to reach deep into multi-step reaction pathways and interrogate specific steps of reactions (which actors are present, what are their stoichiometries, how quickly do they come and go) without a need for population synchronization and while also avoiding the deleterious effects of population averaging.

In vitro single-molecule experiments provide key new insights into the functions of proteins and molecular motors for instance associated with nucleic acids (polymerases [21], helicases [22], translocases [23], topoisomerases [24], recombinases [25], …). *In vivo* single-molecule experiments allow one to observe diffusion of proteins as they search for and bind to their targets including DNA lesions, polymerases, RNA, chromatin, and so on. They allow one to understand how these systems are stable yet responsive to their environment, engaging for instance in DNA repair on average in a timely and robust manner, despite the obligate sampling of off-target sites during diffusion in a heterogeneous environment [26,27]. They also allow one to begin to appreciate how these systems compete with all of the other systems in the cell [28].

In this review, we will first discuss and put in context the new *in vitro* and *in vivo* single-molecule results that have been obtained on TCR. We then provide a brief historic overview of this phenomenon and finally discuss how the single-molecule data help illuminate the apparently antagonistic roles of Mfd *in vivo*.

Mechanistic Insights from Single Molecules *in Vitro*

Single-molecule analysis of bacterial Mfd is built upon nearly two decades of expertise in the single-molecule study of RNAP. This expertise has been developed across numerous groups using a range of single-molecule techniques including single-molecule nanomanipulation (first using the optical trap [29], and then shortly thereafter using the magnetic trap [13,30]) and single-molecule fluorescence (first using FRET to observe conformational changes within polymerase [31], and then shortly thereafter using multi-color colocalization of single-molecule studies, or CoSMoS) to study mechanisms of transcription and the dynamic assembly and disassembly of factors on RNAP [14,32,33]).

We will not go into technical details about the exact nature of the single-molecule signals used to observe TCR in single-molecule assays as these were recently discussed in a detailed review in this Journal [34]. Instead, we simply resume the mechanistic insights gained using these tools. By irreversibly stalling RNAP on DNA in the presence of Mfd, it was possible to observe that Mfd could bind RNAP and DNA and, in a slow and multi-step reaction, use the energy derived from ATP hydrolysis to displace damage-stalled RNAP from the DNA [18]. Displacement of RNAP from DNA was accompanied by loss of nascent RNA, indicating that the RNAP was indeed catalytically disengaged from the DNA template [35]. Surprisingly, RNAP was observed to remain in complex with Mfd after displacement and as part of a long-lived Mfd–RNAP complex, which slowly translocated along DNA in the same direction as initial transcription. The translocating complex was observed to have a maximum velocity of about 5 nt/s and displayed very high affinity for ATP (K_M ~ 20 μM) [19,35], and could reliably be observed to translocate over several thousands of base pairs [18,35].

The observation of continual translocation after remodeling of stalled RNAP suggests that it is precisely *via* a translocation mechanism that Mfd succeeds at remodeling stalled RNAP in the first place. One possibility is that Mfd forward-translocates (or hypertranslocates) RNAP off the 3′ end of its RNA without concomitant nucleotide addition, causing RNAP to dissociate from DNA. It is also noteworthy that formation of a complex between Mfd and stalled RNAP, each of which also bind DNA, likely results in creation of a DNA topological domain constrained by the two DNA-binding proteins. Because *E. coli* Mfd is a monomer, its translocation along DNA likely requires it to track the DNA helix [23,36–38]. As per the original twin-domain model for supercoiling proposed by Liu and Wang [39], this would result in Mfd generating positive supercoiling and torque ahead of itself (with negative supercoiling and torque behind), helping to mechanically close the transcription bubble and remove RNAP from DNA as proposed by Savery [40]. Whether Mfd displaces RNAP *via* pure translocation, or by coupling translocation and torque, remains an open question. At the same time, the observation of slow

translocation by Mfd also explains why it displaces stalled RNAP: elongating RNAP moves too quickly (at least 25 nt/s *in vitro* [41]) for Mfd to catch up to it and displace it by force and/or torque.

It could be pointed out that in retrospect the original biochemistry of Selby and Sancar [8] was in fact sensitive to Mfd–RNAP translocation, as they noted that there needed to be a minimal ~100 bp DNA *downstream* of the DNA lesion (i.e., before the 5′ end of the DNA template strand) for the transcription–repair coupling reaction to occur. One could imagine that, based on this model of Mfd–RNAP translocation, if too short a damaged substrate is used then the translocating Mfd–RNAP complex will simply "walk off" the end of the DNA before coupling with downstream stages of repair.

In vitro, absent UvrA, the translocating Mfd–RNAP complex was able to move thousands of base pairs along DNA [18,19,35]. Its translocation can then be interrupted by the recruitment of UvrA or UvrAB, which displayed surprisingly high diffusion-limited binding in the range of 10^8–10^9/M·s [19]. This elevated rate is likely due to the fact that UvrA is engaged in diffusional search for a subdomain of Mfd, a much larger feature to search for than a minuscule DNA lesion. The translocating complex was thus arrested by the downstream components for approximately 10–20 s and was then released from the DNA in an ATP-hydrolysis dependent fashion. Both Mfd and RNAP were released in this reaction [19]. The single-molecule assays used were also able to observe the kinetics of incision of damaged DNA, and found as already observed in the literature that it was approximately 3-fold higher for TCR than for GGR [9].

One may wonder why Mfd retains RNAP even after remodeling it. The answer to this is likely based on the structural properties of Mfd and relates to the regulation of Mfd. Mfd is a multi-domain protein with domains connected *via* flexible linkers [42,43]. Domains 1 and 2 form a UvrB homology module (BHM) with which Mfd can recruit UvrA; domain 3 has an as-yet undefined role; domain 4 is the so-called RNAP-interaction domain (RID), which allows Mfd to specifically dock to a conserved patch on RNAP β subunit. Domains 5 and 6 are DNA translocation modules similar to those observed in RecG. They couple ATP hydrolysis to DNA translocation and are homologous to the so-called TRG motifs of RecG [44,45]. Although the exact functions of domain D7 remains unspecified, it docks to an extensive surface of D2 *via* three key residues on D7 (E1045, D1048, and R1049) and two on D2 (R165, R185) [42] [43]. This helps to maintain Mfd in a compact and repressed state in solution, whereas disrupting the D2-D7 interface is associated with unrepressed and unregulated (i.e., RNAP-independent) DNA binding and translocase activity and a constitutive ability to interact with UvrA [9].

Binding of Mfd to RNAP *via* the RID is expected to disrupt the D2-D7 interface and "unpack" the enzyme [42,43,46], enabling it to latch onto DNA *via* its translocation motifs and begin translocating toward the RNAP. It is thus possible that maintaining the Mfd–RNAP interface is necessary to allow Mfd to remain unpacked and tightly bound to DNA so that it can accomplish its RNAP-remodeling task. Above and beyond this initial task, however, Mfd must still remain on the DNA long enough so as to ensure it can recruit UvrA to the lesion; if Mfd were to rapidly dissociate from DNA after displacing RNAP, it would likely fail to couple transcription to repair as noted by Selby and Sancar [8] and mentioned above. The stable Mfd–RNAP interaction therefore enables Mfd to remain engaged on the DNA so that it may, first, stay in the vicinity of the lesion and, second, recruit UvrA. This would allow the Mfd–RNAP complex to itself be a reliable marker for DNA damage and further provide UvrA with a larger target than a simple lesion, potentially enhancing diffusive search of UvrA for Mfd–RNAP relative to that of UvrA for a lesion [19].

Recently, additional single-molecule experiments have been performed using optical tweezer assays and have observed the translocation of Mfd alone along DNA [47]. The mode of association of Mfd alone to DNA is unknown, as the crystal structure of Mfd alone indicates that both DNA binding and UvrA binding should not be possible when Mfd is in the repressed state (i.e., absent RID–RNAP interaction) [42]. At the same time, it is also known that Mfd on its own can weakly bind DNA as well as pull some UvrA out of solution [7]. To reconcile the two observations, one can imagine that there is a small, steady-state fraction of spontaneously "unpacked" Mfd, which has fluctuated into an open state and from there can transiently bind to DNA and translocate along it. However, absent RID–RNAP interactions it is likely that such an Mfd molecule can spontaneously "repack" itself and dissociate from DNA.

Indeed although the optical trapping experiments obtain a velocity for Mfd identical to that observed earlier in magnetic trapping experiments, it also appears as though the processivity of Mfd alone is significantly lower than for the fully reconstituted TCR reactions described above. The fully reconstituted Mfd–RNAP complex is routinely and reliably able to translocate over thousands of base pairs [19,35], whereas the processivity of translocating Mfd alone appears to be only on the range of a few hundred base pairs [47]. This enhanced stability of the Mfd–RNAP complex on DNA, relative to that for Mfd alone, would be consistent with the role of the complex as a stable and reliable damage-signaling complex as discussed in Ref. [19].

These *in vitro* single-molecule measurements have recently been complemented by *in vivo* single-molecule measurements of fluorescently labeled Mfd interacting with RNAPs in live bacteria even in the absence of exogenous DNA damage [28]. The authors observed 30-s-long interactions between Mfd and

RNAP if UvrA was absent, but 20-s-long interactions if UvrA was present. These experiments showed that the kinetics of the process *in vivo* were quite similar to what had been described *in vitro* [18,19], and confirmed that the presence of UvrA shortens the lifetime of Mfd–RNAP complexes observed on DNA. These interactions took place absent exogenous DNA damage but were enhanced by inhibiting RNAP elongation, leading the authors to propose the interactions could involve naturally stalled RNAP. It is at least possible to know that the RNAPs targeted in this case are in the elongation phase rather than bound to their promoter, as RNAP engaged in promoter recognition is not a target of Mfd [48], most likely for steric structural reasons. The Mfd–RNAP interactions were also specific to Mfd functions: they were dramatically reduced in frequency by mutating the RID, whereas inhibiting ATP hydrolysis increased their frequency. Endogenous DNA damage, stalling and pausing of elongating RNAP can offer explanations as to why Mfd is observed to associate with RNAP absent exogenous DNA damage. Interestingly, Mfd association with actively elongating RNAP is another explanation [18,19]. These results highlight the temporal challenge that Mfd may face in searching for a *bona fide* stalled RNAP *in vivo*.

In addition to identifying the chemo-physical basis for RNAP displacement (translation and/or torque), ongoing challenges in the study of TCR include for instance understanding the stoichiometry of the UvrAB complex in TCR and GGR. Indeed, it is most likely that a $UvrA_2UvrB$ complex is deposited on DNA by Mfd–RNAP, whereas a $UvrA_2UvrB_2$ complex would be operant in GGR. Indeed some form of symmetry breaking is required for the UvrAB complex to be able to direct repair to the transcribed strand even after Mfd–RNAP are no longer there to mark the strand. It will thus be of great interest to observe in detail, both *in vitro* and *in vivo*, the downstream steps of UvrA/UvrB recruitment.

The Genetics of TCR

In 1928, Frederick L. Gates stated "the reciprocal of the bactericidal curve matches the [UV] absorption curves for … cytosine, thymine and uracil" [49]. UV absorption by DNA results in the formation of cyclopyrimidine dimers (CPDs), nucleic acid lesions which, as discussed above, inhibit transcription by stalling RNAP and generate mutations during error-prone lesion bypass by DNA polymerase. To prevent such mutations from arising, the lesion must be repaired *via* excision by the UvrA/UvrB/UvrC machinery (UvrC nicks the damaged strand twice to generate a 13-base ssDNA oligo carrying the lesion) and removal of the resulting oligo from DNA by UvrD [50].

In 1943, Luria and Delbruck [51] used *E. coli* B strain to study spontaneous mutation rates. B strain died upon filamentation when exposed to UV, and in 1946, Witkin [52] described a mutant of the *E. coli* B strain named B/r, which does not filament and die upon UV exposure. This made B/r a particularly useful strain for the study of UV-induced mutagenesis and its repair. From B/r, Witkin [53] isolated a series of amino acid auxotrophs, including the WP2 strain unable to grow in the absence of tryptophane and the WU36-10 strain unable to grow in the absence of tyrosine. Witkin then used UV light to generate lesions in the auxotrophs' genomes; if the lesions were not repaired, they led to mutations, which allowed the bacteria to revert to prototrophic behavior. Such mutated bacteria could be isolated on culture plates lacking the appropriate amino acid (e.g., tryptophane or tyrosine). [We note for the sake of completeness that WU36-10 is also a leucine auxotroph but that it was not selected against this trait in these experiments [5].]

Intriguingly, this procedure resulted in a maximum of prototrophs (i.e., mutants) when the bacteria were driven to divide immediately after UV exposure by shifting them to rich nutrient conditions. However, if the cells were made to wait just for a few minutes before plating on rich nutrient the number of prototrophs dropped precipitously. This was the first description of the temporal phenotype known as "Mutation Frequency Decline" (or MFD). Witkin then used another positive selection screen to delineate this phenotype, isolating living bacteria from culture plates lacking tryptophane despite conditions in which the UV-irradiated bacteria had been given a bit of time to wait before dividing. From this, Witkin isolated the WP2-S strain, which lacked the rapid, precipitous drop in mutants in the minutes just following UV irradiation, that is, displaying the MFD phenotype [5]. Similar results were obtained with WU36-10-45, a WU36-10 substrain lacking the MFD phenotype [5]. [It should be noted that WU36-10-45 and, to a lesser extent, WP2-S showed a gradual decline in prototrophs when they were made to wait before plating onto rich nutrient; this is a signature of ongoing GGR]. When the genetic locus associated with this change in WP2-S was ultimately identified, biochemical examination showed it encoded a gene which was given the name Mfd.

Over the course of the following decades, the protein Mfd was identified as the so-called transcription–repair coupling factor [54,55] shown separately to be responsible for promoting enhanced repair of transcription-inhibiting DNA lesions located on the template strand of actively transcribed genes [2,56,57]. As discussed earlier, this so-called TCR pathway is intertwined with the so-called global genome repair (GGR) pathway, which provides an alternate, transcription-independent means of repair [58]. First of all, UvrA also interacts with Mfd during TCR [7,9]. Second, the two pathways can compete with and complement each other in terms of lesion identification and incision [50,59–61]. Third, they both

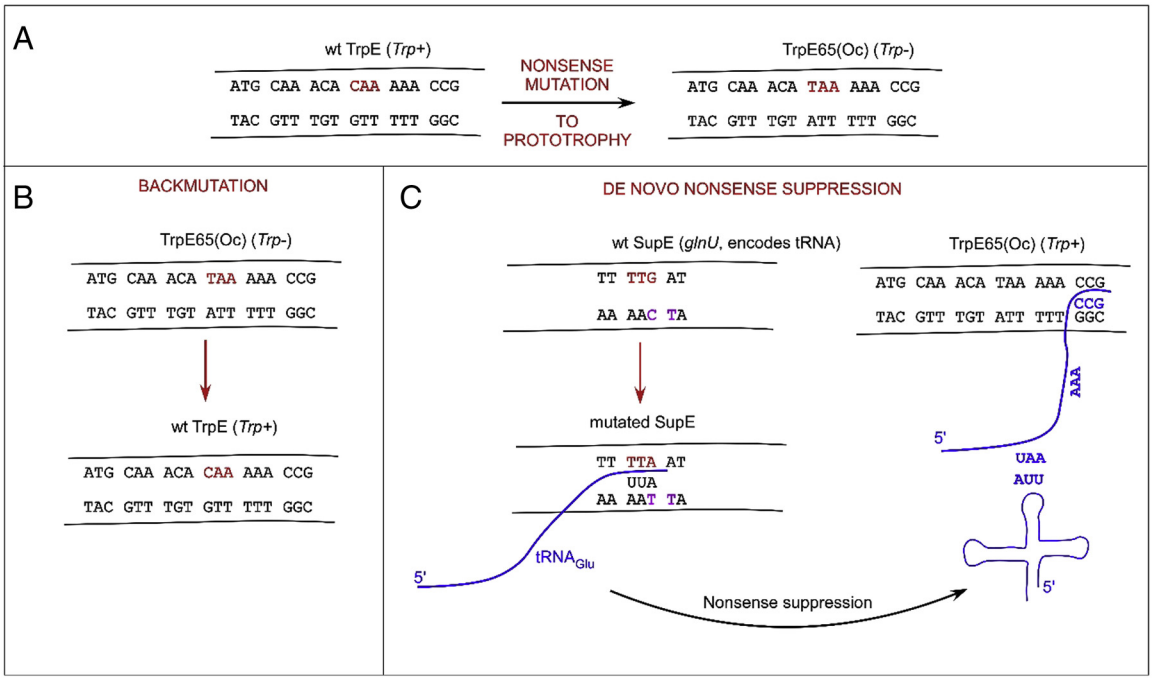

Fig. 1. Classical genetics of the WP2 *TrpE65(Oc)* tryptophane auxotroph's (A) spontaneous appearance, (B) backmutation (a.k.a. true reversion), and (C) *de novo* suppression. Nonsense codons and their top-strand mutations are in red, and neighboring pyrimidines involved in *de novo* nonsense suppression are in purple.

require UvrD helicase at the end to unwind and remove the damaged DNA prior to resynthesis of double-strand DNA over the single-strand patch [50]. Thus, while Mfd and its associated TCR are responsible for the precipitous decline in mutants seen in WP2 and WU36-10, UvrA/UvrB/UvrC/UvrD are responsible for the gradual decline in mutants still observed in WP2-S and WU36-10-45. Of course, removing any one of these four factors also abolishes TCR. These interconnections illustrate some of the original complexities in picking apart these two DNA repair processes.

These interconnections also explain a widely misinterpreted feature of MFD, namely, the comparatively mild nature of the phenotypes with which its defects are associated. This is due in part to the fact that even absent TCR there can still be GGR taking place in the background. It is also due to the fact that the MFD phenotype is a transient one which essentially affects how quickly bacteria recover the ability to grow after having been hit with UV radiation [5,28,62]. Fast outgrowth after environmental stress is likely an essential selective advantage in the wild, but likely less so in a clonal population of laboratory-grown bacteria.

Thus, in her original work, Witkin notes that "Among auxotrophic substrains of *E. coli* B/r, isolated at random, only about 20 to 30% give relatively high yields [of the MFD phenotype]" [5]. In 1977, Bockrath and Palmer write "In general, conditions favouring MFD have little effect on overall survival" [59]. Indeed in their comparison of WU36-10 (MFD+) and WU36-10-45 (MFD −) revertants [59], it is apparent that only a fraction of revertants—and in particular *de novo* nonsense suppressors—are affected by the MFD phenotype. Other revertants—and in particular back-mutations—are repaired by mechanisms present at all time-points in the reaction (i.e., GGR).

A detailed analysis of auxotrophs helps to understand the genetic basis for these effects (Fig. 1). Witkin and colleagues worked with strains made auxotrophic by the appearance of a nonsense mutation in a key gene of the amino acid biosynthetic pathway [60,61]. A nonsense mutation corresponds to the appearance of a stop codon in a protein-coding RNA—UAA ("ochre"), UGA ("opal"), or UAG ("amber")—encoded by DNA "top-strand" sequences TAA, TGA, or TAG, respectively. In the case of the WP2 strain, this corresponded to an ochre stop codon at the beginning of the *TrpE* gene required for tryptophane synthesis [61] (denoted *TrpE65(Oc)*, Fig. 1A). For the WU36-10 strain, an ochre stop codon was found in the *TyrA* gene necessary for tyrosine synthesis (*TyrA14(Oc)* [60]). These auxotrophs can revert to prototrophy if the nonsense mutation is itself undone by another mutation, and UV was a standard mutagen for such reversion assays, provided the base-pair change needed for reversion involves adjacent pyrimidines (TT, TC, CT, or CC), which are susceptible to UV. We note that different wavelengths of UV result in different ratios of photoproducts, but that is beyond the scope of this review, and in Witkin's experiments (using 254-nm light), no single CPD is favored.

Thus, in the proper sequence context, the nonsense mutation can be undone by an unrepaired, UV-induced lesion and the error-prone replication it causes. Such UV-induced premutational lesions typically result in either a direct backmutation to prototrophy (Fig. 1B; in this case, one speaks of a "true revertant") or nonsense suppressor mutations in the anticodon loop of certain tRNAs (Fig. 1C). However, premutational lesions are also repaired by the pathways under discussion, and this repair leads to a reduction of the number of bacteria, which revert to prototrophy (i.e., MFD). Premutational lesions located on the coding strand can only be repaired by GGR, whereas premutational lesions located on the template strand may be repaired in a manner which displays MFD in addition to GGR.

Because the sequence context does not involve adjacent pyrimidines, UV-induced backmutation (or "true reversion") from the *TrpE65*(Oc) TAA back to CAA should not take place (Fig. 1B). As mentioned above, nonsense mutations are also often found to be undone by a compensatory (or suppressor) mutation in the anticodon loop of a tRNA; these are so-called *de novo* nonsense suppressor mutations (Fig. 1C) [61]. For each stop codon, there are nine tRNA anticodon loops that are a single base-pair change away from becoming a *de novo* nonsense suppressor. [In reality, a few of these cannot become nonsense suppressors as they simply result in the formation of another stop codon. We further note that these mutations are not lethal to bacteria only because tRNA genes are found in multiple copies (see http://lowelab.ucsc.edu/GtRNAdb/Esch_coli_K12/Esch_coli_K12-by-isotype.html).].

For instance, the GlnU gene can become an ochre nonsense suppressor *via* UV exposure if a CPD is formed at the transcribed strand "CT" sequence, which then transitions, post-replication, to a "TT" (Fig. 1C, purple). Because CPDs and mutations located at the coding (nontranscribed) strand "TT" cannot result in an ochre nonsense suppressor, a nonsense suppressor screen will only pick up the UV-induced lesions and mutations located on the transcribed strand. Because CPDs formed on the transcribed strand are prone to GGR as well as TCR, both repair pathways can, if given enough time, prevent those lesions from becoming a mutation and cause fewer prototrophs to appear. The exact balance between GGR and TCR at this locus will depend in particular on the transcriptional load at this locus. It is likely that the necessary transcription of this housekeeping gene ensures a sufficient level of RNAP on the gene to make it a good locus in which to observe MFD/TCR as opposed to just GGR.

It is interesting to note that, in the case of the tyrosine auxotroph WU36-10 resulting from the *TyrA14(Oc)* mutation, it is the same GlnU gene that mediates *de novo* nonsense suppression as for the W2 tryptophane auxotroph, and these *de novo* nonsense suppressors also clearly display the fast MFD phenoytype [5,59]. Unlike the *TrpE65(Oc)* mutation, however, the *TyrA14(Oc)* mutation can undergo true reversion *via* UV-induced lesions in a pyrimidine dimer on the coding strand, and also standard reversion *via* lesions in a pyrimidine dimer on the transcribed strand of the *TyrA14(Oc)* locus [59]. Indeed the coding strand sequence at this locus is GG**C *T*AA** TTA, where the nonsense codon resulting from appearance of a T is in italics and the coding strand shows adjacent pyrimidines in bold [60]. This explains why Bockrath and Palmer [59] observe a slow GGR phenotype for true revertants (coding strand TAA➔AAA) in the MFD+ strain. Furthermore, it also likely explains why Witkin observed a slow GGR phenotype in WU36-10-45 (MFD−), but essentially no GGR phenotype in WP2-S (MFD−) [5].

Although the transcriptional load and pyrimidine contents on the coding or transcribed strands are already two parameters which must be instrumental in balancing the ratio between GGR and TCR, a third confounding factor has also recently emerged: pervasive transcription [63–65]. Pervasive transcription refers to the fact that transcripts from both strands of entire genomes can be detected, albeit at highly differing levels. As a result, it is quite likely that some TCR of a gene's *coding* strand may also take place, depending on whether that locus sustains any level of antisense transcription from downstream genomic regions [50]. These recent genome-wide studies of TCR have nevertheless been able to show that Mfd is indeed the key component coupling transcription to repair.

Non-Canonical Functions of Mfd

Given the evolutionary conservation of Mfd across all kingdoms of life and the minimal phenotype of slight UV-sensitivity observed in MFD− *E. coli* strains, much work has been devoted to the role of Mfd in non-typical contexts, where perhaps other major roles exist. Indeed although the original function ascribed to Mfd was to repair UV-induced premutational lesions in DNA, there is also mounting evidence that Mfd can enhance prokaryotic virulence and survival *via* the promotion of mutagenesis. Currently, however, there is no consensus on the mechanism underlying this potential mutagenic role of Mfd, the growth phase in which Mfd acts, or potential protein partners required. This topic has become of wide interest in recent years for both fundamental and applied reasons, as it opens the possibility that Mfd inhibition could be a part of clinical antimicrobial strategies. It has led to the conclusion that Mfd can also be a pro-evolutionary factor, conferring upon this protein a Janus-like nature, which makes its analysis inevitably more complex.

We first consider examples in which a standard understanding of Mfd (i.e., its anti-mutagenic

properties) explains the role it plays in the virulence of certain prokaryotes. Thus, in the so-called nitric oxide (NO) response, infected eukaryotic cells produce reactive NO species to induce DNA lesions in the invading bacteria. In consequence, organisms such as *Shigella flexneri* and *Bacillus cereus* up-regulate Mfd to survive [66,67]. Similarly, in *Helicobacter pylori* Mfd is important for DNA break repair, potentially *via* recombination, with MFD− strains being more susceptible to mitomycin C [68]. Lastly, in *Vibrio cholerae*, SOS induction is MFD−dependent [69]. Here, the proposed model is independent of UvrA and involves Mfd removing stalled RNAP (RNAP stalls more under aminoglycoside treatment, due to lesion formation), and somehow an R-loop remains. An R-loop is an RNA−DNA hybrid formed when complementary RNA invades the DNA double helix, leaving the displaced DNA as a single strand [70]. This R-loop can prime origin-independent replication, and the resultant collapse of the replication fork at a ssDNA nick produces DNA breaks, leading to SOS-induction *via* the RecBCD pathway.

At the same time, a number of examples suggest that Mfd may also have a pro-mutagenic role. In the soil bacteria *Pseudomonas putida*, Mfd plays a role in UV-induced and stationary phase mutagenesis but does not affect mutation frequency in exponential phase [71]. In *Campylobacter jejuni*, antibiotic treatment itself induces Mfd expression, and it has been shown that in this context Mfd over-expression increases spontaneous mutations and leads to antibiotic resistance [10], whereas MFD− strains are 100-fold less likely to become antibiotic resistant. In nutritionally stressed stationary-phase *Bacillus subtilis*, Mfd can be mutagenic even absent DNA lesions [11].

Also in *B. subtilis*, the Merrikh group showed that Mfd can work with polY1, increasing mutation frequency on genes on the lagging strand [72]. Models to explain this include the following:: head-on RNAP-replisome conflicts create exposed ssDNA that can be damaged, and the repair, involving Mfd and PolY1, is error-prone; or post-replication, RNAPs may encounter more damage than usual (as discontinuous replication creates more lesions), also resulting in error-prone TCR. More recently, the Merrikh group has probed Mfd's mutagenic role in *Shigella typhirium*, *Pseudomonas aeruginosa*, *B. subtilis*, and *Mycobacterium tuberculosis* subjected to antibiotic stress [12]. Consistent with the earlier findings of the Zhang laboratory [10], they find that MFD+ bacteria evolve resistance to antibiotics at rates 2- to 5-fold higher than MFD− strains, supporting a role for Mfd as a general evolvability factor. Finally, the Merrikh group also showed that species cross-complementation was possible to maintain Mfd's mutagenic properties and thus proposed that this mutagenic role of Mfd is common and evolutionarily conserved.

It should be noted that these observations stand in contrast to the original studies in the field, in which appearance of resistance to chloramphenicol was nearly systematically examined, but with no apparent difference between MFD+ and MFD− strains [5]. This may be due to the fact that the classical experiments exposed the bacteria to both UV and antibiotic, possibly titrating available Mfd onto stalled RNAP for DNA repair and thus away from the partners or pathways involved in action as an evolvability factor.

In a related fashion, the Hastings group showed that mutations are over-represented in highly-transcribed regions for *E. coli*, which enter stationary phase upon starvation stress [73]. Mfd was required for this phenotype, and because overproduction of RNase H1 reduced this phenotype, it was proposed to involve R-loop formation. They proposed that Mfd acts on RNAP, whose RNA has formed an R-loop in the region upstream (we now know from Ho *et al*. [28] that Mfd can associate with elongating RNAP, which expands this model), and the RNAP is removed from the DNA, but somehow the R-loop remains. This R-loop can prime origin-independent replication that, in similar fashion to the model proposed by Baharoglu *et al*. [69], can lead to double-strand breaks if the replisome collapses upon encountering ssDNA nicks. Point mutations and amplifications result from the recombinative repair of the double-strand ends [73].

Discussion and Conclusion: A Simple New Model for Mfd-Mediated Mutagenesis and Evolution

Taking these recent developments further, we propose here a specific and testable mechanism for Mfd-driven mutagenesis *via* an R-loop dependent model. Per the observations of Ho *et al*. [28], Mfd can associate with elongating RNAP *in vivo* even absent exogenous DNA lesions. When this happens, Mfd binds RNAP *via* its RID and opens its structure allowing translocation motifs to bind DNA. All the while RNAP is still elongating, as only stalled RNAP is a substrate for displacment by Mfd. Now, there are two associated proteins on DNA, traveling at different velocities which generates a domain of negative supercoiling between the proteins, with positive supercoiling outside as per Liu and Wang's classical model for twin-domain supercoiling [39]. Negative supercoiling between Mfd and RNAP will favor R-loop formation, *via* the nascent RNA from the still elongating RNAP [74]. Transcription termination or dissolution of the complex would eliminate the twin-domains yet global negative supercoiling of the bacterial genome is expected to leave the R-loop intact [74]. The R-loop is the cause of genetic instability as many studies have shown [75−77].

Furthermore, this model posits that UvrA is a regulator of R-loops: considering that under normal conditions UvrA is freely diffusing or engaged on DNA

as part of a damage-search complex, it is mostly available to limit R-loop formation by dissolving the translocating Mfd–RNAP complex, in a similar fashion to how UvrA dissolves the Mfd–RNAP intermediate complex found in TCR. Thus UvrA can limit evolution *via* Mfd-mediated mutagenesis. However, under more stressed conditions (antibiotic attack, for example) where UvrA is quenched on DNA engaged in GGR, Mfd–RNAP complexes can travel for longer along DNA, generating more substantial R-loops, thus contributing more to genetic instability. Thus, UvrA, in response to cellular DNA damage, may fine-tune the mutagenic properties of Mfd. This is in contrast to the findings of the Merrikh group that the Mfd–UvrA interaction interface is required for the mutagenic properties of Mfd. However, as they point out, it is possible that the mutation (R165A) carried out to test this interface weakens the D2-D7 latch [9,42]. This is suspected to destabilize the closed Mfd structure [43], pushing the solution equilibrium of Mfd toward the open state in which it may interact non-specifically with DNA [9,42,43,47]. This would titrate Mfd onto undamaged genomic DNA, reducing its likelihood of interacting stably with RNAP, and resulting in the observed decrease in mutagenesis.

In this review, we have attempted to remind the reader about the complexities involved in classical genetic analysis of TCR and underscore the power of classical genetics in isolating novel phenomenon. We have furthermore attempted to demonstrate the fruits that may be borne by exchange of viewpoints between the pathway dissection of classical genetics, and the mechanistic and kinetic insights obtained from single-molecule experiments *in vitro* and *in vivo*. We believe that this is particularly true in relation to highly dynamic and complex multicomponent pathways in which the detailed kinetics of the actors play a key role in balancing the ultimate outome of the pathway.

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TCR, transcription-coupled repair; RNAP, RNA polymerase; RID, RNAP-interaction domain; CPD, cyclobutane pyrimidine dimer; GGR, global genome repair.

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