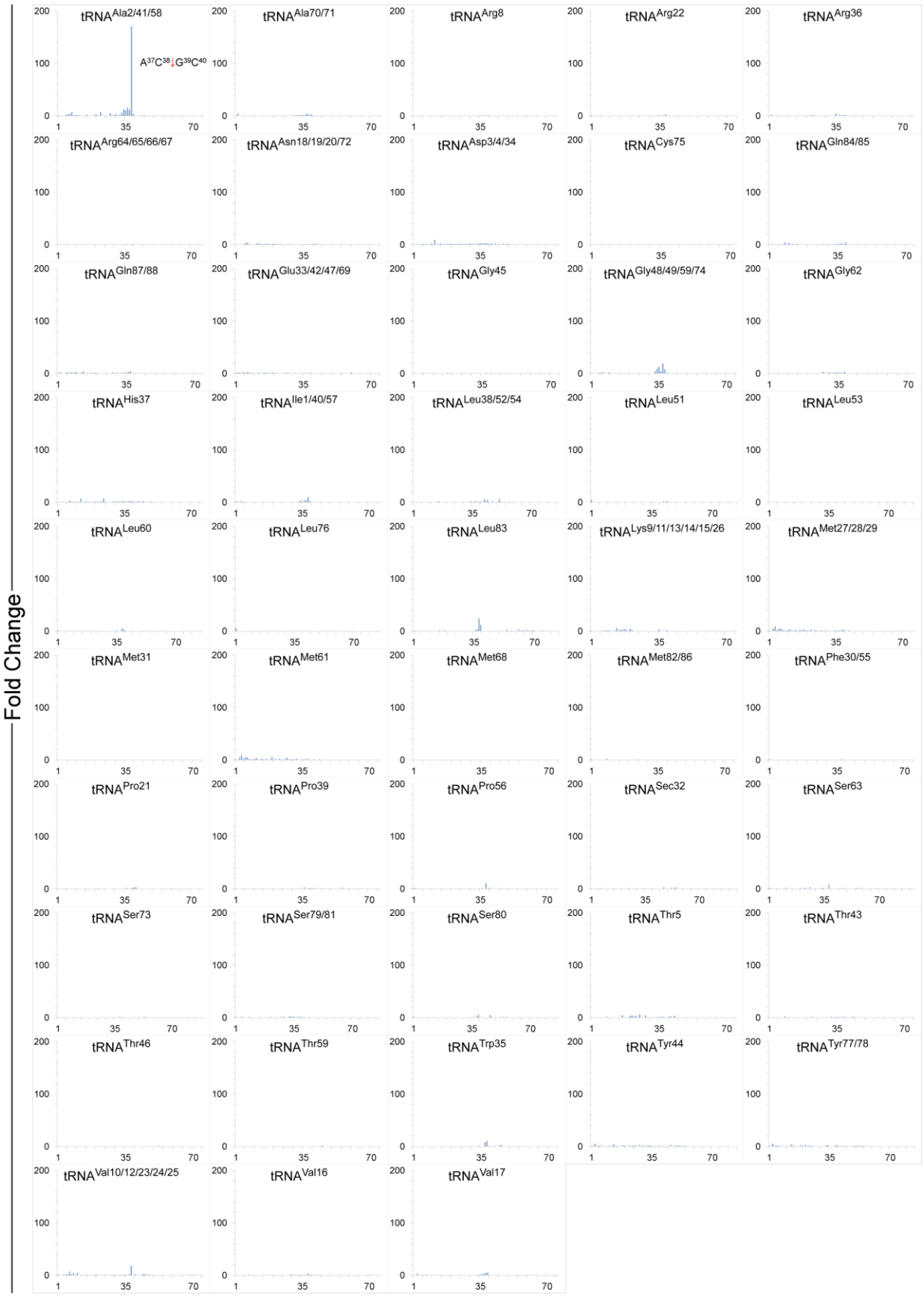
Fold Change
Nucleotide Position
tRNA^Ala2/41/58
A37C38↓G39C40
tRNA^Ala70/71
tRNA^Arg8
tRNA^Arg22
tRNA^Arg36
tRNA^Arg64/65/66/67
tRNA^Asn18/19/20/72
tRNA^Asp3/4/34
tRNA^Cys75
tRNA^Gln84/85
tRNA^Gln87/88
tRNA^Glu33/42/47/69
tRNA^Gly45
tRNA^Gly48/49/59/74
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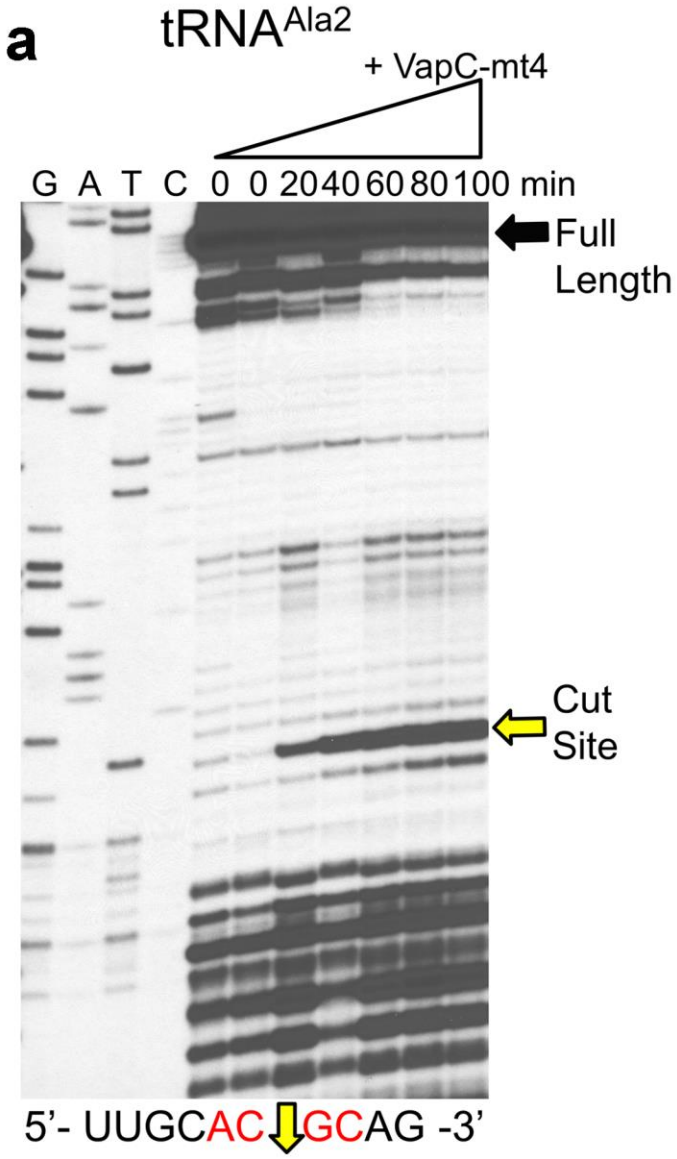
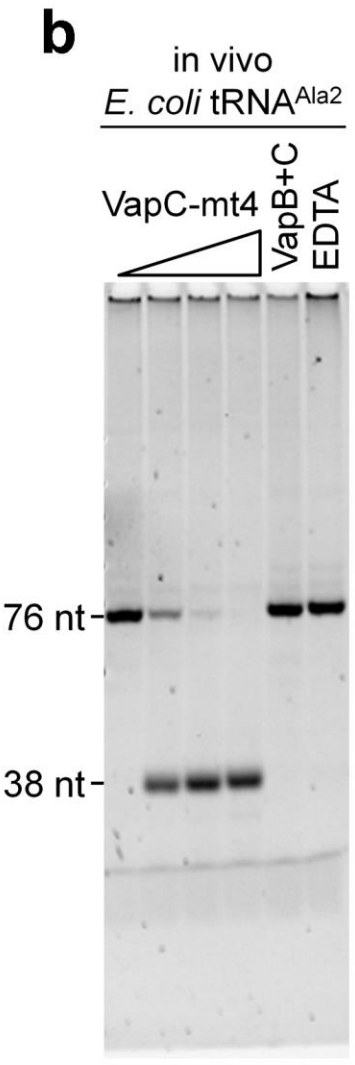
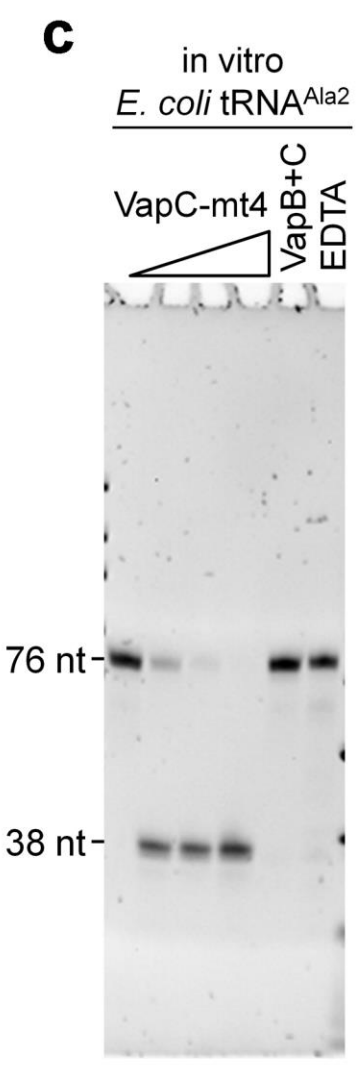
tRNA^His37
tRNA^Ile1/40/57
tRNA^Leu38/52/54
tRNA^Leu51
tRNA^Leu53
tRNA^Leu60
tRNA^Leu76
tRNA^Leu83
tRNA^Lys9/11/13/14/15/26
tRNA^Met27/28/29
tRNA^Met31
tRNA^Met61
tRNA^Met68
tRNA^Met82/86
tRNA^Phe30/55
tRNA^Pro21
tRNA^Pro39
tRNA^Pro56
tRNA^Sec32
tRNA^Ser63
tRNA^Ser73
tRNA^Ser79/81
tRNA^Ser80
tRNA^Thr5
tRNA^Thr43
tRNA^Thr46
tRNA^Thr59
tRNA^Trp35
tRNA^Tyr44
tRNA^Tyr77/78
tRNA^Val10/12/23/24/25
tRNA^Val16
tRNA^Val17
0
100
200
1
35
70

Supplementary Figure 1. VapC-mt4 cleaves tRNA^{Ala2} in *E. coli*. Histograms representing the fold change in reads observed in tRNAs from the analysis of RNAs carrying a 5'-OH ends isolated from cells induced to express VapC-mt4 versus uninduced cells. The sequence surrounding the site of cleavage in tRNA is shown in the corresponding histogram with the position of cleavage indicated by a red arrow.

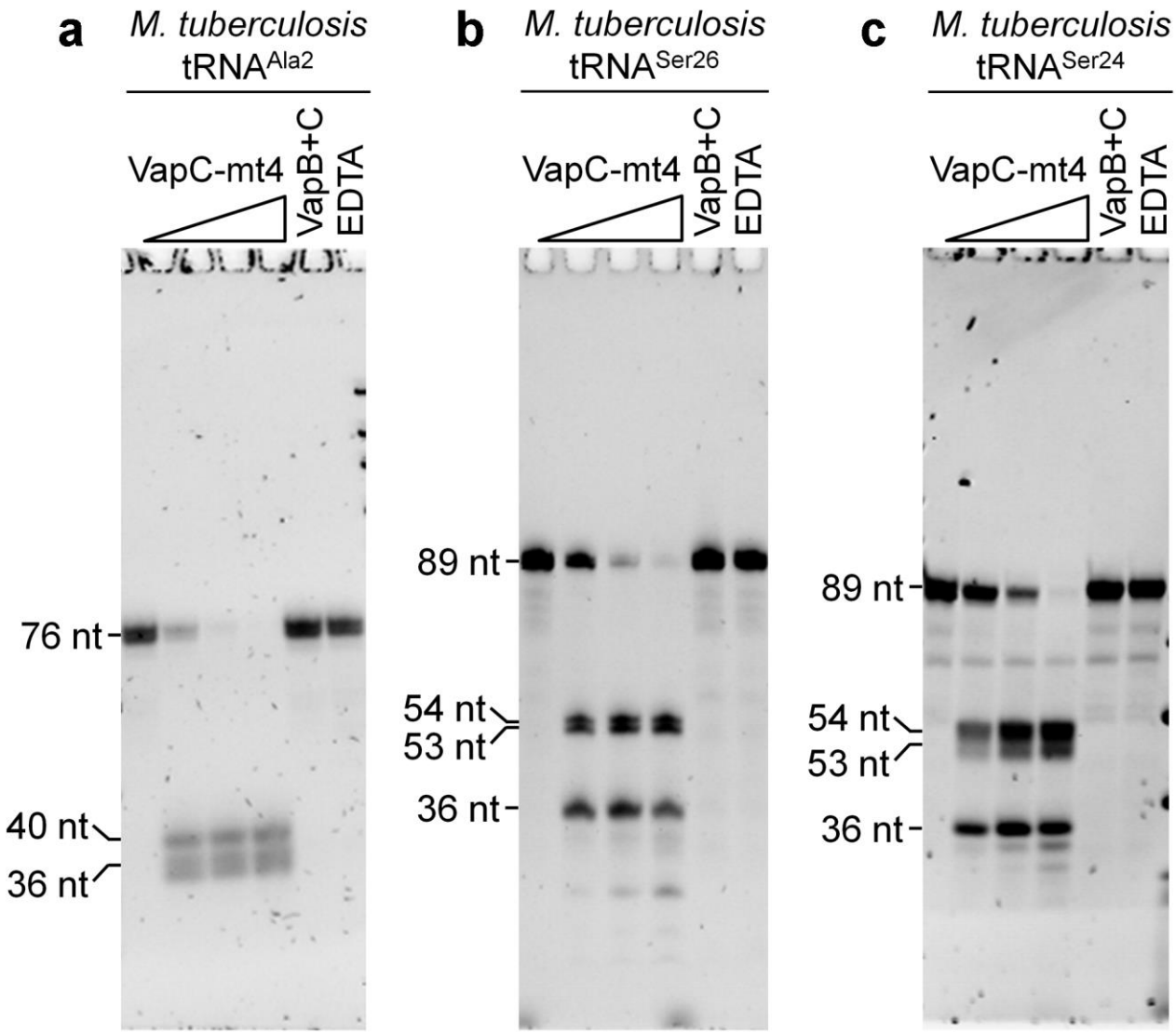






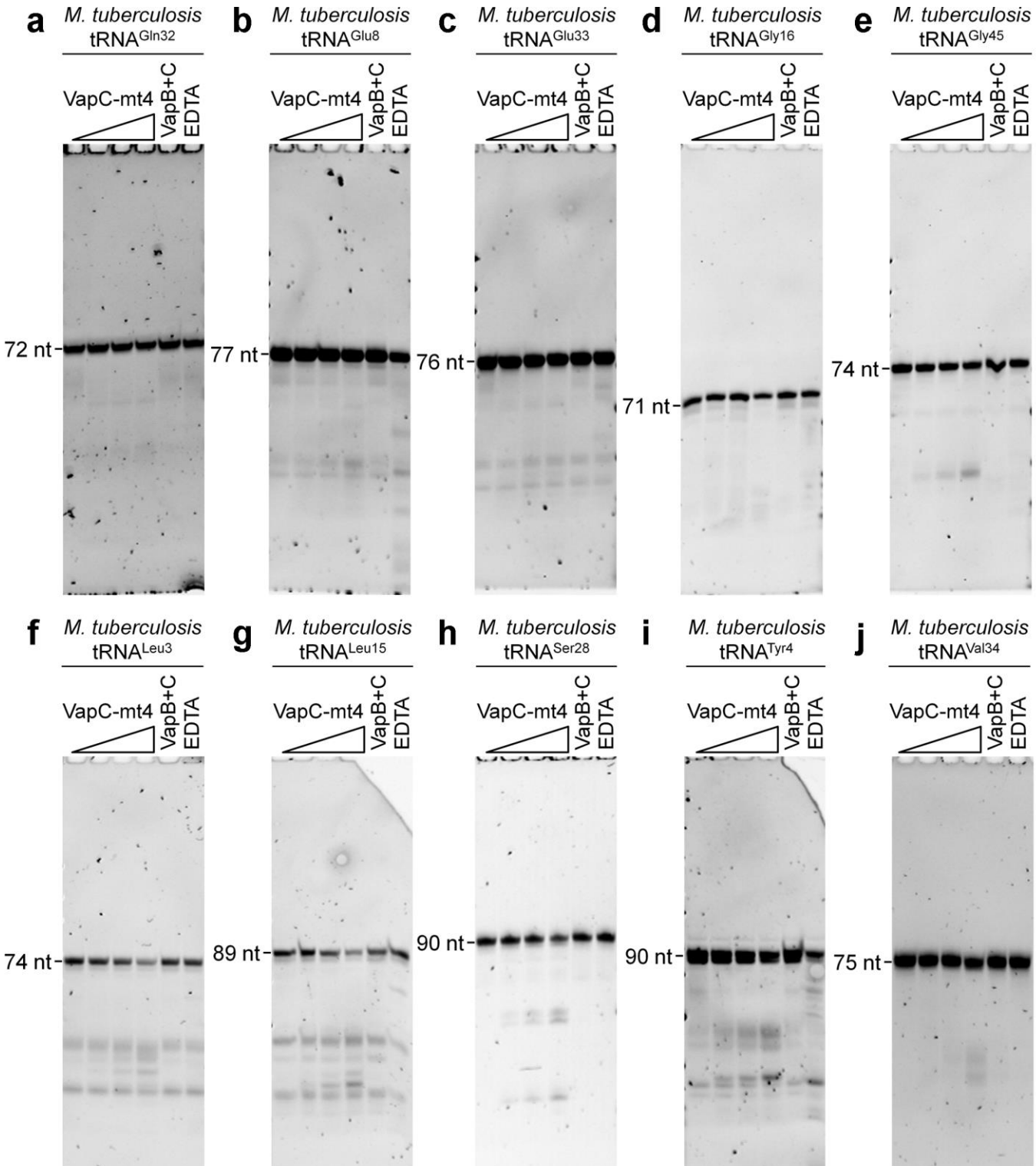
Supplementary Figure 2. VapC-mt4 Cleaves tRNA^{Ala2} in *E. coli* (complete gel images of Fig. 1b, d, e). (**a**) Primer extension analysis with total *E. coli* RNA following induction of VapC-mt4 for the times indicated. The 0 minute time point was loaded twice. The RNA sequence (ACGC consensus in red) and positions of cleavage

(yellow arrow) shown below the gel image. G, A, T, and C lanes correspond to DNA sequencing ladders using the same primer and a $tRNA^{Ala2}$ DNA template. The major primer extension band migrates between the G and T residues in the sequencing ladder instead of aligning exactly to the G residue. We attribute this to the repeatable aberrant migration of the sequencing ladder below this tRNA sequence. (**b,c**) Cleavage assay with in vivo purified $tRNA^{Ala2}$ (**b**) or in vitro synthesized $tRNA^{Ala2}$ (**c**) and increasing amounts of VapC-mt4 (ratios of toxin to RNA were 0:1, 1.25:1, 2.5:1, and 5:1). Control reactions on the right contained the highest concentration of VapC-mt4 preincubated with VapB antitoxin or EDTA before addition of the respective tRNAs. Reactions were incubated at 37°C for 3 h. Sizes of full length and cleaved tRNA products on the left.

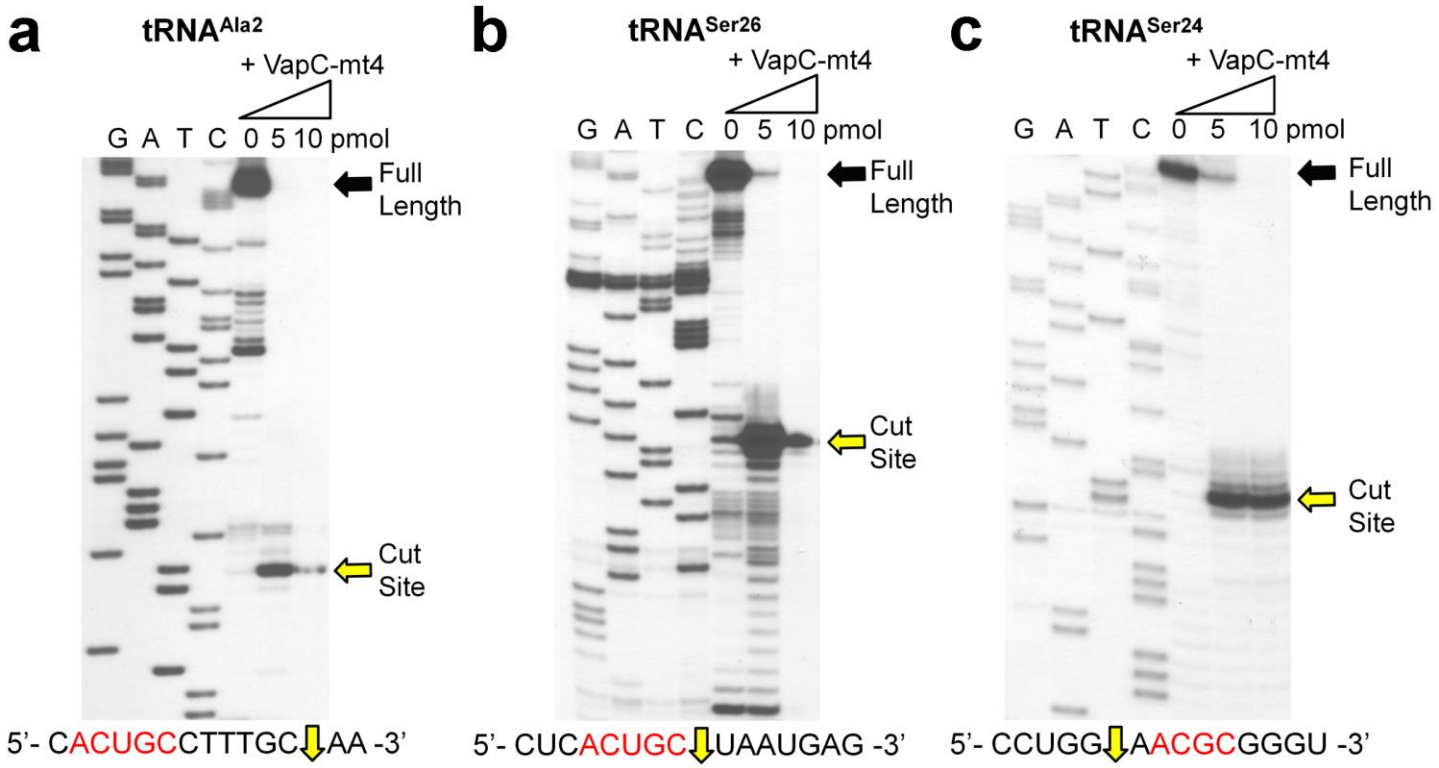


Supplementary Figure 3. VapC-mt4 targets a specific subset of tRNAs in *M. tuberculosis* (complete gel

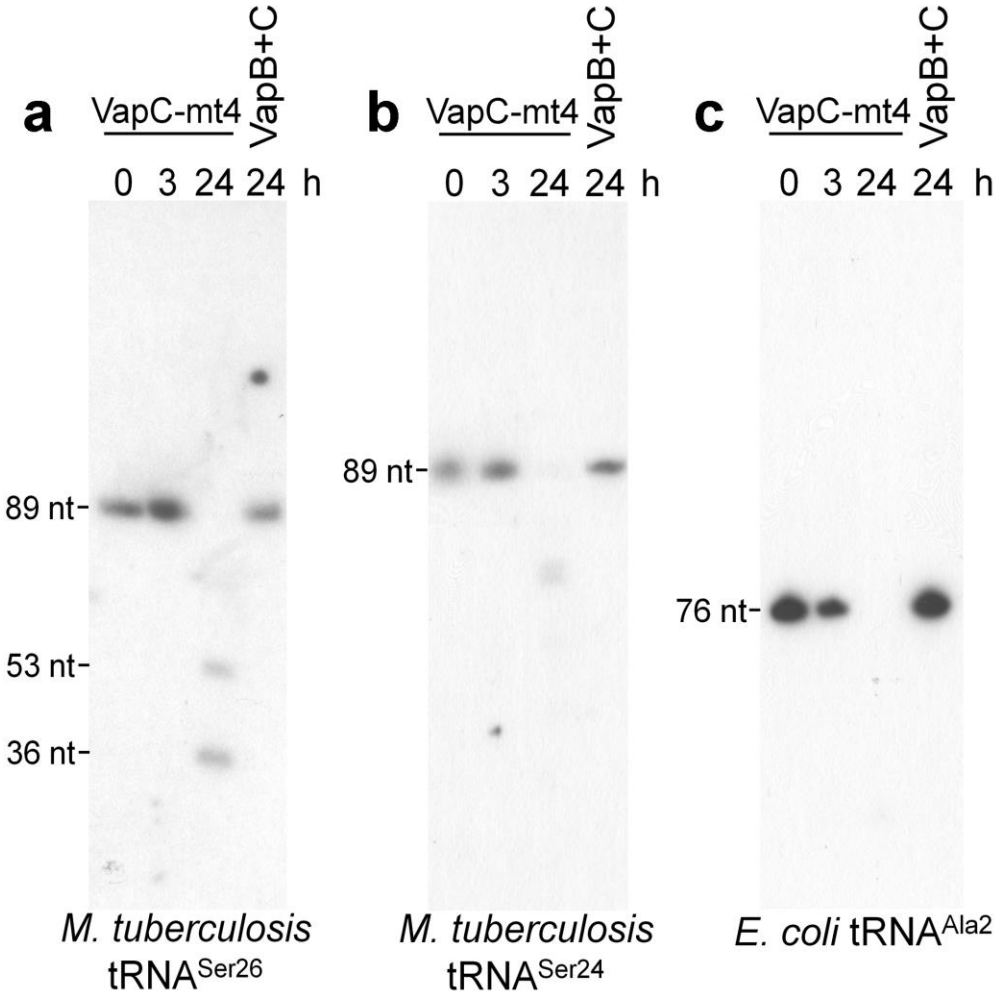
images of Fig. 2 a, b and c). (**a-c**) In vitro VapC-mt4 cleavage assays showing the three tRNAs from among the 13 consensus-containing *M. tuberculosis* tRNAs tested that are cleaved to completion: $tRNA^{Ala2}$ (**a**), $tRNA^{Ser26}$ (**b**), and $tRNA^{Ser24}$ (**c**). In vitro synthesized tRNAs were incubated with increasing amounts of VapC-mt4 (ratios of toxin to RNA were 0:1, 1.25:1, 2.5:1, and 5:1). Control reactions on the right contained the highest concentration of VapC-mt4 preincubated with VapB antitoxin or EDTA before addition of the respective tRNAs. Reactions were incubated at 37°C for 3 h. Sizes of full length and cleaved tRNA products on the left.



Supplementary Figure 4. VapC-mt4 does not cleave all consensus-containing tRNAs in *M. tuberculosis*. In vitro VapC-mt4 cleavage assays showing the 10 tRNAs from among the 13 consensus-containing *M. tuberculosis* tRNAs tested that are not cleaved: $tRNA^{Gln32}$ (**a**), $tRNA^{Glu8}$ (**b**), $tRNA^{Glu33}$ (**c**), $tRNA^{Gly16}$ (**d**), $tRNA^{Gly45}$ (**e**), $tRNA^{Leu3}$ (**f**), $tRNA^{Leu15}$ (**g**), $tRNA^{Ser28}$ (**h**), $tRNA^{Tyr4}$ (**i**), and $tRNA^{Val34}$ (**j**). In vitro synthesized tRNAs were incubated with increasing amounts of VapC-mt4 (ratios of toxin to RNA were 0:1, 1.25:1, 2.5:1, and 5:1). Control reactions on the right contained the highest concentration of VapC-mt4 preincubated with VapB antitoxin or EDTA before addition of the respective tRNAs. Reactions were incubated at 37°C for 3 h. Sizes of full length and cleaved tRNA products on the left.

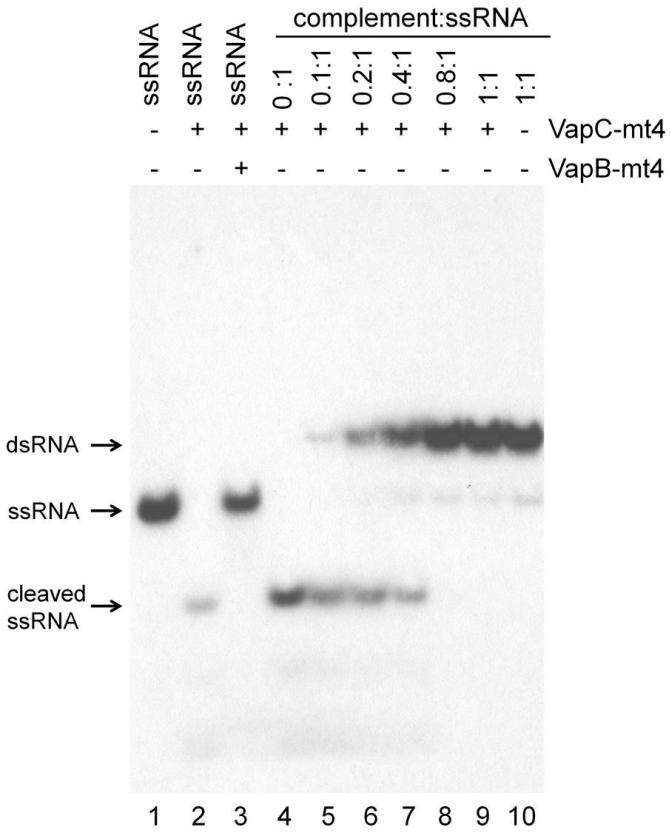


Supplementary Figure 5. VapC-mt4 cleaves three *M. tuberculosis* tRNAs near consensus sequences (complete gel images of Fig. 3a, c, e). (**a-c**) Primer extension analysis with in vitro synthesized *M. tuberculosis* $tRNA^{Ala2}$ (**a**), $tRNA^{Ser26}$ (**b**), and $tRNA^{Ser24}$ (**c**). Full gels are shown for each tRNA. The RNA sequence (ACGC or ACUGC consensus in red) and positions of cleavage (yellow arrow) shown below gel images in left panels. G, A, T, and C lanes correspond to DNA sequencing ladders using the same primer and matched tRNA DNA template as the corresponding primer extension. tRNAs were incubated with increasing amounts of VapC-mt4 (ratios of toxin to RNA were 0:1, 2.5:1, and 5:1) for 3h at 37°C.

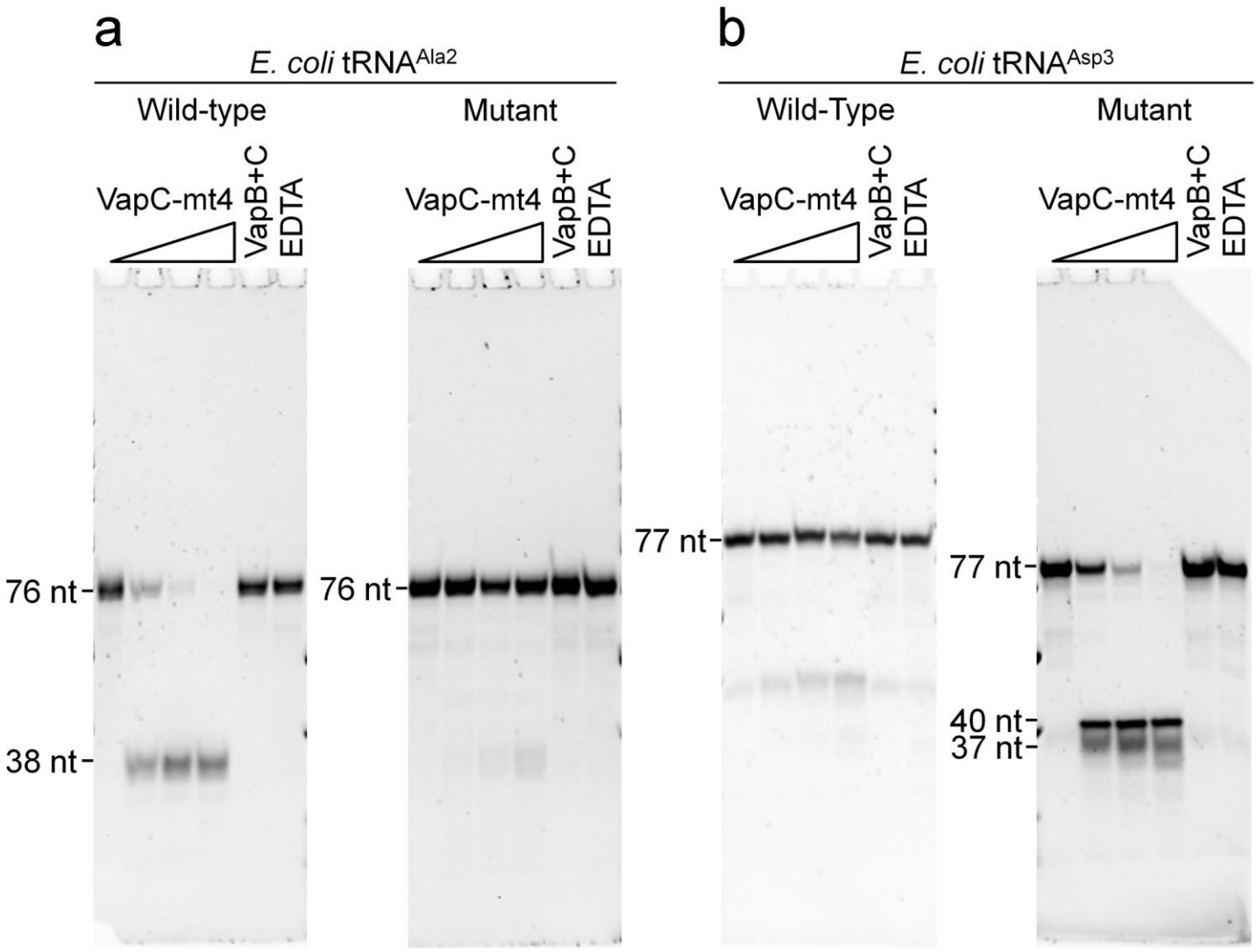


Supplementary Figure 6. VapC-mt4 also cleaves modified *M. tuberculosis* tRNAs (complete blot images of Fig. 4). (a, b) *M. tuberculosis* total RNA (2 µg) was incubated with VapC-mt4 (20 pmol) for the times indicated and northern analysis was performed using isoacceptor-specific oligonucleotides complementary to each ASL. VapB+C samples denote preincubation of the VapC-mt4 toxin (20 pmol) with VapB-mt4 antitoxin (120 pmol) before addition of total RNA. (**c**) Analogous experiment for *E. coli* $tRNA^{Ala2}$ using *E. coli* total RNA and an

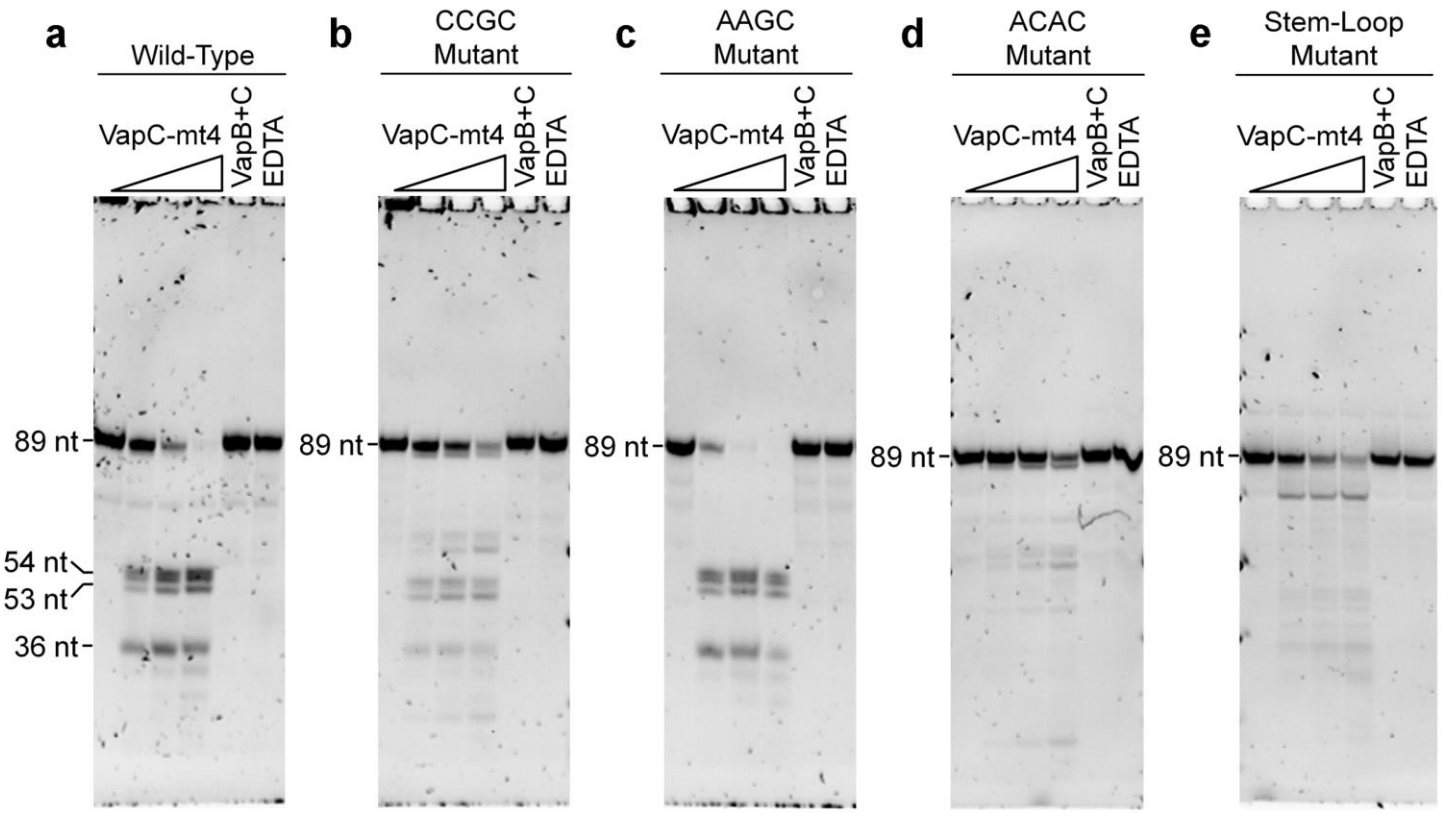
isoacceptor-specific oligonucleotide complementary to the ASL.



Supplementary Figure 7. VapC-mt4 cleavage requires a single-stranded RNA template (complete gel image of Fig. 5). A 20 nt 5'-end labeled RNA containing an ACGC consensus sequence alone (lane 1), after incubation with VapC-mt4 (lane 2), or after incubation with VapB-mt4 and VapC-mt4 (lane 3). This 20 nt RNA was also preincubated with increasing amounts of an RNA complement lacking an ACGC consensus (lanes 4-10) followed by incubation with VapC-mt4. The positions of the dsRNA, ssRNA and cleaved ssRNA are shown on the left. The ratio of toxin to RNA was 64.2:1 and the assay was incubated at 37^{o}C for 3 h.



Supplementary Figure 8. VapC-mt4 cleavage requires an ACGC in the proper context (complete gel images of Fig. 6a, b). (**a**) *left panel*, complete cleavage of wild-type $tRNA^{Ala2}$ by VapC-mt4; *right panel*, no cleavage after mutation of two bases in the $tRNA^{Ala2}$ anticodon to match those in $tRNA^{Asp3}$ (**b**) *left panel*, no cleavage of wild-type $tRNA^{Asp3}$ by VapC-mt4; *right panel*, complete cleavage after mutation of two bases in the $tRNA^{Asp3}$ anticodon to match those in $tRNA^{Ala2}$. In vitro synthesized tRNAs were incubated with increasing amounts of VapC-mt4 (ratios of toxin to RNA were 0:1, 1.25:1, 2.5:1, and 5:1). Control reactions on the right contained the highest concentration of VapC-mt4 preincubated with VapB antitoxin or EDTA before addition of the respective tRNAs. Reactions were incubated at 37°C for 3 h. Sizes of full length and cleaved tRNA products on the left. The sizes of the $tRNA^{Asp3}$ mutant cleavage products are predicted based on the site of cleavage in $tRNA^{Ala2}$.



Supplementary Figure 9. tRNA cleavage by VapC-mt4 requires both structure and sequence determinants (complete gel images of Fig. 7). (**a-e**) Cleavage assays comparing wild-type VapC-mt4 cleavage (**a**) to mutants that alter the consensus sequence but not ASL base pairing (**b,c**), a mutant that alters the consensus and disrupts one base pair in the stem (**d**), a mutant that retains the consensus sequence but removes all base pairing in the stem (**e**). In vitro synthesized tRNAs were incubated with increasing amounts of VapC-mt4 (ratios of toxin to RNA were 0:1, 1.25:1, 2.5:1, and 5:1). Control reactions on the right contained the highest concentration of VapC-mt4 preincubated with VapB antitoxin or EDTA before addition of the respective tRNAs. Reactions were incubated at 37°C for 3 h. Sizes of full length and cleaved tRNA products on the left.