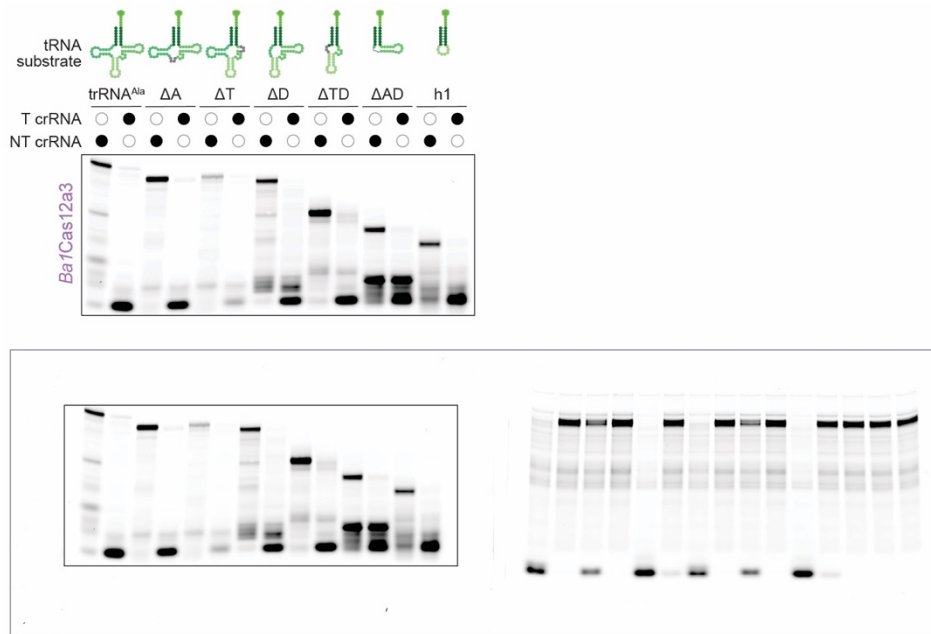
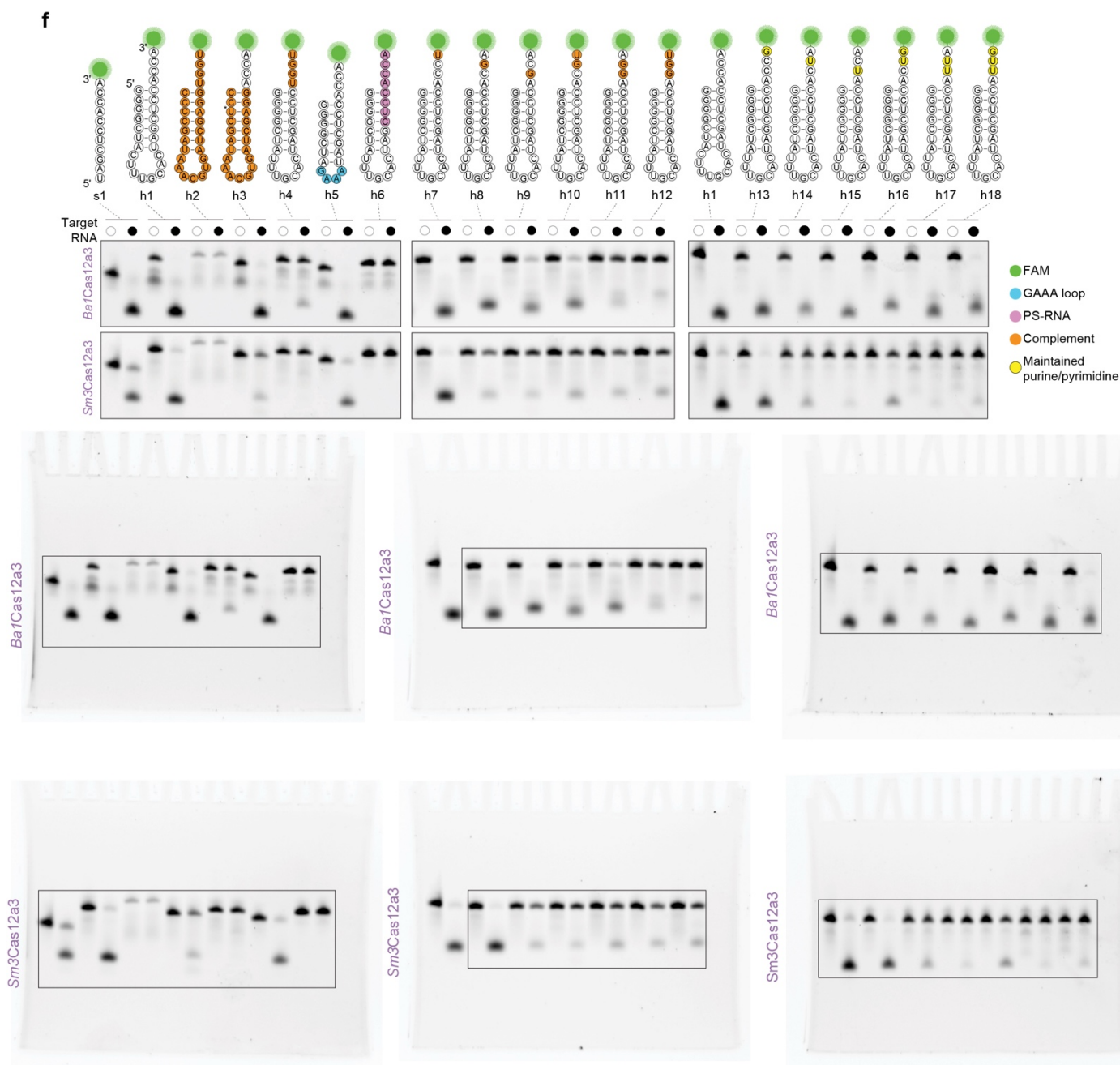

Supplementary information

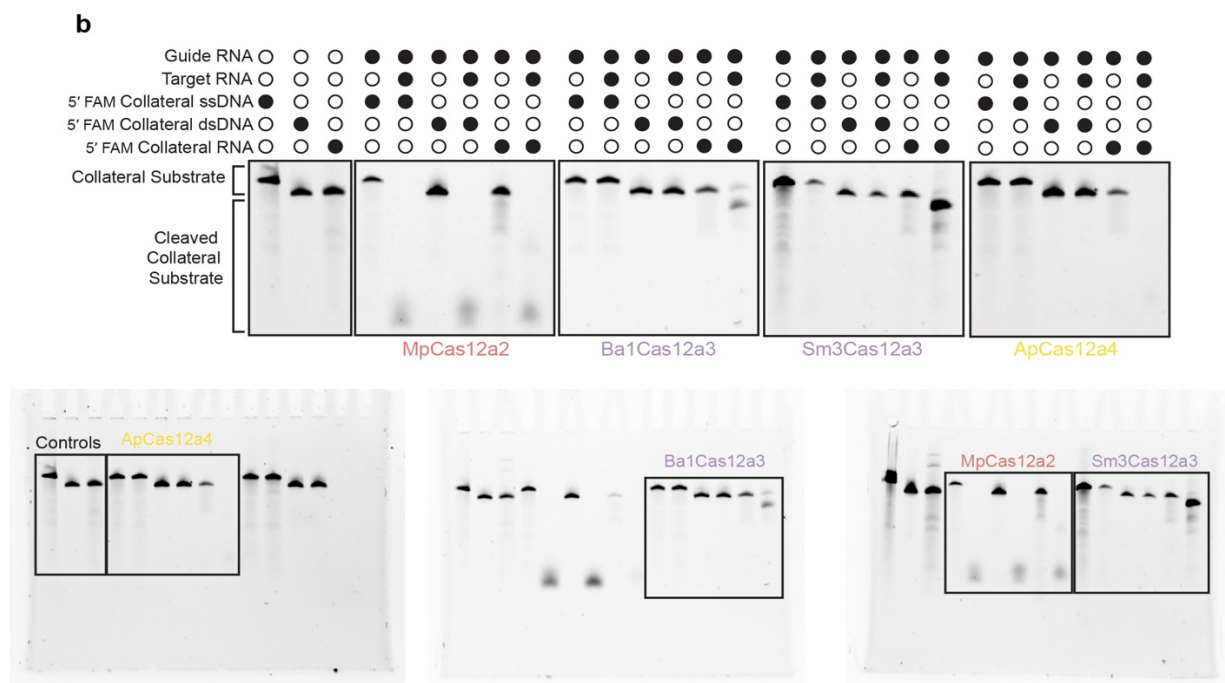
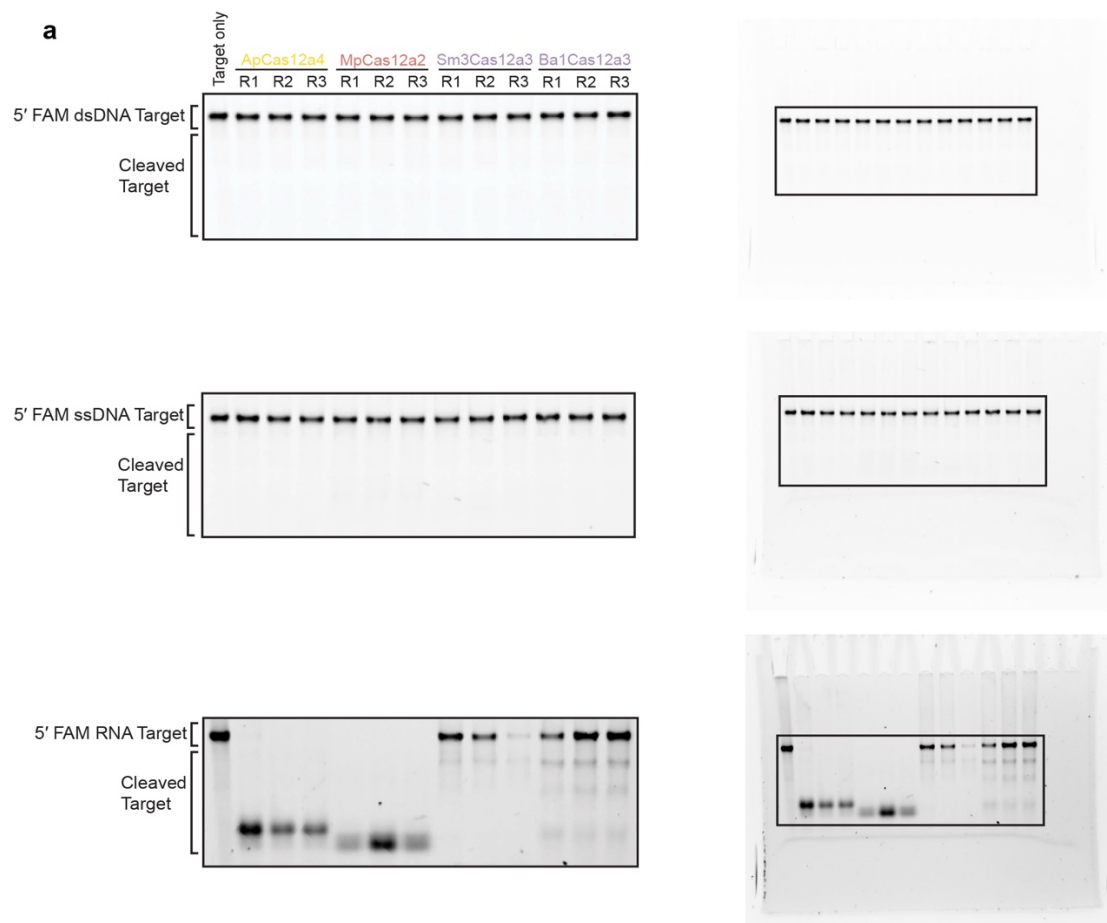
RNA-triggered Cas12a3 cleaves tRNA tails to execute bacterial immunity

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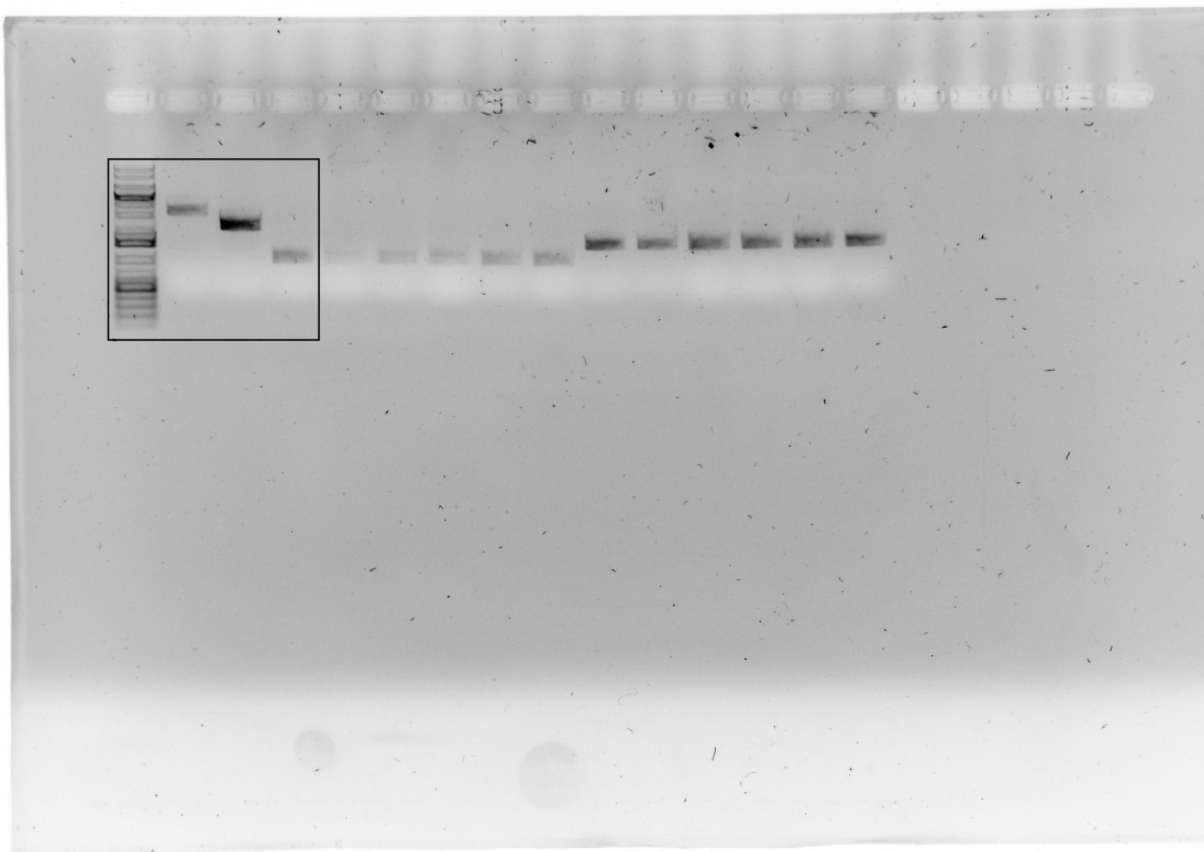
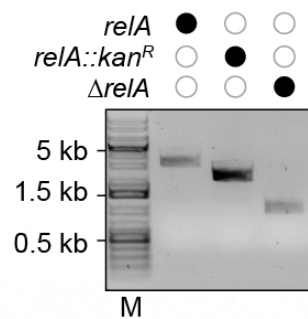


Supplementary Fig. 1. Original gel images cont'd. Original gel shown for Figure 3e.

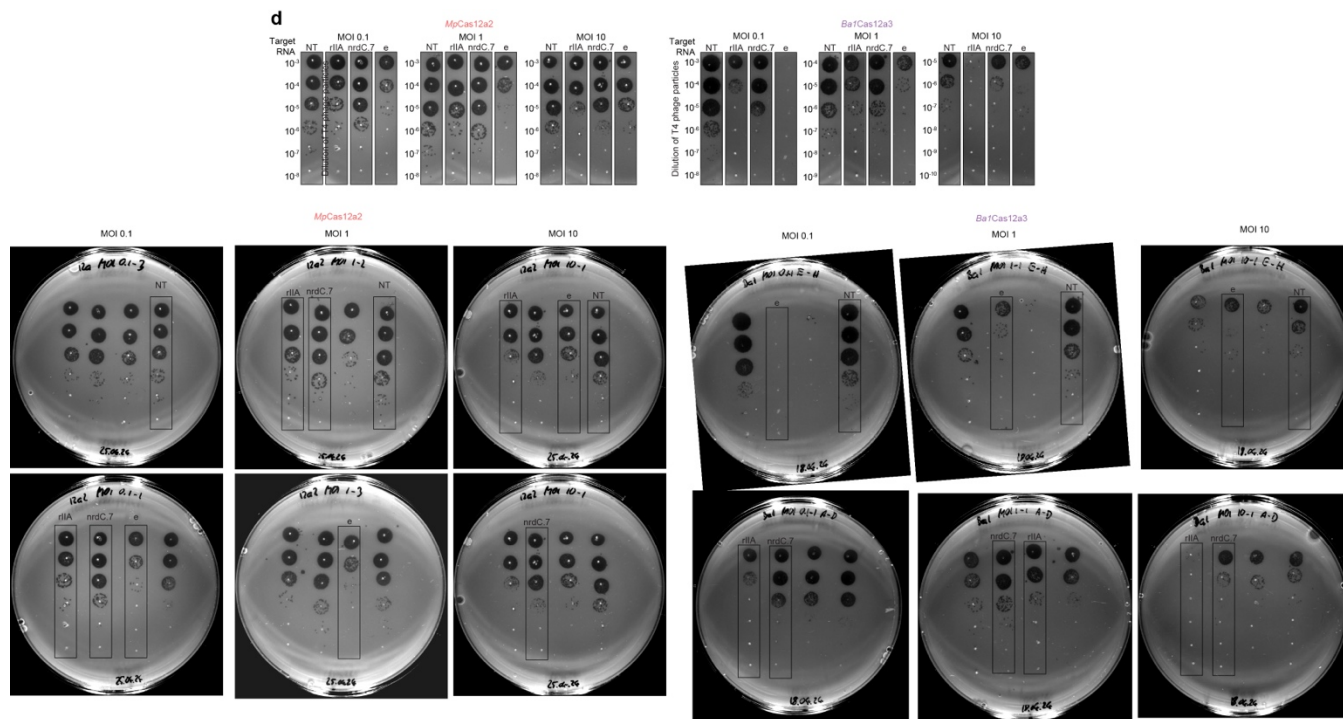




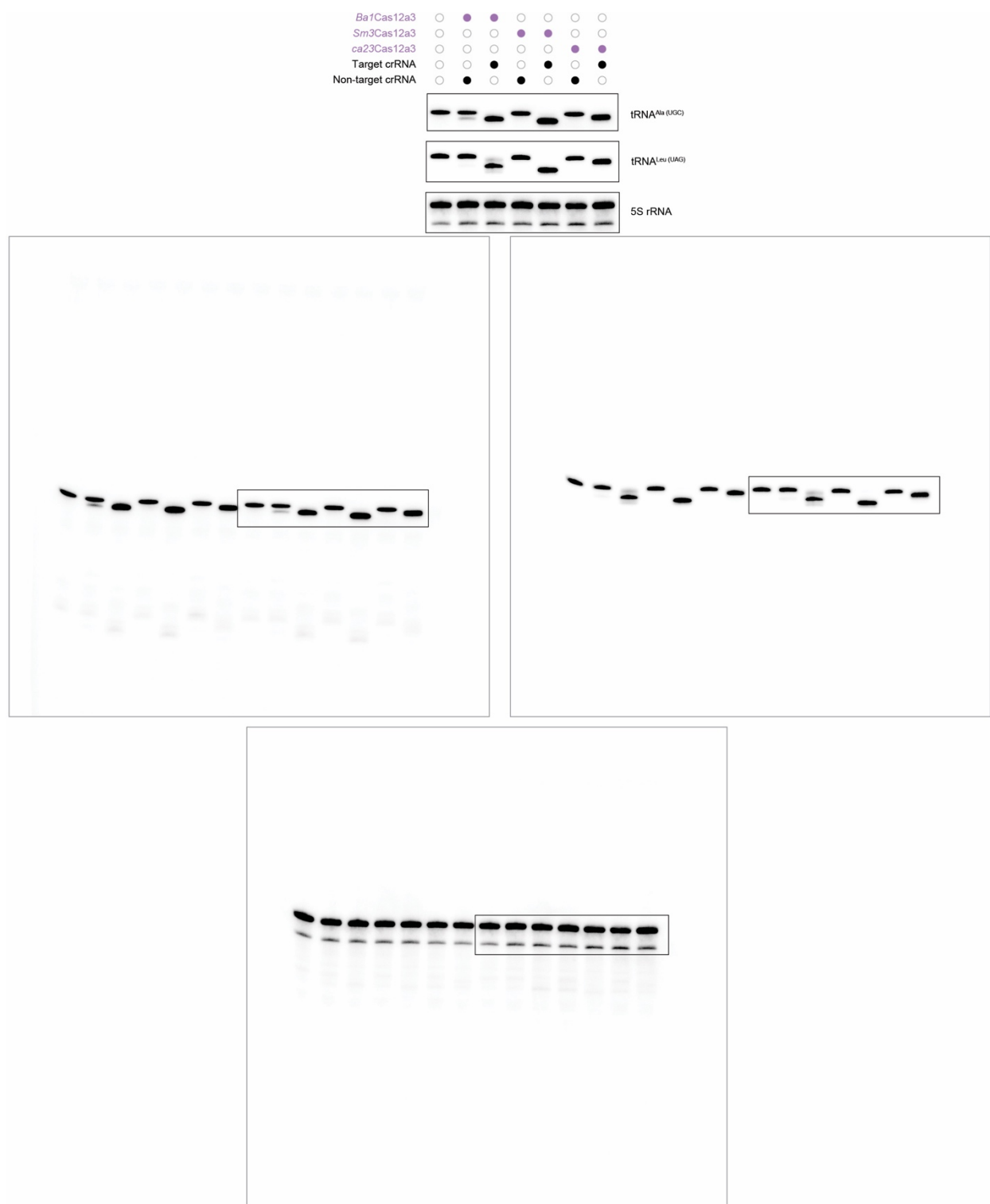
Supplementary Fig. 1. Original gel images cont'd. Original gels shown for Extended Data Figure 2a,b.



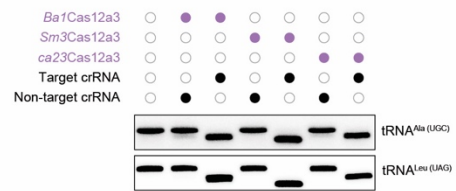
Supplementary Fig. 1. Original gel images cont'd. Original gels shown for Extended Data Figure 4a.



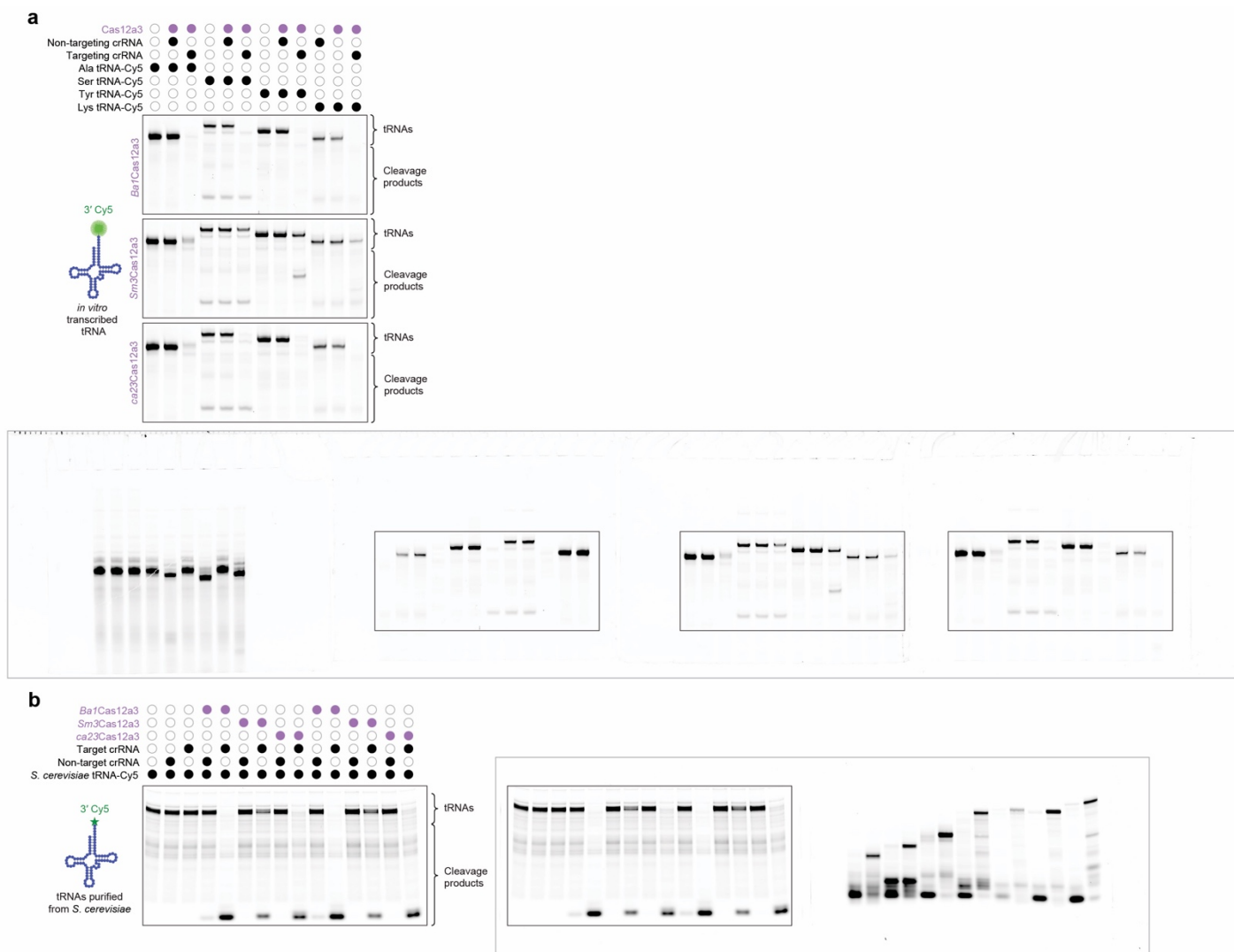
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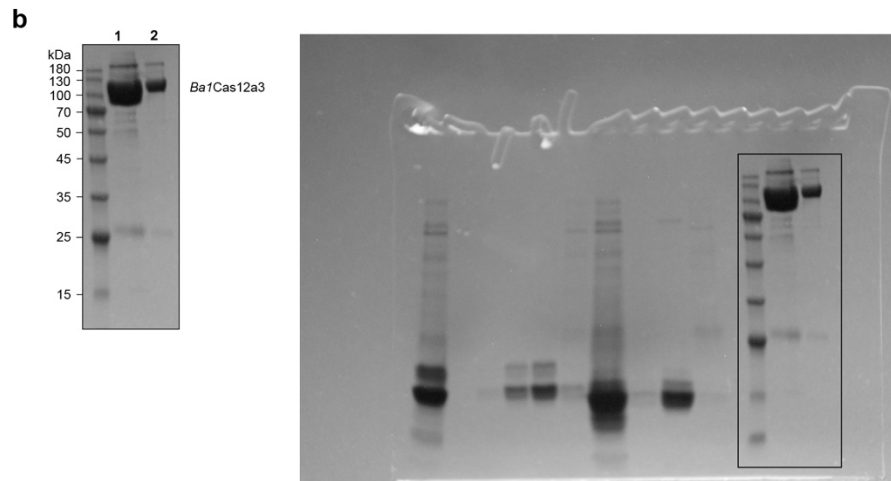
Supplementary Fig. 1. Original gel images cont'd. Original gels shown for Supplementary Figure 4b.



Supplementary Fig. 1. Original gel images cont'd. Original gels shown for Supplementary Figure 6.

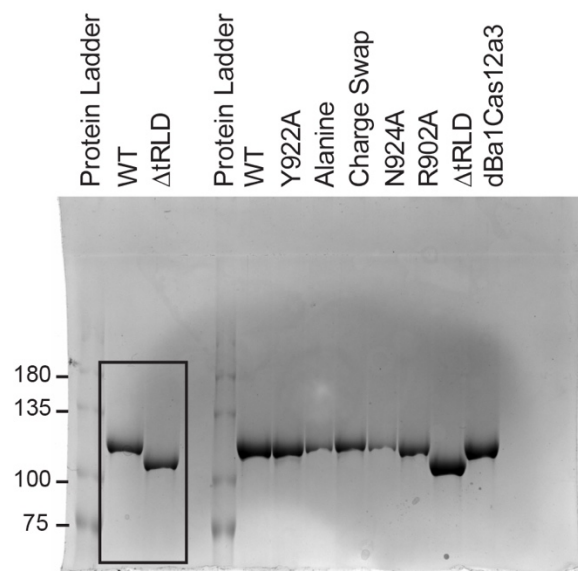
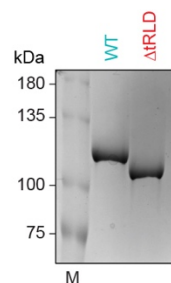


Supplementary Fig. 1. Original gel images cont'd. Original gels shown for Supplementary Figure 7a,b.

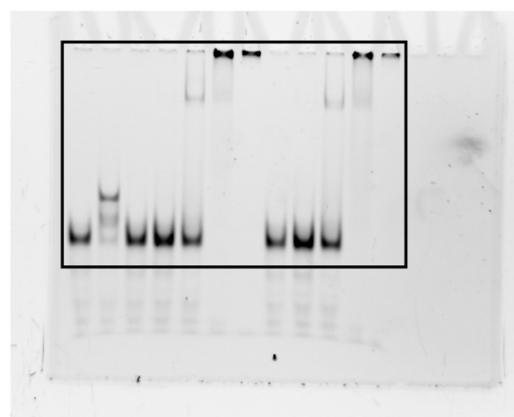
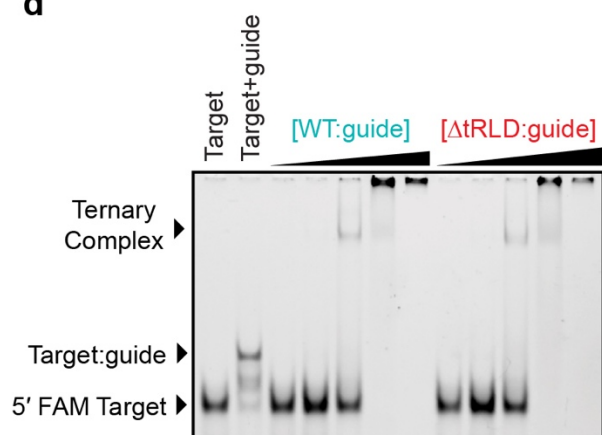


Supplementary Fig. 1. Original gel images cont'd. Original gels shown for Supplementary Figure 8b.

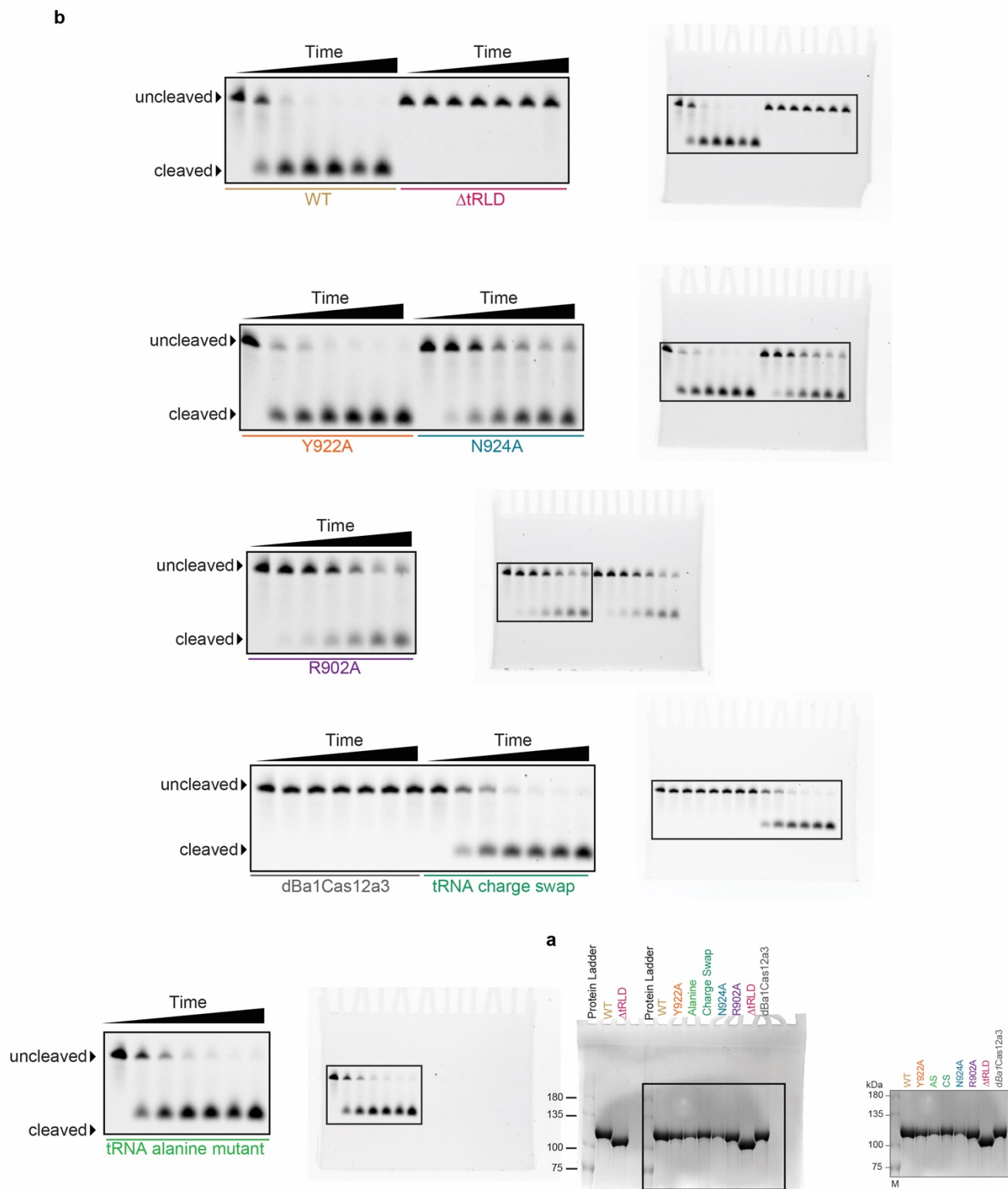
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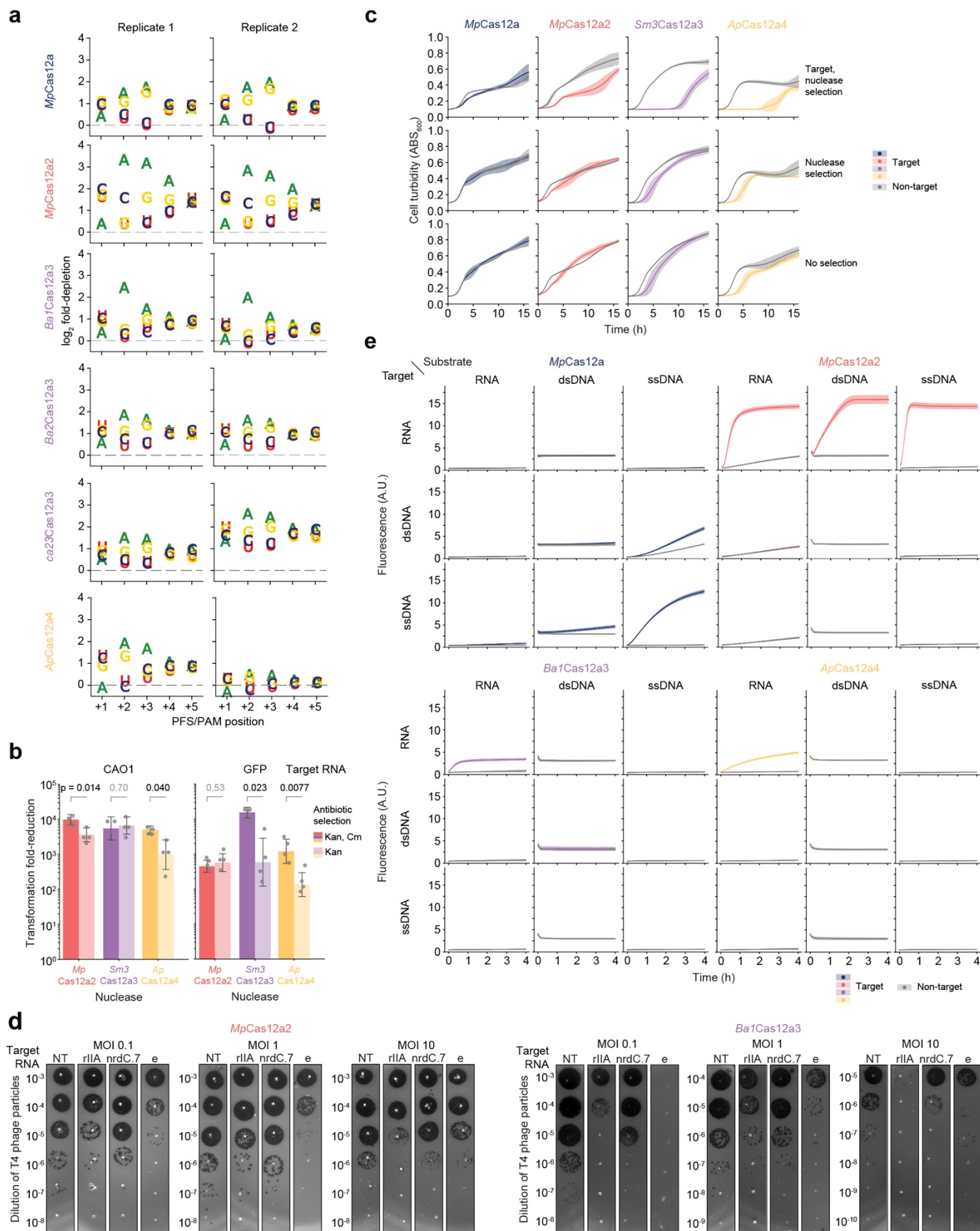
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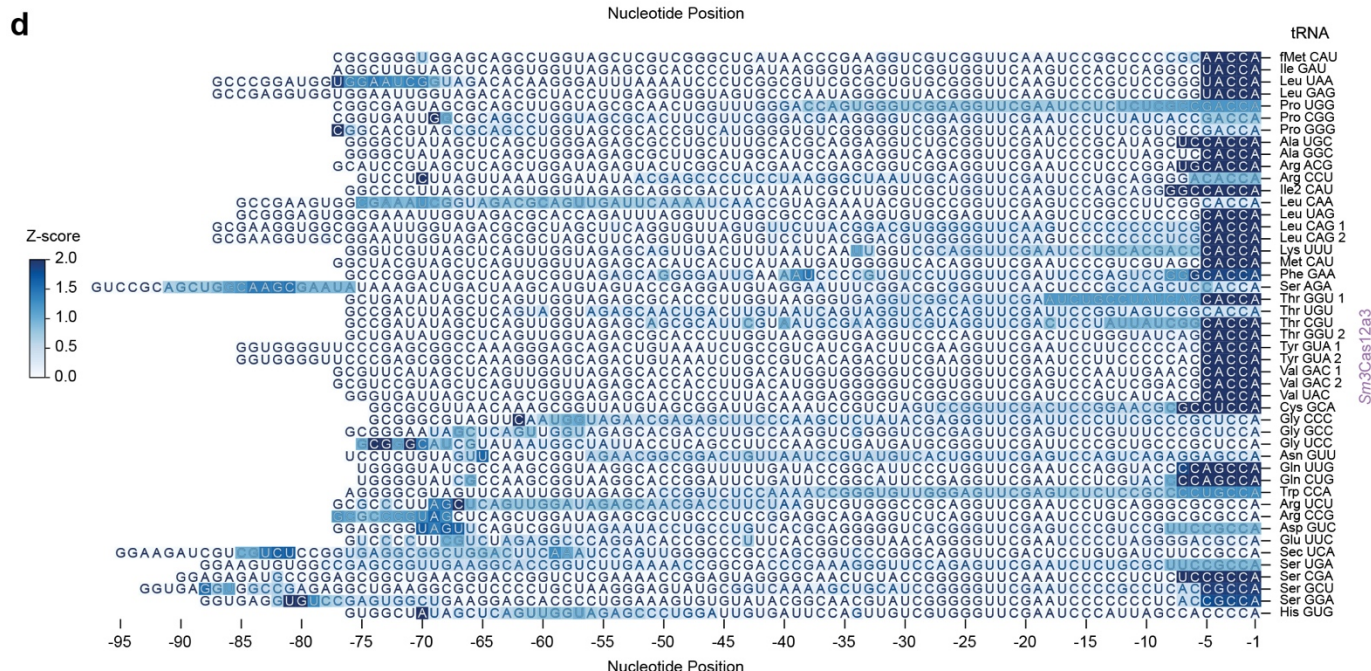
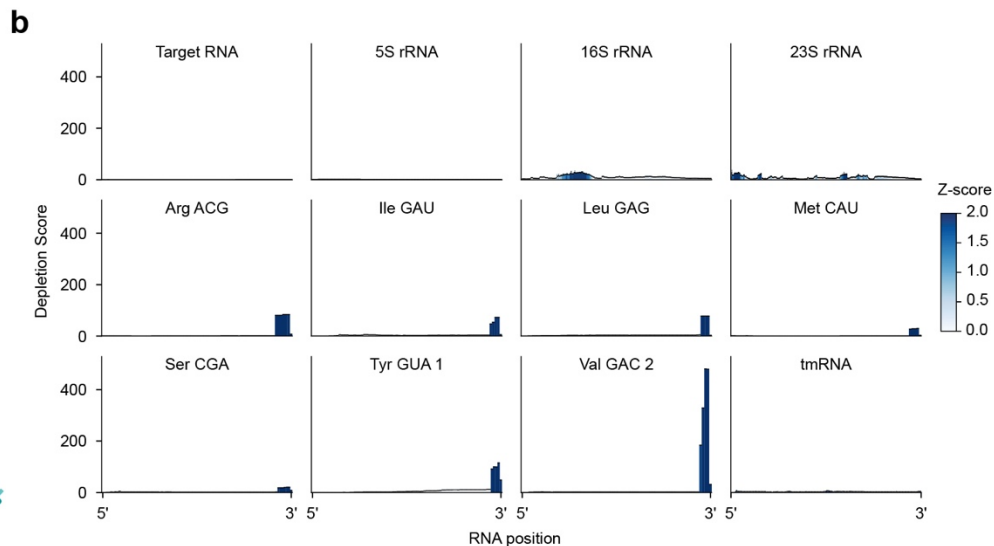
Supplementary Fig. 1. Original gel images cont'd. Original gels shown for Supplementary Figure 12b,d.



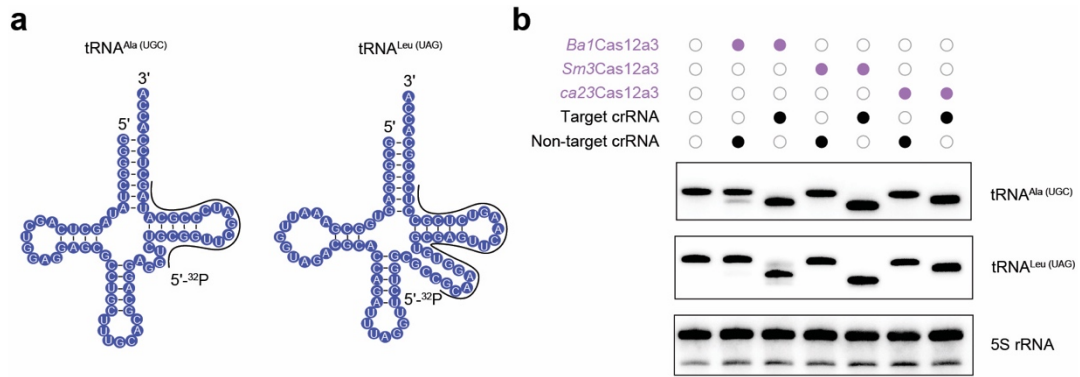
Supplementary Fig. 1. Original gel images cont'd. Original gels shown for Supplementary Figure 14a,b.



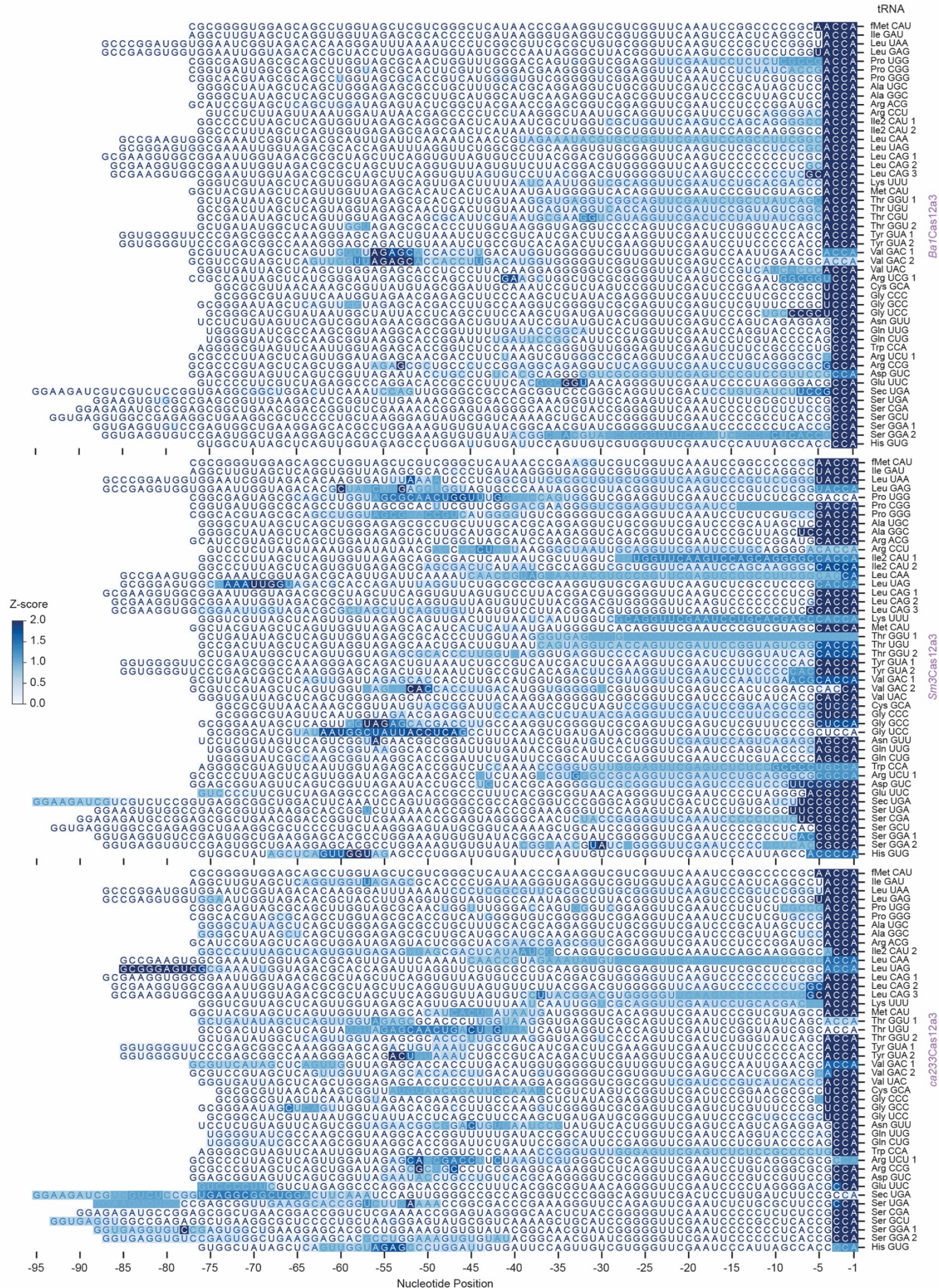
Supplementary Fig. 2. Extended characterization of Cas12a3 and Cas12a4 nucleases in *E. coli* and *in vitro*. (a) Nucleotide depletion plots following the screen of the 5-nucleotide PFS/PAM library. Note that the PAM for *MpCas12a* is the reverse complement of the sequence normally reported for Cas12a nucleases (*i.e.*, YYV). Each plot shows the mean of independent screens in *E. coli* ($n = 2$). The PFS screen for *Sm3Cas12a3* is not shown due to insufficient sequence coverage. (b) Plasmid interference assay in *E. coli* targeting *CAO1* and *GFP* transcripts. See **Figure 1d** for a diagram of the assay. Plasmid clearance and growth arrest are differentiated based on selection for the target plasmid. Bars and error bars represent the geometric mean $\pm$ geometric standard deviation of independent experiments starting from separate colonies, with gray dots representing each measurement ($n = 4$). (c) Growth curves (OD_{600}) of *E. coli* expressing each nuclease (*MpCas12a*, *MpCas12a2*, *Sm3Cas12a3*, and *ApCas12a4*), with either target or non-target crRNAs and target RNA. Expression was induced at $t = 0$ under the indicated selection conditions. Scatter plots with error bars represent the mean $\pm$ standard deviation of independent experiments ($n = 4$) started from separate colonies. (d) Protection conferred by *MpCas12a2* and *Ba1Cas12a3* against T4 phage in *E. coli*. Bacterial cultures of *E. coli* carrying nuclease expression plasmids, alongside plasmids encoding either a non-target (NT) crRNA or a crRNA targeting T4 transcripts, were infected with T4 at the indicated multiplicities of infection (MOIs) and grown overnight. The following day, the phages in these cultures were quantified with a plaque assay. The plaques illustrate the degree of protection conferred by the nucleases. Images are representative of independent experiments ($n = 3$) starting from separate colonies. (e) *In vitro* collateral substrate cleavage in response to different target types. Scatter plots and error bars show the mean $\pm$ standard deviation of independent reactions ($n = 3$ or 4). The corresponding initial linear rates, normalized per each nuclease, are shown in **Figure 1f**. Statistical analyses were performed using two-tailed Welch's t-tests. P-values that are not significant ($p \geq 0.05$) are shown in gray.



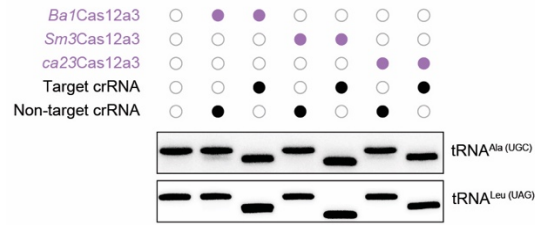
Supplementary Fig. 3. Direct RNA Nanopore sequencing of target RNA, rRNA, tmRNA, and tRNA under RNA-target and non-target conditions with *Ba1Cas12a3* and *Sm3Cas12a3* in TXTL. (a) Schematic of the RNA sample preparation protocol for direct RNA sequencing via Nanopore, using tRNA as an example. (b) Plots showing sequencing coverage depletion scores of selected rRNA, tRNA, and tmRNA at each nucleotide position under non-target (NT) versus target (T) conditions with *Sm3Cas12a3*. Data represent averages from independent TXTL experiments ($n = 3$), in which RNAs were barcoded with WDX adapters for sequencing and demultiplexed following high-fidelity basecalling. The color map indicates the Z-score (number of standard deviations from the mean) of depletion scores at each position. (c) tRNA sequences identified from independent TXTL experiments ($n = 3$) with *Ba1Cas12a3* under NT and T conditions, showing NT/T depletion Z-scores for each nucleotide as a color map. (d) tRNA sequences identified from independent TXTL experiments ($n = 3$) with *Sm3Cas12a3* under NT and T conditions, likewise displayed as NT/T depletion Z-scores at each nucleotide position.



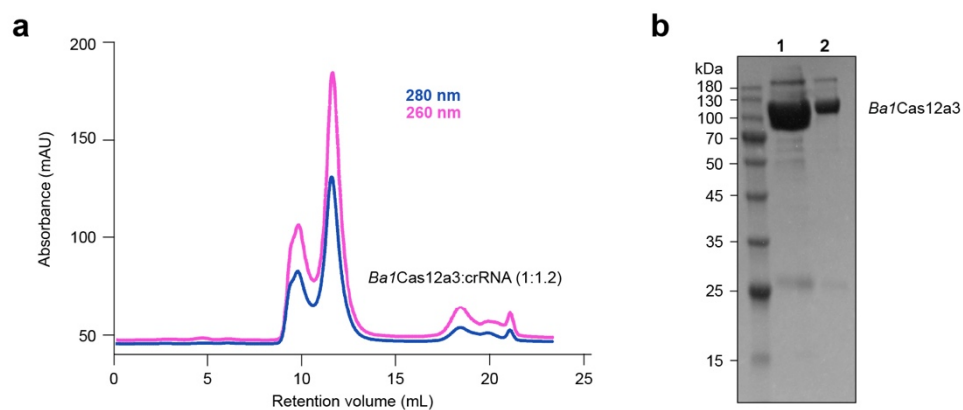
Supplementary Fig. 4. Northern blotting analysis of selected tRNAs cleaved with Cas12a3 in TXTL. **(a)** Probing of tRNA^{Ala}(UGC) and tRNA^{Leu}(UAG) by northern blotting analysis. The binding location of the oligonucleotide probes are indicated. **(b)** Blots for the two probed tRNAs as well as 5S rRNA. The indicated Cas12a3 RNP was incubated in TXTL, and the extracted total RNA was subjected to northern blotting analysis. Gel images are representative of independent biological replicates (n = 3). For gel source data, see Supplementary Figure 1.



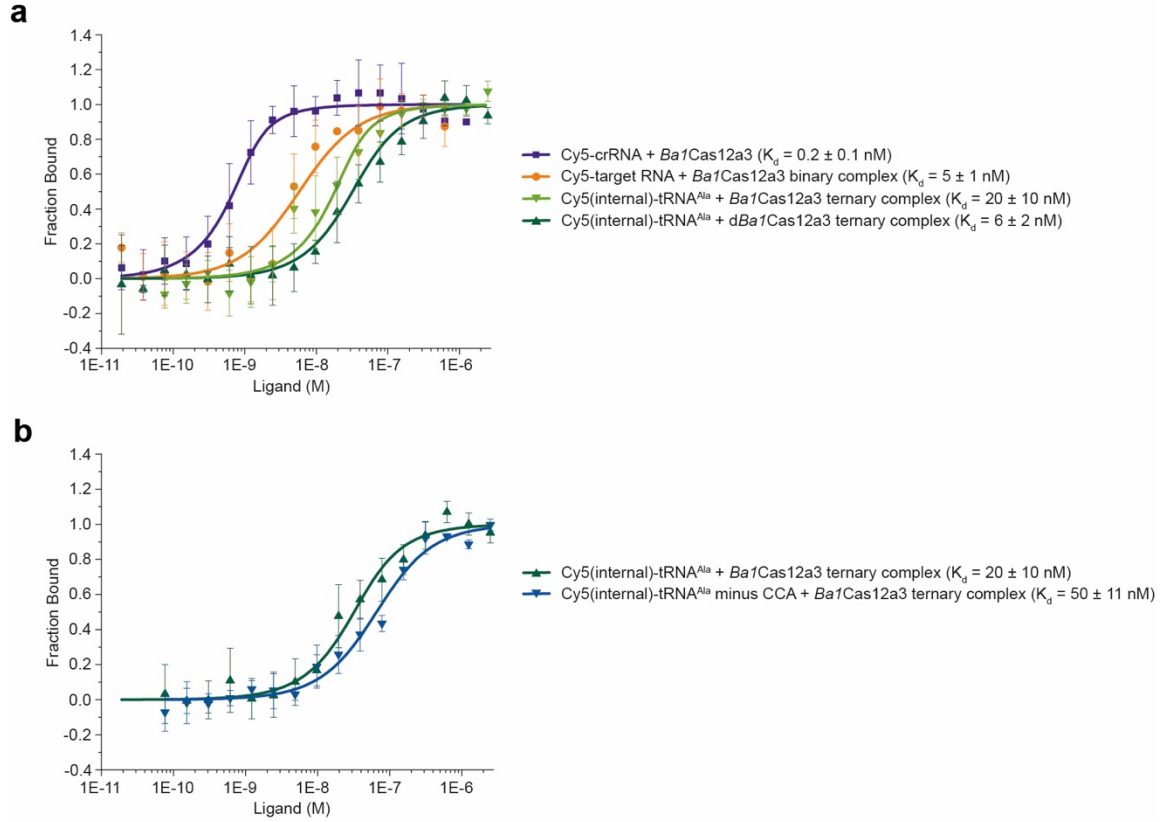
Supplementary Fig. 5. Direct RNA Nanopore sequencing of purified tRNA from *E. coli* MRE600 under RNA-target and non-target conditions with *Ba1Cas12a3* and *Sm3Cas12a3* *in vitro*. Only the tRNA sequences (labeled with the corresponding anticodons) identified in independent *in vitro* experiments are shown (n = 3). The color map shows NT/T depletion Z-scores for each nucleotide position.



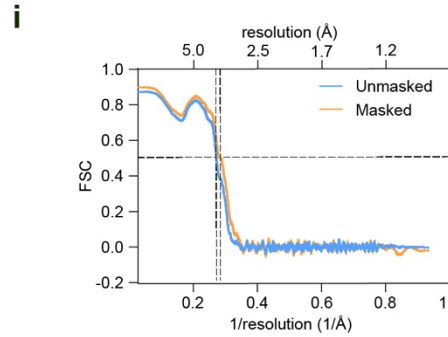
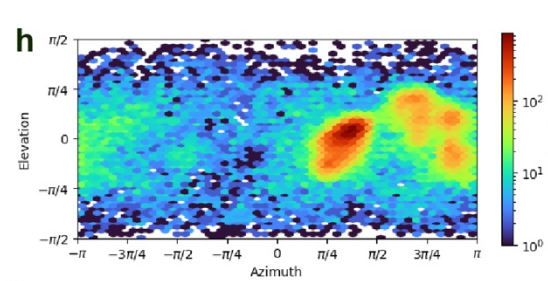
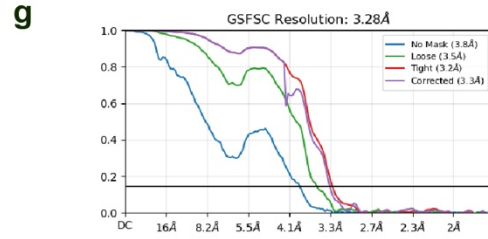
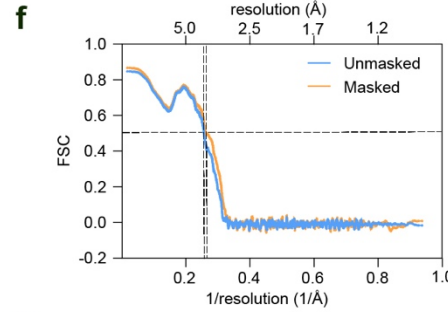
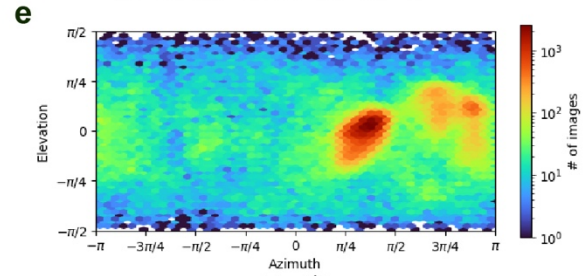
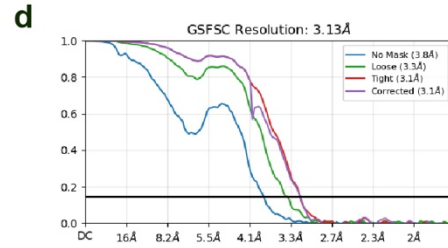
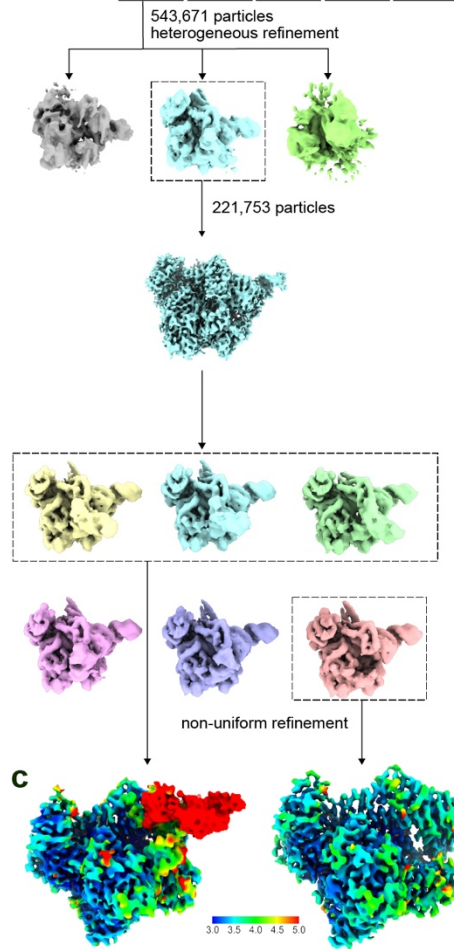
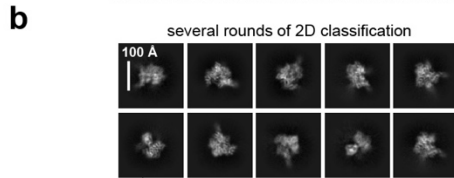
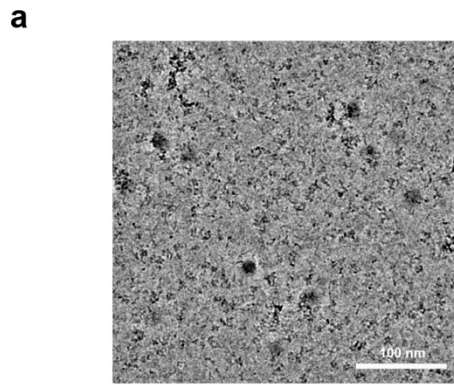
Supplementary Fig. 6. Northern blotting analysis of selected tRNAs cleaved with Cas12a3 *in vitro*. Blots for the two probed tRNAs as well as 5S rRNA. The indicated Cas12a3 RNP was incubated with purified *E. coli* tRNAs *in vitro*, and the extracted total RNA was subjected to northern blotting analysis. Gel images are representative of independent biological replicates (n = 3). See Supplementary Fig. 4a for the probe locations. For gel source data, see Supplementary Figure 1.



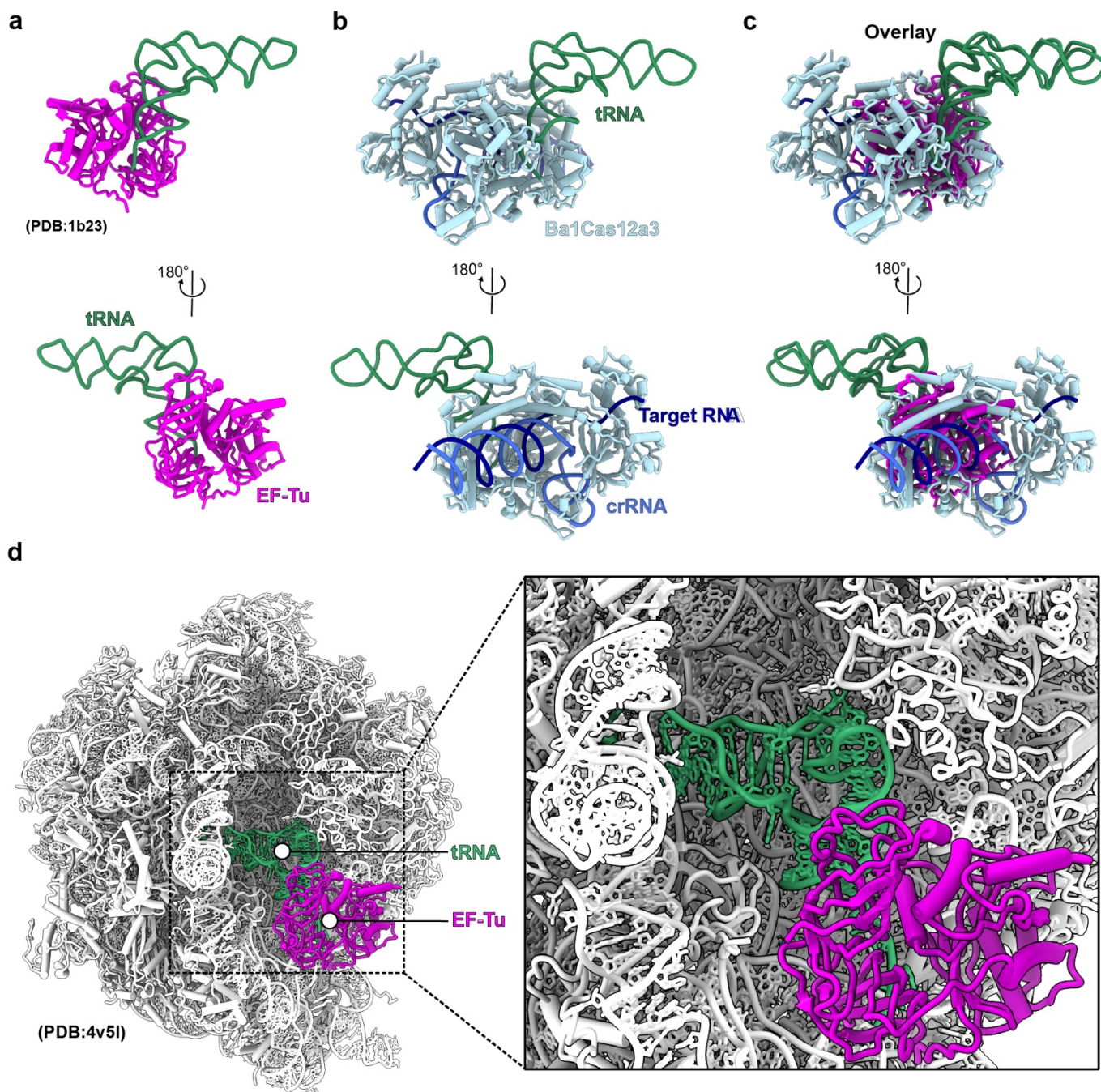
Supplementary Fig. 8. Sample preparation for *Ba1Cas12a3* binary complex. (a) Size-exclusion chromatography (SEC) profile of *Ba1Cas12a3* in the presence of crRNA at a molar ratio of 1:1.2. (b) SDS-PAGE of purified *Ba1Cas12a3* binary complex. Lane 1: Sample before SEC; lane 2: Peak fraction from SEC. For gel source data, see Supplementary Figure 1.



Supplementary Fig. 9. Microscale thermophoresis (MST) measurements of *Ba1Cas12a3* binding to crRNA, target RNA, and tRNA^{Ala}(UGC). (a) MST-based quantification of *Ba1Cas12a3* binding to a targeting crRNA, target RNA, and tRNA^{Ala}(UGC). d*Ba1Cas12a3* contains the E1065A mutation in the RuvC endonuclease domain to render it catalytically inactive. (b) *Ba1Cas12a3* binding of tRNA^{Ala}(UGC) with and without the 3' CCA tail. Symbols and error bars represent the mean $\pm$ standard deviation of independent experiments ($n = 3$).

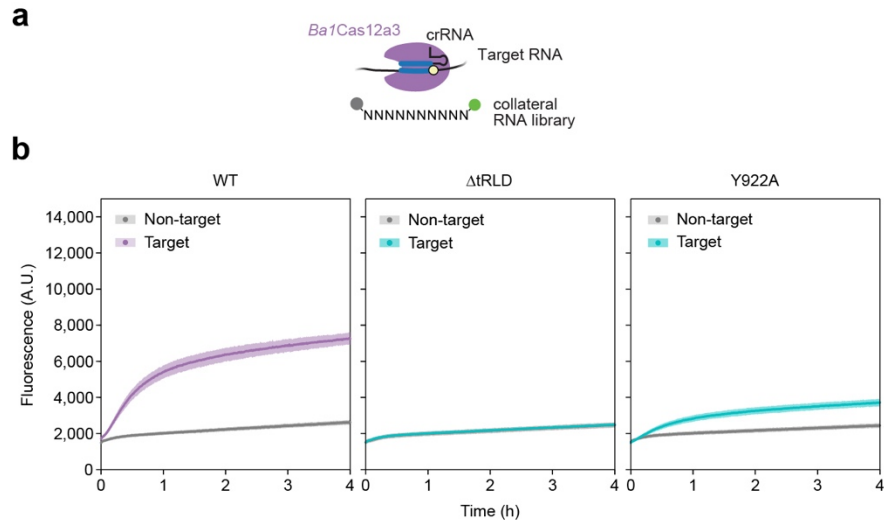


Supplementary Fig. 10. Data processing scheme of *Ba1Cas12a3* quaternary complexes. (a) Representative cryo-EM micrograph of *Ba1Cas12a3* quaternary complexes. (b) Data processing scheme for *Ba1Cas12a3* quaternary complexes at pre-cleavage and post-cleavage stages. (c) Cryo-EM structure of *Ba1Cas12a3* quaternary complexes colored by local spatial resolution in Å (left panel: pre-cleavage stage; right panel: post-cleavage stage). (d) Gold standard Fourier Shell Correlation (FSC) curves for *Ba1Cas12a2* quaternary complex at pre-cleavage stage. (e) Euler plot for *Ba1Cas12a3* quaternary complex at pre-cleavage stage. (f) Map-to-model FSC curves for *Ba1Cas12a3* quaternary complex at pre-cleavage stage. (g) FSC curves for *Ba1Cas12a2* quaternary complex at post-cleavage stage. (h) Euler plot for *Ba1Cas12a3* quaternary complex at the post-cleavage stage. (i) Map-to-model FSC curves for *Ba1Cas12a3* quaternary complex at post-cleavage stage.

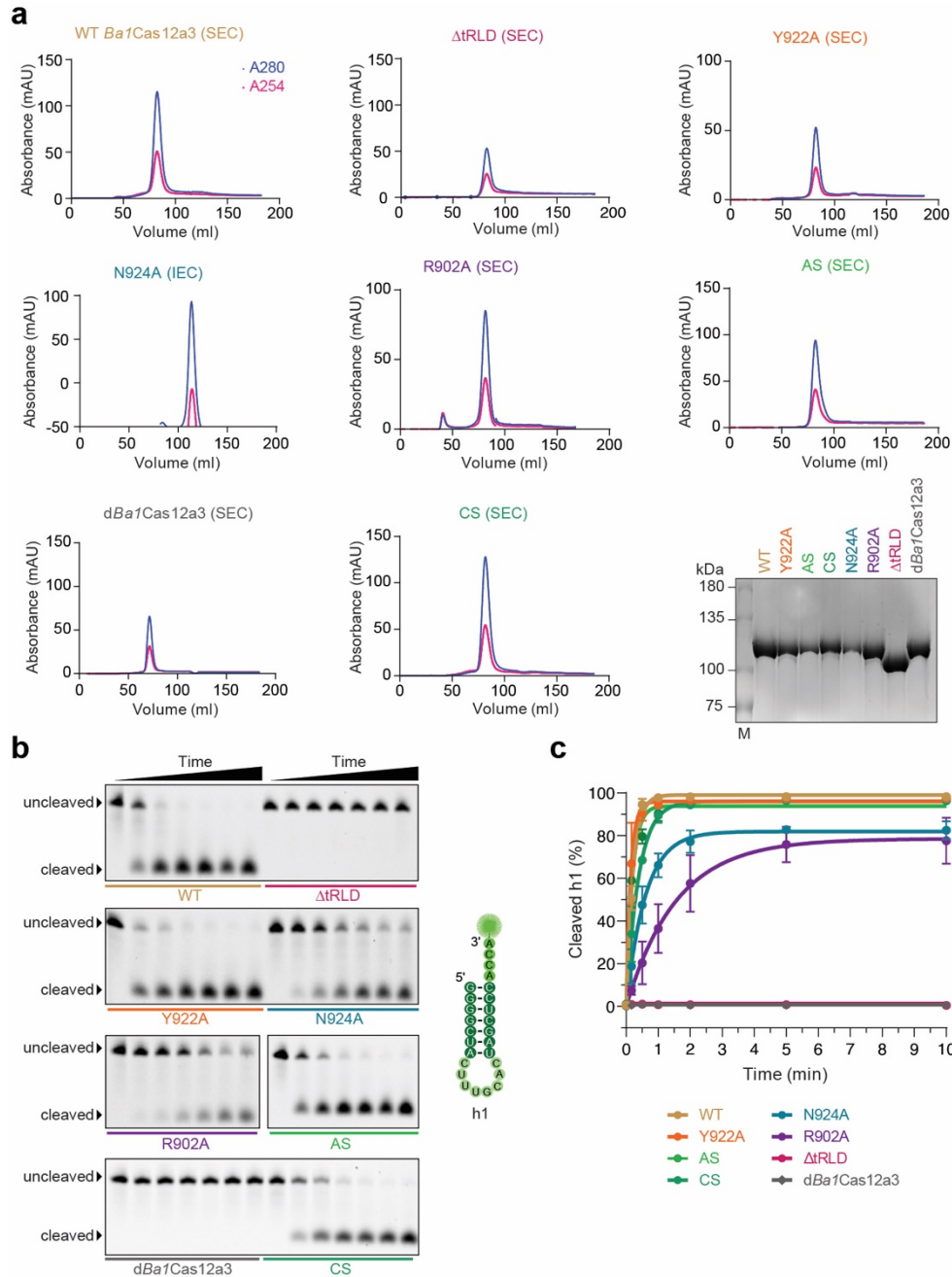


Supplementary Fig. 11. Structural comparison between the *Ba1Cas12a3* quaternary complex and the EF-Tu-tRNA complex. (a) Structure of the EF-Tu-tRNA complex (PDB ID: 1b23). EF-Tu is shown in magenta and tRNA in green. **(b)** Structure of the *Ba1Cas12a3* quaternary complex. *Ba1Cas12a3* is shown in light blue, tRNA in green, crRNA in royal blue and the target RNA in dark blue. **(c)** Superimposition of the *Ba1Cas12a3* quaternary complex and the EF-Tu-tRNA complex, showing massive steric clashes between both proteins. The structures

were aligned on tRNA. The structures were colored as in panels a and b. **(d)** Structure of EF-Tu-tRNA bound to the ribosome (PDB ID: 4v5l), showing the tRNA being deeply buried inside the ribosome.

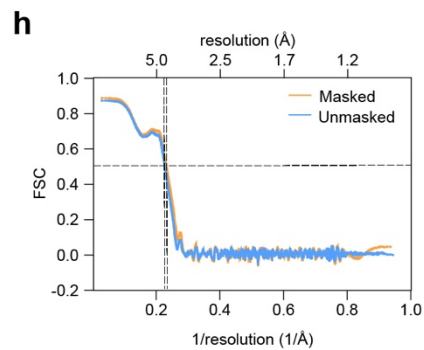
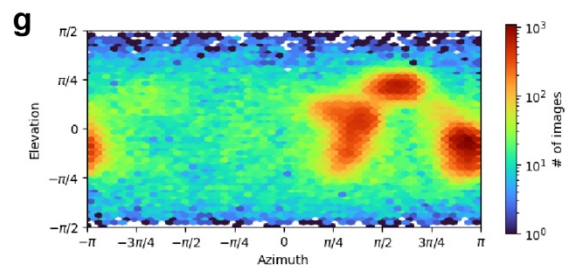
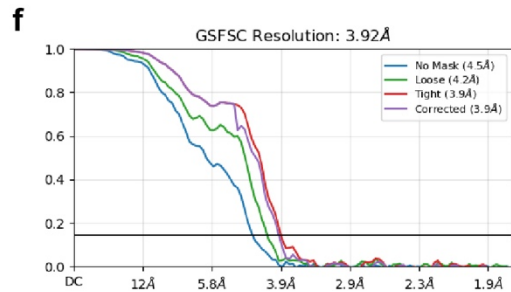
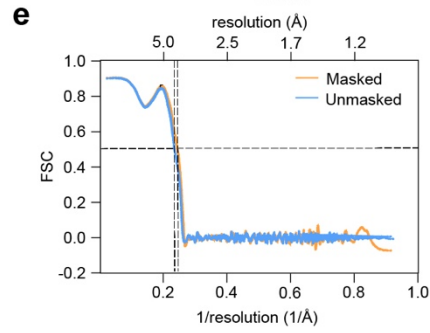
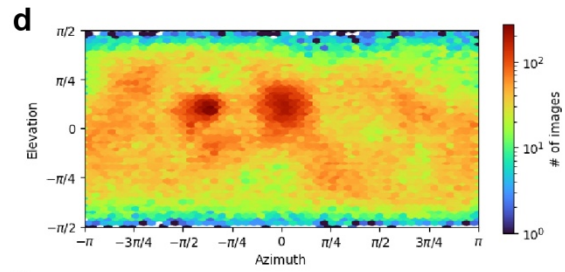
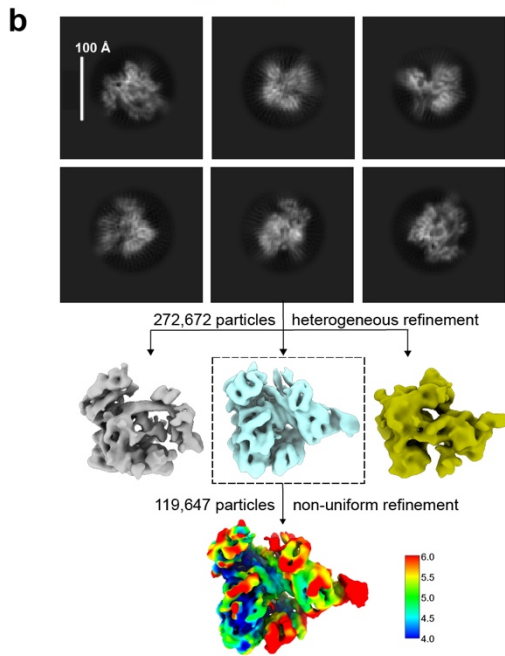
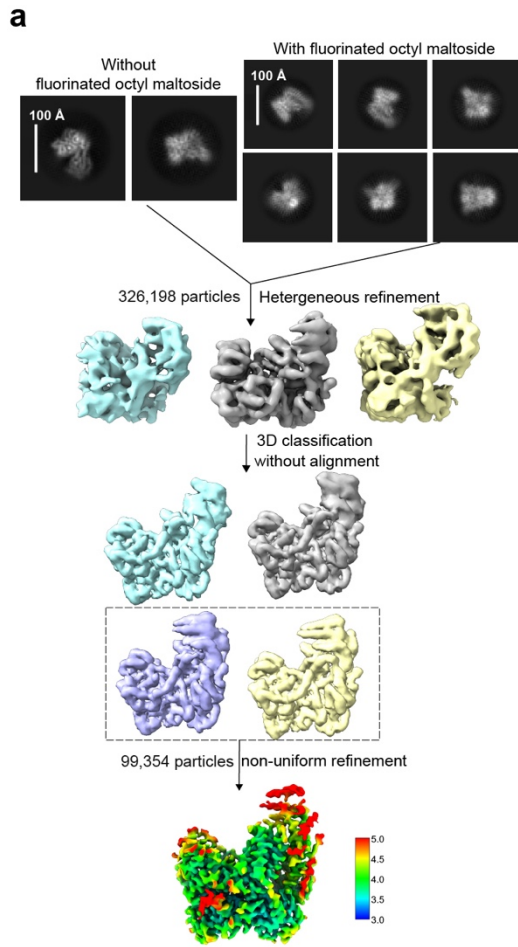


Supplementary Fig. 13. Assessing *in vitro* cleavage of the collateral RNA library by the *Ba1Cas12a3* mutants Δ tRLD and Y922A. (a) General setup to monitor cleavage of the collateral RNA library by *Ba1Cas12a3*. **(b)** Fluorescence time-course measurements using a target or non-target RNA. Dotted curves and shaded regions represent the mean $\pm$ standard deviation of independent cleavage reactions ($n = 3$ or 4).

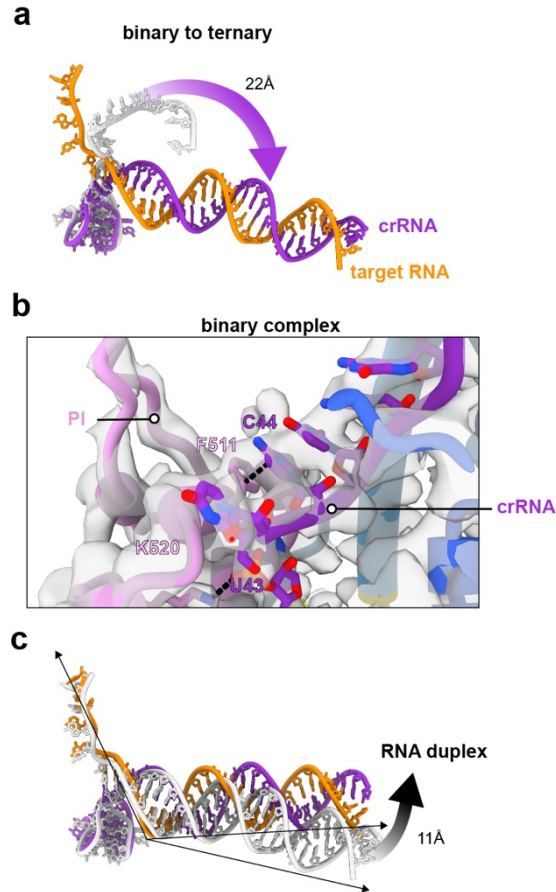


Supplementary Fig. 14. *Ba1Cas12a3* tRLD and T-arm interacting mutants cleave tRNA mimics at different rates. (a) Size exclusion and ion exchange chromatograms and SDS-PAGE of the *Ba1Cas12a3* mutants. AS: alanine swap, R251A/N253A/K256A/K257A; CS: charge swap, R251E/N253D/K256E/K257E; *dBa1Cas12a3*, D712A/E1032A/D1137A. M, marker. **(b)** Collateral cleavage time course assay with the tRNA^{Ala} mimic (h1). **(c)** The percent of h1 cleavage determined using gel densitometry of three independent replicates represented in panel **b**. The rate constants (k_{obs}) reported in **Figure 3f** were determined by fitting the curves of individual

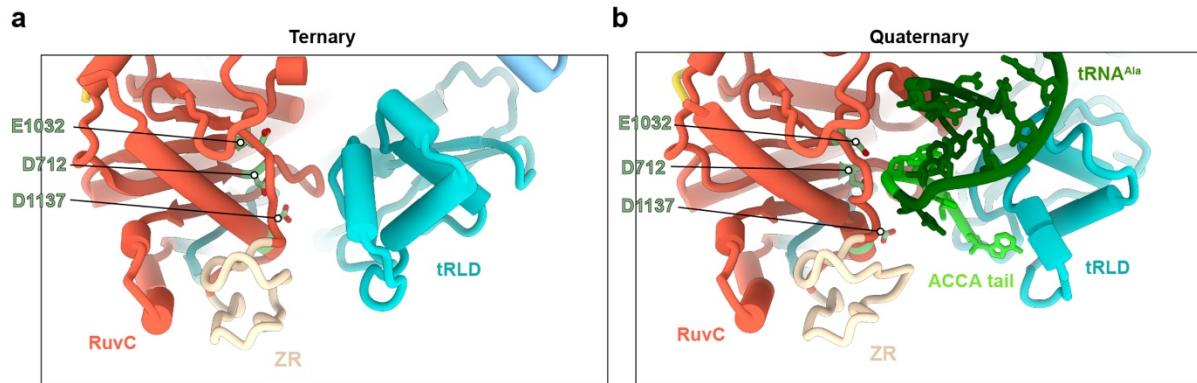
experiments using a one-phase association pseudo-first order equation. Circles and error bars represent the mean $\pm$ standard deviation of independent experiments ($n = 3$). For gel source data, see Supplementary Figure 1.



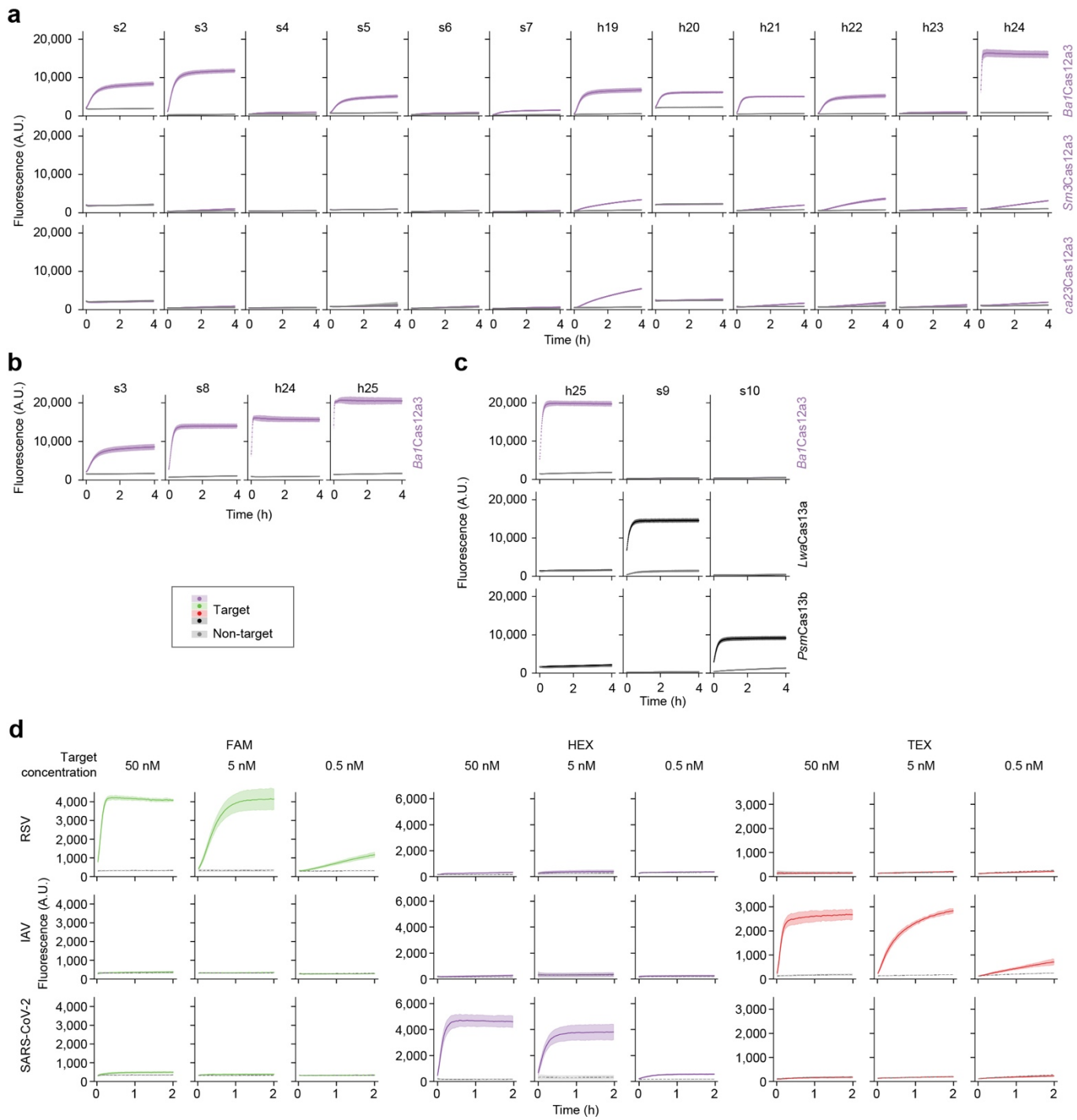
Supplementary Fig. 15. Data-processing scheme of *Ba1Cas12a3* binary and ternary complexes. (a) Data processing flow-chart of *Ba1Cas12a3* binary complex. W/O:without; FOM: fluorinated octyl maltoside. (b) Data processing flow-chart of *Ba1Cas12a3* ternary complex. 3D heterogeneous refinement separated the dataset into three particle classes (43,544; 119,647; and 109,481 particles, left to right). Only the middle class displayed well-defined guide-target RNA duplex densities, indicative of a fully assembled ternary complex. Subsequent non-uniform refinements in cryoSPARC produced a better-resolved EM map exclusively for the middle class, enabling reliable model building. (c) FSC curves for *Ba1Cas12a3* binary complex. (d) Euler plot for *Ba1Cas12a3* binary complex. (e) Map-to-model FSC curves for the *Ba1Cas12a3* binary complex. (f) FSC curves for the *Ba1Cas12a3* ternary complex. (g) Euler plot for *Ba1Cas12a3* ternary complex. (h) Map-to-model FSC curves for *Ba1Cas12a3* ternary complex.

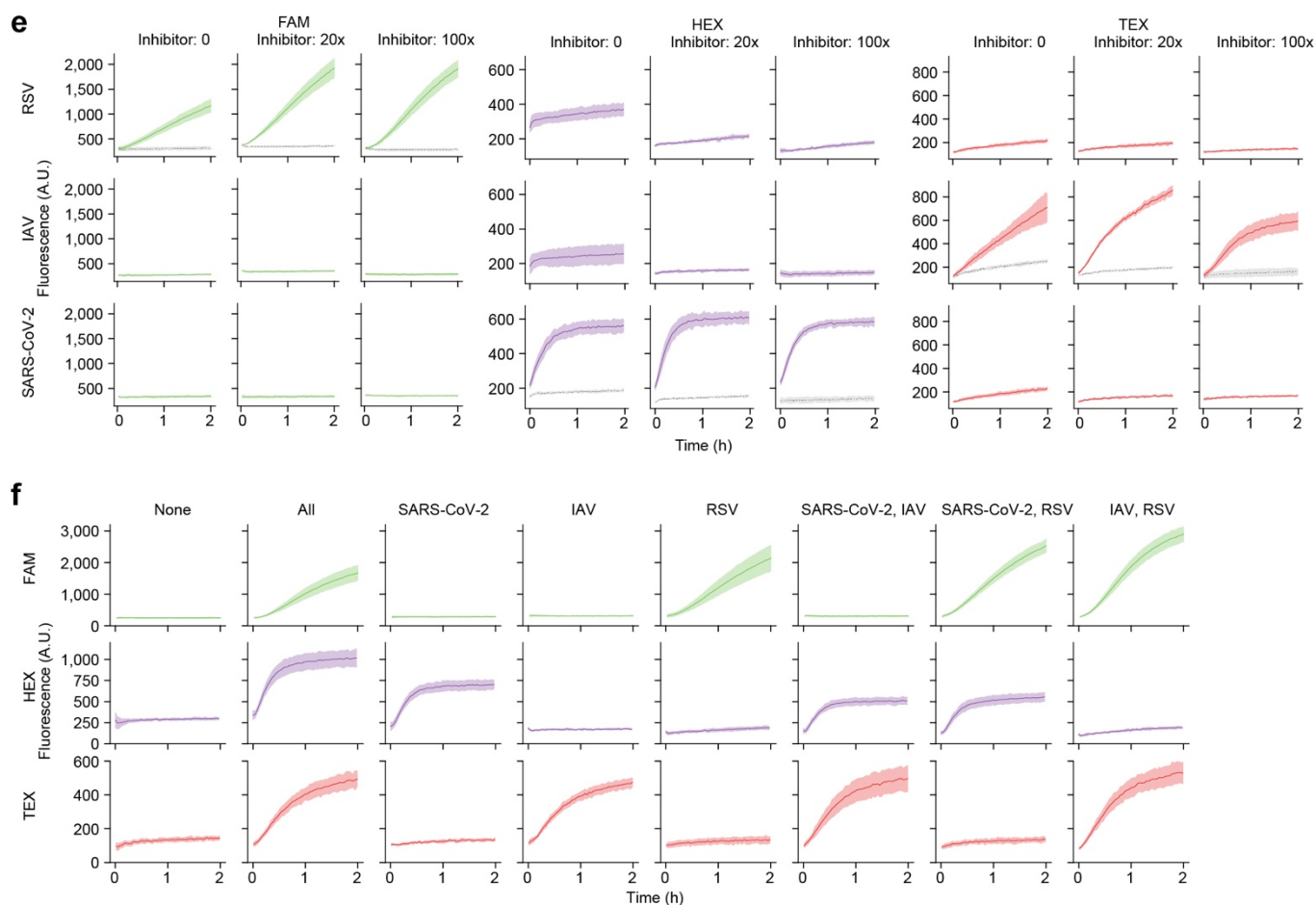


Supplementary Fig. 16. crRNA and target RNA rearrangement during *Ba1Cas12a3* nuclease activation. (a) Detailed interactions between the PI domain of *Ba1Cas12a3* and crRNA. (b) 3' of crRNA released from the PI domain forming a guide-target RNA duplex with 5' target RNA after target RNA recognition. (c) Guide-target RNA duplex shifted up to 11 Å after tRNA loading.



Supplementary Fig. 17. Zoomed-in view of the RuvC and tRLD domains before and after tRNA binding. (a) Close proximity of tRLD and RuvC domains before tRNA loading. The three catalytic residues responsible for substrate cleavage are shown. **(b)** Separation of tRLD and the RuvC domain upon tRNA binding.





Supplementary Fig. 18. Fluorescence formation over time from the cleavage of tRNA-mimic reporters shown in Figure 5. (a) Time-course of the fluorescence signal generated by cleavage of FAM-FQ-labeled single-stranded (s2–7) and hairpin (h14–19) reporters by *Ba1Cas12a3*, *Sm3Cas12a3*, and *ca23Cas12a3* nucleases under target and non-target conditions *in vitro*. Scatter plots with error bars represent the mean $\pm$ standard deviation of independent experiments ($n = 4$). The corresponding initial fluorescence formation rates, normalized to each nuclease and the rates calculated as a function of nuclease concentration, are shown in **Figure 5a**. **(b)** Time-course of the fluorescence signal associated with **Figure 5b** ($n = 3$ or 4). **(c)** Time-course of the fluorescence signal associated with **Figure 5c** ($n = 4$). **(d)** Time-course of the fluorescence signal associated with **Figure 5e** ($n = 4$). **(e)** Time-course of the fluorescence signal associated with **Figure 5f** ($n = 4$). **(f)** Time-course of the fluorescence signal associated with **Figure 5g** ($n = 6$).