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A DinB variant reveals diverse physiological consequences of incomplete TLS extension by a Y-family DNA polymerase

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The only Y-family DNA polymerase conserved among all domains of life, DinB and its mammalian ortholog pol κ , catalyzes proficient bypass of damaged DNA in translesion synthesis (TLS). Y-family DNA polymerases, including DinB, have been implicated in diverse biological phenomena ranging from adaptive mutagenesis in bacteria to several human cancers. Complete TLS requires dNTP insertion opposite a replication blocking lesion and subsequent extension with several dNTP additions. Here we report remarkably proficient TLS extension by DinB from *Escherichia coli*. We also describe a TLS DNA polymerase variant generated by mutation of an evolutionarily conserved tyrosine (Y79). This mutant DinB protein is capable of catalyzing dNTP insertion opposite a replication-blocking lesion, but cannot complete TLS, stalling three nucleotides after an N^2 -dG adduct. Strikingly, expression of this variant transforms a bacteriostatic DNA damaging agent into a bactericidal drug, resulting in profound toxicity even in a *dinB*⁺ background. We find that this phenomenon is not exclusively due to a futile cycle of abortive TLS followed by exonucleolytic reversal. Rather, gene products with roles in cell death and metal homeostasis modulate the toxicity of DinB(Y79L) expression. Together, these results indicate that DinB is specialized to perform remarkably proficient insertion and extension on damaged DNA, and also expose unexpected connections between TLS and cell fate.

MazEF | proofreading | TonB

Y-family DNA polymerases are found in virtually all organisms and possess the remarkable ability to copy over lesions that otherwise stall DNA replication, a process termed translesion synthesis (TLS) (1). Such broadened substrate specificity comes at a mutagenic cost, however, as these enzymes display lower fidelities on undamaged DNA than their replicative counterparts (2–4). Organisms have therefore developed elaborate regulatory systems to restrict the access of Y-family DNA polymerases to appropriate primer termini (3–16). In *E. coli*, activity of both DinB (DNA pol IV) and UmuD₂C (DNA pol V) is strongly influenced by interactions with the β processivity clamp of DNA polymerase III (pol III) (10, 17, 18) and the *umuD* gene products, UmuD₂ and UmuD₂' (5, 19–22). Other protein–protein interactions, notably with single stranded DNA binding protein (SSB) (19, 21), RecA (23–25), and RecFOR (26) also play critical roles in regulating TLS. In eukaryotes, TLS is similarly controlled by association with the PCNA processivity clamp (9, 13, 16, 27, 28), interaction with recombination proteins (29, 30), and by ubiquitylation of both PCNA and the polymerases themselves (7, 8, 13, 16, 27, 31, 32).

Several Y-family DNA polymerases display marked preference for catalysis on particular damaged substrates. Pol η from yeast and humans preferentially replicates cyclobutane pyrimidine dimers (33–35), whereas *E. coli* DinB and its ortholog mouse DNA polymerase κ (pol κ) display strikingly elevated catalytic proficiency for dC insertion opposite N^2 -dG lesions (36). Diverse structural features contribute to these catalytic capabilities. Relatively accommodating active sites (37–39) and divergent C-terminal domains (40) facilitate TLS by several Y-family DNA polymerases. Specialized TLS function does not arise exclusively from open active sites,

however. The active sites of both pol κ (41) and DinB (22) are somewhat closed under many conditions. This may occur at least in part through interaction with other proteins.

Although relatively little is known about the detailed molecular basis of intrinsic preference for replication of particular damaged substrates, specific active site residues in both pol η and DinB are critical for TLS function. In *E. coli* DinB and yeast pol η , mutation of F13 or F34, respectively, generates polymerase variants that are proficient for normal DNA synthesis but cannot catalyze TLS (36, 42). Similarly, mutation of Y52 in human pol η profoundly reduces UV-induced mutagenesis, but does not alter catalysis of canonical DNA synthesis (43). Other such molecular determinants of TLS function assuredly exist (40). Some may be common to all Y-family DNA polymerases and others may be restricted to certain polymerases according to their distinct substrate specificities.

Complete TLS involves insertion of a nucleotide opposite an adducted base (i.e., lesion bypass) and extension by several subsequent additions. In eukaryotes these steps are often catalyzed by two distinct DNA polymerases (16). Replication by the TLS polymerase must continue beyond the DNA lesion, >5 nucleotides in the case of *E. coli* pol V, to avoid reversal by the proofreading activity of the replicative DNA polymerase (11). Consistent with a role in surveying the products of TLS, the *E. coli* *dnaQ*⁺ gene, which encodes the ϵ proofreading subunit of pol III, affects several *dinB*⁺-dependent mutagenic phenomena (44–46). Thus, regulating the length of the products formed by TLS seems to be of critical importance. Those that are too small risk being removed by proofreading, and those that are too long carry undue mutagenic potential.

More broadly, recent studies hint that TLS may be connected to cellular stress response networks in unexpected ways (47–49). Lethality induced by hydroxyurea (HU)-mediated dNTP depletion can be abrogated by certain UmuC variants, and this requires *dinB*⁺ (47). Cytotoxicity from HU exposure also requires the toxin-antitoxin modules *mazEF* and *relBE* (47); in a wild-type (wt) background, deletion of these genes and *tonB* substantially prevents HU-induced cell death (50).

Here we report that DinB catalyzes strikingly proficient extension after dC incorporation opposite an N^2 -furfuryl-dG (N^2 -FG) DNA lesion. Preferential TLS extension of this magnitude has not previously been observed for any DNA polymerase. Moreover, a cluster of aromatic hydrophobic residues surrounding DinB's steric gate dictate its function in TLS extension. Specifically, we demon-

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Table 1. Steady state kinetic parameters for staggered primer extension assays

Primer	V_{\max} , G	V_{\max} , N^2 -FG	K_m , G	K_m , N^2 -FG	V_{\max}/K_m , G	V_{\max}/K_m , N^2 -FG	N^2 -FG/G preference
G-5	557.8	668.9	83.2	27.9	6.7×10^6	2.4×10^7	3.6
G-4	755.9	2492.7	173.9	129.3	4.4×10^6	1.9×10^7	4.4
G-3	464.2	304.6	9.6	5.1	4.8×10^7	6.0×10^7	1.2
G-2	1081.7	1149.6	109.0	51.7	9.9×10^6	2.2×10^7	2.2
G-1	894.3	990.5	231.6	16.0	3.9×10^6	6.2×10^7	16.0
G	619.8	886.3	42.2	2.35	1.5×10^7	3.8×10^8	25.7
G + 1	240.4	167	67.1	41.7	3.6×10^6	4.0×10^6	1.1
G + 2	188.2	150.3	24.9	8.4	7.6×10^6	1.8×10^7	2.4
G + 3	446.1	438.2	59.8	19.6	7.5×10^6	2.2×10^7	3.0

V_{\max} units are $\text{pmol min}^{-1} \text{mg}^{-1}$; K_m units are μM ; V_{\max}/K_m units are $\text{pmol min}^{-1} \text{mg}^{-1} \text{M}^{-1}$. Undamaged template designated G; N^2 -furfuryl-dG abbreviated as N^2 -FG.

strate that Y79, which is invariant among DinB orthologs, critically influences the extension steps of DinB-catalyzed TLS. Expression of DinB(Y79) mutant proteins reveals profound toxicity that is mediated by DnaQ, MazEF, and the iron import protein TonB. Together, our data suggest that survival may be coupled to proper execution and completion of TLS through unexpected aspects of cellular physiology.

Results

DinB Catalyzes Preferential Extension on a Damaged DNA Template. We systematically analyzed DinB activity around a site-specific N^2 -FG lesion using staggered standing start primer extension assays. Primer termini initiated four base pairs before and ended four base pairs after the lesion (Table 1; primers described in Table S1 and Fig. S1). The apparent catalytic proficiency of DinB (V_{\max}/K_m) on the damaged template is similar to an undamaged control for the first several additions we tested. As we previously reported, DinB is ≈ 15 -fold more proficient at catalyzing dC insertion opposite this lesion than opposite dG; this is largely due to a reduced K_m for dCTP (36).

Strikingly, we found that DinB is even more proficient (≈ 25 -fold relative to an undamaged control) at extending from dC opposite the N^2 -FG lesion (Table 1). This effect arises primarily from a low K_m for dATP addition. Subsequent extension steps proceed with comparable efficiencies on both damaged and undamaged templates. These observations reveal that both highly efficient insertion opposite an N^2 -FG lesion and highly efficient addition of the next nucleotide are specialized functions of DinB. Modest preferential extension activity has previously been observed for pol η extending from a cyclobutane pyrimidine dimer (33). However, the exceptionally large preference we observed for both DinB-catalyzed insertion and extension during TLS over an N^2 -FG lesion supports our hypothesis that at least one of DinB's key biological roles is to replicate over ubiquitously occurring classes of N^2 -dG adducts (36, 51, 52). As described below, an independent line of investigation pointed to the further physiological importance of later extension steps that proceed with more normal proficiency.

Aromatic Residues Surrounding DinB's Steric Gate Are Critical for Extension. We previously reported that the steric gate (53) residue of DinB (F13) is indispensable for its TLS function (36). This insight grew out of our efforts to develop a structural model of DinB encountering an N^2 -FG lesion. We examined this model in detail to ascertain whether additional residues might contribute to preferential activity of DinB on a damaged substrate. F13 is surrounded by a cluster of aromatic residues: F12, F76, and Y79 (Fig. 1A). F12 is universally conserved among Y-family DNA polymerases (Fig. S2A) and corresponds to F34 of yeast pol η (42). F76, in contrast, is comparatively weakly conserved among DinB orthologs. Y79 is invariant among DinB orthologs, including pol κ . *E. coli* UmuC shares a tyrosine at this position, whereas the related but function-

ally distinct enzymes Rev1 and Rad30 possess a phenylalanine (Fig. S2A).

To gain further insights into the molecular basis of DinB's properties as a TLS DNA polymerase, we mutated each of F12, F76, and Y79 to valine and examined the ability of these variants to complement the sensitivity of a ΔdinB strain to the DNA damaging agents nitrofurazone (NFZ) and 4-nitroquinoline-1-oxide (4-NQO) (36). Mutation of F12 generates a DinB variant that cannot complement NFZ sensitivity, but does not confer the hypersensitivity caused by expression of *dinB*(F13V) (36) (Fig. S2B). Mutation of F76 also impairs complementation (Fig. S2B), but again does not confer NFZ hypersensitivity. In contrast, Y79 mutations (Y79A, Y79V, and Y79L) have a very large effect on NFZ and 4-NQO sensitivity (Fig. 1B and Fig. S2C). A plasmid expressing DinB(Y79L) not only cannot complement NFZ sensitivity, but also confers a further 10- to 50-fold increase in sensitivity beyond that of a vector control. These observations suggest a critical role for Y79 in TLS over N^2 -dG lesions in vivo. We also tested whether the function of Y79 requires its hydroxyl group. Expression of DinB(Y79F) fully complements the NFZ sensitivity of a ΔdinB strain (Fig. S3).

DinB(Y79) Catalyzes Proficient Lesion Bypass But Is Deficient in Subsequent Extension. We initially suspected that the inability of the DinB(Y79L) mutant protein to complement NFZ or 4-NQO sensitivity would be due to inability to catalyze TLS over N^2 -dG lesions they produce. Unexpectedly, we discovered that in vitro

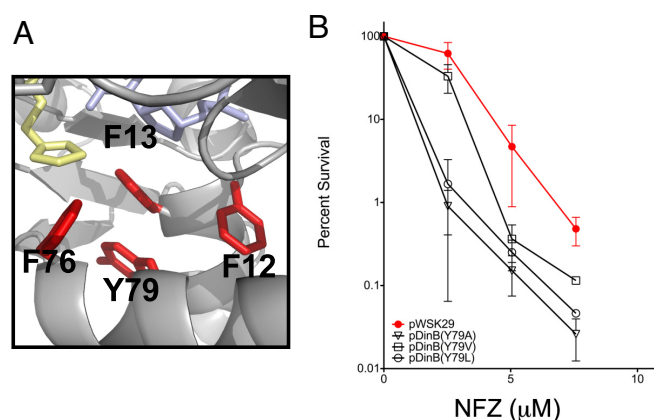


Fig. 1. A pocket of aromatic hydrophobic residues in DinB's active site modulates its TLS function. (A) Structural model of conserved residues in the active site of DinB showing conserved residues (red), the N^2 -furfuryl moiety (yellow), and the incoming nucleotide (blue). (B) Mutation of Y79 eliminates the ability of DinB to complement NFZ sensitivity. Error bars, the standard deviation determined from three independent cultures.

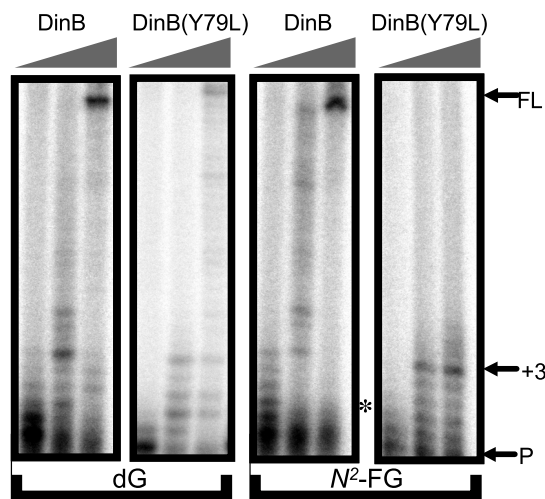


Fig. 2. DinB(Y79L) bypasses N^2 -FG and stalls three nucleotides following the lesion. Primer extension assays with 1, 5, and 50 nM DinB and DinB(Y79L) show that DinB(Y79L) catalyzes lesion bypass, but cannot complete TLS extension. Symbols are as follows: (*) site of N^2 -FG lesion; (P) radiolabeled primer; (+3) products extended three nucleotides past the lesion; (FL) full-length products. Ratios of stalled to full-length products at 5 and 50 nM enzyme are 120 and 0.2, respectively, for DinB on the undamaged template; 31 and 0.1 for DinB(Y79L) on the undamaged template; 0.9 and 0.02 for DinB on the damaged template; and 107 and 97 for DinB(Y79L) on damaged template.

DinB(Y79L) is proficient at both inserting a nucleotide opposite an N^2 -FG lesion and adding the next nucleotide. DinB(Y79L) also has comparable activity to wt DinB on undamaged DNA (Fig. 2). However, products synthesized by DinB(Y79L) replicating a damaged template are shorter than those produced by wt-DinB. The majority arise from stalled synthesis three nucleotides after the lesion. At 5-nM enzyme concentration, the ratio of G + 3 paused-compared to full-length products is 4-fold higher for DinB(Y79L) than for wt DinB. However, this is insignificant relative to the same comparison on damaged DNA, where the ratio is 125-fold. As even wt DinB shows modest accumulation of this product at lower enzyme concentrations, this may arise from exacerbation of a natural pause site at the + 3 position. Notably, DinB has also been observed to stall three nucleotides following an N^2 -dG DNA-protein cross-link in a different sequence context from the one we used (54). This suggests that such pausing may be an intrinsic property of DinB rather than an isolated effect of sequence context.

To examine the nature of DinB(Y79L)'s defect in TLS extension we examined its activity on primers synthetically elongated either two or three base pairs beyond the N^2 -FG lesion. At the + 2 position, DinB(Y79L) showed similar activity to wt DinB on both undamaged ($V_{\max} = 654.8$ pmol/min/mg; $K_M = 217.7$ μ M; $V_{\max}/K_M = 3.0 \times 10^6$ pmol/min/mg/ μ M) and damaged templates ($V_{\max} = 442.9$ 8 pmol/min/mg; $K_M = 118.5$ μ M; $V_{\max}/K_M = 3.7 \times 10^6$ pmol/min/mg/ μ M). In contrast, DinB(Y79L) showed a pronounced defect on the damaged template when extending from the primer synthetically elongated three base pairs beyond the N^2 -FG lesion. Whereas DinB(Y79L) retains roughly half the activity of wt DinB for this addition on undamaged DNA, it is impaired by >20-fold on the damaged substrate (Table 2). Thus, the stalling behavior of DinB(Y79L) likely arises from communication between the already bypassed lesion and DinB's active site, now at the + 3 position. There is an emerging precedent for such enzyme/template interactions. Catalysis of -1 frameshift mutagenesis in polyG regions by the archaeal DinB ortholog Dbh requires specific interactions between enzyme and template at the + 3 position to stabilize an extrahelical base (55). Similarly, contacts between

Table 2. Steady state kinetic parameters for extension from the G + 3 primer by DinB(Y79L)

Template	Enzyme	K_M (μ M)	V_{\max}	V_{\max}/K_M	Fold change
G-control/(G + 3)	WT DinB	59.8	446.1	7.5×10^6	—
	DinB(Y79L)	330.0	1072.0	3.2×10^6	2.3
N^2 -FG/(G + 3)	WT DinB	19.6	438.2	2.2×10^7	—
	DinB(Y79L)	1551.0	1416.0	0.9×10^6	24.0

Units as in Table 1.

enzyme and template allow replicative DNA polymerases to couple distal mismatch recognition to synthesis in the active site (56).

DinB(Y79L) expression does not increase the frequency of mutation to rifampicin resistance upon NFZ treatment ($4.2 \pm 2.0 \times 10^{-8}$ for pDinB(Y79L) vs. $5.5 \pm 3.0 \times 10^{-8}$ for pDinB; average and standard deviation from five independent cultures), suggesting that it is relatively accurate. Thus, DinB(Y79L) is a type of DNA polymerase mutant that can catalyze formal lesion bypass but cannot finish the subsequent extension steps that are required to complete TLS. The strong toxicity of DinB(Y79L) expression indicates that the extension steps of TLS have at least as much physiological importance as lesion bypass itself.

Expression of DinB(Y79L) Changes NFZ from a Bacteriostatic to a Bactericidal Drug. Not only do DinB(Y79L) and DinB(F13V) confer NFZ hypersensitivity in a Δ *dinB* background, but even more strikingly they do so in a *dinB*⁺ background (Fig. 3A). In contrast, a catalytically deficient DinB mutant protein does not similarly increase NFZ sensitivity under these conditions (Fig. S4). We expressed wt DinB, DinB(Y79L), and DinB(F13V) from a low copy number plasmid (≈ 5 copies per cell) under the control of *dinB*'s native promoter (Plasmids described in Table S1). Such modest expression of wDinB does not result in the lethality that has been observed when it is overexpressed 30- to 140-fold in the AB1157 strain background (57) or when DinB is overexpressed to a lesser extent in the BW27786 strain background (58).

The extreme phenotypes arising from modest expression of DinB(Y79L) and DinB(F13V) led us to investigate the underlying cause of the increased NFZ sensitivity in greater detail.

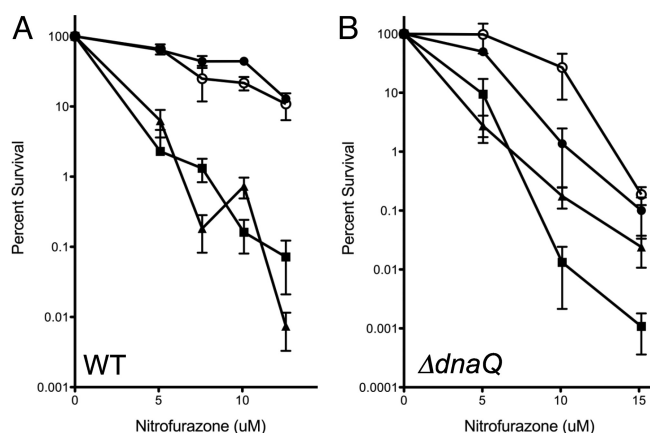


Fig. 3. DinB(Y79L) confers hypersensitivity to NFZ and genetic interactions between *dinB* and *dnaQ*. (A) Expression of pDinB(Y79L) (closed triangles) or pDinB(F13V) (closed squares) results in profound toxicity to NFZ relative to pDinB (closed circles), or an empty vector control (pWSK29; open circles). Chromosomal genotype is *dinB*⁺. In this and all graphs in this figure, error bars represent the standard deviation determined from three independent transformants or four independent transductants. (B) Dominance of pDinB(Y79L) and pDinB(F13V) in a Δ *dnaQ* *dinB*⁺ background. Symbols and error bars as in A.

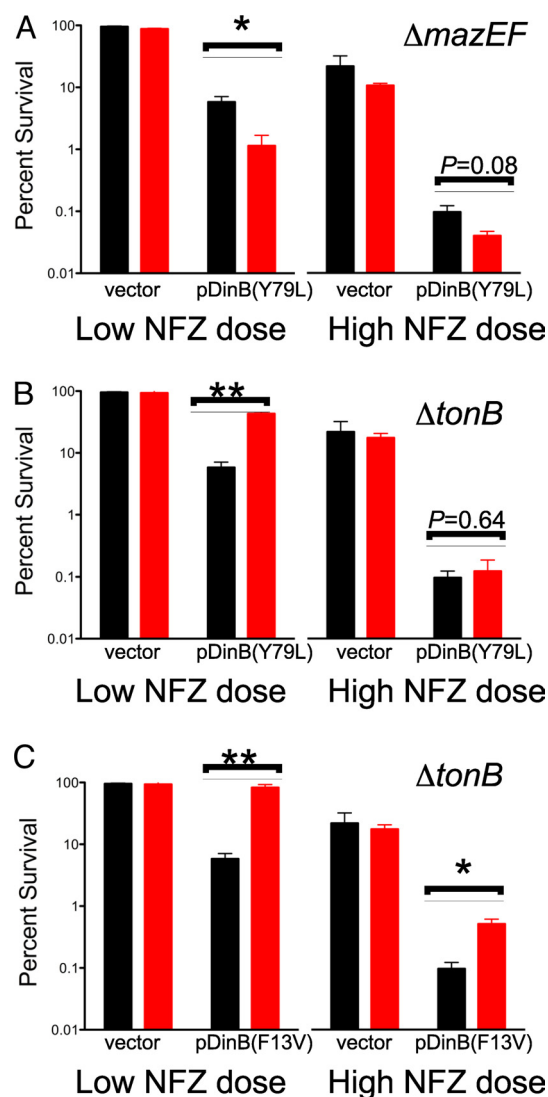
Consistent with the bacteriostatic rather than bactericidal mechanism of NFZ (59), we recovered an expected number of colony forming units from regions between colonies on NFZ agar plates for strains expressing DinB. In contrast, we were unable to recover any bacteria from strains expressing DinB(Y79L) or DinB(F13V), indicating that NFZ is transformed from a bacteriostatic into a bactericidal agent in the presence of these DinB mutants that are specifically unable to carry out normal TLS.

Interaction between *dinB* and the ϵ Proofreading Subunit of DNA Pol III. These observations, along with the biochemical properties of DinB(Y79L) and DinB(F13V) raised the possibility that these mutant proteins may induce a futile cycle in which the immature TLS intermediates they produce are repeatedly destroyed by the proofreading activity of pol III. A similar behavior has been observed for pol V (11), where extension of a TLS product by a nucleotide can rescue it from degradation by the ϵ proofreading subunit of pol III, encoded by *dnaQ*⁺ (12).

We therefore examined DinB(Y79L) and DinB(F13V) expression in a Δ *dnaQ* *dinB*⁺ background. Deletion of *dnaQ* does not prevent DinB(Y79L) or DinB(F13V) expression from conferring NFZ sensitivity in a *dinB*⁺ background (Fig. 3*A* and *B*). However, toxicity from DinB(Y79L) expression is partially reduced by *dnaQ* deletion (Fig. 3*A* and *B*; Fig. S5*A*). Thus, NFZ killing brought about by DinB(Y79L), but not DinB(F13V), expression is at least partially due to a futile cycle of abortive TLS followed by proofreading. In *E. coli*, *dnaQ* deletion leads to suppressor mutations in *dnaE* (60, 61); these do not affect the phenotypes of any *dinB* allele used in this study.

Given the abundant reports of genetic interactions between *dnaQ*⁺ and TLS polymerases, including *dinB*⁺ (11, 44–46), we were surprised that *dnaQ* deletion had only a modest effect on NFZ sensitivity from DinB(Y79L) expression and none at all for DinB(F13V) expression. We therefore examined combinations of *dinB* deletion with a *dnaQ* deletion or *dnaQ903*, which encodes an ϵ variant lacking an exonuclease domain and the C-terminal amino acids that mediate its interaction with pol III (61, 62). Both *dnaQ* alleles confer NFZ sensitivity, although the magnitude differs somewhat (Fig. S5*B*). In a *dnaQ903* mutant the N-terminal portion of DnaQ is present, providing a possible explanation for the differing NFZ sensitivities. This may point to additional DnaQ functions, other than proofreading, that are important for survival in NFZ. We observed an epistatic relationship with respect to NFZ sensitivity for each *dnaQ* allele and Δ *dinB* (Fig. S5*B*) in which the double mutants behave most similarly to the *dnaQ* mutants alone. These observations confirm that *dnaQ*⁺ affects *dinB*⁺ function under conditions of NFZ exposure. However, this interaction does not entirely explain the NFZ sensitivity observed upon DinB(Y79L) or DinB(F13V) expression.

Toxin-Antitoxin Pairs and Metal Homeostasis Modulate Toxicity of Abortive TLS. We also examined the effects of DinB(F13V) and DinB(Y79L) expression in a variety of mutant backgrounds. The *mazEF* and *relBE* genes encode toxin-antitoxin modules that alter cellular physiology and metabolism in response to environmental stress, such as antibiotic exposure (63, 64). Inactivation of *mazEF* or *relBE* increases resistance to replication inhibition by HU (47). We considered whether toxin-antitoxin pairs might also mediate lethality associated with incomplete TLS. Strains bearing deletions of *mazEF*, *relBE*, or an alternative toxin-antitoxin pair *hipAB* are not themselves sensitive to NFZ (Fig. S6), and the effects of DinB(Y79L) and DinB(F13V) expression are unchanged in Δ *relBE* and Δ *hipAB* strains. However, DinB(Y79L) expression confers increased cell death in a Δ *mazEF* strain at low NFZ doses (Fig. 4*A*), suggesting that MazEF may protect the cell from abortive TLS intermediates. We do not observe this effect at higher doses of NFZ (Fig. 4*A*),



consistent with NFZ's highly dose-dependent mode of action (59, 65–69). In contrast, there is no statistically significant difference in NFZ-mediated killing promoted by DinB(F13V) expression in a Δ *mazEF* strain (Fig. S7), indicating that there is an important physiological difference between a failure to insert opposite a lesion [DinB(F13V)] and a failure to sufficiently extend past a lesion [DinB(Y79L)].

We also examined the effect of *tonB* deletion on cytotoxicity from DinB(Y79L) and DinB(F13V) expression. The *tonB* gene encodes a ferric iron import protein that contributes to cellular lethality from oxidative stress (70) and HU (50), but not bactericidal drugs (71). At low NFZ doses *tonB* deletion completely suppresses lethality associated with DinB(F13V) expres-

sion and partially suppresses that associated with DinB(Y79L) expression (Fig. 4 *B* and *C*), indicating that incomplete TLS induces cellular toxicity linked to iron homeostasis. In contrast, at higher NFZ doses *tonB* deletion does not fully suppress the toxicity of DinB(F13V) expression and has no effect on DinB(Y79L) mediated killing (Fig. 4 *B* and *C*), suggesting that additional mechanisms contribute to lethality under those conditions. Thus, participation of both MazEF and TonB in modulating the toxicity of incomplete TLS appears to be regulated by threshold levels of DNA damage.

Discussion

Our previous observation that mutation of DinB's steric gate residue selectively abolishes its TLS activity stimulated us to investigate molecular determinants of DinB function. In so doing, we discovered that DinB is specialized for TLS extension, catalyzing the first nucleotide addition after an N^2 -FG lesion with 25-fold greater catalytic proficiency than on undamaged DNA. Preferential extension of this magnitude has not been reported for any Y-family DNA polymerase. In contrast to models for eukaryotic TLS that involve two DNA polymerases (16, 28, 72), *E. coli* DinB appears capable of catalyzing both insertion and extension with remarkable proficiency.

To explain these findings, we revisited our model of DinB encountering an N^2 -FG lesion. We noted that F12, F76, and Y79 along with F13 comprise a conserved core of aromatic residues. Each contributes to *dinB*⁺-dependent NFZ resistance. Mutation of F76 or F12 creates *dinB* variants that cannot complement the NFZ sensitivity of a Δ *dinB* strain. However, mutation of Y79 results in variants that confer NFZ hypersensitivity by as much as 50-fold. Based on similar behavior by *dinB*(F13V) in vivo, we anticipated that DinB(Y79L) would be unable to catalyze lesion bypass in vitro. Instead, we found that DinB(Y79L) is entirely proficient at dNTP insertion opposite an N^2 -FG adduct and three subsequent nucleotide additions. However, it is unable to catalyze the final extension steps of TLS. The striking toxicity of DinB(F13V) and DinB(Y79L) expression indicates that cellular survival is coupled to successful completion of TLS, and suggests that the extension steps of TLS are at least as physiologically important as dNTP insertion opposite an adducted base.

DinB(Y79L) or DinB(F13V) expression fails to complement the NFZ sensitivity of a Δ *dinB* strain and exerts a dominant effect on survival in *dinB*⁺ backgrounds. This effect is not seen with a catalytically deficient DinB variant under these conditions, and could be explained in part by the unique features of each mutant protein. The defect of a catalytically deficient DinB variant is manifested before dNTP insertion opposite a replication blocking lesion ever takes place. In contrast, both DinB(F13V) and DinB(Y79L) could participate in the initial steps of TLS that precede nucleotide insertion opposite a damaged base. The relatively late manifestation of their TLS deficiencies may restrict alternative mechanisms the cell can employ (Fig. S8). The inability of chromosomally expressed DinB to negate the effects of DinB(F13V) or DinB(Y79L) expression is especially unexpected. This finding suggests that, once TLS is initiated, dynamic exchange of DinB at the primer terminus may be considerably reduced, a commitment that may necessitate increased regulation. Further investigation of this point may prove interesting, especially in light of recent studies regarding competition between DNA polymerases III and IV (57, 58).

Remarkably, expression of DinB(F13V) or DinB(Y79L) also transforms NFZ from a bacteriostatic into a bactericidal agent. Further investigation revealed complex genetic interactions that mediate the lethality of incomplete TLS. Loss of proofreading function partially suppresses the effects of DinB(Y79L) expression, but has no effect on toxicity induced by DinB(F13V) expression. Inactivation of the toxin-antitoxin pair *mazEF* enhances lethality from expression of DinB(Y79L) but not DinB(F13V), suggesting that it may serve to

specifically protect cells from the consequences of incomplete TLS extension. Immature TLS intermediates produced by both DinB(Y79L) and DinB(F13V) lead to cell death through a mechanism involving the TonB iron import protein. Thus, the mechanisms of lethality differ somewhat depending on the nature of the abortive TLS, suggesting that these diverse cellular stress responses may be coupled to the precise execution of TLS at several stages.

How is lethality from abortive TLS related to other types of replication stress? Deletion of *tonB* partially mitigates killing due to replication arrest induced by HU (50). Similarly, the effects of DinB(F13V) and DinB(Y79L) expression are suppressed in a Δ *tonB* strain. We previously observed that Δ *mazEF* strains are strikingly resistant to HU (47). In contrast, deletion of *mazEF* increases NFZ sensitivity upon DinB(Y79L), but not DinB(F13V) expression. Thus, although common factors govern cellular responses to these different replication stresses, their precise roles differ considerably.

Together, our data point to the critical importance of the extension phase of TLS. DinB itself is especially proficient at dNTP addition immediately following an N^2 -FG lesion. Subsequent TLS extension requires communication between the bypassed lesion and the active site that proceeds through an evolutionarily conserved tyrosine. Failure to complete further extensions results in pronounced lethality upon NFZ exposure, suggesting that TLS extension is as physiologically important as lesion bypass itself. Our observations strongly indicate that TLS is far more nuanced than mere insertion of a dNTP opposite a damaged base and subsequent extension. Indeed, the precise execution of TLS appears to be connected to a variety of cellular processes with roles extending well beyond DNA metabolism. DinB's exceptional catalytic proficiency, extreme cellular abundance (73), and elaborate regulation point to its critical and conserved function in TLS over ubiquitous DNA lesions.

Materials and Methods

Bacterial Strains and Plasmids. Strains, plasmids, and primers used in this study are described in Table S1. Plasmid borne *dinB* variants were produced either from a low-copy number plasmid containing *dinB*⁺ under its own promoter (74) or from a DinB expression plasmid (36) using site-directed mutagenesis. Plasmids were maintained with ampicillin (100 μ g/mL).

Sensitivity to DNA damaging agents was determined as described in refs. 36 and 75. Saturated cultures were diluted 1,000-fold, grown to exponential phase in LB medium, and plated on LB agar containing 0–20 μ M NFZ or 4-NQO. Survival was determined relative to growth in the absence of DNA damaging agent. Mutation frequency was measured by reversion to rifampicin resistance as described in refs. 36 and 75. Procedures for assessing bacteriostatic vs. bactericidal behavior are described in the SI Text accompanying this article.

Oligonucleotide Synthesis. Synthesis of oligonucleotides used as templates is described in detail in ref. 36. Nested primers for extension assays were purified by gel electrophoresis before use and are described in Table S1.

Protein Purification and Primer Extension Assays. Purification of wt DinB and DinB(Y79L) was performed as previously (75), and is described in detail in SI Text accompanying this manuscript. Primer extension assays were performed as described in ref. 36, using equivalent concentrations of wt DinB and DinB(Y79L) (1, 5, and 50 nM) as indicated in the figure legends. Running start assays were performed in 10- μ L volumes with 10 nM primer/template and 250 μ M dNTPs. Reactions were carried out in 50 mM Hepes (pH 7.5), 100 mM KCl, 7.5 mM MgCl₂, 5% glycerol, and 0.1% BSA. Standing-start experiments were performed in triplicate with 5 nM DinB and 0-, 1000 μ M dNTP (Fig. S1). Reactions were quenched with formamide after 15 min. Products were separated by 16% denaturing polyacrylamide gel and quantified using a phosphorimager. When nucleotide addition resulted in multiple products, the sum of all products was used to calculate an apparent V_{max} and K_m .

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