Supplemental Table 1. Bacterial strains used in this study.

For the sake of simplicity, Mtb DnaE1 numbering was used throughout the paper.
Mtb DnaE1 D23 = Msmeg DnaE1 D25
Mtb DnaE1 D226 = Msmeg DnaE1 D228

M. smegmatis
All strains are derivatives of mc^2155 with the exception of the protein production strain, which is a derivative of $mc^2$4517.

Strain #	Genotype
1	wild-type mc^2155
19	*ΔdnaE1::Hyg dnaE1::L5(Zeo) [Ms3178]*
22	*ΔdnaE1::Hyg dnaE1::L5(Kan) [Ms3178]*
30	*ΔdnaE1::Hyg dnaE1::L5(Kan) [Rv1547]*
48	*dnaE1-MYC::L5(Kan) [Ms3178]*
58	*dnaE1-D228N-MYC::L5(Kan) [Ms3178]*
62	*$P_{UV15\text{-}Tet}$-dnaE1-MYC::L5(Kan) [Ms3178]*
90	*$P_{UV15\text{-}Tet}$-dnaE1-D228N-MYC::L5(Kan) [Ms3178]*
121	*dnaE1-D25N-MYC::L5(Kan) [Ms3178]*
128	*groELΔC P_{acet}-T7 RNA polymerase::L5(Kan)*
215	*$P_{UV15\text{-}Tet}$-dnaE1-MYC::L5(Kan) [Rv1547]*
217	*$P_{UV15\text{-}Tet}$-dnaE1-D23N-MYC::L5(Kan) [Rv1547]*
221	*$P_{UV15\text{-}Tet}$-dnaE1-D226N-MYC::L5(Kan) [Rv1547]*
223	*$P_{UV15\text{-}Tet}$-dnaE1-D25N-MYC::L5(Kan) [Ms3178]*
248	*ΔdnaE1::Hyg dnaE1-K95N::L5(Kan) [Rv1547]*
251	*ΔdnaE1::Hyg dnaE1-T249I::L5(Kan) [Rv1547]*
285	*ΔdnaE1::Hyg dnaE1-A149V::L5(Kan) [Rv1547]*
CF1	*ΔdnaQ (Ms6275)*
CF2	*ΔMs4259*
CF3	*ΔdnaQ (Ms6275) ΔMs4259*
MA3	*ΔdnaE1::Hyg dnaE1-D228N+silent::L5(Kan) [Ms3178]*
MA4	*ΔdnaE1::Hyg dnaE1-D25N+silent::L5(Kan) [Ms3178]*

M. tuberculosis **(all strains are derivatives of H37Rv)**

CF4	wild-type H37Rv
CF5	*ΔdnaQ (Rv3711c)*

E. coli

BL21-Gold(DE3)	*E. coli* B F^- *ompT hsdS*(r_8^- m_8^-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte

Supplemental Table 2. Plasmids generated for this study.

Plasmid #	Description
14	*dnaE1::L5(Kan) [Rv1547]*
17	*ΔdnaE1::lox-Hyg-lox [Ms3178]*
23	*dnaE1::L5(Kan) [Ms3178]*
30	*dnaE1::L5(Zeo) [Ms3178]*
40	*dnaE1-D228N::L5(Kan) [Ms3178]*
42	*dnaE1-MYC::L5(Kan) [Ms3178]*
50	*dnaE1-D228N-MYC::L5(Kan) [Ms3178]*
53	$P_{UV15\text{-}Tet}$*-dnaE1-MYC::L5(Kan) [Ms3178]*
57	*dnaE1-D25N-MYC::L5(Kan) [Ms3178]*
65	$P_{UV15\text{-}Tet}$*-dnaE1-D228N-MYC::L5(Kan) [Ms3178]*
84	*dnaE1-MYC::L5(Kan) [Rv1547]*
87	*dnaE1-D228N+silent::L5(Kan) [Ms3178]*
113	*dnaE1-D25N::L5(Kan) [Ms3178]*
143	$P_{UV15\text{-}Tet}$*-dnaE1-MYC::L5(Kan) [Rv1547]*
144	$P_{UV15\text{-}Tet}$*-dnaE1-D23N-MYC::L5(Kan) [Rv1547]*
146	$P_{UV15\text{-}Tet}$*-dnaE1-D226N-MYC::L5(Kan) [Rv1547]*
147	$P_{UV15\text{-}Tet}$*-dnaE1-D25N-MYC::L5(Kan) [Ms3178]*
161	*dnaE1-D25N+silent::L5(Kan) [Ms3178]*
UFL1	*6xHis-dnaE1 [Rv1547;* pYUB28b-Hyg]
UFL2	*6xHis-dnaE1-D23N [Rv1547;* pYUB28b-Hyg]
UFL3	*6xHis-dnaE1-D226N [Rv1547;* pYUB28b-Hyg]
UFL4	*PolIII alpha (E. coli)* [pET3c-Amp]
UFL5	*6xHis-epsilon (E. coli)* [pET28a-pp-Kan]
UFL6	*6xHis-dnaQ [Rv3711c;* pET28a-pp-Kan]

Supplemental Table 6. DNA substrates used in this study

Red letters in bold/underline indicate position of mismatched bases. Blue, lower case 's' indicates position of the phosphorothioate linkage.

Real-time primer extension assay

matched	5′ TAGGACGAAGGACTCCCAACTTTAGGTGCG 3′ ATCCTGCTTCCTGAGGGTTGAAATCCACGCCCCCCCCC-[6FAM]
mismatched	5′ TAGGACGAAGGACTCCCAACTTTAGGTGC**T** 3′ ATCCTGCTTCCTGAGGGTTGAAATCCACGCCCCCCCCC-[6FAM]
matched, phosphorothioate	5′ TAGGACGAAGGACTCCCAACTTTAGGTGCsG 3′ ATCCTGCTTCCTGAGGGTTGAAATCCACG-CCCCCCCCC-[6FAM]
mismatched, phosphorothioate	5′ TAGGACGAAGGACTCCCAACTTTAGGTGCs**T** 3′ ATCCTGCTTCCTGAGGGTTGAAATCCACG-CCCCCCCCC-[6FAM]

Gel analysis primer extension assay

matched	[6FAM]-5′ GAGTCCTTCGTCCTT 3′ CTCAGGAAGCAGGAATTTTGCTGTCTGTGAA
mismatched -1 TG	[6FAM]-5′ GAGTCCTTCGTCCTT 3′ CTCAGGAAGCAGGA**G**TTTTGCTGTCTGTGAA
mismatched -1 TC	[6FAM]-5′ GAGTCCTTCGTCCTT 3′ CTCAGGAAGCAGGA**C**TTTTGCTGTCTGTGAA
mismatched -2 TG	[6FAM]-5′ GAGTCCTTCGTCCTT 3′ CTCAGGAAGCAGG**G**ATTTTGCTGTCTGTGAA
mismatched -2 TC	[6FAM]-5′ GAGTCCTTCGTCCTT 3′ CTCAGGAAGCAGG**C**ATTTTGCTGTCTGTGAA
ara-A inhibition assay	[6FAM]-5′ GAGTCCTTCGTCCTT 3′ CTCAGGAAGCAGGAAGTCAGTCAGTCAGTCA

Gel analysis exonuclease assay

single-stranded, with 5′ label	[6FAM]-5′ GTTCACGAGACCTACTGACACTGA
single-stranded, with 3′ label	5′ GTTCACGAGACCTACTGACACTGA -[6FAM]