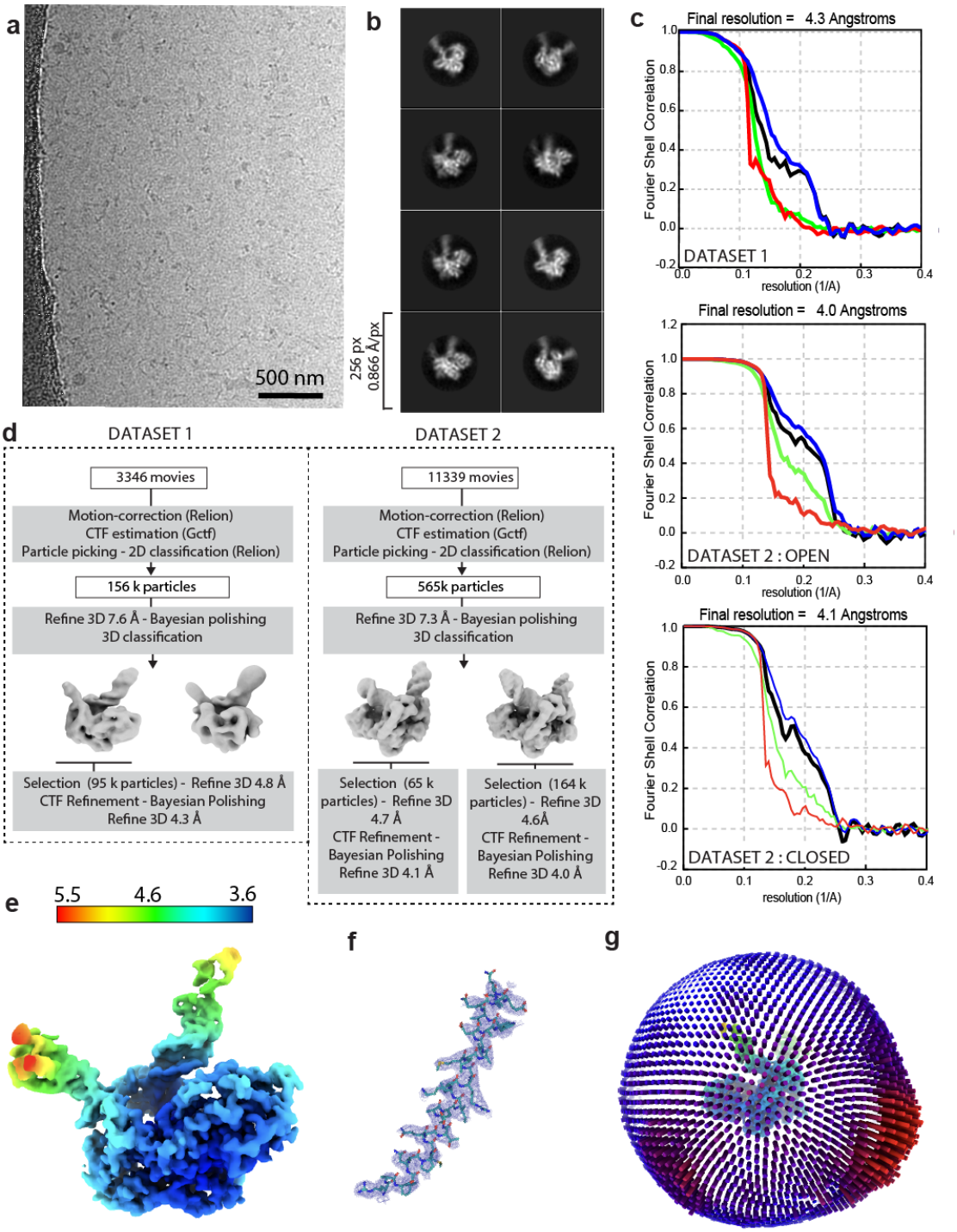
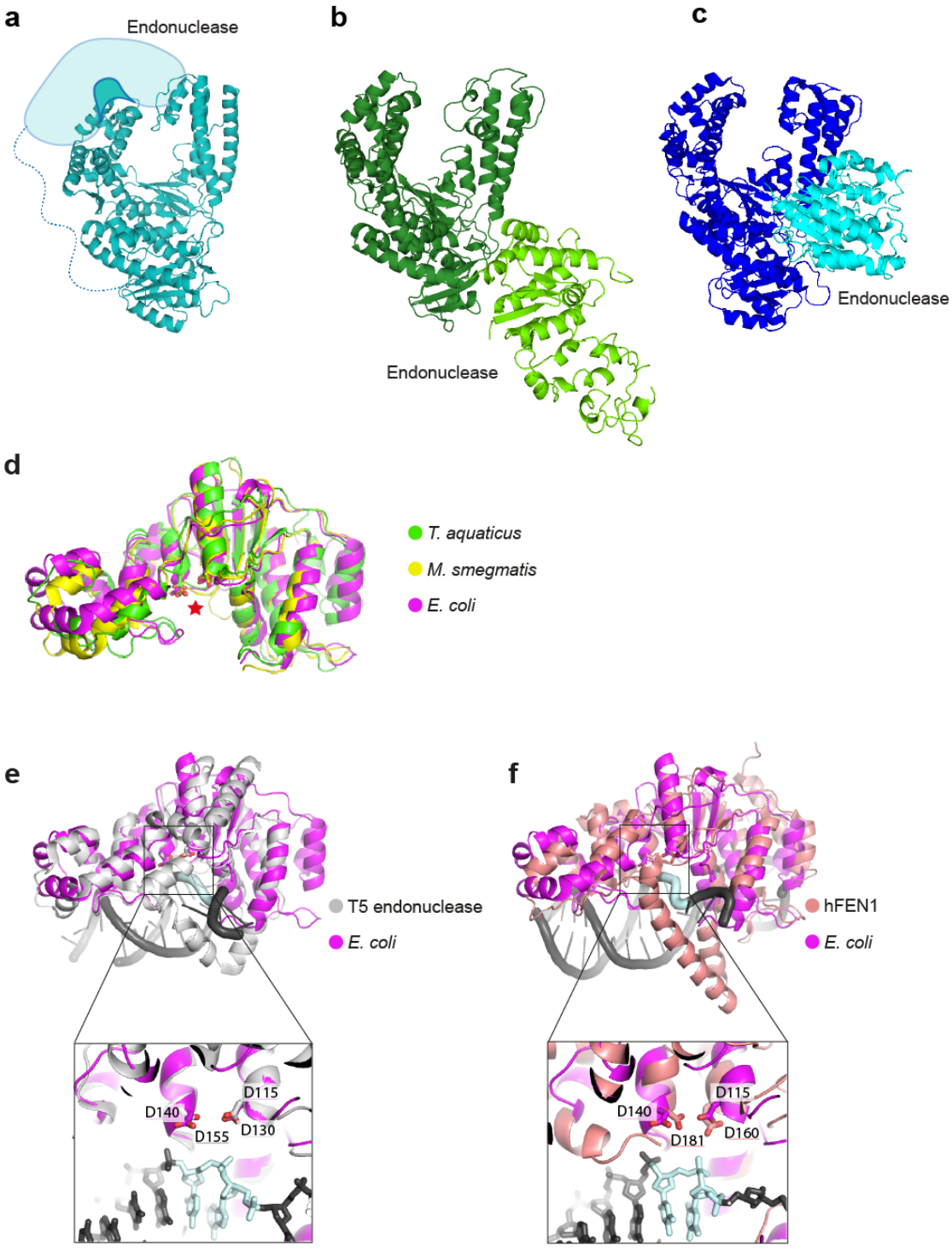


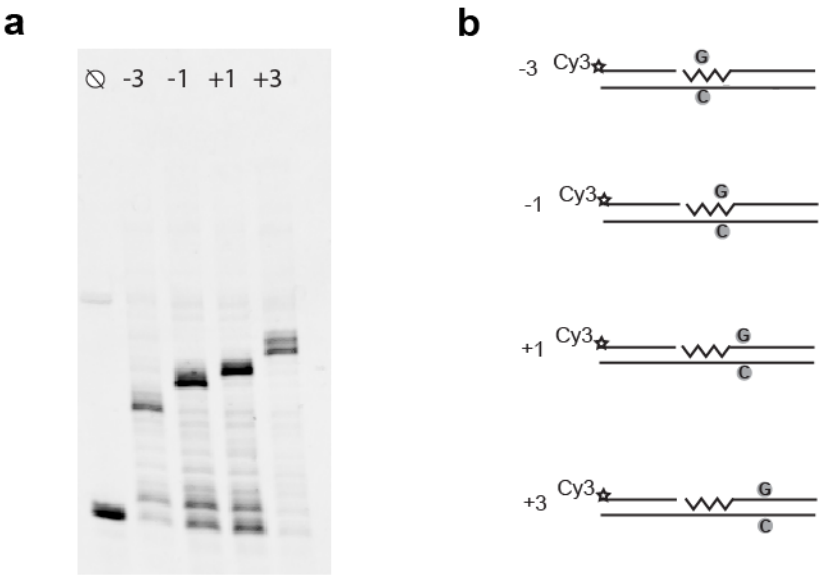
Extended Data Fig. 1 | Cryo-EM data collection and data processing details. **a**, Representative micrograph. **b**, 2D class averages from full dataset. **c**, Fourier Shell Correlation between half-maps from final refinement. Green line: unmasked. Blu line: masked. Red line: phase randomized. Black line: corrected. **d**, Schematic

representation of main data processing procedures. See methods section for more details. **e**, Final map obtained applying SuperEM code to Relion post-processed map from dataset 1 and colored by local resolution. **f**, Detail of model fitted to the final cryo-EM map from dataset 1. **g**, Orientational distribution of final set of refined particles from dataset 1.

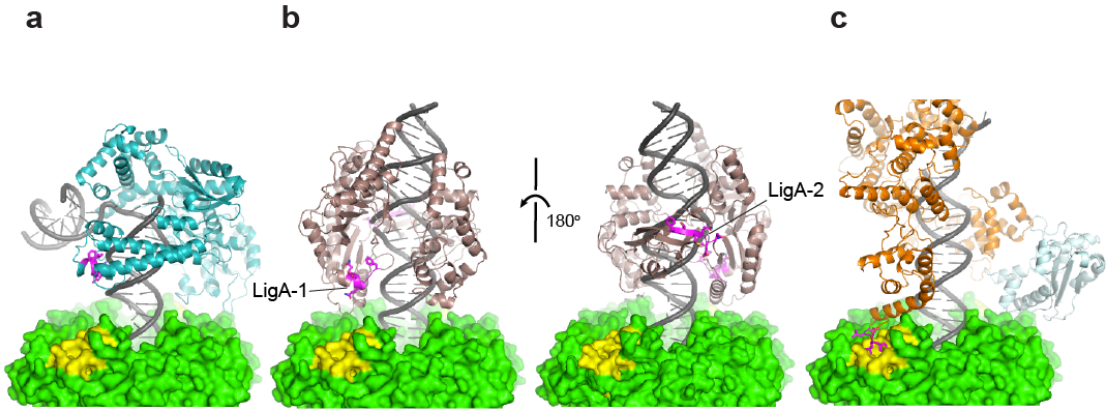


Extended Data Fig. 2 | Positional modelling of the endonuclease domain in Pol I. **a**, *E. coli* Pol I with predicted position of the endonuclease domain on top of the fingers domain, based on the position of the single stranded flap (see also main Fig. 3a). **b**, *Thermus aquaticus* Pol I with its endonuclease domain adjacent to the 3'-5'

exonuclease domain[33]. **c**, *Mycobacterium smegmatis* Pol I with its endonuclease domain adjacent to the thumb domain[31]. **d**, Superposition of three Pol I endonuclease domains from *T. aquaticus*, *M. smegmatis*, and *E. coli*, (modelled using AlphaFold[34]). Red star marks the endonuclease active site. **e**, Superposition of *E. coli* Pol I endonuclease domain and T5 endonuclease bound to its substrate DNA[35]. The square marks the enlarged area shown below. Site of incision is marked by the two nucleotides in light blue. Two catalytic aspartates are shown in sticks. **f**, Similar comparison with human FEN1[36]. Structures of T5 endonuclease and FEN1 were used to model the position of Pol I endonuclease in main Fig. 3b.



Extended Data Fig. 3 | Pol I does not progress past a C in the template strand in absence of dGTP. a, Pol I activity in absence of LigA using the templates shown in panel b, using only the three nucleotides dATP, dCTP, and dTTP. Experimental conditions are the same as in Fig. 5b. **b**, Substrates used for experiment in panel a.



d

Pol I	587	QNSR–F	591
LigA-1	297	QEQLGF	302
LigA-2	318	QEQMTF	323
Pol IIIα	920	QLDL–F	924

Extended Data Fig. 4 | The location of β-binding motifs of Pol I, LigA and Pol IIIα. **a**, Model of Pol I and β-clamp. The β-clamp is shown in green surface, with binding pocket highlighted in yellow. The predicted β-binding motif of Pol I is shown in magenta sticks and is located in a helix of the thumb domain that interacts with the minor groove of the DNA. **b**, Model of LigA[79] and β-clamp shown in two views rotated by 180°. The two predicted β-binding motifs are shown in magenta sticks. Motif LigA-1 is located in a helix that precedes a loop that interacts with the DNA. Motif LigA-2 is located on a strand that is part of the OB-domain that also interacts with the DNA. **c**, Cryo-EM structure of Pol IIIα bound to the β-clamp, exonuclease ε, and DNA[52]. The Pol IIIα β-binding motif is shown in magenta sticks and located on a loop at the end of the fingers domain and interacts with the binding pocket of the β-clamp. **d**, Alignment of β-binding motifs from Pol I, LigA and Pol IIIα that are highlighted in magenta in panels a-c.

Primer name	Length	5'-3' sequence
ASP115ALA Fw	26	GCGTAGAAGCGGCCGACGTTATCGGT
ASP115ALA Rv	26	ACCGATAACGTCGGCCGCTTCTACGC
ASP140ALA Fw	28	GCACTGGCGATAAAGCTATGGCGCAGCT
ASP140ALA Rv	28	AGCTGCGCCATAGCTTTATCGCCAGTGC
Third Stop Codon Pol1 Fw	24	TGCTGATGTCTAAGCGGGCAAATG
Third Stop Codon Pol1 Rv	24	CATTTGCCCGCTTAGACATCAGCA

Supplementary Table 1 Primers used for cloning and mutagenesis

Figure n°	Panel	Sequences
1	A	5′ Cy3-GGTAACGCCAGGGTTTTCCCAGTC 3′ 3′ CCATTGCGGTCCCAAAAGGGTCAGTGAAGTGCTTAGATCTGCTGCAACATTTTGCTGCCGGTCACTCGC 5′
	B	5′ Cy3-GGTAACGCCAGGGTTTTCCCAGTC ACGACGTTGTAAAACGACGGCCAGTGAGCG 3′ 3′ CCATTGCGGTCCCAAAAGGGTCAGTGAAGTGCTTAGATCTGCTGCAACATTTTGCTGCCGGTCACTCGC 5′
	C	5′ Cy3-GGTAACGCCAGGGTTTTCCCAGTC *ACGACGTTGTAAAACGACGGCCAGTGAGCG 3′ 3′ CCATTGCGGTCCCAAAAGGGTCAGTGAAGTGCTTAGATCTGCTGCAACATTTTGCTGCCGGTCACTCGC 5′
3	D	5′Cy3-GGTAACGCCAGGGTTTTCCCAGTC 3′ 3′CCATTGCGGTCCCAAAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCACTCGC 5′
	E	5′Cy3-GGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCG 3′ 3′CCATTGCGGTCCCAAAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCACTCGC 5′
	F	5′GGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCG-Cy5 3′ 3′CCATTGCGGTCCCAAAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCACTCGC 5′ 5′GGTAACGCCAGGGTTTTCCCAGTCGCGACGTTGTAAAACGACGGCCAGTGAGCG-Cy5 3′ 3′CCATTGCGGTCCCAAAAGGGTCAGCGCTGCAACATTTTGCTGCCGGTCACTCGC 5′ 5′GGTAACGCCAGGGTTTTCCCAGTCGTGACGTTGTAAAACGACGGCCAGTGAGCG-Cy5 3′ 3′CCATTGCGGTCCCAAAAGGGTCAGCACTGCAACATTTTGCTGCCGGTCACTCGC 5′ 5′GGTAACGCCAGGGTTTTCCCAGTCGTAACGTTGTAAAACGACGGCCAGTGAGCG-Cy5 3′ 3′CCATTGCGGTCCCAAAAGGGTCAGCATTGCAACATTTTGCTGCCGGTCACTCGC 5′ 5′GGTAACGCCAGGGTTTTCCCAGTCGTACCGTTGTAAAACGACGGCCAGTGAGCG-Cy5 3′ 3′CCATTGCGGTCCCAAAAGGGTCAGCATGGCAACATTTTGCTGCCGGTCACTCGC 5′ 5′GGTAACGCCAGGGTTTTCCCAGTCGTACGGTTGTAAAACGACGGCCAGTGAGCG-Cy5 3′ 3′CCATTGCGGTCCCAAAAGGGTCAGCATGCCAACATTTTGCTGCCGGTCACTCGC 5′
4	A	5′CTTAACTCCAACCTTTTCCCAATCACTTCACGAACCACATTCTAAAACCACTTCCATTCACCT-Cy5 3′ 3′GAATTGAGGTTGGAAAAGGGTTAGTGAAGTGCTTGGTGTAAGATTTTGGTGAAGGTAAGTGGA 5′ 5′CTTAACTCCAACCTTTTCCCAATC**ACUUCACGAA**CCACATTCTAAAACCACTTCCATTCACCT-Cy5 3′ 3′GAATTGAGGTTGGAAAAGGGTTAGTGAAGTGCTTGGTGTAAGATTTTGGTGAAGGTAAGTGGA 5′
	B	5′CTTAACTCCAACCTTTTCCCAATC**ACUUCACGAA**CCACATTCTAAAACCACTTCCATTCACCT-Cy5 3′ 3′GAATTGAGGTTGGAAAAGGGTTAGTGAAGTGCTTGGTGTAAGATTTTGGTGAAGGTAAGTGGA 5′
	C	5′CTTAACTCCAACCTTTTCCCAATC**ACUUCACUAA**GCACATTCTAAAACCACTTCCATTCACCT-Cy5 3′ 3′GAATTGAGGTTGGAAAAGGGTTAGTGAAGTGATTGGCGTAAGATTTTGGTGAAGGTAAGTGGA 5′ 5′CTTAACTCCAACCTTTTCCCAA **ACUUCACUAA**GCACATTCTAAAACCACTTCCATTCACCT-Cy5 3′ 3′GAATTGAGGTTGGAAAAGGGTTAGTGAAGTGATTGGCGTAAGATTTTGGTGAAGGTAAGTGGA 5′ 5′CTTAACTCCAACCTTTTCCC **ACUUCACUAA**GCACATTCTAAAACCACTTCCATTCACCT-Cy5 3′ 3′GAATTGAGGTTGGAAAAGGGTTAGTGAAGTGATTGGCGTAAGATTTTGGTGAAGGTAAGTGGA 5′ 5′CTTAACTCCAACCTTTT **ACUUCACUAA**GCACATTCTAAAACCACTTCCATTCACCT-Cy5 3′ 3′GAATTGAGGTTGGAAAAGGGTTAGTGAAGTGATTGGCGTAAGATTTTGGTGAAGGTAAGTGGA 5′

5	A	5’Cy3-GGTAACGCCAGGGTTTTCCCAGTC*ACGACGTTGTAAAACGACGGCCAGTGAGCG 3’ 3’CCATTGCGGTCCCAAAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCACTCGC 5’ 5’Cy3-GGTAACGCCAGGGTTTTCCCAGTC***A**CGACGTTGTAAAACGACGGCCAGTGAGCG 3’ 3’CCATTGCGGTCCCAAAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCACTCGC 5’ 5’Cy3-GGTAACGCCAGGGTTTTCCCAGTC*A**CG**ACGTTGTAAAACGACGGCCAGTGAGCG 3’ 3’CCATTGCGGTCCCAAAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCACTCGC 5’ 5’Cy3-GGTAACGCCAGGGTTTTCCCAGTC*A**CGACGUUGUAAAACGACGGCCAGUGAGCG** 3’ 3’CCATTGCGGTCCCAAAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCACTCGC 5’
	B	5’Cy3-CTTAACTCCAACCTTTTCCCAATC**ACUUCACGAA**CCACATTCTAAAACCACTTCCATTCACCT 3’ 3’GAATTGAGGTTGGAAAAGGGTTAGTGAAGTGCTTGGTGTAAGATTTTGGTGAAGGTAAGTGGA 5’ 5’Cy3-CTTAACTCCAACCTTTTCCCAATC**ACUUCACUAG**CCACATTCTAAAACCACTTCCATTCACCT 3’ 3’ GAATTGAGGTTGGAAAAGGGTTAGTGAAGTGATCGGTGTAAGATTTTGGTGAAGGTAAGTGGA 5’ 5’Cy3-CTTAACTCCAACCTTTTCCCAATC**ACUUCACUAA**GCACATTCTAAAACCACTTCCATTCACCT 3’ 3’ GAATTGAGGTTGGAAAAGGGTTAGTGAAGTGATTCGTGTAAGATTTTGGTGAAGGTAAGTGGA 5’ 5’Cy3-CTTAACTCCAACCTTTTCCCAATC**ACUUCACUAA**GCACATTCTAAAACCACTTCCATTCACCT 3’ 3’ GAATTGAGGTTGGAAAAGGGTTAGTGAAGTGATTCGTGTAAGATTTTGGTGAAGGTAAGTGGA 5’ 5’Cy3-CTTAACTCCAACCTTTTCCCAATC**ACUUCACUAA**CCGCATTCTAAAACCACTTCCATTCACCT 3’ 3’ GAATTGAGGTTGGAAAAGGGTTAGTGAAGTGATTGGCGTAAGATTTTGGTGAAGGTAAGTGGA 5’
6	A	5’Cy3-GGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCG 3’ 3’CCATTGCGGTCCCAAAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCAC 5’

Supplementary Table 2 Primers used for primer extension assays. RNA highlighted in bold. Underlined letters indicate the position of the nick. Phosphorylation of substrate at 5’ end indicated with an asterisk.

Substrate name	Sequences
Continuous	5’GGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCG-B 3’ 3’B-CCATTGCGGTCCCAAAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCACTCGC 5’
Primed	5’ACGACGTTGTAAAACGACGGCCAGTGAGCG-B 3’ 3’B-CCATTGCGGTCCCAAAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCACTCGC 5’
Nicked	5’GGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCG-B 3’ 3’B-CCATTGCGGTCCCAAAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCACTCGC 5’

Supplementary Table 3 Primers used for biolayer interferometry assays. Underlined letters indicate the position of the nick. All primers contain a biotin at 3’ end.