Supplementary Information for:

Retron Eco2 breaks tRNAs for antiphage defense

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Content:

Source data for this manuscript include:

Source Data 1: Maximum Likelihood phylogenies of RT-TOPRIM fused sequences type I-C, RT-TOPRIM fused sequences with XI and XII type sequences, RT-only sequences, TOPRIM-only sequences.

Source Data 2: Sequence alignments used for estimating Maximum Likelihood phylogenies.

Source Data 3: Annotated code for Maximum Likelihood phylogenetic estimations and HMM profile searches.

Source Data 4: HMM profiles.

Source Data 5: HDX-MS results.

Source Data 6: MS results.

Materials and Methods

Phylogenetic analysis of Eco2

We retrieved the RT-TOPRIM sequences listed in table S1 in Rodriguez-Mestre *et al.*, [1] as subtypes I-C1, I-C2, I-C3, XI, and XII, directly from NCBI, UniParc, and PATRIC. We increased sequence diversity by retrieving protein sequences predicted by Millman *et al.*, [2] as "RT/Predicted OLD family endonuclease (TOPRIM) fusion", which we download from the Integrated Microbial Genomes (IMG) system (Chen *et al.*) [3]. Additionally, we ran a blastp search querying the RT-TOPRIM sequence obtained by this study, excluding *E. coli* from the subject database, and requesting 1,000 maximum target sequences. We retrieved a total of 1,003 sequences from the databases, accessed between November 2024 and January 2025.

To reduce redundancy of sequence diversity, we clustered all sequences into 412 clusters using CD-HIT v4.8.1 [4] and a 70% sequence identity threshold. We then aligned the representative sequences from each cluster using MAFFT v7.525 G-INS-I algorithm [5]. We evaluated alignments by eye using JalView v.2.11.4 [6] and Geneious vR11 (https://www.geneious.com). We use IQtree v2.3.6 [7] to infer the maximum Likelihood phylogenies of concatenated RT-TOPRIM and separated RT from TOPRIM alignments (for the tanglegram), using Blosum62 for the amino acid substitution model. We visualized the phylogenies using the python library baltic v0.3.0 (https://github.com/evogytis/baltic).

We used HMMER profiles to predict and confirm the presence of RT-like and TOPRIM-like domains in sequences of the I-C, XI, and XII types. We downloaded the alignments for the RT-TOPRIM fused domain, RT-like, TOPRIM-like, TIR-like, and protease-like domains from NIH's Conserved Domains Database (CDD; https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml; last used on January 2025). We used the commands hmmbuild and hmmsearch from HMMER v3.4 (http://hmmer.org/) to build the profiles and query the 412 sequences used in this study, respectively. The code for all analyses and visualizations is available and commented in the supplementary material file ec67-seq-analysis.html.

BASEL phage plaque assay screen

E. coli MG1655ΔRM [8] were transformed either with the empty control vector pACYC184 (NEB) or the Eco2-carrying plasmid pLG006 [9] (Addgene ID:157884) and plated on LB-agar-

chloramphenicol (Cm, 30 µg/mL), prior to incubation overnight at 37 °C. 4 mL LB-Cm cultures were subsequently inoculated and incubated for 16 h at 37 °C to prepare overnight cultures for double-layer agar plaque assays. Double-layer agar plates were prepared using 1.5% agar LB-Cm (Cm, 30 µg/mL) for the bottom layer and LB-Cm (Cm, 30 µg/mL) 0.75% agar mixed with 200 µL of overnight *E. coli* culture for the top layer. For effective infection, 20 mM $MgCl_2$ and 5 mM $CaCl_2$ were supplemented into the LB-agar. 10-fold serial phage dilutions were spot-plated in 3.5 µL volumes onto the double-layer plates, prior to incubation at 37 °C for 16 h.

Isolation of T2 phage mutants

To generate Eco2-escaper phages, *E. coli* DH5α (Thermo Scientific) were transformed with plasmid pLG006 [9] (Addgene ID:157884), plated on LB-Agar (5 g/L yeast extract, 10 g/L tryptone, agar 15 g/L, 10 g/L NaCl) plates containing chloramphenicol (Cm, 30 µg/mL) and incubated for 16 h at 37 °C. 4 mL LB-Cm cultures were subsequently inoculated and incubated for 16 h at 37 °C to prepare overnight cultures for double-layer agar plaque assays. Double-layer agar plates were prepared by layering 30 mL 1.5% LB-agar-Cm for the bottom layer and 5 mL 0.75% LB-agar-Cm mixed with 500 µL overnight culture for the top layer. Subsequently, 10-fold dilutions series of T2 were spot plated in 3.5 µL volumes onto the top layer, prior to incubation for 16 h at 37 °C. Emerging escaper phage candidates, appearing to propagate well in presence of Eco2 as indicated by large plaques at high dilution, were subsequently isolated by scraping spots for transfer into 1 mL phage buffer (50 mM Tris pH 7.4, 10 mM NaCl, 100 mM $MgCl_2$). Phages were incubated for 1 h at room temperature prior to repeated vortexing for phage release. The agar was then removed by centrifugation at 3,200 g for 10 min and the phage supernatant was transferred to a new tube. Enriched escaper phages were then replated as serial dilutions on fresh Eco2-expressing *E. coli* DH5α double layer plates (LB-agar-Cm) and incubated for 16 h at 37 °C. Enriched escaper phage spots were subsequently used for iterative phage isolation until the escaper phages were purified. The final purification step was performed by scraping individual plaques for transfer in 4 mL LB-Cm containing *E. coli* DH5α at an OD_{600} ≈0.5. Phages were subsequently incubated at 37 °C, shaking at 250 r.p.m. for ~20 h. To separate phage from bacteria, cells were sedimented by centrifugation at 6,000 r.p.m. for 5 min. The phage supernatant was then filtered using a 0.22 µm filter and stored at 4 °C.

Phage DNA extraction

Prepared phage escaper solutions were propagated on a 10 mL *E. coli* DH5α culture in LB-Cm (Cm, 30 µg/mL) at 37 °C overnight. The next day, cultures were centrifuged for 2 min at 6,000 g, and the supernatant was filtered through a 0.22 µm pore filter to remove cell debris and unlyzed cells. Filtered supernatants were centrifuged at 12,000 g for 1 h, the supernatant was discarded and phages were resuspended in 400 µL of 30 mM Tris-HCl pH 7.5, 50 mM $MgCl_2$, 0.5 mM $CaCl_2$, prior to incubation overnight at 4 °C. 1 µL of RNase A (10 mg/mL) and 5 µL of DNase I (1 U/µL) were added the next day and samples were incubated at 37 °C for 2 h. Subsequently, 15 µL of proteinase K (0.65 mg/mL) and EDTA (final concentration of 62.5 mM) were added to the mix and incubated at 56 °C for 2 h. Phage genomic DNA was then purified using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific), according to the manufacturer's protocol.

Phage genome sequencing and escaper analysis

DNA libraries were prepared using the Colibri ES PCR Library Prep Kit (Invitrogen) according to the manufacturer's protocol and sequenced in a 150 paired-end mode on a Miniseq (Illumina). Adapters were trimmed with Bbduk from the BBmap package v38.18 (https://sourceforge.net/projects/bbmap/ktrim = r k=15 mink = 11 hdist = 1 tpe tbo). Reads were subsampled to 40,000 per dataset using seqtk –s 123, as Breseq requires coverage of ~100. The initial WT T2 analysis was performed using breseq v0.37.1 with default parameters on our laboratory strain T2 sequence data and reference genome (GenBank accession: LC348380.1). The reference genome of bacteriophage T2 was refined using gdtools APPLY command to reflect the genetic composition of our laboratory T2 strain. This revised genome sequence served as the reference for subsequent breseq analyses using default parameters.

To identify DenB (T2, YP_010073922.1) and A1 (T5, AAU05157.1) homologs across BASEL phage genomes, 3 iterations of psi-blast against the NCBI clusteredNR database (2025-07-21 version) were performed, selecting sequences with 30-90% homology and >70% query coverage (expected threshold 0.05, BLOSSUM62 substitution matrix). The sequences were clustered to 80% sequence identity for DenB and 70% sequence identity for A1; and 80% alignment coverage using mmseqs2 algorithm (MPI Bioinformatics tools kit, Version: c552cce6c3194c06bc0bba84f04c4ef13d62f0a5, https://toolkit.tuebingen.mpg.de/tools/mmseqs2). Sequences were aligned using Ugene v51.0 default MAFFT algorithm v7.520 (gap opening penalty 1.53). The alignment was visually inspected to remove sequences with long insertion and

deletions, and realigned to remove the unaligned N and C terminal stretches. The HMM profiles were built using the hmmbuild command from HMMER v3.4 (http://hmmer.org/). Resulting HMM profiles were used to search the proteomes by the hmmsearch command (http://hmmer.org/ E-value 0.001).

E. coli growth assay

E. coli MG1655 were transformed either with the empty control vector pACYC184 (NEB), or the Eco2 plasmid pMJ001, and plated on LB-agar-chloramphenicol (Cm, 25 µg/mL), prior to incubation overnight at 37 °C. 4 mL LB-Cm (25 µg/mL Cm) media were subsequently inoculated and incubated at 37 °C, 200 r.p.m. until the optical density at 600 nm (OD_{600}) reached ≈ 0.7. Subsequently, cultures were diluted in 90 µL LB-Cm (25 µg/mL) and transferred into clear 96-well PS F-bottom plates (Greiner), prior to addition of phages, or medium, to a total volume of 100 µL and final OD_{600} of 0.1. Phage titers were estimated by spot plating prior to the experiment. Growth was monitored every 5 min for 16 h by OD-measurement in a CLARIOstar plus plate reader (BMG Labtech, software version 5.70 R2), at 37 °C, 200 r.p.m.. Data were analyzed and plotted in Prism (software version 10.5.0 (673), Graphpad).

Plasmid construction

Constructs for expression and affinity purification were cloned by fusing a Strep-tag II encoding sequence downstream of the *rt-toprim* gene encoded on pLG006 (Addgene plasmid number: #157884), using Golden Gate (GG)-assembly. Mutations encoding amino-acid substitutions were subsequently introduced by GG-mutagenesis. For expression of the N-terminally Strep tag-II tagged apo *rt-toprim*, the gene was cloned into pRSFDuet-1 For Bacterial Adenylate Cyclase Two-Hybrid (BACTH) system experiments, genes were cloned by GG-assembly upstream of *t18* into pUT18.

Protein production and purification from *E. coli* cultures

All proteins were purified at a constant temperature of 4 °C and kept on ice.

For small scale purification of Eco2 and its variants, Eco2 (ncRNA- and RT-TOPRIM-strep-tag-encoding) plasmids were transformed in *E. coli* DH5α (Thermo Scientific), plated on LB-agar plates containing chloramphenicol (Cm, 25 µg/mL) and incubated at 37 °C overnight. To express Eco2, or variants thereof, 30 mL TB-Cm (12 g/L trypton, 24 g/L yeast extract, 4 mL/L glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4, 25 µg/mL Cm) were directly inoculated from

transformation plates with multiple colonies and incubated at 37 °C shaking at 200 r.p.m. for 16 h. Cells were subsequently harvested and resuspended in 30 mL lysis buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) prior to cell disruption at 10,000 PSI using an LM10 Microfluidizer (Microfluidics) equipped with an H10Z interaction chamber. Lysates were clarified by centrifugation at 20,000 g for 20 min at 4 °C. Supernatants were loaded onto 100 µL Strep-Tactin XT resin (Cytiva) and incubated for 10 min on ice while shaking. Subsequently, the resin was centrifuged at 4,000 g for 5 min at 4 °C and the supernatant was discarded. The resin was then resuspended in 700 µL lysis buffer and transferred to a Zymo-Spin P1 column (Zymo research). To remove non-specific contaminants, the resin was washed 3 times with 700 µL lysis buffer prior to incubation with 100 µL elution buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1mM EDTA, 50 mM biotin) for 5 min on ice. Proteins were eluted by centrifugation 4,000 g for 1 min at 4 °C. Concentrations were subsequently estimated based on the absorbance at 280 nm using a NanoDrop Eight spectrophotometer (Thermo Scientific). Protein samples were analyzed by SDS-PAGE and SYPRO Ruby (Thermo Scientific) staining.

For preparative purifications, Eco2-encoding (ncRNA- and RT-TOPRIM-strep-encoding) plasmids were transformed in *E. coli* DH5α (Thermo Scientific) plated on LB-agar plates containing chloramphenicol (Cm, 25 µg/mL) and incubated at 37 °C overnight. To express Eco2, or variants thereof, 2 L TB-Cm (25 µg/mL Cm) were directly inoculated from transformation plates with multiple colonies and incubated at 37 °C shaking at 200 r.p.m. for 16 h. Cells were subsequently harvested and resuspended in 40 mL lysis buffer (c) prior to cell disruption using an LM10 Microfluidizer (Microfluidics). Lysates were clarified by centrifugation at 20,000 g for 40 min at 4 °C. The supernatants were subsequently loaded onto 1 mL StrepTrap XP columns (Cytiva) pre-equilibrated in lysis buffer and washed with 20 column volumes (CV) of 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA buffer prior to elution with 6 CV elution buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 50 mM biotin). Samples were further purified by size exclusion chromatography (SEC) using either Superose 6 10/300 (Cytiva) columns for Eco2 and variants thereof, or a Superdex 200 10/300 (Cytiva) column for the Eco2 dRT variant, pre-equilibrated in SEC buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl). Peak fractions were concentrated to approximately 50 µL and concentrations were estimated based on the absorbance at 280 nm using a NanoDrop Eight spectrophotometer (Thermo Scientific). Samples were split in aliquots, snap frozen in liquid nitrogen and stored at -70 °C prior to usage for assays.

For expression of Strep-tagged apo RT-TOPRIM, pRSF-Duet-derived plasmids encoding the *strep-rt-toprim* gene were transformed in *E. coli* BL21 Star (DE3) (Invitrogen) and plated on LB-agar plates containing kanamycin (Kan, 50 µg/mL). To express the *strep-rt-toprim* gene, 10 mL LB-Kan overnight cultures (50 µg/mL Kan), grown shaking at 37 °C, were used to inoculate 2 L TB-Kan (50 µg/mL Kan) and incubated shaking at 200 r.p.m and 37 °C until the optical density at 600 nm (OD_{600}) reached ≈ 0.6. Gene expression was induced with 0.5 mM IPTG, prior to incubation for 16 h at 16 °C. Cells were subsequently collected by centrifugation and resuspended in lysis buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) prior to cell disruption using a microfluidizer. The lysate was clarified by centrifugation at 20,000 g for 40 min at 4 °C. The supernatant was then loaded on a 5 mL StrepTrap XP column (Cytiva) pre-equilibrated in lysis buffer, subsequently washed with 5 CV lysis buffer and eluted with 2 CV elution buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA and 50 mM biotin). The elution was then applied to a 5 mL HiTrap Q HP column (Cytiva), pre-equilibrated in a buffer containing 20 mM NaCl and 100 mM Tris-HCl (pH 8.0). After a 4 CV column wash with 20 mM NaCl and 100 mM Tris-HCl (pH 8.0) buffer, a 15 CV gradient from 20 mM to 2 M NaCl in 100 mM Tris-HCl (pH 8.0) was applied for elution. The peak fraction was concentrated and further purified by SEC using a Superose 6 10/300 (Cytiva) column pre-equilibrated in SEC buffer. The protein was then concentrated to approximately 50 µL and the concentration was estimated based on the absorbance at 280 nm using a NanoDrop Eight spectrophotometer (Thermo Scientific). The sample was split in aliquots, snap frozen in liquid nitrogen and stored at -70 °C.

Urea-PAGE analysis of nucleic acids

10 µL of protein-nucleic acid complex samples (0.5-7 µM) were combined with 1 µL Proteinase K (~20 mg/mL, Thermo Fisher Scientific), and optionally 1 µL DNase I (1 U/µL, Thermo Fisher Scientific) and/or 1 µL RNase A (10 mg/mL, Thermo Fisher Scientific) for 30 min at 37 °C in DNAse I reaction buffer (Thermo Fisher Scientific). Subsequently, samples were mixed with one volume of 2X RNA Loading Dye (Thermo Fisher Scientific), incubated 70 °C for 10 min, and cooled down on ice before separation on a 12.5% Urea-PAGE. Gels were stained with SYBR Gold (Thermo Fisher Scientific) prior to visualisation using a GelDoc Go imaging system (Bio-Rad, software version 3.0.0.07).

For Urea-PAGE analysis of DenB cleavage products, the nucleic acids underwent a two-step treatment process. First, nucleic acids were treated with alkaline phosphatase (FastAP,

Thermo Scientific) in a 10 µL reaction containing 0.5X PNK A buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine), 500 nM DNA, and 1.5 µL FastAP enzyme (1 U/µL). To stop this reaction, 5 µL of 15 mM EDTA was added (final concentration 5 mM) and heated at 65 °C for 15 minutes. The samples were then supplemented with 2 µL of 10X PNK A buffer, 2 µL of 3.3 µM γ-[^{32}P]-ATP (6,000 Ci/mmol), and 1 µL of T4-PNK enzyme (10 U/µL, Thermo Scientific). This mixture was incubated at 37 °C for 30 minutes. The reaction was stopped by adding 5 µL of 50 mM EDTA and heating at 75 °C for 10 minutes (25 µL final volume). Parallel reactions were prepared following the same protocol, with the addition of RNase A (Thermo Scientific) to a final concentration of 0.4 mg/mL during the phosphorylation step. 5 µL of the reaction mixtures were analyzed by Urea-PAGE. Samples were separated on a 15% (29:1 acrylamide and bis-acrylamide) sequencing gel with 7 M of urea in 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). Gels were run for 2 hours at 60 °C. The gel was visualized by phosphor imaging on a Amersham Typhoon scanner (GE Healthcare, software v. 2.0.0.6) .

Cryo-EM grid preparation and data acquisition

Eco2 complexes were purified as described above either w/o or with 10 mM $MgCl_2$ supplementation in the SEC buffer. The activated Eco2 sample was purified as described in the 'Protein purification from CFEs' section below, $MgCl_2$ was added to the thawed sample to a final concentration of 10 mM. Cryo-EM grids were prepared by applying 3 µL of Eco2 sample (7 µM) to Quantifoil R 1.2/1.3 300 copper mesh grids with a 2 nm continuous carbon support layer, which were glow discharged at 20 mA for 7 s (GLOQUBE PLUS, Quorum) or at 12 W for 5 s (Zepto-W6, Diener) before sample application. The grids were flash frozen in liquid ethane using Vitrobot Mark IV (Thermo Scientific) with 100% relative humidity at 4 °C, with no preincubation, blotting time 5 s.

Datasets were collected on a Glacios Cryo-TEM (Thermo Scientific) operated at 200 kV and equipped with a Falcon III directed electron detector (Thermo Scientific) at x92,000 magnification, pixel size of 1.1 Å, 30 frames with a total dose of 30 $e^-/Å^2$, and defocus range of -2.2 to -1.0 µm. Data collection was performed using EPU v3.2 and v3.10 software (Thermo Fisher Scientific).

Cryo-EM data processing

Eco2 without $MgCl_2$ data was processed using CryoSPARC (v. 4.3) [10]. A dataset of 3,826 micrographs was collected, patch motion correction and CTF estimation were done using on-the-fly processing in CryoSPARC Live. Blob auto-picking (box side 360) resulted in 2,914,940 particles, which were sorted by 2D classification. An initial model was generated from the best 2D classes with 583,457 particles. The particles were further classified using heterogeneous refinement. The best class with 508,057 particles was refined using homogeneous refinement and the data were further polished by Global and local CTF refinement and Reference-based motion correction. The final reconstruction on polished particles contained 504,525 particles and reached a global resolution of 2.76 Å.

For the Eco2 sample in presence of $MgCl_2$, a dataset of 2,309 movies was collected. Data processing was performed using CryoSPARC (v. 4.6) [10]. The blob auto-picking resulted in 1,037,763 particles extracted with a box size of 360 pixels, which were sorted by 2D classification. The best class containing 339,226 particles was selected following several rounds of 2D classification and heterogenous refinement. The dataset was further polished using per-particle CTF, reference-based motion correction. A symmetry expansion job was run for C3 symmetry and local refinement was performed using a mask covering a single Eco2 monomer. After refinement, a final reconstruction with global resolution of 3.04 Å was obtained containing 1,017,678 C3 symmetry expanded particles. The final map was sharpened using sharpening tools in CryoSPARC (v. 4.6).

A dataset of 2,968 micrographs was collected for DenB activated Eco2. Patch motion correction and CTF estimation were done using on-the-fly processing in CryoSPARC Live, data processing was performed using CryoSPARC (v. 4.7) [10]. Template picking was performed using a template generated from the map of Eco2 in absence of $MgCl_2$ resulting in 1,430,679 particles which were sorted by 2D classification. An initial model was generated from the best 2D classes and further classified using heterogeneous refinement. Global and local CTF refinement, Reference-based motion correction, and Rebalance orientations jobs were performed, followed by non-uniform refinement. A symmetry expansion job was run for C3 symmetry and local refinement was performed using a mask covering a single Eco2 monomer. A final reconstruction with global resolution of 2.90 Å was obtained containing 617,364 C3 symmetry expanded particles. The final map was sharpened using sharpening tools in CryoSPARC (v. 4.6).

Model building, refinement, and figure preparation

The initial RT-TOPRIM model was generated using AlphaFold2 [11] under the ColabFold [12] framework with default parameters and fit into the cryo-EM map in UCSF ChimeraX (v.1.6.1) [13]. The model was manually modified using Coot (v.0.9.8.7) [14] to fit the map. RNA and DNA nucleotides observed in the map were built manually in Coot. Further model refinement and evaluation were performed using Phenix real space refine (v.1.20.1–4487) [15] against sharpened maps. Secondary structure restraints were generated for nucleic acid base pairs. Global minimization and local grid search strategies were used. The resulting model was then used as a starting model for Eco2 in presence of $MgCl_2$ and activated Eco2 and was fitted into the experimental maps in UCSF ChimeraX (v.1.8). The model was manually modified using Coot (v.0.9.8.95) and refined using Phenix (v.1.21.2-5419) as described above. Figures were prepared in UCSF ChimeraX.

AlphaFold structure prediction

DenB and RT-TOPRIM structures in presence of ligands were predicted using AlphaFold 3 [16] (default settings) via the AlphaFold Server (https://deepmind.google/technologies/alphafold/alphafold-server/). pLDDT values were mapped on structures using UCSF ChimeraX.

Sample preparation for proteomic analysis

Protein bands of interest were excised from SDS-PAGEs, stained with colloidal coomassie (0.08% [w/v] Coomassie Brilliant Blue G250, 10% [w/v] citric acid, 8% [w/v] ammonium sulfate, 20% [v/v] methanol) overnight. For all following steps, buffers were exchanged by two consecutive 15 min incubation steps of the gel pieces with 200 µL of acetonitrile (ACN) whereby ACN was removed after each step. Proteins were reduced by the addition of 200 µL of a 10 mM DTT solution in 100 mM ammonium bicarbonate (AmBiC, Sigma Aldrich, A6141), samples were incubation at 56 °C for 20 min, 180 µL ACN were added, and samples were incubated for 15 min incubation at room temperature. Proteins were alkylated for 20 min by the addition of 200 µL of a 55 mM chloroacetamide (CAA) solution in 100 mM AmBiC. Gel pieces were incubated twice with 200 µL ACN for 15 min at room temperature.

In-gel enzymatic digest

A 0.2 µg/µL stock solution of trypsin (Promega, V511A) in resuspension buffer (Promega, V542A) was diluted with ice-cold freshly prepared 50 mM AmBiC buffer to achieve a final concentration of 2 ng/µL. 50 µL thereof were added to gel pieces, which were incubated for 30 min on ice and thereafter over night at 37°C. Gel pieces were sonicated for 15 min, spun down and the supernatant was transferred into a glass vial (VDS Optilab, 93908556). Remaining gel pieces were washed with 50 µL of an aqueous solution of 50% (v/v) ACN and 1% (v/v) formic acid and sonicated for 15 min. The combined supernatants were dried in a speedvac and reconstituted in 10 µL of an aqueous solution of 0.1% (v/v) formic acid.

LC-MS/MS analysis of peptides

Peptides were analyzed by LC-MS/MS on an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) as previously described [17]. To this end, peptides were separated using an Ultimate 3000 nano RSLC system (Dionex) equipped with a trapping cartridge (Precolumn C18 PepMap100, 5 mm, 300 µm i.d., 5 µm, 100 Å) and an analytical column (Acclaim PepMap 100. 75 × 50 cm C18, 3 mm, 100 Å) connected to a nanospray-Flex ion source. The peptides were loaded onto the trap column at 30 µL per min using solvent A (0.1% [v/v] formic acid in water) and peptides were eluted using a gradient from 2 to 85% solvent B (0.1% [v/v] formic acid in ACN) over 30 min at 0.3 µL per min (all solvents were of LC-MS grade). The Orbitrap Fusion Lumos was operated in positive ion mode with a spray voltage of 2.2 kV and capillary temperature of 275 °C. Full scan MS spectra with a mass range of 375–1200 m/z were acquired in profile mode using a resolution of 120,000 (maximum injections time of 50 ms, AGC target was set to 400% and a max injection time of 86 ms. Precursors were isolated using the quadrupole with a window of 1.2 m/z and fragmentation was triggered by HCD in fixed collision energy mode with fixed collision energy of 34%. MS2 spectra were acquired with the Orbitrap with a resolution of 30.000 and a max injection time of 86 ms.

The Orbitrap Fusion Lumos was operated in positive ion mode with a spray voltage of 2.2 kV and capillary temperature of 275 °C. Full scan MS spectra with a mass range of 350–1500 m/z were acquired in profile mode using a resolution of 120,000 (maximum injection time of 100 ms), AGC target was set to standard and a RF lens setting of 30%. Precursors were isolated using the quadrupole with a window of 1.2 m/z and Fragmentation was triggered by HCD in fixed collision

energy mode with fixed collision energy of 30%. MS2 spectra were acquired in Ion Trap normal mode. The dynamic exclusion was set to 5 s.

Acquired data were analyzed using FragPipe [18] and a *E. coli* Uniprot fasta database (UP000000625, ID83333, 4.402 entries, date: 27.10.2022, downloaded: 11.01.2023) including common contaminants as well as the protein sequence of our protein of interest. The following modifications were considered: Carbamidomethyl (C, fixed), TMT10plex (K, fixed), Acetyl (N-term, variable), Oxidation (M, variable) and TMT10plex (N-term, variable). The mass error tolerance for full scan MS spectra was set to 10 ppm and for MS/MS spectra to 0.02 Da. A maximum of 2 missed cleavages were allowed. A minimum of 2 unique peptides with a peptide length of at least seven amino acids and a false discovery rate below 0.01 were required on the peptide and protein level [19].

Bacterial two-hybrid assay

Protein-protein interactions were assayed using the Bacterial Adenylate Cyclase Two-Hybrid (BACTH) System kit (Euromedex, Souffelweyersheim, France). In brief, the *s1* gene and its truncated variants were fused with the T25 adenylate cyclase fragment in plasmid vector pKNT25. WT Eco2, dRT Eco2 and apo RT-TOPRIM loci were fused downstream of the *rt-toprim* gene with the T18 adenylate cyclase fragment in pUT18. The BTH101 cells were co-transformed with plasmid pairs and grown overnight at 30 °C in LB medium containing 100 µg/mL ampicillin (Amp) and 50 µg/mL kanamycin (Kan). 2 mL LB-Amp-Kan was inoculated with 40 µL of overnight culture and grown to $OD_{600} \approx 0.8$. For spot tests, 7 µL of fresh cultures were spotted on M9 minimal medium (47.7 mM $Na_2HPO_4 \times 7H_2O$, 22 mM KH_2PO_4, 8.5 mM NaCl, 18.7 mM NH_4Cl, 0.2% [w/v] glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.5 mM IPTG, 60 µg/mL X-Gal, 100 µg/mL ampicillin and 50 µg/mL kanamycin) agar plates, prior to incubation at 30 °C for 20 h. Spots were then assessed for blue color, indicative of protein-protein interactions.

Hydrogen/deuterium exchange mass spectrometry (HDX-MS)

HDX-MS experiments for investigation of the impact of msDNA on Eco2 conformation and topology were conducted essentially as described previously [20]. The msDNA-bound Eco2 and apo RT-TOPRIM were purified as described above. HDX reactions were automatically set up with a two-arm robotic autosampler (LEAP technologies) by dispension of 6.5 µL of Eco2 sample (40 µM) in a 96 well plate followed by addition of 58.5 µL HDX buffer (20 mM Tris-Cl pH 8.0, 5 mM

383 $MgCl_2$, 150 mM NaCl) prepared with 99.9% D_2O. After incubation at 25 °C for various time-
384 points (10 100, 1,000 or 10,000 seconds), 55 µL of the HDX reaction were withdrawn, added to
385 55 µL of pre-dispensed quench buffer (400 mM KH_2PO_4/H_3PO_4, pH 2.2, 2 M guanidine-HCl)
386 temperated at 1 °C, and 95 µL of the resulting mixture injected into an ACQUITY UPLC M-Class
387 System with HDX Technology (Waters) [21]. The preparation of non-deuterated samples was carried
388 out similarly (incubation for approximately 10 s at 25 °C) by 10-fold dilution of samples with
389 HDX buffer prepared with H_2O. The injected samples were delivered with H_2O + 0.1% (v/v)
390 formic acid (100 µL/min) to a column (2 mm x 2 cm, 12 °C) containing porcine pepsin
391 immobilized to beads, and the resulting peptic peptides trapped on an AQUITY UPLC BEH C18
392 1.7 µm 2.1 x 5 mm VanGuard Pre-column (Waters) kept at 0.5 °C. After 3 min of digestion and
393 trapping, the trap column was placed in line with an ACQUITY UPLC BEH C18 1.7 µm 1.0 x 100
394 mm column (Waters), and the peptides eluted at 0.5 °C with a gradient of H_2O + 0.1% (v/v) formic
395 acid (eluent A) and acetonitrile + 0.1% (v/v) formic acid (eluent B) at 30 µL/min as follows: 0-7
396 min: 95-65% A; 7-8 min: 65-15% A; 8-10 min: 15% A; 10-11 min: 5% A; 11-16 min: 95% A. The
397 peptides were guided to a G2-Si HDMS mass spectrometer with ion mobility separation (Waters)
398 and ionized with an electrospray ionization source (250 °C capillary temperature, 3.0 kV spray
399 voltage), and mass spectra acquired in positive ion mode over a range of 50 to 2,000 m/z in
400 enhanced high definition MS ($HDMS^E$) or high definition MS (HDMS) mode for non-deuterated
401 and deuterated samples, respectively [22]. Lock-mass correction was implemented with [Glu1]-
402 Fibrinopeptide B standard (Waters). During separation of the peptide mixtures on the C18 column,
403 the protease column was washed three times with 80 µL of wash solution (0.5 M guanidine
404 hydrochloride in 4% (v/v) acetonitrile), and blank injections performed between each sample to
405 reduce peptide carry-over. Measurements were conducted in technical triplicates (individual HDX
406 reactions).
407 ProteinLynx Global SERVER (PLGS, software version 3.0.1, Waters) and DynamX
408 (software version 3.0, Waters) facilitated peptide identification and analysis of deuterium
409 incorporation essentially as described previously [20]. In brief, peptides were identified with PLGS
410 from the non-deuterated samples acquired with $HDMS^E$ by employing low energy, elevated
411 energy, and intensity thresholds of 300, 100 and 1,000 counts, respectively. Identified ions were
412 matched to peptides with a database containing the amino acid sequence of Eco2, porcine pepsin,
413 and their reversed sequences with the following search parameters: peptide tolerance = automatic;

fragment tolerance = automatic; min fragment ion matches per peptide = 1; min fragment ion matches per protein = 7; min peptide matches per protein = 3; maximum hits to return = 20; maximum protein mass = 250,000; primary digest reagent = non-specific; missed cleavages = 0; false discovery rate = 100. Only peptides identified in at least three non-deuterated samples with a minimum intensity of 10,000 counts, a peptide length of 5-40 residues, a minimum number of two products, a maximum mass error of 25 ppm, and retention time tolerance of 0.5 minutes were considered for further analysis. All spectra were manually inspected with DynamX 3.0 and, if necessary, peptides omitted (e.g., in case of low signal-to-noise ratio or presence of overlapping peptides). Residue-specific HDX was calculated from overlapping peptides with DynamX 3.0 by employing the shortest peptide covering a residue. Where multiple peptides were of the shortest length, the peptide with the residue closest to the peptide's C-terminus was utilized. Numerical peptide and residue-specific HDX data are contained within the related source data file.

RNase activity assay

Reactions were assembled by combining 1 µM protein and 200 nM RNase Alert (IDT) FQ probe in reaction buffer (10 mM HEPES pH 7.5, 250 mM KCl, 5 mM $MgCl_2$, 5% [v/v] glycerol) in a total volume of 30 µL in 384-well flat bottom black polystyrene assay plates (Thermo Fisher Scientific). Reactions to assess the effect of DNA degradation additionally contained 16.7 mU/µL DNase I (Thermo Fisher Scientific). The reactions were monitored by fluorescence measurements (λ_{ex}: 489 nm; λ_{em}: 522 nm) in a CLARIOstar plus plate reader (BMG Labtech, software version 5.70 R2) at 37 °C every 30 sec. Data were analyzed and plotted in Prism (software version 10.4.0 (527), Graphpad).

Cell-free extract (CFE) preparation

Cell-free extracts (CFEs) were prepared following a protocol adapted from Dopp *et al.*, 2019 [23]. Briefly, a saturated overnight culture of *E. coli* BL21 Star (DE3) (Invitrogen) was inoculated into sterile 2 L baffled flasks, containing 400 mL of 2x YTPG media (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 22 mM KH_2PO_4, 40 mM K_2HPO_4, 100 mM D-(+)-glucose, pH 7.2). The initial inoculation density was adjusted to an OD_{600} of 0.05, and cultures were incubated at 37 °C, 200 r.p.m.. To induce T7 RNA polymerase expression, IPTG was added to a final concentration of 1 mM once the cultures reached an (OD_{600}) ≈ 0.5. Cells were harvested during the mid-exponential phase (OD_{600} ≈ 2.5 – 3.0) via centrifugation and washed three times with ice-cold filtered S30A

buffer (50 mM Tris-acetate pH 7.8, 14 mM magnesium L-glutamate, 60 mM potassium L-glutamate, 2 mM DTT). The resulting biomass pellet were collected by centrifugation, decanted, weighed, flash-frozen in liquid nitrogen, and stored at –80 °C. Next, the biomass pellet was thawed on ice and resuspended in ice-cold filtered S30A buffer (1 mL of buffer per 1 g of biomass). Cells were then lysed using a Sonics VibraCell ultrasonic liquid processor (Sonics and Materials) equipped with a 13 mm probe. Lysis was performed in an ice bath at 50% amplitude, with a total lysis time of 30 s per 1 g of biomass, using a pulsing cycle of 3 s on and 6 s off. The resulting lysate was cleared by centrifugation at 48,384 r.c.f. for 30 min at 4 °C, followed by incubation for 1 h at 37 °C and centrifugation at 200 r.p.m. for 1 hour. To remove any remaining debris the subsequent centrifugation step was performed for 10 min at 3,113 r.c.f., 4 °C. The total protein concentration of the lysate was determined using the Pierce™ Bradford Plus Protein Assay Kit (Thermo Fisher Scientific). The lysate was aliquoted, flash-frozen in liquid nitrogen, and stored at –80 °C.

Preparation of templates for expression in CFEs

Linear DNA templates for *denB* wild type (wt), the *denB*-G71R escaper mutant and mCherry expression in CFEs were generated by two-step fusion PCR [24]. In the first step, the target genes were amplified by PCR, attaching a GC-rich fusion sequence at the 5' ends. In the second step, the amplified genes were combined with a short DNA cassette, similar to the ones described by Garamella *et al.*, 2016 [25], containing the native *E. coli* RNA polymerase P70a or T7 RNA polymerase PT714 promoters, a ribosome-binding site, a StrepII-tag, and the GC-rich fusion sequence at the 3' end. These components were fused via PCR using flanking ORG13 and ORG113 oligonucleotides. PCR products were analysed by agarose gel electrophoresis and the resulting fusion products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific).

The pCI-T7Max-UTR1-deGFP-8xHis-T500 reporter plasmid was a gift from Kate Adamala (Addgene plasmid #178422) [26]. The plasmid was propagated in *E. coli* DH5α (Thermo Scientific) and purified using a ZymoPURE II Plasmid Maxiprep Kit (Zymo Research).

Transcription-translation interference assay in CFEs

Transcription-translation interference (TXTLi) experiments were performed following the guidelines adopted from Garenne et al., 2021 and Marshall et al., 2020 [27,28]. TXTLi reactions were

assembled on ice, and aliquoted in 9 µL volumes to a Nunc™ 384 well ShallowWell Standard Height Black microplate (Thermo Fisher #264705). Each reaction contained 10 mg/mL *E. coli* BL21 Star (DE3) CFE, 57 mM HEPES-K pH 8.0, 0.2 mg/mL tRNA mix from *E. coli* MRE600 (Sigma Aldrich), 1.5 mM ATP, 1.5 mM GTP, 0.9 mM CTP, 0.9 mM UTP, 0.75 mM cAMP, 30 mM 3-PGA, 0.33 mM NAD, 0.26 mM coenzyme A, 68 µM folinic acid, 1 mM spermidine, 30 mM D-ribose, 60 mM maltodextrin, 2 mM of each of the 20 L-amino acids, 7 mM magnesium L-glutamate, 50 mM potassium L-glutamate, 1 mM DTT, 20 g/L PEG 8000, 2.5 µM GamS inhibitor, 5 nM pCI-T7Max-UTR1-deGFP-8xHis-T500 reported plasmid, 20 nM of linear P70a DNA expression templates encoding either mCherry, DenB or dDenB (G71R) and 1 µM purified Eco2 or dTOPRIM. The plate was centrifuged for 1 min at 200 r.c.f., 4 ºC, equilibrated at room temperature for 10 min, sealed with MicroAmp Optical Adhesive Film (Thermo Fisher Scientific) and transferred to the CLARIOstar Plus microplate reader (BMG Labtech, software v. 5.70 R2) for measurements. The fluorescence intensities of GFP (λ_{ex}: 470 nM ± 15 nM, λ_{em}: 515 nM ± 20 nM) and mCherry (λ_{ex}: 570 nM ± 15 nM, λ_{em}: 620 nM ±20 nM bandpass window) were measured every 5 min for 16 h at 30 ºC with double orbital shaking at 200 r.p.m. for 30 s before every measurement.

Since fluorescence signals plateaued after 4 h, data from this time point were used for further analysis. The GFP fluorescence in each reaction was first normalized to background noise by subtracting the fluorescence of corresponding negative control reactions lacking the reporter plasmid. To obtain the relative values, each normalized GFP value was divided by the fluorescence of a corresponding positive control reaction containing no linear DNA templates. Data were analyzed and plotted in Prism (software version 10.4.0 (527), Graphpad).

RNA isolation from CFEs and Urea-PAGE analysis

CFE reactions were performed as described above. Reactions were mixed with 50 µL of TRIzol™ reagent (Thermo Fisher Scientific) and total RNA was extracted according to the manufacturer's recommendations. The ssRNA concentration in each sample was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific). 5 µL 100 ng/µL ssRNA from each sample was mixed with 5 µL of 2x RNA loading dye (NEB), denatured for 10 min at 75 ºC and separated on a denaturing urea polyacrylamide gel (8 M Urea, 12% polyacrylamide (29:1 acrylamide/bis-acrylamide ratio) in 1x TBE (89 mM Tris-borate pH 8.4, 2 mM EDTA)), pre-run for 30 min at 35 mA. The electrophoresis was performed for ~2.5 h at 35 mA. The gel was subsequently stained in

1x TBE (89 mM Tris-borate pH 8.4, 2 mM EDTA) supplemented with 1x SYBR Gold dye (Thermo Fisher Scientific) on an orbital shaker for 10 min at room temperature and imaged with the Amersham Typhoon scanner (GE Healthcare, control software v. 2.0.0.6) using the SYBR Gold channel analyzed with Fiji (software v. 2.14.0/1.54f).

Protein purification from CFEs

DenB was synthesized using NEBExpress (NEB) CFEs following the manufacturer's recommendations. A total of 250 µL of reaction mixture, containing 50 nM of linear PT714-Strep-DenB expression template and 2.5 µM homemade GamS was prepared. The RecBCD inhibitor GamS was purified from *E. coli* for CFE-supplementation as described before [29]. The reaction mix was divided into 50 µL reactions in 1.5 mL tubes and incubated in a thermomixer (Eppendorf) for 18 h at 37 ºC, 800 r.p.m.. Lysates were cleared by centrifugation for 5 min at 17,000 r.c.f., 4 ºC. The reactions were pooled, combined with 250 µL of 2x binding buffer (200 mM Tris-HCl pH 8.0, 300 mM NaCl, 2 mM EDTA, 10 mM 2-mercaptoethanol) and loaded to Zymo-spin P1 columns (Zymo Research, P2003-1) preloaded with dry Strep-Tactin XT Sepharose chromatography resin (Cytiva, 29401324). Prior to use, 100 µL of the resin solution was loaded into the column, washed 3 times with the 1x binding buffer (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol) and decanted by centrifugation (1-2 min at 1,000 r.c.f., 4 ºC). The bottom of the column was covered with parafilm and it was incubated for 30 min at room temperature, shaking vigorously on a 3D shaker (Biosan). Subsequently, the flowthrough was collected by centrifugation, and the beads were washed 3 times with the wash buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM 2-mercaptoethanol) and decanted by centrifugation. Subsequently 100 µL of elution buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM 2-mercaptoethanol, 50 mM D-biotin) was added to dry aspirated beads, the bottom of the column was covered with parafilm and the mix was incubated for 15 min at room temperature, shaking vigorously on a Multi Bio 3D shaker (Biosan). The elution was collected by centrifugation. DenB synthesis and purification was assessed by SDS-PAGE and Western blotting. The concentration of the purified WT DenB protein was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific). The protein was aliquoted, flash frozen in liquid nitrogen and stored at -80 ºC.

For the Eco2 activation and tRNA cutting assay, WT DenB was synthesized using NEBExpress (NEB) cell-free expression systems (CFEs) following the manufacturer's recommendations. A reaction mixture containing 100 nM linear PT714-His_6-DenB-wt expression

template, 2.5 μM homemade GamS, and the NEBExpress CFE mix was prepared to a total volume of 500 μL. The reaction was divided into 50 μL aliquots in 1.5 mL tubes and incubated at 37 °C for 18 h, shaking at 800 r.p.m. in a thermomixer (Eppendorf). Lysates were clarified by centrifugation at 17,000 r.c.f. for 10 min at 4 °C. Pooled lysates were split into two aliquots. Each aliquot was mixed with 25 μL of 15.76 μM purified Eco2 or dTOPRIM, respectively, and incubated at 37 °C for 1 h in a thermomixer at 800 r.p.m. (Eppendorf). Subsequently, 238 μL of each reaction was mixed with an equal volume of 2× binding buffer (200 mM Tris-HCl, pH 8.0, 300 mM NaCl, 2 mM EDTA, 10 mM 2-mercaptoethanol) and loaded onto Zymo-spin P1 columns (Zymo Research, P2003-1) preloaded with dry Strep-TactinXT Sepharose chromatography resin (Cytiva, 29401324). Prior to loading, 100 μL of the resin solution was loaded into the column, washed three times with 1× binding buffer (100 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA; 5 mM 2-mercaptoethanol), followed by centrifugation (1–2 min at 500–1,000 r.c.f., 4 °C). After adding the lysate-resin mixture, the bottom of each microcolumn was sealed with parafilm, and the mixture was incubated for 1 h at room temperature with vigorous shaking on a 3D orbital shaker (Biosan). The flowthrough was collected by centrifugation. The beads were washed three times with wash buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 5 mM 2-mercaptoethanol), removing the supernatant after each wash via centrifugation. For elution, 50 μL of elution buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 5 mM 2-mercaptoethanol; 50 mM D-biotin) was added to the aspirated beads. The microcolumns were sealed with parafilm and incubated for 30 min at room temperature with vigorous shaking on a 3D orbital shaker (Biosan). The eluates were collected by centrifugation and analyzed by SDS-PAGE and Western blotting. Protein concentrations of the DenB-activated and purified Eco2 variants were determined using the Qubit Protein Assay (Thermo Fisher). Proteins were aliquoted, flash-frozen in liquid nitrogen, and stored at −80 °C. The thawed sample was analyzed by running SEC on Superdex 200 3.2/300 column preequilibrated with buffer containing 100 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM $MgCl_2$.

tRNA cleavage assay

The tRNA mix from *E. coli* MRE 600 (Sigma Aldrich) was dephosphorylated in a 10 μL reaction containing 0.5× PNK Buffer A (Thermo Fisher), 250 nM tRNA mix, and 0.15 U/μL FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher). The reaction was incubated at 37 °C for 10 min and subsequent enzyme inactivation was achieved by adding 5 μL of 15 mM EDTA, followed by heating the reaction mixture at 75 °C for 10 min. Dephosphorylated tRNA was

subsequently 5'-radiolabeled in a 20 µL reaction. The radiolabeling reaction mixture contained 1.25× PNK Buffer A (Thermo Fisher), 125 nM tRNA mix, 5 U/µL T4 Polynucleotide Kinase (Thermo Fisher), 125 nM γ-[^{32}P]-ATP (6000 Ci/mmol), 3.75 mM EDTA, and 0.075 U/µL heat-inactivated FastAP Thermosensitive Alkaline Phosphatase. The reaction was incubated at 37 °C for 30 min, after which 5 µL of 50 mM EDTA was added, and the reaction mixture was heated at 75 °C for 10 min to stop the reaction. Following heating, the thermoblock containing the reaction mixture was allowed to cool to room temperature to facilitate proper folding of tRNA molecules. The labeled tRNA was aliquoted and stored at −20 °C.

DenB-activated Eco2 and dTOPRIM were produced as described above. Activated Eco2 was combined with radiolabeled tRNA mix in a 30 µL reaction (final composition: 25 mM Tris-HCl pH 8.0, 250 mM NaCl, 5 mM MgCl2, 5 % (v/v) glycerol, 0.05 nM ^{32}P-tRNA mix, 877 nM DenB-activated and purified Eco2/dTOPRIM) and subsequently incubated at 37 ºC. At 1 h, 2 h and 17 h timepoints 10 µL of the reactions were extracted and mixed with 10 µL of phenol/chloroform/isoamyl alcohol solution (ROTH). The mixtures were centrifuged for 5 min 17,000 r.c.f. and the water fraction was mixed with 10 µL of 2x RNA loading dye (Thermo Fisher Scientific), followed by heating for 10 min at 75 ºC. The denatured samples were analyzed by 12 % Urea-PAGE. The gel was dried, exposed to a phosphor screen overnight and read using an Amersham Typhoon scanner (GE Healthcare, software v. 2.0.0.6) via the phosphor channel and analyzed with Fiji (v. 2.14.0/1.54f).

Protein analyses by SDS PAGE and Western blotting

12 µL of samples were mixed with 4 µL of 4x SDS dye (10% glycerol [v/v], 2% SDS [w/v], 63 mM Tris-HCl pH 6.8, 0.1% 2-Mercaptoethanol, 0.01% bromophenol blue [w/v]) and denatured for 5 min at 95 ºC. Samples were separated in homemade 12% or 15% Tris-Glycine SDS polyacrylamide gels and stained with Coomassie solution (0.1% Coomassie R-250, 40% [v/v] EtOH, 10% [v/v] acetic acid) or used for Western blotting.

For Western blot analysis, the samples were separated in 12% or 15% Tris-Glycine SDS polyacrylamide gels and transferred onto a 0.22 or 0.45 µm PVDF membrane (Thermo Fisher Scientific) in a semi-dry transfer with a Pierce Power Station blotter (Thermo Fisher Scientific). The membrane was blocked (5% [w/w] BSA, 1x PBS, 0.2% [v/v] Tween-20 for 30 min – 1 h shaking at room temperature. A 1:4,000 dilution of Strep•Tag II Antibody HRP Conjugate (Merc, 71591) in blocking buffer was incubated with the membrane overnight, shaking at 4 ºC. The

membrane is subsequently washed 3-5 times (1x PBS, 0.2% [v/v] Tween-20) and developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) for visualisation on a Chemidoc system (Biorad). The images were analyzed with Fiji (v. 2.14.0/1.54f).

RNA sequencing and analysis of cell free extracts

10 µL cell free extracts were transferred into Trizol for RNA extraction according to the manufacturer's protocol (Thermo Fisher Scientific). cDNA libraries were prepared using a Small RNA-Seq Library Prep Kit (Lexogen) with 500 ng RNA input material, according to the manufacturer's protocol. Libraries were subsequently pooled and prepared for nanopore sequencing using the Oxford Nanopore Ligation Sequencing Kit V14 (SQK-LSK114). The resulting libraries were sequenced on the Oxford Nanopore platform (R10.4.1 flow cell) and base calling was performed using dorado v0.8.0, sup v5.0.0 (Oxford Nanopore Technologies. Dorado. https://github.com/nanoporetech/dorad). Datasets were demultiplexed and adapters trimmed using dorado v0.8.0 with Illumina barcodes as custom input. To identify RNA cleavage positions, reads were mapped to the *E. coli* MG1655 genome (GenBank: U00096.3) using minimap2 v2.17 [30] (-ax map-ont), and samtools v1.13 [31] was used to remove ambiguously mapped reads (-F 256 -F 2048). Data was analyzed using custom Python scripts. Read end positions were identified using python pysam package v0.22.1 (https://github.com/pysam-developers/pysam) and counts were normalized according to the sample with fewer reads. Positions without coverage were assigned a pseudocount of 1. The read end ratio was calculated at each position to identify read ends enriched in the WT Eco2 sample (cutoff 20). Hits present in the genomic regions without gene annotations were not further analyzed.

Sequencing and analysis of protein-bound ncRNA

Protein-nucleic acid complexes were purified in the preparative scale as described above. 100 µL samples (14-17 µM) were transferred into Trizol for RNA extraction according to the manufacturer's protocol (Thermo Fisher Scientific). cDNA libraries were prepared using a Small RNA-Seq Library Prep Kit (Lexogen) with 300 ng RNA input material, according to the manufacturer's protocol. Libraries were subsequently barcoded using Oxford Nanopore Native Barcoding Kit V14 (SQK-NBD114.96), sequenced on the Oxford Nanopore platform (R10.4.1 flow cell) and base calling was performed using dorado v0.8.0, sup v5.0.0 (Oxford Nanopore

Technologies. Dorado. https://github.com/nanoporetech/dorado). Reads were aligned to *E. coli* DH5α genome (GenBank: CP026085.1) and to the retron operon by minimap2 v2.17 [30] using default parameters. The dataset was filtered to exclude ambiguously mapped reads by samtools v1.13 [31] (-F 256 -F 2048). Reads were visualized using the IGV viewer [32] and custom Python scripts. The bed file with genome features was prepared from the gff3 file using awk and *E. coli* gene coverage was evaluated using bedtools v2.29.2 [33] with default parameters.

Deep RNA sequencing and analysis of infected *E. coli*

E. coli MG1655 were transformed with plasmids pMJ001, or pMJ003, and plated on LB-agar-chloramphenicol (Cm, 25 µg/mL), prior to incubation overnight at 37 °C. 4 mL LB-Cm (25 µg/mL Cm) media was subsequently inoculated with 6-7 colonies and incubated at 37 °C, 200 r.p.m., until the optical density at 600 nm (OD_{600}) reached ≈ 0.7. Bacteria cultures were transferred into sterile 1,5 mL Eppendorf tubes, normalized by dilution in LB-Cm to OD_{600} of 0.7, and infected with T2 or T5 at an estimated MOI of ~10 in a final volume of 660 µL. Cultures were incubated at 37 °C, 200 r.p.m.. 150 µL samples were taken 10 min, 30 min, 1 h and 3 h after infection and immediately centrifuged for 30 s at 13,000 r.p.m.. The supernatant was rapidly decanted, and cell pellets were flash-frozen in liquid nitrogen. For RNA-extraction, the pellets were resuspended in 400 µL Trizol and RNA was extracted according to the manufacturer's manual (Thermo Fisher Scientific). Concentrations were estimated based on the absorbance at 260 nm using a NanoDrop Eight spectrophotometer (Thermo Scientific).

Small RNA fractions were enriched from purified RNA (0.5-1 µg) using the SPLIT RNA extraction kit (Lexogen) according to the manufacturer's manual. Concentrations were estimated based on the absorbance at 260 nm using a NanoDrop Eight spectrophotometer (Thermo Scientific).

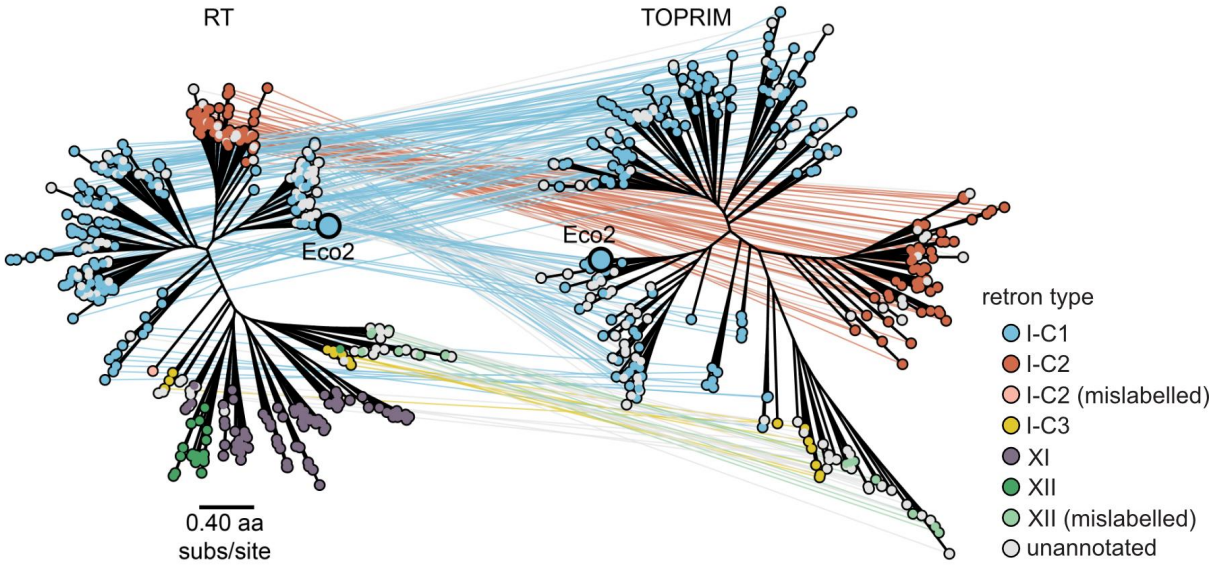
To identify cleaved RNA positions an adapter ligation-based RNA library preparation method was used. Sequencing libraries were prepared using the Small RNA-Seq Library Prep Kit for Illumina (Lexogen) according to the manufacturer's manual, using 200-500 ng of RNA as input. Library quality was evaluated by Bioanalyzer using DNA high sensitivity kit (Agilent) and Qubit 4 fluorimeter (Thermo Fisher Scientific). The library was sequenced using Illumina NextSeq P3 flow cell in 100 PE mode (EMBL Genomics Core facility, Heidelberg, Germany). Data quality was evaluated using fastqc v0.12.1 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed using Bbduk v38.18 (https://sourceforge.net/projects/bbmap/ ktrim=r k=20 mink=10

hdist=1 tpe tbo minlen=17 minavgquality=30). *E. coli* MG1655 genome (GenBank ID: NZ_LR881938.1) was joined with phage T5 (GenBank ID: NC_005859.1) and T2 (GenBank ID: NC_054931.1) genomes using Bash cat command. Annotation files .gff and. gbff were created the same way. The corresponding reads were aligned to the joined genome files using bwa-mem2 v2.2.1 [34] with the default parameters and filtered using samtools v1.13 (-F 260 -f 2). For indexing and sorting samtools was used. The data was further analyzed using custom Python scripts. In brief: read end positions were identified by pysam v0.23.3 (read.reference_end and read.reference_start methods), genomic positions without read ends were assigned pseudo count of 1. Read end ratios (WT Eco2/dTOPRIM) at each genomic position were calculated and enriched positions were selected by setting the filtering cutoff 100. Finally, only the enriched positions present in both biological replicates were further analyzed. Corresponding transcripts and sequences were extracted from the annotation files and the most prominent putative cleavage positions were verified manually by IGV Viewer [32].

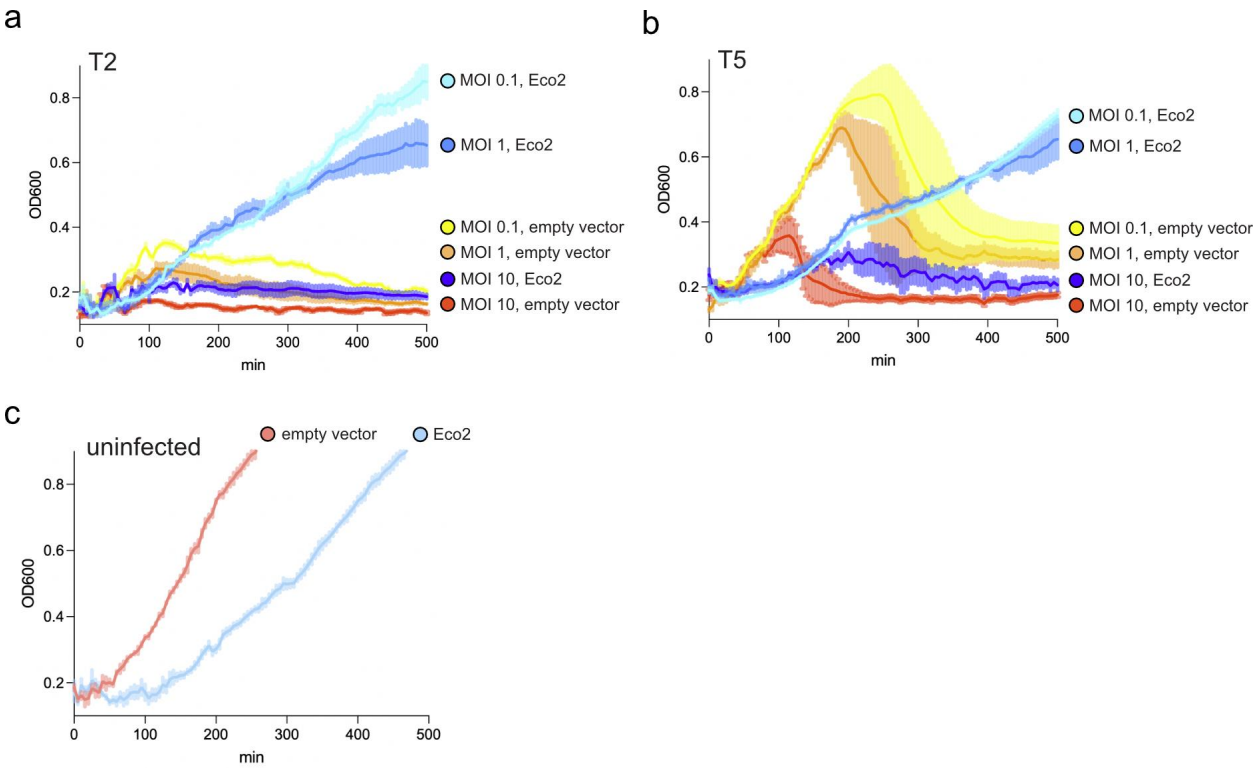
Data availability

Cryo-EM maps and model coordinates were deposited to the EMDB (52583, 52584, 54448) and PDB (9I2F, 9I2G, 9S1F). HDX-MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD060313. Source data are provided with this paper.

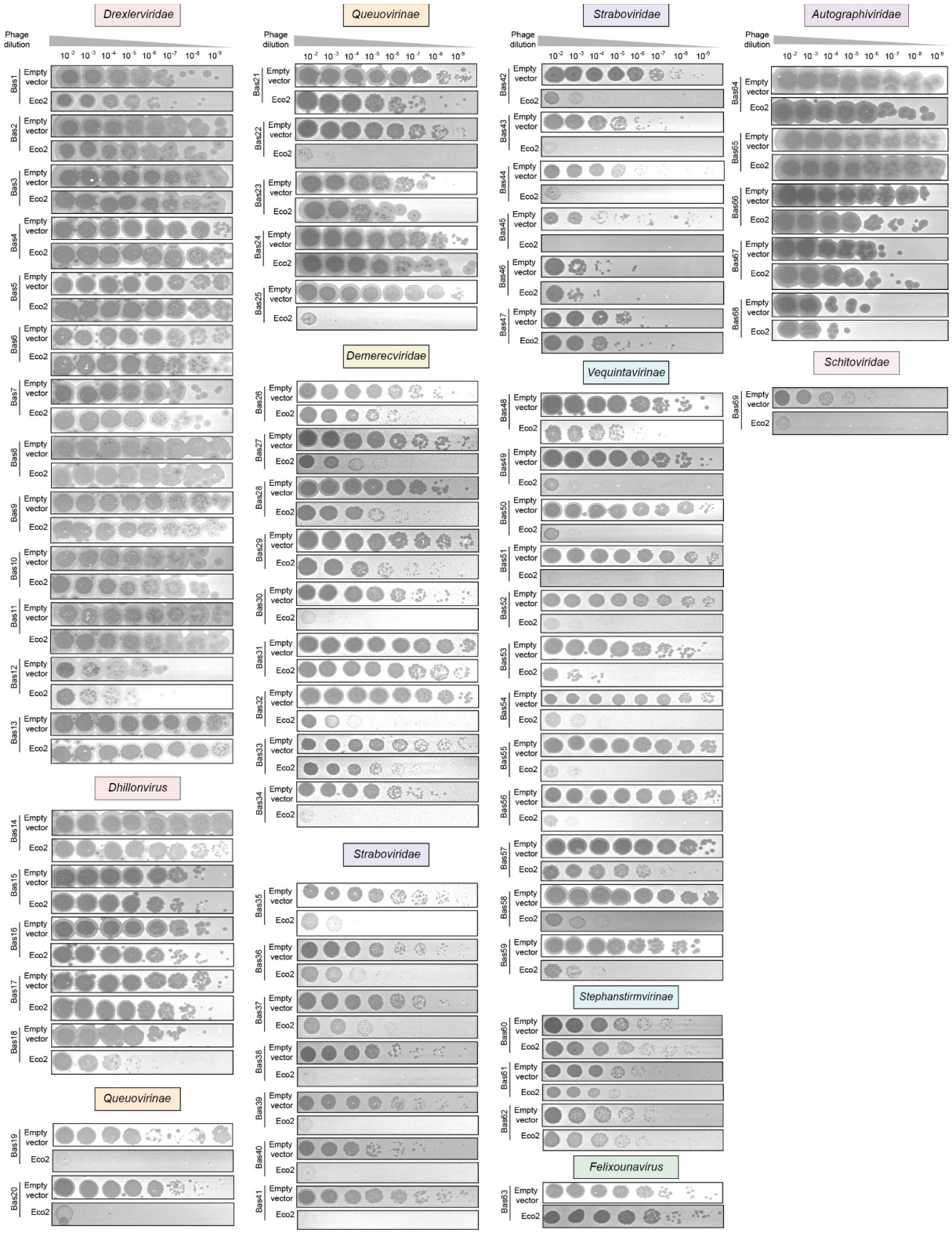
Supplementary Figures



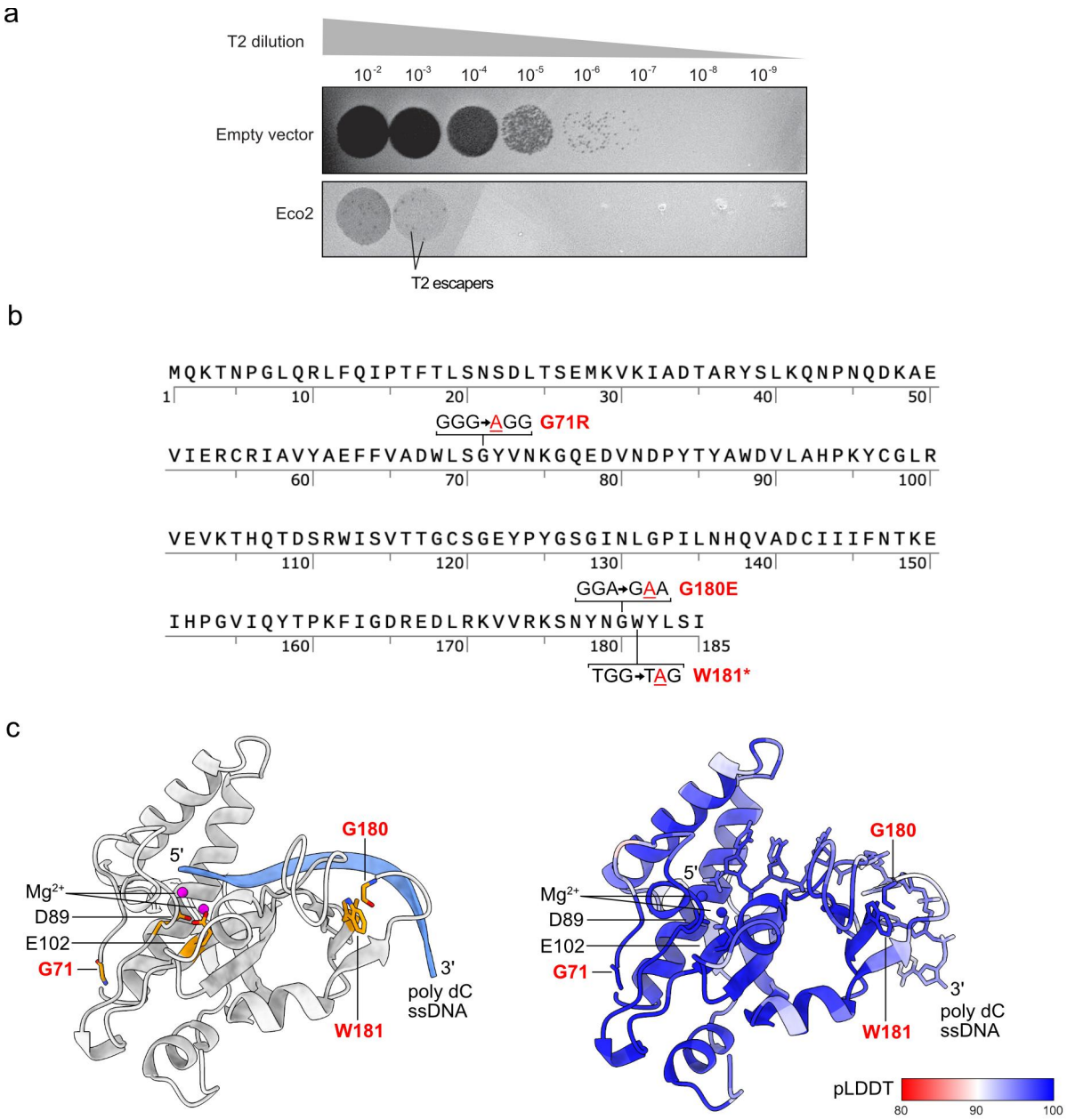
Supplementary Fig. 1: Phylogenetic relationships of type I-C RT and TOPRIM domains. Separate phylogenetic trees for RT (left) and TOPRIM (right) domains from retron types I-C, XI, and XII are shown (color-coded according to legend). Linking lines in the tanglegram connect RT and TOPRIM domains from the same protein.



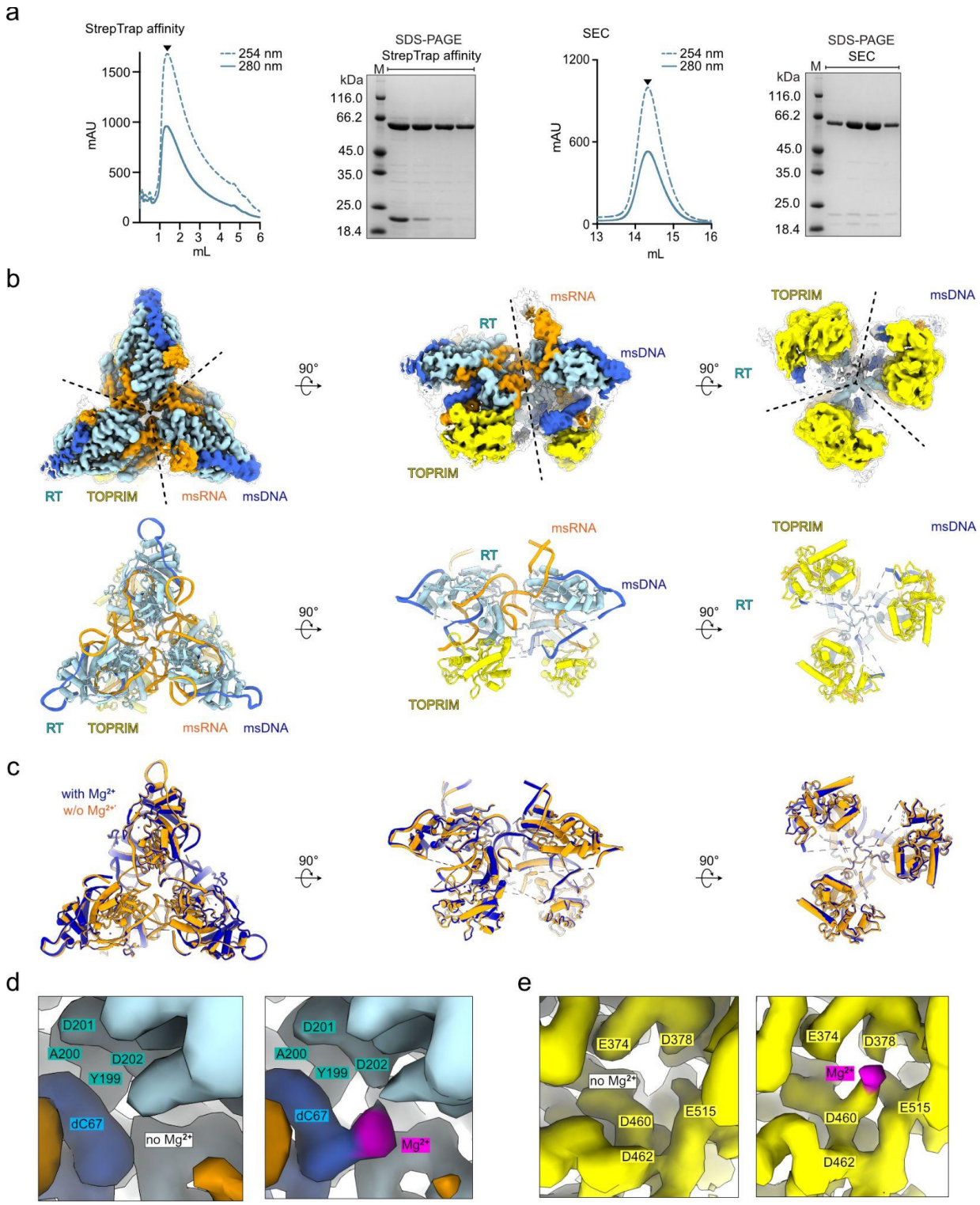
Supplementary Fig. 2: Eco2 defends against phages. a-c, *E. coli* growth curves in dependence of Eco2 infection at varying MOI; for phages T2 (**a**), T5 (**b**), and in the absence of phage (**c**). n = 3 technical replicates; mean ± s.d..



Supplementary Fig. 3: Eco2 defense specificity across the BASEL collection. Shown are representative spot plating results for BASEL phages [8] propagating on *E. coli*, either in presence of an empty vector control (pACYC184) or a vector producing Eco2 (pLG006 [9]).



Supplementary Fig. 4: T2 escapes by mutation of *denB*. a, Representative spot plating for T2 on *E. coli*, either in presence of an empty vector control (pACYC184) or a vector expressing Eco2 (pLG006). Escaper plaques are marked with lines. **b**, DenB protein sequence highlighting escaper mutations in bold red. Corresponding DNA mutations are shown. **c**, Left: AlphaFold3-predicted structure of T2-DenB (grey) with catalytic magnesium ions (purple spheres) and poly-C DNA (blue band). The amino acids mutated in the escaper variant are highlighted in orange and labeled in red. Right: pLDDT values mapped on the structure (color code), indicating prediction confidence (>90 highly confident).



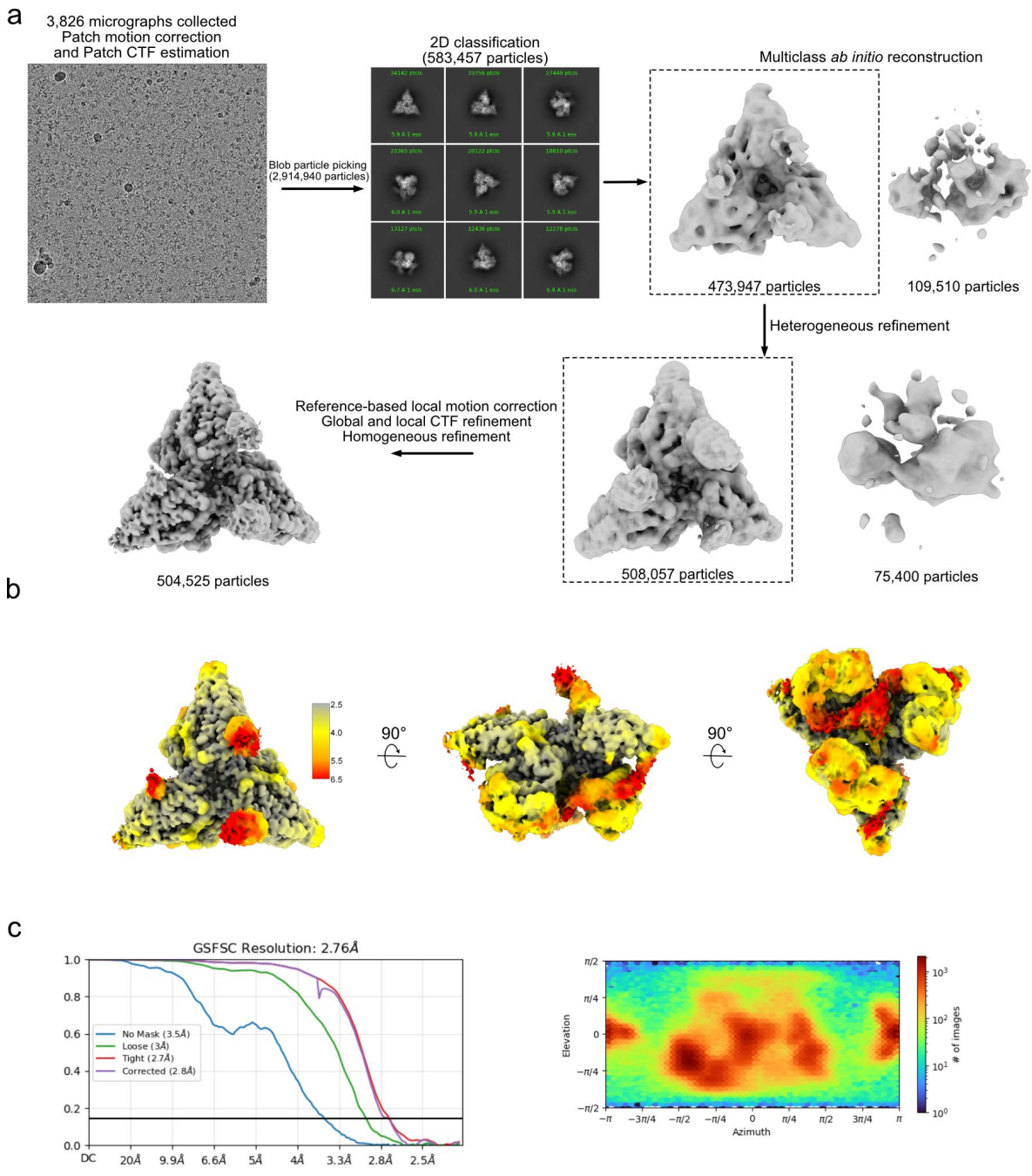
715

716 **Supplementary Fig. 5: Purification of Eco2 and cryo-EM structure in absence of magnesium.**

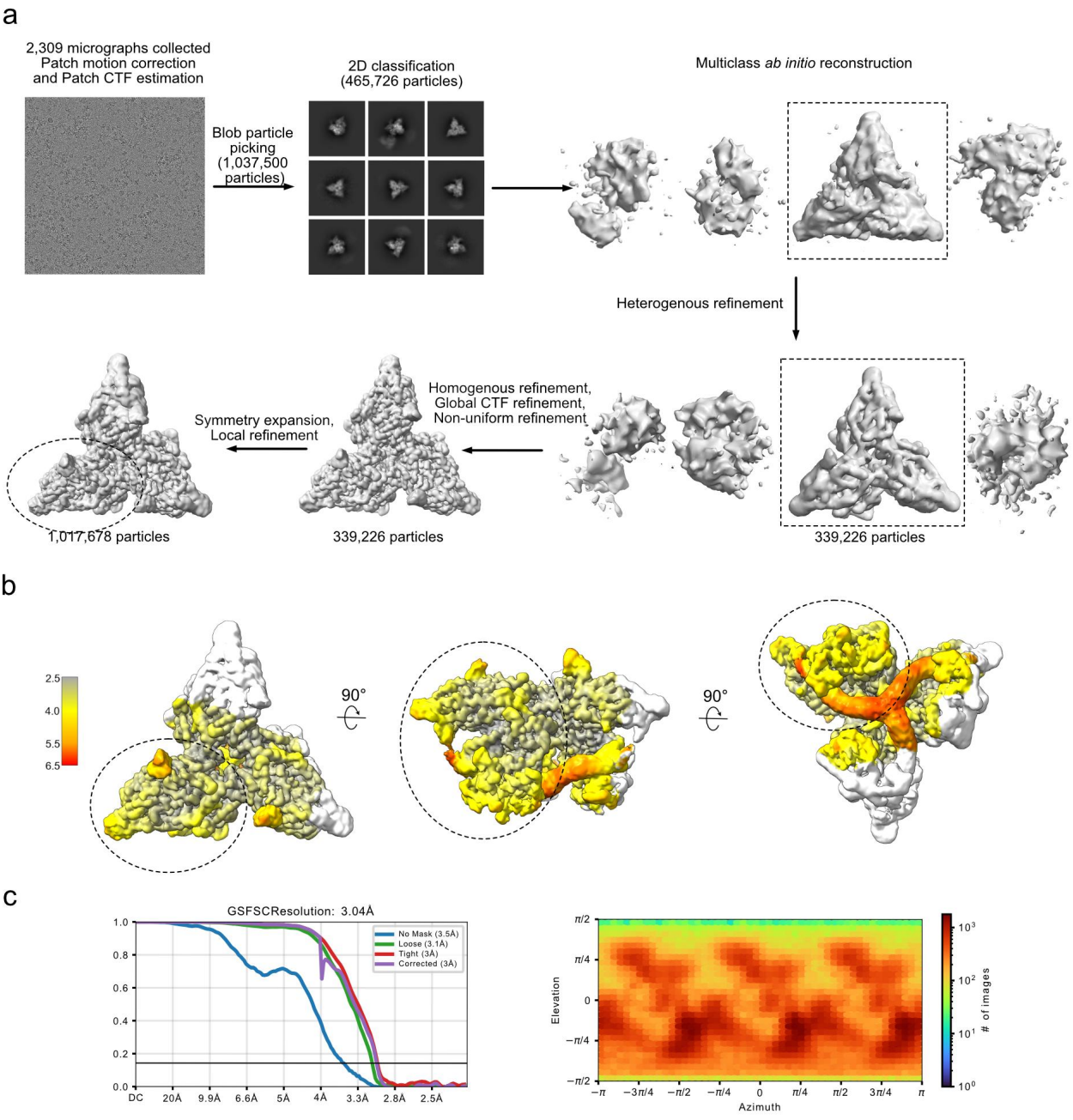
717 **a**, Strep-trap affinity and SEC chromatograms (traces) with corresponding analytical SDS-PAGEs.

718 **b**, Cryo-EM maps (above) and corresponding model (below) of Eco2 trimers in absence of

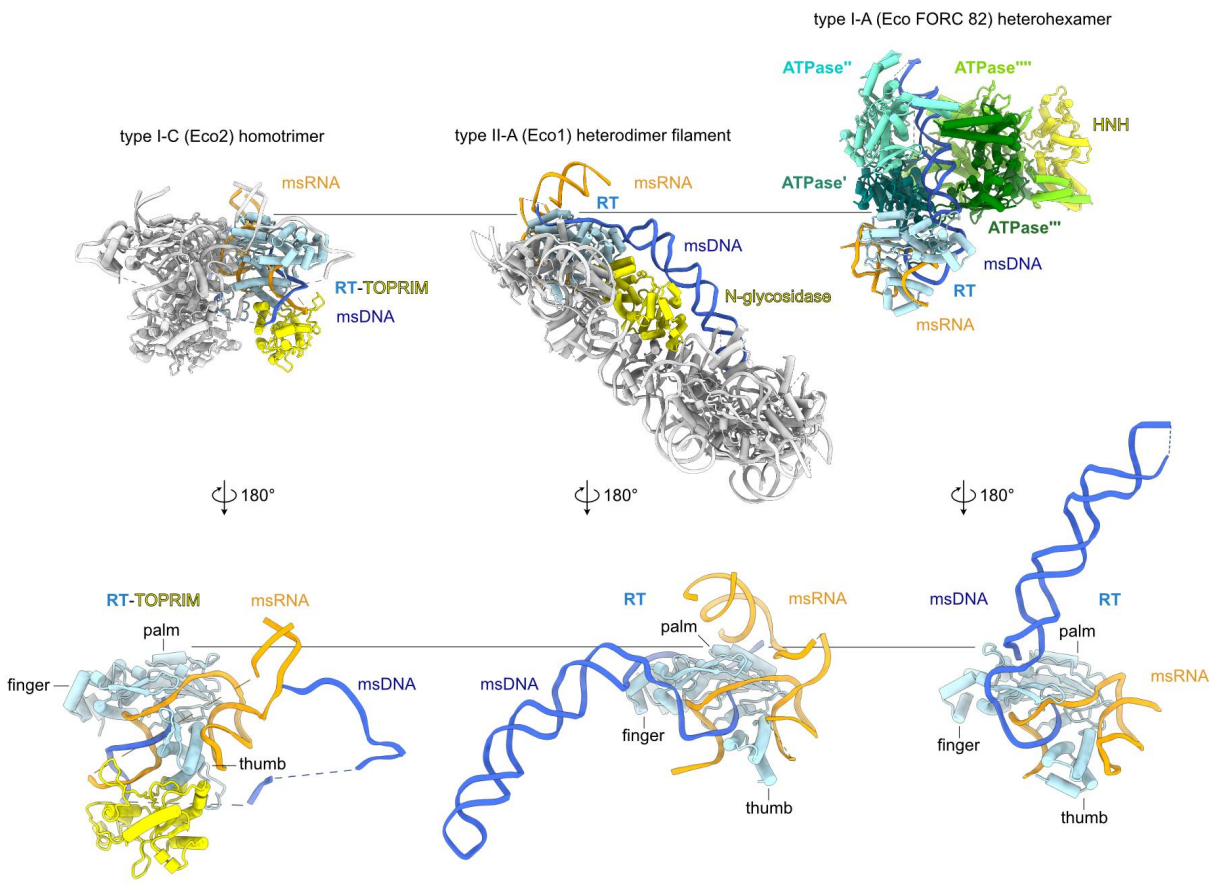
magnesium, shown in three 90°-rotations. Maps and models are color-coded according to **Fig. 1**, with unfiltered maps shown as translucent surfaces. **c**, Superimposition of Eco2 structures determined in absence (orange) and presence (blue) of magnesium, shown in three 90°-rotations. **d and e**, Close-up side-by-side comparison of the RT (**d**) and TOPRIM (**e**) active sites in absence (left) and presence (right) of magnesium (purple). The sharpened cryo-EM maps are shown.



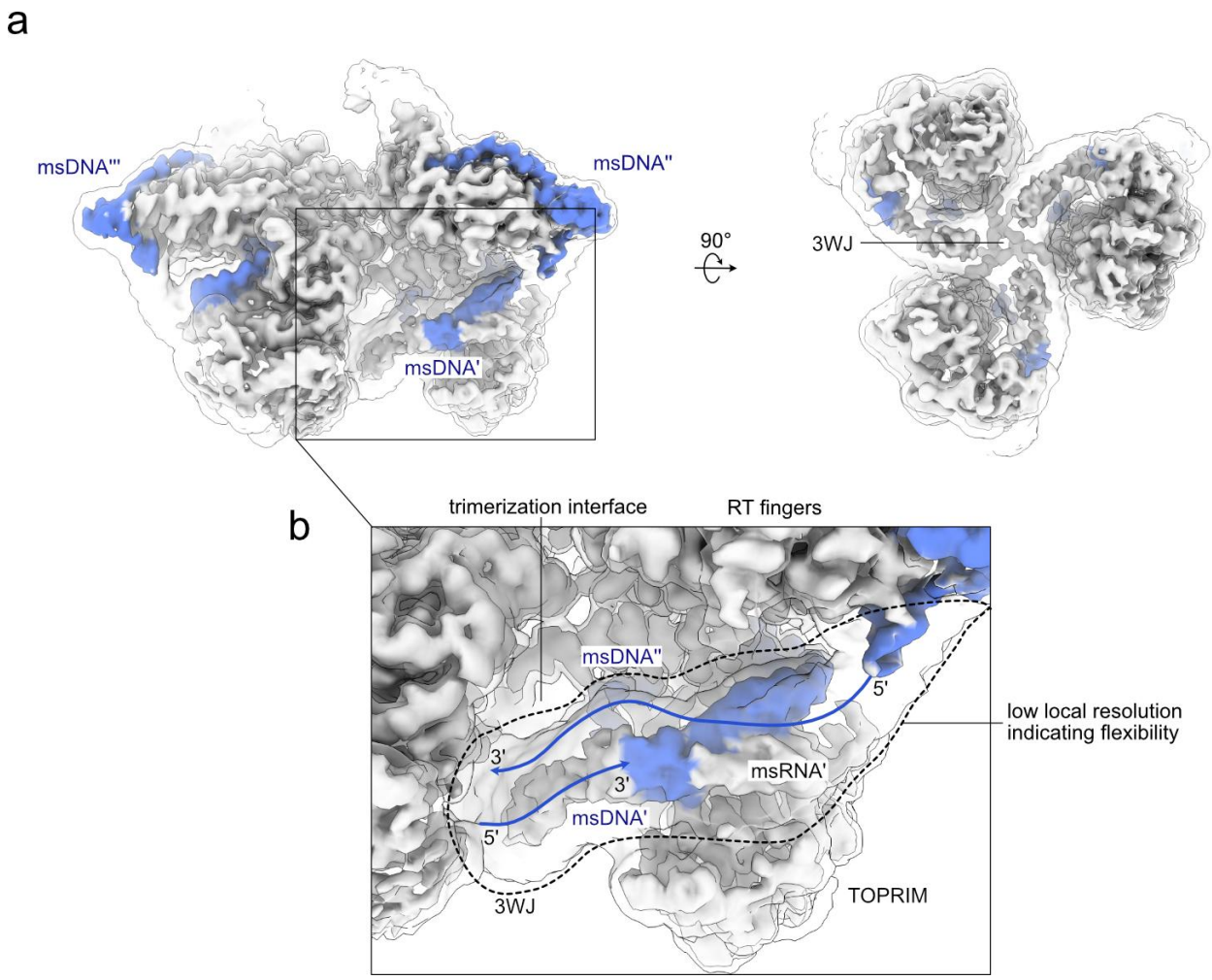
Supplementary Fig. 6: Cryo-EM data processing for Eco2 in absence of magnesium. a, Processing workflow as performed in CryoSPARC. **b**, Local resolution map. **c**, FSC curves and angular distribution plots calculated in CryoSPARC.



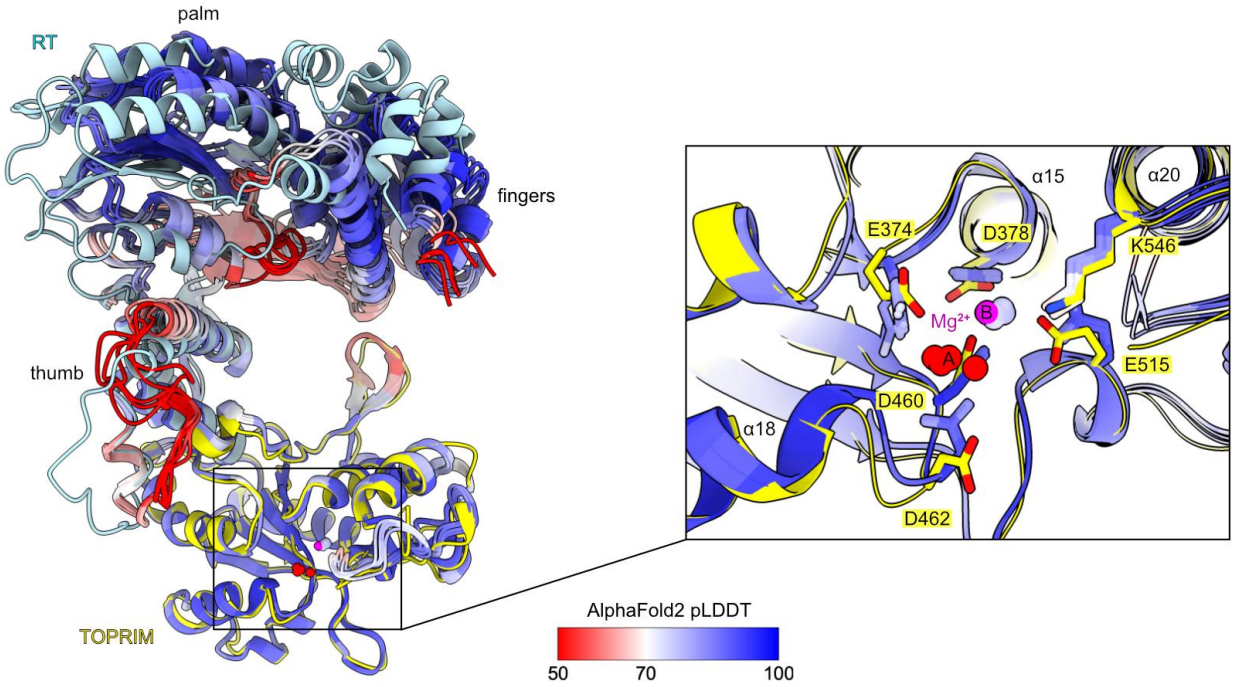
Supplementary Fig. 7: Cryo-EM data processing for Eco2 in presence of magnesium. a, Processing workflow as performed in CryoSPARC. **b**, Local resolution map. Dashed outlines indicate the locally refined subunit. **c**, FSC curves and angular distribution plots calculated in CryoSPARC.



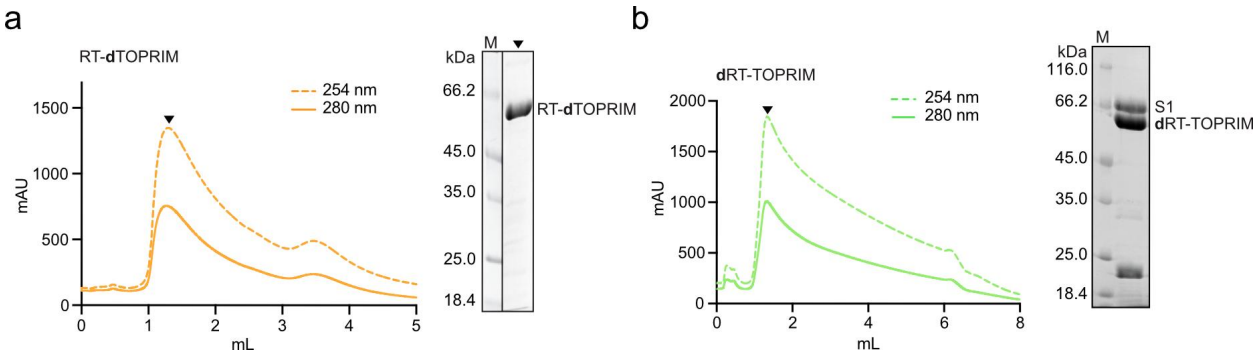
Supplementary Fig. 8: Comparison of retron types I-C, II-A and I-A. a and b, Multimeric structures (**a**) and individual RT:msRNA:msDNA structures (**b**) of Eco2 (type I-C, this study), Eco1 (type II-A, PDB-ID 8QBK [35]) and Eco FORC 82 (type I-A, coordinates provided by B. Wiedenheft [36]). Structures were aligned via the RT and are displayed side-by-side. Coloring according to subunits; only a single RT, its nucleic acid product, and the associated effector are colored for simplicity.



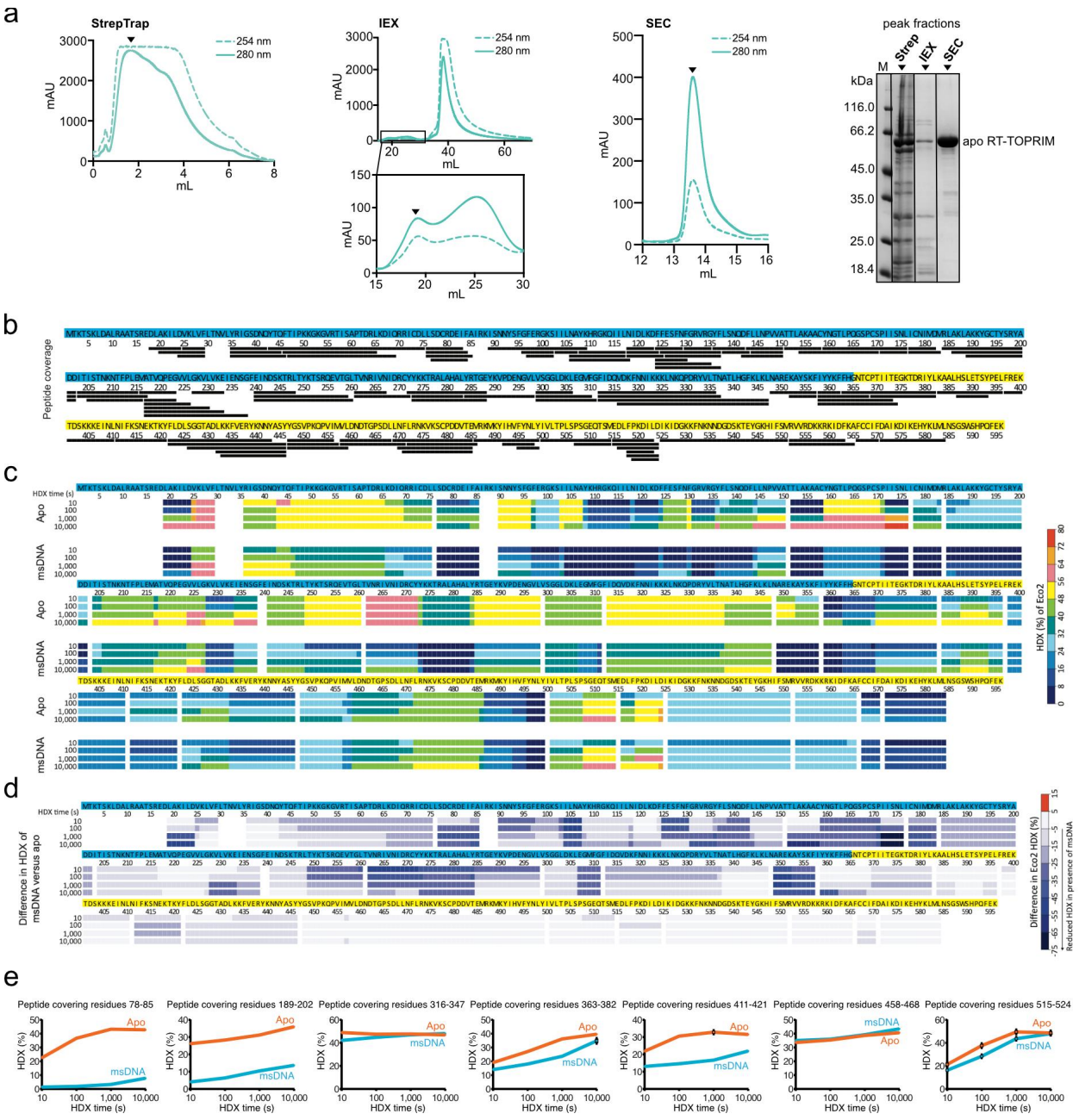
Supplementary Fig. 9: Conformational flexibility of the msDNA. a, Cryo-EM maps of magnesium-bound Eco2 in two 90*-rotated orientations. The sharpened map is colored in gray and blue (msDNA). The unfiltered map is shown as a translucent surface. **b**, Close-up on the msDNA segments that lie adjacent to the trimerization interface. Blue arrows indicate msDNA-paths and polarity. The dotted outline highlights local low-resolution areas.



Supplementary Fig. 10: Comparison of the experimental and AlphaFold models. Left: Superimposition of five AlphaFold2-predicted magnesium-bound RT-TOPRIM models (color-coded from red to blue according to pLDDT scale). The experimental model is colored according to the protein domains (light blue: RT, and yellow: TOPRIM) and bound magnesium (magenta). Right: Close-up on the TOPRIM active site. Active site residues are shown as sticks. A and B indicate magnesium binding sites.

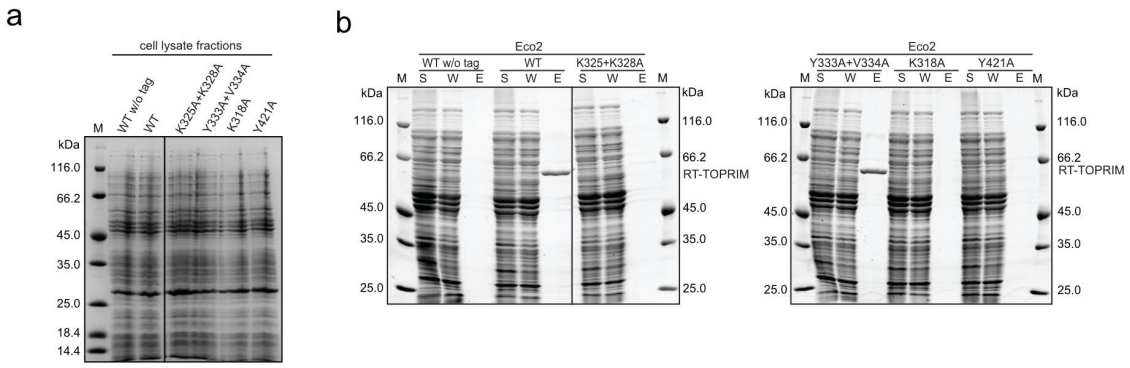


Supplementary Fig. 11: StrepTrap affinity-purification of dRT and dTOPRIM Eco2 variants. a and b, StrepTrap affinity-purification chromatograms (traces), as well as the corresponding analytical SDS-PAGEs for the dTOPRIM (**a**) and dRT (**b**) variants.

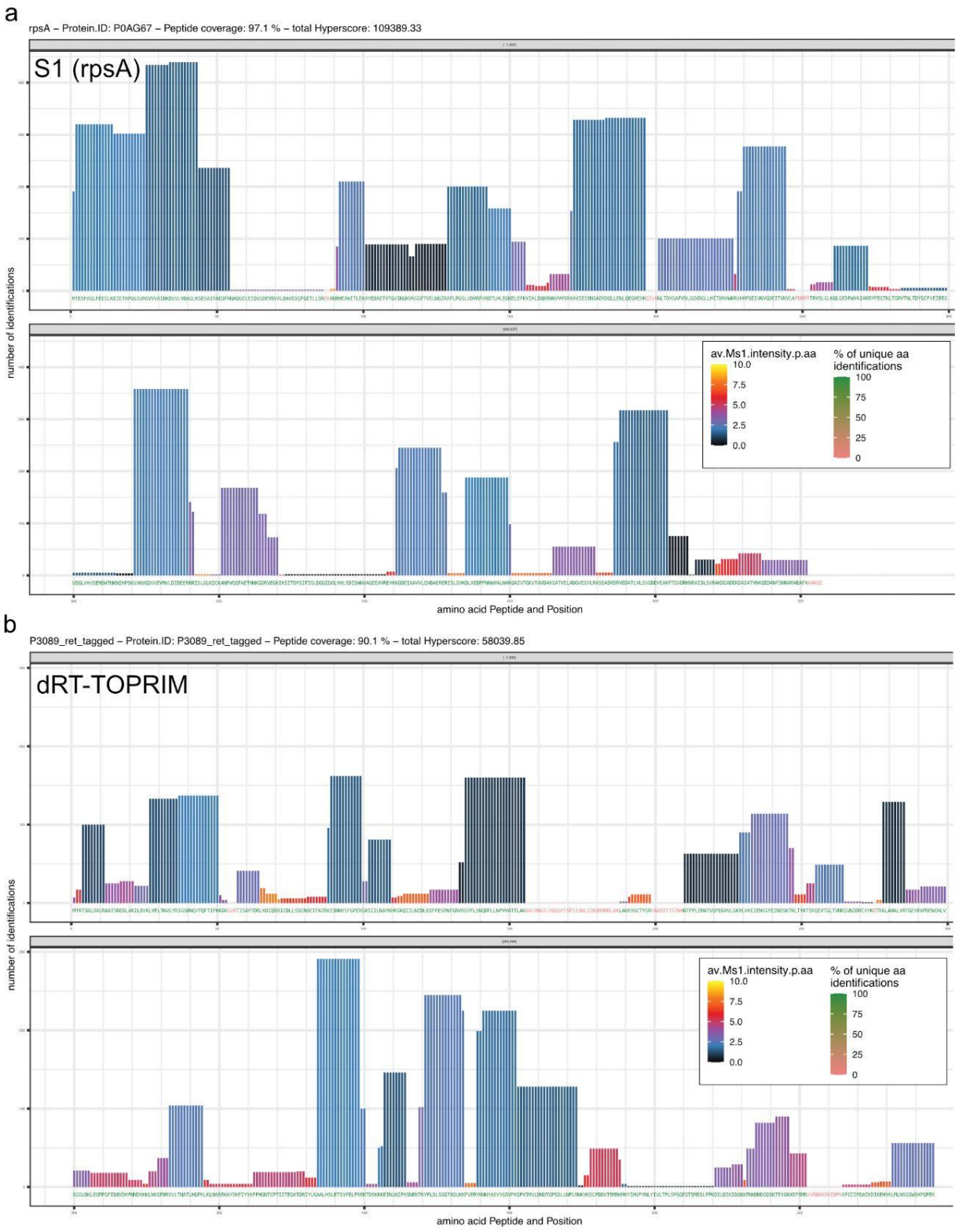


Supplementary Fig. 12: HDX-MS of Eco2. a, StrepTrap affinity, ion-exchange (IEX) and SEC chromatograms (traces), as well as the corresponding analytical SDS-PAGEs of the apo RT-TOPRIM protein purification. **b**, HDX-MS peptide coverage of the RT-TOPRIM protein. Each black bar denotes a peptide identified in the HDX-MS experiments. Blue and yellow highlighted sequences correspond to the RT and TOPRIM domains, respectively. **c**, Residue-specific HDX of the apo state or when bound to msDNA, derived from HDX analysis of peptides from **b**. **d**, Difference in residue-specific HDX of the RT-TOPRIM between the msDNA-bound and apo

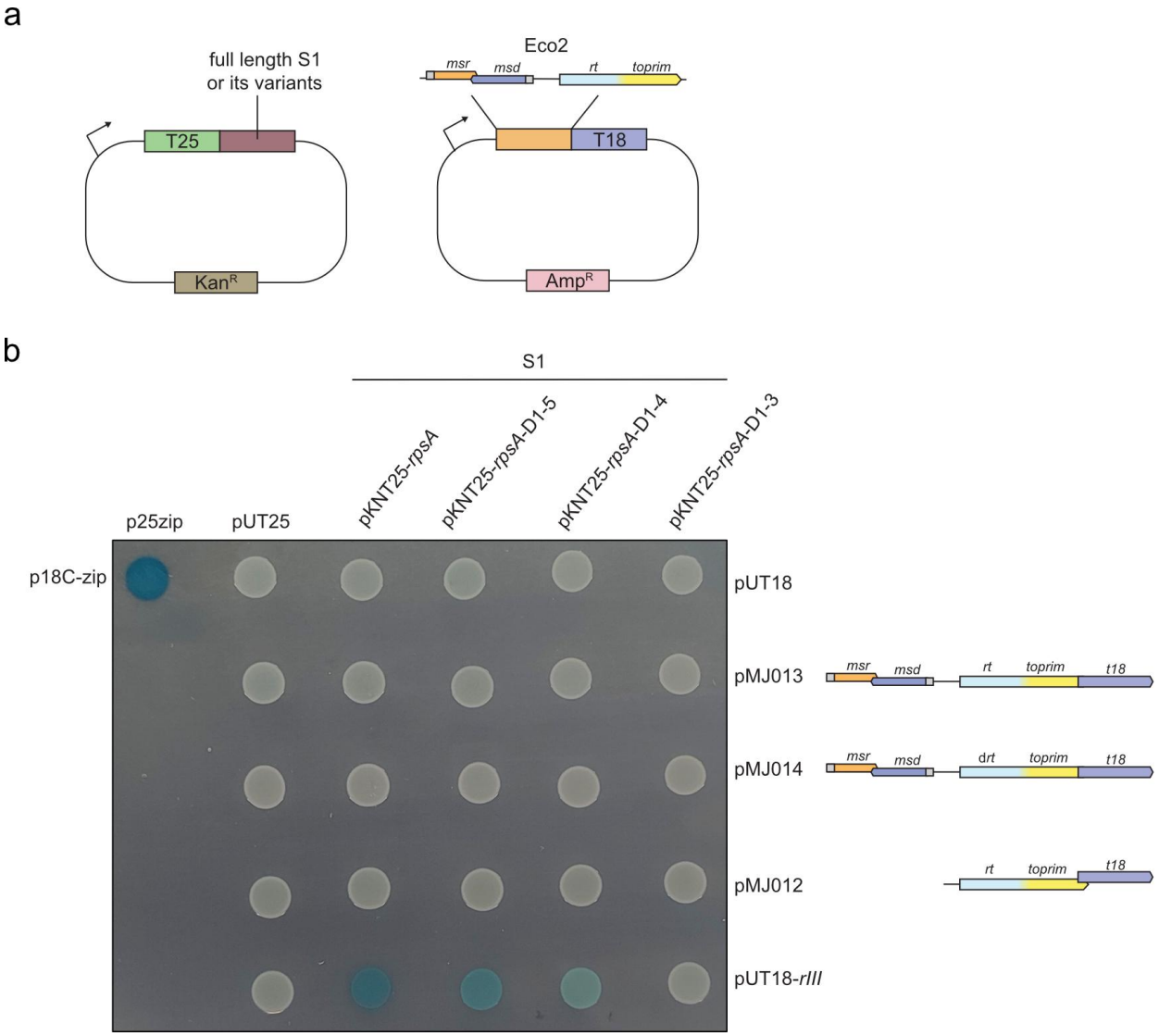
795 states. Blue color denotes reduced HDX in presence of msDNA. **e**, Progression of HDX of selected
796 representative peptides. Data represent the mean ± s.d. of $n = 3$ replicates.



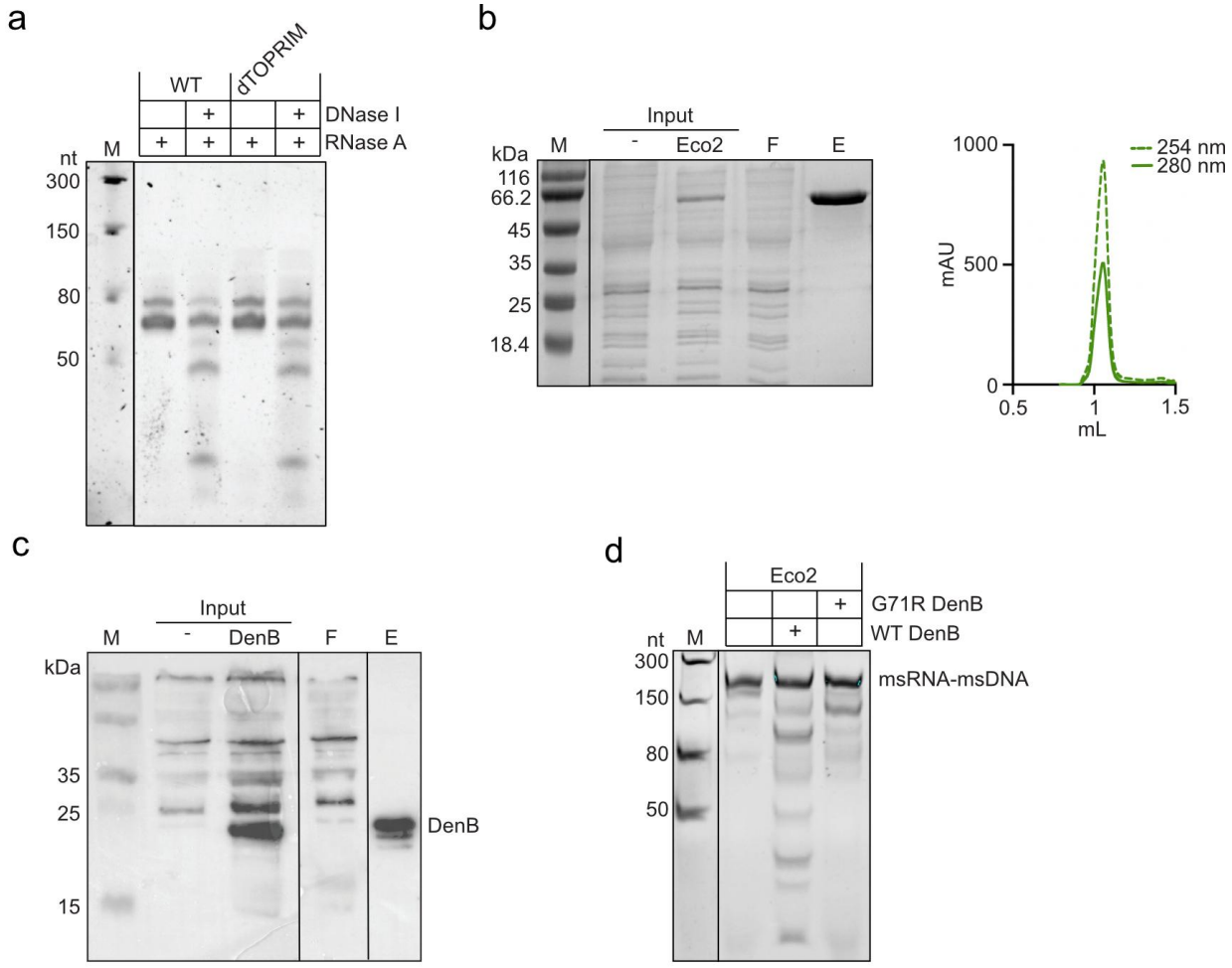
Supplementary Fig. 13: Small-scale expression and purification of Eco2 variants. a, SDS-PAGE analysis of whole cell protein content after overnight incubation. **b**, SDS-PAGE of soluble (S), wash (W) and elution (E) StrepTrap affinity-chromatography fractions.



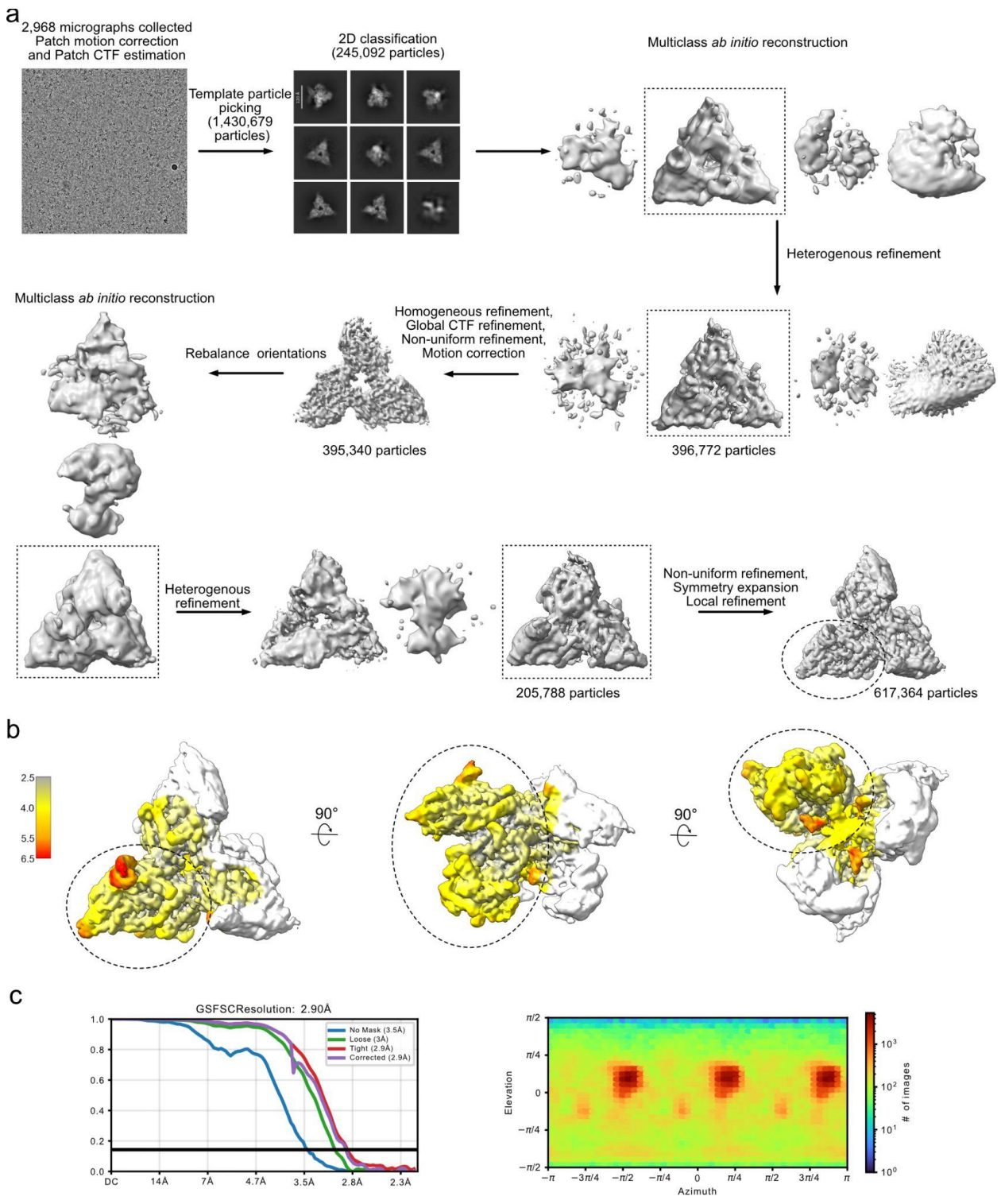
Supplementary Fig. 14: Mass spectrometry analysis of dRT-purifications. a and b, shown are the number of peptide identifications, intensities and % unique identifications for the S1 (**a**) and dRT-TOPRIM (**b**) samples.



Supplementary Fig. 15: Bacterial Adenylate Cyclase Two-Hybrid assay. a, Scheme illustrating the BACTH plasmid setup. **b**, Representative BACTH result (n = 3). Blue spots indicate interaction, while white spots indicate no interaction. The scheme next to the Eco2 constructs (pMJ012-014) illustrates the different tested *rt-toprim-t18* constructs.



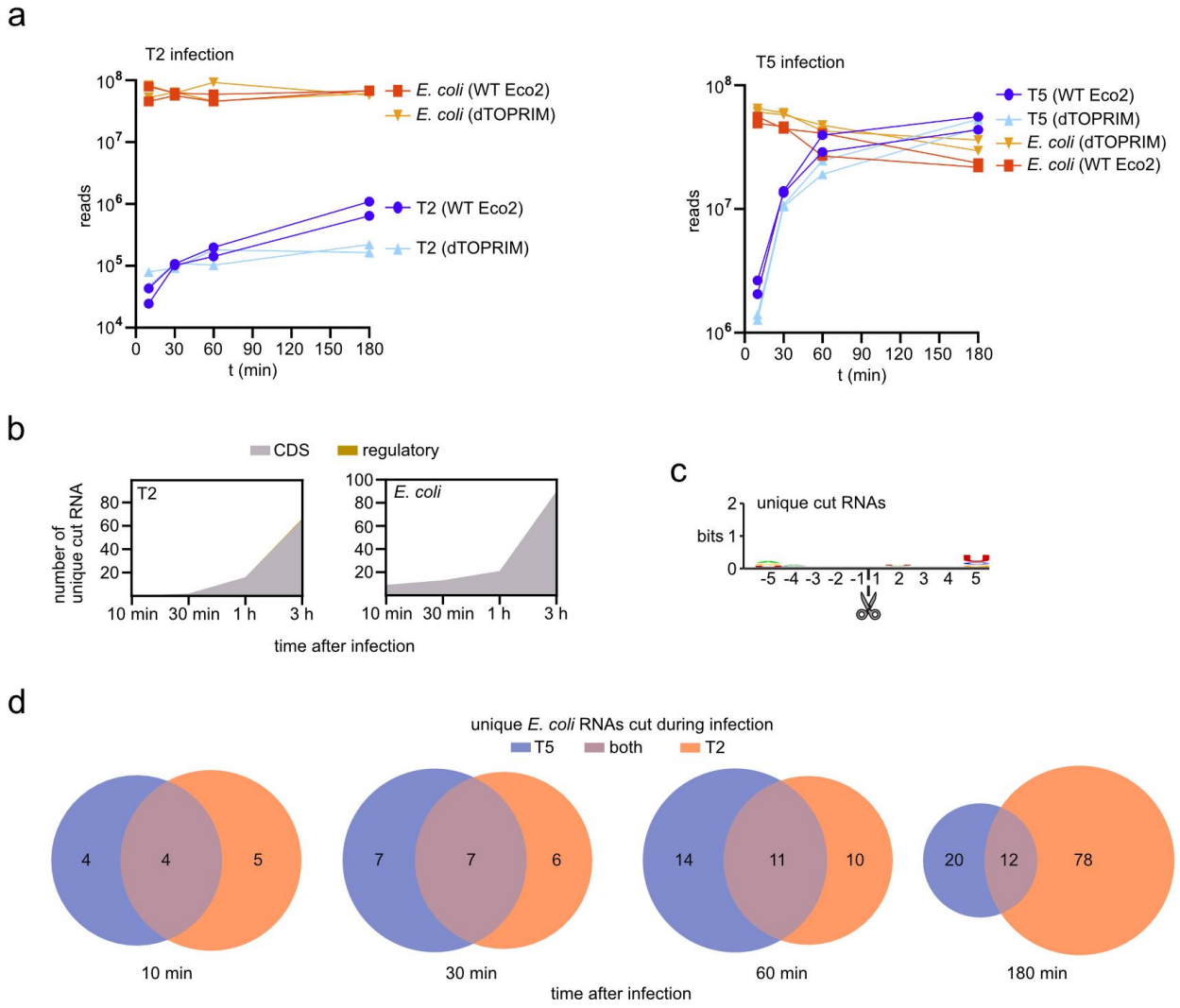
Supplementary Fig. 16: The msDNA is cut by DNases. a, Urea-PAGE analysis showing cleavage products of RT-TOPRIM:msDNA complexes treated with DNase I. RNase I digestion was performed after nucleic acid extraction. **b**,Left: SDS-PAGE of in CFE by DenB activated and subsequently StrepTrap-affinity purified Eco2. Right: Size-exclusion chromatography trace of DenB-activated Eco2. The sample was run on an S200 3.2 x 300. **c**, Western blot of CFE-expressed and subsequently Strep-bead purified DenB. **d**, Urea-PAGE separating DenB cleavage products.



Supplementary Fig. 17: Cryo-EM data processing for activated Eco2. a, Processing workflow as performed in CryoSPARC. **b**, Local resolution map. Dashed outlines indicate the locally refined subunit. **c**, FSC curves and angular distribution plots calculated in CryoSPARC.

ratio WT/dTOPRIM	RNA	cut sequence
176.9	tRNA-Val	GGGUGAUUAGCUCAGCUGGGAGAGCACCUCCCUUACAAGGAGGGGGUCGGCGGUUCGAUCCCGUCAUCAC | CCACCA
126.0	tRNA-Val	GGGUGAUUAGCUCAGCUGGGAGAGCACCUCCCUUACAAGGAGGGGGUCGGCGGUUCGAUCCCGUCAUCAC | CCACCA
46.8	tRNA-Trp	A | GGGGCGUAGUUCAAUUGGUAGAGCACCGGUCUCCAAAACCGGGUGUUGGGAGUUCGAGUCUCUCCGCCCCUGCCA
32.2	tRNA-Trp	AGGGGCGUAGUUCAAUUGGUAGAGCACCGGUCUCCAAAACCGGGUGUUGGGAGUUCGAGUCUCUCCGCC | CCUGCCA
26.3	tRNA-Ser	GGUGAGGUGUCCGAGUGGCUGAAGGAGCACGCCUGGAAAGUGUGUAUACGGCAACGUAUCGGGGGUUCGAAUCCCCCCCUCA | CCGCCA
56.0	tRNA-His	GGUGGCUAUAGCUCAGUUGGUAGAGCCCUGGAUUGUGAUUCCAGUUGUCGUGGGUUCGAAUCCCAUUAGC | CACCCCA
26.3	tRNA-His	GGUGGCUAUAGCU | CAGUUGGUAGAGCCCUGGAUUGUGAUUCCAGUUGUCGUGGGUUCGAAUCCCAUUAGCCACCCCA
45.3	tRNA-Gly	GCGGGAAUAGCU | CAGUUGGUAGAGCACGACCUUGCCAAGGUCGGGGUCGCGAGUUCGAGUCUCGUUUCCCGCUCCA
36.5	tRNA-Gly	GCGGGAAUAGCUCAGUUGGUAGAGCACGACCUUGCCAAGGUCGGGGUCGCGAGUUCGAGUCUCGUUUCC | CGCUCCA
30.7	tRNA-Gly	GCGGGAAUAGCUCAGUUGGUAGAGCACGACCUUGCCAAGGUCGGGGUCGCGAGUUCGAGUCUCGUUUCCC | GCUCCA
24.1	tRNA-Gly	GCGGGAAUAGCUCAGUUGGUAGAGCACGACCUUGCCAAGGUCGGGGUCGCGAGUUCGAGUCUCGUUUCCC | GCUCCA
23.4	tRNA-Gly	GCGGGAAUAGCUCAGUUGGUAGAGCACGACCUUGCCAAGGUCGGGGUCGCGAGUUCGAGUCUCGUUUCCC | GCUCCA
20.5	tRNA-Gly	GCGGGAAUAGCU | CAGUUGGUAGAGCACGACCUUGCCAAGGUCGGGGUCGCGAGUUCGAGUCUCGUUUCCCGCUCCA
60.7	tRNA-Gln	UGGGGUAUCGCCAAGCGGUAAGGCACCGGUUUUUGAUACCGGCAUUCCCUGGUUCGAAUCCAGGUACCC | CAGCCA
59.9	tRNA-Gln	UGGGGUAUCGCCAAGCGGUAAGGCACCGGUUUUUGAUACCGGCAUUCCCUGGUUCGAAUCCAGGUACC | CCAGCCA
50.4	tRNA-Gln	UGGGGUAUCGCCAAGCGGUAAGGCACCGGUUUUUGAUACCGGCAUUCCCUGGUUCGAAUCCAGGUACC | CCAGCCA
33.3	tRNA-Gln	UGGGGUAUCGCCAAGCGGUAAGGCACCGGUUUUUGAUACCGGCAUUCCCUGGUUCGAAUCCAGGUACCC | CAGCCA
27.8	tRNA-Gln	UG | GGGUAUCGCCAAGCGGUAAGGCACCGGUUUUUGAUACCGGCAUUCCCUGGUUCGAAUCCAGGUACCCCAGCCA
25.0	tRNA-Gln	UGGGGUAUCGCCAAGCGGUAAGGCACCGGAUUCUGAUUCCGGCAUUCCGAGGUUCGAAUCCUCGUACC | CCAGCCA
25.0	tRNA-Gln	UGGGGUAUCGCCAAGCGGUAAGGCACCGGAUUCUGAUUCCGGCAUUCCGAGGUUCGAAUCCUCGUACCC | CAGCCA
23.0	tRNA-Gln	UGGGGUAUCGCCAAGCGGUAAGGCACCGGAUUCUGAUUCCGGCAUUCCGAGGUUCGAAUCCUCGUACC | CCAGCCA
23.0	tRNA-Gln	UGGGGUAUCGCCAAGCGGUAAGGCACCGGAUUCUGAUUCCGGCAUUCCGAGGUUCGAAUCCUCGUACCC | CAGCCA
20.0	tRNA-Gln	UG | GGGUAUCGCCAAGCGGUAAGGCACCGGUUUUUGAUACCGGCAUUCCCUGGUUCGAAUCCAGGUACCCCAGCCA
21.9	tRNA-Asp	GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCCUGUCACGCAGGGGGUCGCGGGUUCGAGUCCCGUCCGUU | CCGCCA
24.9	sRNA OxyS	AGUUUCUCAACUCGA | AUAACUAAAGCCAAC
21.0	16S rRNA	CGGCAGGCCUAACAC | AUGCAAGUCGAACGG
20.5	16S rRNA	CGGCAGGCCUAACAC | AUGCAAGUCGAACGG

Supplementary Fig. 18: RNA sequencing results. Shown are the most frequently cleaved RNAs (color-coded according to their identity, middle column) in the presence of WT Eco2 and WT DenB, as well as their enrichment compared to the sample containing deactivated dTOPRIM (left column).



Supplementary Fig. 19: RNA sequencing of infected *E. coli*. **a**, Total number of reads aligned to bacteria and phage genomes during T2 (left) and T5 (right) infection. $n = 2$ biological replicates. **b**, Number of unique cleaved phage T2 and bacterial transcripts during T2 infection. **c**, Weblogo illustrating the sequence of 10 most prominently cut T5 RNAs, other than tRNA. **d**, Comparison of bacterial transcripts cut during infection by T5 and T2 phages.

Supplementary tables

Supplementary Tab. 1: Cryo-EM data collection, refinement and validation statistics

	Eco2 in absence of magnesium (EMD-52583, PDB-ID: 9I2F)	**Eco2 in presence of magnesium** (EMD-52584, PDB-ID: 9I2G)	**Activated Eco2 in presence of magnesium** (EMD-54448, PDB-ID: 9S1F)
Data collection and processing			
Magnification	×92000		
Voltage (kV)	200		
Exposure (e^-/Å^2)	30		
Defocus range (μm)	-2.0 to -1.0		
Pixel size (Å)	1.1		
Initial particles	2,914,940	1,037,763	2,271,864
Final particles	504,525	1,017,678	617,364
Symmetry imposed	C1	C1	C1
Resolution (Å)	2.76	3.04	2.90
FSC cut-off	0.143	0.143	0.143
Map resolution range (Å)	2.5-7	2.5-6.5	2.5-6.5
Refinement			
Model resolution (Å)	3.2	3.3	3.2
0.143 FSC Threshold (Å)	2.7	3.1	2.9
Map sharpening *B* factor	121.6	119.2	130.3
Model composition			
Non-hydrogen atoms	19,847	19,824	18,445
Protein residues	1738	1738	1713
Nucleotides	277	275	218
Ligands	0	MG: 21	MG: 6
***B* factors (Å^2)**			
Protein	137.02	133.60	132.65
Nucleotide	156.81	151.90	128.88
Ligands	-	30	30
Validation			
Bonds (r.m.s. deviations)			
Length (Å) (# > 4s)	0.003 (0)	0.004 (0)	0.004 (0)
Angles (°) (# > 4s)	0.818 (0)	0.852 (1)	0.869 (2)
MolProbity score	1.23	1.18	1.40
Clash score	4.59	3.98	7.22
Poor rotamers (%)	0.00	0.00	0.00
Ramachandran plot (%)			
Outliers	0.12	0.12	0.06
Allowed	0.41	0.46	0.41
Favored	99.48	99.42	99.53

Rama-Z score, whole (r.m.s. Rama-Z)	-1.40 (0.20)	-1.47 (0.20)	-1.34 (0.20)
CC (mask)	0.80	0.72	0.68
CC (box)	0.83	0.83	0.72

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859 **Supplementary Tab. 2: Plasmids**

ID	Purpose	Features	Selection marker
pLG006 (Gao *et al.* 2020 [9], Addgene #157885)	Phage plaque assays; expression of Eco2	pACYC-derived vector encoding the Eco2 locus.	Chloramphenicol
pACYC184 (NEB)	Phage plaque assays; empty vector control	p15A-ori, cloning vector	Chloramphenicol
pMJ001	Protein purification, activity assays *in vitro* and *in vivo*	Derivative of pLG006. Contains a Strep-tag II encoding sequence fused downstream to the *rt-toprim* gene of the Eco2 locus	Chloramphenicol
pMJ002	Protein purification, activity assays *in vitro* and *in vivo,* phage plaque assays	Derivative of pLG006. Contains a Strep-tag II-encoding sequence fused downstream to the *rt-toprim* gene (D201A, D202A mutant) of the Eco2 locus.	Chloramphenicol
pMJ003	Protein purification, activity assays *in vitro* and *in vivo,* phage plaque assays	Derivative of pLG006. Contains a Strep-tag II-encoding sequence fused downstream to the *rt-toprim* gene (D460A, D462A, E374A, D378A mutant) of the Eco2 locus.	Chloramphenicol
pMJ004	Protein purification, activity assays *in vitro* and *in vivo*	Derivative of pLG006. Contains a Strep-tag II-encoding sequence fused downstream to the *rt-toprim* gene (Y333A, V334A mutant) of the Eco2 locus.	Chloramphenicol
pMJ005	Protein purification, activity assays *in vitro* and *in vivo*	Derivative of pLG006. Contains a Strep-tag II-encoding sequence fused downstream to the *rt-toprim* gene (K325A, K328A mutant) of the Eco2 locus.	Chloramphenicol

pMJ006	Protein purification, activity assays *in vitro* and *in vivo*	Derivative of pLG006. Contains a Strep-tag II-encoding sequence fused downstream to the *rt-toprim* gene (K421A mutant) of the Eco2 locus.	Chloramphenicol
pMJ007	Protein purification, activity assays *in vitro* and *in vivo*	Derivative of pLG006. Contains a Strep-tag II-encoding sequence fused downstream to the *rt-toprim* gene (K318A mutant) of the Eco2 locus.	Chloramphenicol
pMJ008	Protein purification	pRSF-Duet1 (Novagen, Sigma-Aldrich 71341-3) derived plasmid encoding the *rt-toprim* of Eco2 fused to an N-terminal Strep tag II.	Kanamycin
pMJ009	Phage plaque assays	Derivative of pLG006. Contains a Strep-tag II-encoding sequence fused downstream to the *rt-toprim*gene (D460A mutant) of the Eco2 locus.	Chloramphenicol
pMJ010	Phage plaque assays	Derivative of pLG006. Contains a Strep-tag II-encoding sequence fused downstream to the *rt-toprim*gene (D462A mutant) of the Eco2 locus.	Chloramphenicol
pMJ011	Phage plaque assays	Derivative of pLG006. Contains a Strep-tag II-encoding sequence fused downstream to the *rt-toprim*gene (D460A, D462A mutants) of the Eco2 locus.	Chloramphenicol
pKT25-zip (Euromedex)	BACTH, positive control	Encodes leucine zipper of GCN4 fused to the T25 fragment	Kanamycin
pUT18C-zip (Euromedex)	BACTH, positive control	Encodes leucine zipper of GCN4 fused to the T18 fragment	Ampicillin
pKNT25 (Euromedex)	BACTH, negative control	Encodes T25 fragment (first	Kanamycin

		224 aa of CyaA)	
pUT18 (Euromedex)	BACTH, negative control	Encodes T18 fragment (225 to 399 aa of CyaA)	Ampicillin
pKNT25-*rpsA* (Juškauskas *et al.* 2022 [37])	BACTH	Encodes *E. coli* ribosomal protein S1, fused to the N-terminus of T25.	Kanamycin
pKNT25-*rpsA*-D1-5 (Juškauskas *et al.* 2022 [37])	BACTH	Encodes domains 1-5 of *E. coli* ribosomal protein S1 fused to the N-terminus of T25.	Kanamycin
pKNT25*rpsA*-D1-4 (Juškauskas *et al.* 2022 [37])	BACTH	Encodes domains 1-4 of *E. coli* ribosomal protein S1 fused to the N-terminus of T25.	Kanamycin
pKNT25*rpsA*-D1-3 (Juškauskas *et al.* 2022 [37])	BACTH	Encodes domains 1-3 of *E. coli* ribosomal protein S1 fused to the N-terminus of T25	Kanamycin
pUT18-*rIII* (Juškauskas *et al.* 2022 [37])	BACTH, positive control	Encodes T4 RIII protein fused to the N-terminus of T18 fragment	Ampicillin
pMJ012	BACTH	Encodes apo RT-TOPRIM fused to the N-terminus of T18.	Ampicillin
pMJ013	BACTH	Encodes the Eco2 locus with the RT-TOPRIM fused to the N-terminus of T18.	Ampicillin
pMJ014	BACTH	Encodes the Eco2 locus with the RT-TOPRIM (D201A, D202A variant) fused to the N-terminus of T18.	Ampicillin

Supplementary Tab. 3: Bacterial strains

Strain	Genotype	Purpose	Source
E. coli MG1655 ΔRM	*F– λ– ilvG– rfb-50 rph-1 Δmrr-hsdRMS-mcrBC ΔmcrA*	BASEL library screen	Maffei *et al.* 2021 [8]
E. coli MG1655	K-12 F^- λ^- $ilvG^-$ rfb-50 rph-1	*E. coli* growth assay, tRNA sequencing	Department of Protein-DNA interactions, Life Science Centre, Vilnius University

E. coli DH5α	*F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1*	Cloning, expression	Thermo Scientific (EC0112)
E. coli BL21 Star (DE3)	*F- ompT hsdSB (rB-mB-) gal dcm rne131 (DE3)*	Expression	Invitrogen (C601003)
E. coli BTH101	*F⁻ cys-99 araD139 galE15 galK16 rpsL1 (Str^R) hsdR2 mcrA1 mcrB1.*	BACTH	Euromedex (EUB002)

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863 **Supplementary Tab. 4: Oligonucleotides**

Name	Sequence (5'-3')	Purpose
IR921	GGCGGTAGCGGTGGCGGGATGCAGAAAACGAATCCGGGTTTACAGAG	F oligo for T2-*denB* amplification
IR923	CGAAGCACGATTCTACTCGGTTAAATGGAAAGATACCATCCGTTGTAGTTGCT	R oligo for T2-*denB* amplification
ORG13	GGATGCCCTTACTCTGTTGCTAACG	F oligo for fusion PCR
ORG113	GGATGCCCTTACTCTGTTGCTAACG	R oligo for fusion PCR
ORG330	GGCGGTAGCGGTGGCGGGATGGTCAGCAAGGGAGAGG	F oligo for mCherry amplification
ORG332	CGAAGCACGATTCTACTCGGTTATTTGTATAATTCGTCCATTCCACCTG	R oligo for mCherry amplification
MJ479	CTGCC	sequencing gel ladder
MJ480	TCCTCCTGCC	sequencing gel ladder
MJ481	GCGATTCCTCCTGCC	sequencing gel ladder
MJ482	GGGAGGCGATTCCTCCTGCC	sequencing gel ladder
MJ483	TTTTAGGGAGGCGATTCCTCCTGCC	sequencing gel ladder

MJ484	AAGGATTTTAGGGAGGCGATTCCTCCTGCC	sequencing gel ladder
MJ485	GAATCAAGGATTTTAGGGAGGCGATTCCTCCTGCC	sequencing gel ladder
MJ486	GCTCTGAATCAAGGATTTTAGGGAGGCGATTCCTCCTGCC	sequencing gel ladder
MJ487	GTATAGCTCTGAATCAAGGATTTTAGGGAGGCGATTCCTCCTGCC	sequencing gel ladder
MJ488	CTGCCGTATAGCTCTGAATCAAGGATTTTAGGGAGGCGATTCCTCCTGCC	sequencing gel ladder
MJ489	CACACCTGCCGTATAGCTCTGAATCAAGGATTTTAGGGAGGCGATTCCTCCTGCC	sequencing gel ladder
MJ490	CACAGCACACCTGCCGTATAGCTCTGAATCAAGGATTTTAGGGAGGCGATTCCTCCTGCC	sequencing gel ladder

Supplementary Tab. 5: Linear CFE-expression templates

Name	Sequence	Purpose
P70a-StrepII cassette	https://benchling.com/s/seq-SQYvBip14tuhbvIVXItv?m=slm-ESSTgnFsU5gMQBWl66EJ	Promoter cassette for CFE expression with *E. coli* RNA polymerase
PT714-StrepII cassette	https://benchling.com/s/seq-FBSscKhLFFYdCfW6c5fp?m=slm-7hqdLqo9BDaqy90Jmnx9	Promoter cassette for CFE expression with T7 RNA polymerase
PT714-His6 cassette	https://benchling.com/s/seq-Um41tScIBDlX3mrAWOZL?m=slm-zOhkYyjfS3vsrx4FsWyK	Promoter cassette for CFE expression with T7 RNA polymerase
P70a-StrepII-mCherry	https://benchling.com/s/seq-JZD6FZfH9499g2KINlRF?m=slm-qA2thCVtfYICpqC1QjDU	Linear DNA for mCherry expression in CFEs with an *E. coli* RNA polymerase
P70a-StrepII-DenB-wt	https://benchling.com/s/seq-MjzlpKuXzTeYT28fs11J?m=slm-NxsyAC4R8RrQWKGnhEFl	Linear DNA for WT DenB expression in CFEs with an *E. coli* RNA polymerase
PT714-StrepII-DenB-wt	https://benchling.com/s/seq-hSSZGV5oiOcDsuso7IcY?m=slm-zjQaEae8Nqpo787yXdOR	Linear DNA for WT DenB expression in CFEs with a T7 RNA polymerase
PT714-His_6-DenB-wt	https://benchling.com/s/seq-Ft2EMBa18zl1LNvEAKUJ?m=slm-7TDKSZ1yD9D5OvZA3Vtc	Linear DNA for WT DenB expression in

		CFEs with a T7 RNA polymerase
P70a-StrepII-DenB-G71R	https://benchling.com/s/seq-1rfpVjDqtQfgCo1R5Wfu?m=slm-RrWV9vK7eDWatKM0LNJH	Linear DNA for DenB-G71R expression in CFEs with an *E. coli* RNA polymerase

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