

Supplementary Information

***Bacteroides* expand the functional versatility of a conserved transcription factor and transcribed DNA to program capsule diversity**

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This PDF file includes:

1. Tables

Table S1. Oligonucleotides used in this study	pS3
Table S2. <i>B. fragilis</i> strain construction	pS12
Table S3. Plasmids used in this study	pS14
Table S4. Strains used in this study	pS17

2. Supplementary Figures

Supplementary Fig. 1. <i>ops_X</i> pauses found by NET-seq	pS18
Supplementary Fig. 2. <i>ops_X</i> pause mapping by PIVoT	pS19
Supplementary Fig. 3. Mapping <i>ops_X</i> sites by quantitative comparison to MspI–digested ladders or sequencing ladders created by limiting NTPs	pS20
Supplementary Fig. 4. Y _X and NusA effects at 6 different <i>ops_X</i> sites	pS21
Supplementary Fig. 5. Y _F outcompetes NusG at <i>ops_F</i>	pS22
Supplementary Fig. 6. Confidence metrics from AlphaFold 3 Y _E -Z _A complex structural prediction	pS23
Supplementary Fig. 7. Effects of Y _E and Y _A on WT <i>ops_X</i> and hybrid <i>ops_X</i> sequences in PIVoT assays	pS24
Supplementary Fig. 8. Y _X alignment compared to NusGs and orthologous factors	pS25
Supplementary Fig. 9. Predicted secondary structures of nascent RNA hairpins.	pS26
Supplementary Fig. 10. Effects of <i>ops_E</i> mutants on Y _E or NusA activity	pS27
Supplementary Fig. 11. WT <i>ops_X</i> sequences and variants used for Fig. 5e	pS28

Supplementary Fig. 12. Primary data associated with Fig 6b	pS29
Supplementary Fig. 13. Primary data associated with Fig 6c.	pS30
Supplementary Fig. 14. Modeling of Class 1 Y _x suggest that Y _x provides a larger positively charged surface for usDNA interaction relative to RfaH (<i>E. coli</i>)	pS31
Supplementary Fig. 15. The PSE monoclonal antibody (mAb 16) is specific to PSE	pS32
Supplementary Fig. 16. Nucleic scaffolds used in PIVoT and footprinting assays.	pS33
3. Supplementary References	pS42

Table S1 – Oligonucleotides

Oligo	Sequence (5'-3')	Length (nt)	Purpose/Scaffold
JS145_T	CGTCACGAGTAGTAAGCTCCTGATCCTTACGGGTACGGGCAGCGAA CCAATATTCTTGTTGTTGAGACATTTAGAAGGATATATTCCTGAGTG ATTTACTTAAGTCCGGGTATGCTTCGCAGTTGGGTGAGACTCTTTTTC TCAGCCAACTGCAAGATTTTTTATGACTTATTTTAGGTGGAAACGA ACGGATTTCGATC	200	PIVoT PSE ultramer scaffold
JS146_R	UUUUAACGUUCCAC	15	PIVoT various scaffolds
JS147_NT	GATCGAATCCGTTA <u>ACCGATCT</u> CTAAAATAAGTCATAAAAAATCTTG CAGTTTGGCTGAGAAAAAGAGTCTCACCCAACCTGCGAAGCATACCC GGACTTAAGTAAATCACTCAGGAATATATCCTTCTAAATGTCTCAAC AACAAGAATATTGGTTCGCTGCCCGTACCCGTAAGGATCAGGAGCT TACTACTCGTGACG	200	PIVoT PSE ultramer scaffold
JS510_NT	GCACTGAATTTTTCATTAAAATCATAGAGAAATAACTTG <u>ACCGGGA</u> <u>GGTATGCTTCGCTACTCCGGT</u> GTCCCCAAAGAACATCCTGACGTGAA CGACATGACAACATCCGCATCTATCGAATCTTCGATG	130	PIVoT PSF ultramer scaffold
JS511_T	CATCGAAGATTCGATAGATGCGGATGTTGTCATGTCGTTTCACGTCAG GATGTTCTTTGGGGACACCGGAGTAGCGAAGCATACCTCCCGGTCA AGTTATGTAATGCGGATTTTAATGAAAAATTCAGTGC	130	PIVoT PSF ultramer scaffold
JS191_R	UUUUUCCGCAUAC	15	PIVoT PSF ultramer scaffold
JS708_NT	GATCGAATCCGTTAACCGATCTGTTCAAATCCGGTGCGAATACTTTT TCTGTACCCGATTATCATAAACTTAATTTGTAAATTGCTGAAAATA AGGCATGTTTTTTGAATATTCCTGTTTTTAACAAATTTTCATCCTTAG	200	PIVoT PSC ultramer scaffold

	TCATTACTGAAACTTTTTCTTACGAACGTAGTCTTGGAGACAACAGATAGCGATAAAC		
JS709_T	GTTTATCGCTATCTGTTGTCTCCAAGACTACGTTCGTAAGAAAAAGTTTCAGTAATGACTAAGGATGAAAATTTGTTAAAAACAGGAATATTCAAAAACATGCCTTATTTTCAGCAATTTACAAATTAAGTTTTATGATAATCGGGTACAGAAAAAGTATTCGCACCGGATTTGAACGTGGAAACGAACGGATTTCGATC	200	PIVoT PSC ultramer scaffold
JS551_NT_opsB (+escHP)	GTCCGTTTCGTTTCCACGAATTTGACGGTTTGAAAGAGAAAAGATGTC TCGTTCAAAC <u>CCGCGAAGCGCA</u> CCCGAAATAAAGGAAAATTTCC	90	opsB with pause and escape hp (reconstitute upstream of RNA hp)
JS552_T_opsB(+escHP)	GGAAATTTTCCTTTATTTTCGGGTGCGCTTCGCGGTTTGAACGAGACATCTTTTCTCTTCAAACCGTCAAATTCGTGGAAACGAACGGAC	90	opsB with pause and escape hp (reconstitute upstream of RNA hp)
JS553_NT_opsA (+escHP)	GTCCGTTTCGTTTCCACGTCTTACGGTTTGAATGGGAAAAGATGTCTC GTCCAAAC <u>CCGCGTAGCGCA</u> CCCGAAAGTAACCTCTCGACTTTG	90	opsA with pause and escape hp (reconstitute upstream of RNA hp)
JS554_T_opsA(+escHP)	CAAAGTCGAGAGGTTACTTTTCGGGTGCGCTACGCGGTTTGGACGAGACATCTTTTCCCATTCAAACCGTAAGACGTGGAAACGAACGGAC	90	opsA with pause and escape hp (reconstitute upstream of RNA hp)

JS521_NT_opsE	GTCCGTTTCGTTTCCACGATCTTGCAGTTTGGCTGAGAAAAAGAGTCT CACCCA <u>ACTGCGAAGCATA</u> CCCGGACTTAAGTAAATCACTCAG	90	opsE with pause hairpin (reconstitute upstream of RNA hp)
JS522_T_opsE	CTGAGTGATTTACTTAAGTCCGGGTATGCTTCGCAGTTGGGTGAGAC TCTTTTCTCAGCCAAACTGCAAGATCGTGGAACGAACGGAC	90	opsE with pause hairpin (reconstitute upstream of RNA hp)
JS523_NT_opsF	GTCCGTTTCGTTTCCACGTAACCTG <u>ACCGGGAGGTATGCTTCGCTACT</u> <u>CCGGTGTCCCCA</u> AAGAA <u>CAT</u> CCTGACGTGAACGACATGACAAC	90	opsF with pause hairpin (reconstitute upstream of RNA hp)
JS524_T_opsF	GTTGTCATGTCGTTTCACGTCAGGATGTTCTTTGGGGACACCGGAGTA GCGAAGCATACCTCCCGGTCAAGTTACGTGGAACGAACGGAC	90	opsF with pause hairpin (reconstitute upstream of RNA hp)
JS583_NT_opsH	GTCCGTTTCGTTTCCACGTCACCTGACCGGGAGGTACTTTCGTACTCC GGTGT <u>CCCCAAAGAACAT</u> CCTTTTGTGAAGGAATCCCCAGAGC	90	opsH with pause hairpin (reconstitute upstream of RNA hp)
JS584_T_opsH	GCTCTGGGGATTCTTTCACAAAAGGATGTTCTTTGGGGACACCGGAG TACGAAAGTACCTCCCGGTCAGGTGACGTGGAACGAACGGAC	90	opsH with pause hairpin (reconstitute

			upstream of RNA hp)
JS540_PSE-asDNA_6	CTGCAAGATCGTGGA	15	asDNAs to probe extent of RNA hairpin in PSE
JS541_PSE-asDNA_7	ACTGCAAGATCGTGG	15	asDNAs to probe extent of RNA hairpin in PSE
JS542_PSE-asDNA_8	AACTGCAAGATCGTG	15	asDNAs to probe extent of RNA hairpin in PSE
JS543_PSE-asDNA_9	AAACTGCAAGATCGT	15	asDNAs to probe extent of RNA hairpin in PSE
JS544_PSE-asDNA_10	CAAACCTGCAAGATCG	15	asDNAs to probe extent of RNA hairpin in PSE
JS545_PSE-asDNA_11	CCAAACTGCAAGATC	15	asDNAs to probe extent of RNA hairpin in PSE
JS566_PSE-asDNA_17	GTGCTAGAACGTCAAACCGACTCT	24	negative control asDNA
JS555_NT	GTCCGTTTCGTTTCCACGTCTTACGGTTTGAATGGGAAAAGATGTCTC GTCCAA <u>ACTGCGAAGCATA</u> CCCGAAAGTAACCTCTCGACTTTG	90	HP _A ,[-10:-1] _E (Pause Cycling Observed)

JS556_T	CAAAGTCGAGAGGTTACTTTTCGGGTATGCTTCGCAGTTTGGACGAGACATCTTTTCCCATTCAAACCGTAAGACGTGGAAACGAACGGAC	90	HP _A ,[-10:-1] _E (Pause Cycling Observed)
JS557_NT	GTCCGTTTCGTTTCCACGATCTTGCAGTTTGGCTGAGAAAAAGAGTCTCACCCAACCGCGTAGCGCACCCGGACTTAAGTAAATCACTCAG	90	HP _E ,[-10:-1] _A (Pause Cycling Observed)
JS558_T	CTGAGTGATTTACTTAAGTCCGGGTGCGCTACGCGGTTGGGTGAGACTCTTTTTTCTCAGCCAAACTGCAAGATCGTGGAACGAACGGAC	90	HP _E ,[-10:-1] _A (Pause Cycling Observed)
JS591_NT	GTCCGTTTCGTTTCCACGATCTTGCAGTTTGGCTGAGAAAAAGAGTCTCACCCAAC <u>TGCGtAGCATA</u> CCCGGACTTAAGTAAATCACTCAG	90	-6 opsE>opsA
JS592_T	CTGAGTGATTTACTTAAGTCCGGGTATGCTaCGCAGTTGGGTGAGACTCTTTTTTCTCAGCCAAACTGCAAGATCGTGGAACGAACGGAC	90	-6 opsE>opsA
JS593_NT_mut opsE+hp -1,-2,-10	GTCCGTTTCGTTTCCACGATCTTGCAGTTTGGCTGAGAAAAAGAGTCTCACCCAAC <u>CcGCGAAGCgcA</u> CCCGGACTTAAGTAAATCACTCAG	90	opsE+hp > -10:1 opsB
JS594_T_mut opsE+hp -1,-2,-10	CTGAGTGATTTACTTAAGTCCGGGTgcGCTTCGCgGTTGGGTGAGACTCTTTTTTCTCAGCCAAACTGCAAGATCGTGGAACGAACGGAC	90	opsE+hp > -10:1 opsB
JS595_NT_mut opsE+hp -1,-2,-10,-14	GTCCGTTTCGTTTCCACGATCTTGCAGTTTtGCTGAGAAAAAGAGTCTCACCaAAC <u>CcGCGAAGCgcA</u> CCCGGACTTAAGTAAATCACTCAG	90	opsE+hp > -10:1, -14:-36 opsB
JS596_T_mut opsE+hp -1,-2,-10,-14	CTGAGTGATTTACTTAAGTCCGGGTgcGCTTCGCgGTTtGGTGAGACTCTTTTTTCTCAGCaAAACTGCAAGATCGTGGAACGAACGGAC	90	opsE+hp > -10:1, -14:-36 opsB

JS597_NT_mut opsE+hp -1,-2,-10,insert U-A	GTCCGTTTCGTTTCCACGATCTTGCAGTTTGGCTGAGAAAAAGAGTCT CACCCAAaCcGCGAAGCgcACCCGGACTTAAGTAAATCACTCA	90	opsE+hp > -10:1, -14 ins opsB
JS598_T_mut opsE+hp -1,-2,-10,insert U-A	TGAGTGATTTACTTAAGTCCGGGTgcGCTTCGCgGtTTGGGTGAGACT CTTTTCTCAGCCAAACTGCAAGATCGTGGAACGAACGGAC	90	opsE+hp > -10:1, -14 ins opsB
JS599_NT_mut opsE+hp -1,-2,-10,insert U-A, change var pairing region	GTCCGTTTCGTTTCCACGATCTTGCAGTTTGaaaGAGAAAAAGAGTCTC gttCAAaCcGCGAAGCgcACCCGGACTTAAGTAAATCACTCA	90	opsE+hp > -10:1, -14 ins, -36:-34, - 18:-16 opsB
JS600_T_mut opsE+hp -1,-2,-10,insert U-A, change var pairing region	TGAGTGATTTACTTAAGTCCGGGTgcGCTTCGCgGtTTGaacGAGACTCT TTTTCTCtttCAAACCTGCAAGATCGTGGAACGAACGGAC	90	opsE+hp > -10:1, -14 ins, -36:-34, - 18:-16 opsB
JS672_NT	GTCCGTTTCGTTTCCACGATCTTGCAGTTTGaaaGAGAAAagatGTCTCgtt CAAaCcGCGAAGCgcACCCGGACTTAAGTAAATCACTCA	90	opsE+hp > (mostly opsB now) -14 ins, - 36:-34, -18:-16, - 26:-24 opsB
JS673_T	TGAGTGATTTACTTAAGTCCGGGTgcGCTTCGCgGtTTGaacGAGACatcT TTTCTCtttCAAACCTGCAAGATCGTGGAACGAACGGAC	90	opsE+hp > (mostly opsB now) -14 ins, - 36:-34, -18:-16, - 26:-24 opsB

JS674_NT	GTCCGTTTCGTTTCCACGATCTTGCAGTTTGaaaGAGAAAagatGTCTCggt CAAaCcGCGAAGCgcACCCGaaataaaggaaaattccc	90	opsE+hp > (opsB- escape hairpin) -14 ins, -36:-34, -18:- 16, -26:-24, dsDNA opsB
JS675_T	gggaaatttcctttatttCGGGTgcGCTTCGCgGtTTGaacGAGACatcTTTTCTCtttC AAACTGCAAGATCGTGGAACGAACGGAC	90	opsE+hp > (opsB- escape hairpin) -14 ins, -36:-34, -18:- 16, -26:-24, dsDNA opsB
JS666_R_opsB-3	UUUUUCCGCGAAGC	15	opsB-hp (reconstitute -3)
JS654_NT_opsB	TTTGAAAGAGAAAAGATGTCTCGTTCAAACCGCGAAGCGCACCCGA AATAAAGGAAAATTTCCCAAG	68	opsB-hp (reconstitute -3)
JS655_T_opsB	CTTGGGGAAATTTTCCTTTATTTTCGGGTGCGCTTCGCGGTTTGAACG AGACATCTTTTCTCTTTCAA	68	opsB-hp (reconstitute -3)
JS660_NT_opsB -36-34	TTTGggcGAGAAAAGATGTCTCGTTCAAACCGCGAAGCGCACCCGAA ATAAAGGAAAATTTCCCAAG	68	opsB -36-34 mut to opsE
JS661_T_opsB- 36-34	CTTGGGGAAATTTTCCTTTATTTTCGGGTGCGCTTCGCGGTTTGAACG AGACATCTTTTCTCgccCAA	68	opsB -36-34 mut to opsE
JS662_NT_opsB -26-24	TTTGAAAGAGAAAAaAgGTCTCGTTCAAACCGCGAAGCGCACCCGAA ATAAAGGAAAATTTCCCAAG	68	opsB -26-24 mut to opsE
JS663_T_opsB- 26-24	CTTGGGGAAATTTTCCTTTATTTTCGGGTGCGCTTCGCGGTTTGAACG AGACcTtTTTTCTCTTTCAA	68	opsB -26-24 mut to opsE

JS664_NT_opsB-18-16	TTTGAAAGAGAAAAGATGTCTC <u>cacCAAACCGCGAAGCGCACCCGAA</u> ATAAAGGAAAATTTCCCAAG	68	opsB -18-16 mut to opsE
JS665_T_opsB-18-16	CTTGGGGAAATTTTCCTTTATTTTCGGGTGCGCTTCGCGGTTTGgtgGAG ACATCTTTTCTCTTTCAA	68	opsB -18-16 mut to opsE
JS467_NT-opsE-mut1	GAGAAAAAGAGTCTCACCCA <u>ACTGCGAAGCgc</u> ACCCGGACTTAAGT AAATCACTCAGGAA	60	opsE_mut1(opsA/B-mimic-1(AT□GC))
JS468_T-opsE-mut1	TTCCTGAGTGATTTACTTAAGTCCGGGTgcGCTTCGCAGTTGGGTGAG ACTCTTTTCTC	60	opsE_mut1(opsA/B-mimic-1(AT□GC))
JS469_NT-opsE-mut2	GAGAAAAAGAGTCTCACCCAAC <u>cGCGAAGCATA</u> ACCCGGACTTAAGT AAATCACTCAGGAA	60	opsE_mut2(opsA/B-mimic-2(T□C))
JS470_T-opsE-mut2	TTCCTGAGTGATTTACTTAAGTCCGGGTATGCTTCGCgGTTGGGTGA GACTCTTTTCTC	60	opsE_mut2(opsA/B-mimic-2(T□C))
JS471_NT-opsE-mut3	GAGAAAAAGAGTCTCACCCA <u>ACTGCGtAGCATA</u> ACCCGGACTTAAGTA AATCACTCAGGAA	60	opsE_mut3(opsA-mimic-3(A□T))
JS472_T-opsE-mut3	TTCCTGAGTGATTTACTTAAGTCCGGGTATGCTaCGCAGTTGGGTGA GACTCTTTTCTC	60	opsE_mut3(opsA-mimic-3(A□T))
9563	GGTCAGTACGTCCGGCATAGTTGCGCCCGTAAATTCAGATCTTCCAG TGG	50	consensus pause
8334	CCACTGGAAGATCTGAATTTACGGGCGCAACTATGCCGGACGTACT GACC	50	consensus pause

8342	<u>UUUUUUGGCAUAGUU</u>	15	consensus pause
JS563	GGTCAGTACGTCCTTTTGGTGGTCGTTGTAGTGGGCAGATCTTCCAGT GG	50	anticonsensus pause
8954	CCACTGGAAGATCTGCCCCACTACAACGACCACCAAAAGGACGTACT GACC	50	anticonsensus pause
8952	UUUUUUUUUUGGUGG	15	anticonsensus pause
14047	/5Phos/NNNNNNNNNGCAGCTCTGTAGGCACCATCAATGATCGTCGGA/3ddC/	43	NET-seq
14637	/5Phos/AGATCGGAAGAGCACACGTCTGAAC/iSp18/CACTCA/iSp18/CCTACACGAC GCTCTCCGATCTTCCGACGATCATTGATGGTGCCTACAG	81	NET-seq
14641	AATGATACGGCGACCACCGAGATCTACACAGCGAGCTACACTCTTTCCCTACACG ACGCTCTTCCGATCTTCCGACGATC	80	NET-seq
14645	CAAGCAGAAGACGGCATAAGGATGAGTGACTGGAGTTCAGACGTGTG CTCTTCCGATCT	66	NET-seq

Table S2 – *B. fragilis* Strain Construction

Purpose	Plasmid name	Primers	Sequence
['-10:-1]opsA in PSE' = Clone opsA region into PSE region into pLGB13 - left flank amplified from Δmpi M44	pKF32	opsA-PSE-LF_F	taagattagcattatgagtgccggctgtggaggcaattatc
		opsA-PSE-LF_R	acgcggttggtgagactca
		opsA-PSE-RF_F	agagtctcacccaaccgcgtagegcacccggacttaag
		opsA-PSE-RF_R	cgaattcctgcagcccggggcagtctcctgctcatctatcg
Replacing opsE (+PSE hairpin) with opsA (+PSA hairpin) in pLGB13	pKF52	HPops-LF_F	taagattagcattatgagtgcctgggaacaaaacaaag
		HPops-LF_R	attcaaaccgactgcaagatttttatgac
		HPopsA_F	atcttgcactcggttgaatgggaaaagatg
		HPopsA_R	taagtccgggtgcgctacgcggtttgga
		HPops-RF_F	gcgtagegcacccggacttaagtaaatcac
		HPops-RF_R	cgaattcctgcagcccggggcagtctcctgctcatctatc
Clone rpoC-3X-FLAG in pLGB13 - left flank amplified from Jason's construct pJS039_Bf-rpoC-3X-FLAG, right flank amplified from <i>B. fragilis</i> 9343 genome	pKF36	rpoC-3X_FLAG-LF_fwd	taagattagcattatgagtgcctgtaggtggacacgtag

		rpoC-3X_FLAG-LF_rev	ttgaattgtatcatttgcgtcatcgtc
		rpoC_3X_FLAG-RF_fwd	cgacaaatgatacaattcaattgactatagctgaaaaag
		rpoC_3X_FLAG-RF_rev	cgaattcctgcagcccggggcgcaattcaaccaagacgc

Table S3 – Plasmids

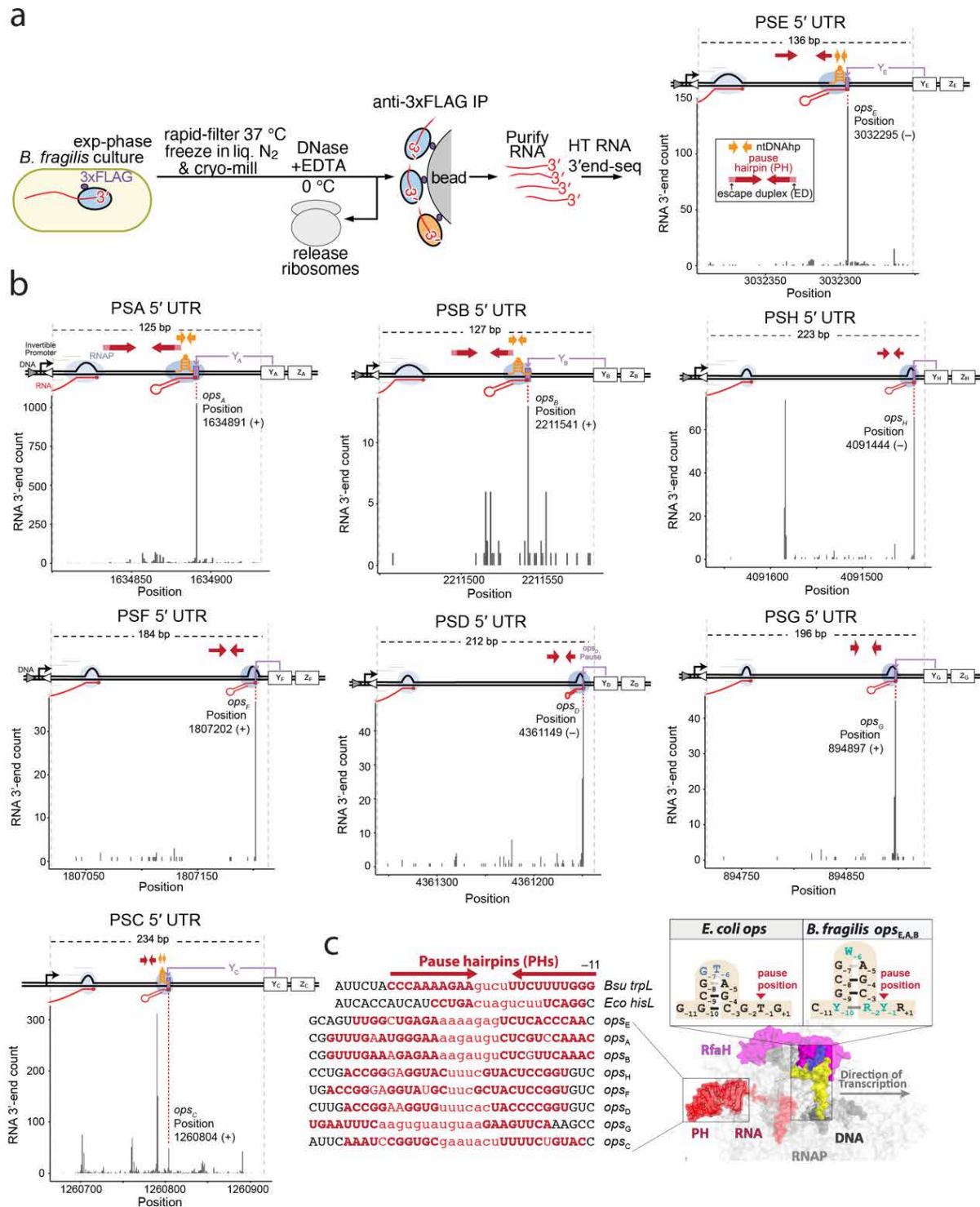
Plasmid	Lab Stock#	Purpose	Properties	Construction	Parent (s)	Anti-biotic
pJS011	5903	T7 OEP	Bf UpaY-intein-CBD	pTYB2 (Stock 182) backbone, UpaY from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pJS015	5906	T7 OEP	BfRNAP, strep-rpoB, rpoC-his10ppx	pRM756 backbone, codon-optimized BfRNAP	pRM756	Kan
pJS019	5910	T7 OEP	Bf UpeY-inteinCBD (CTD Ala insertion for optimal cleavage)	pTYB2 (Stock 182) backbone, UpeY from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pJS021	5912	T7 OEP	Bf UpaZ-inteinCBD (CTD Ala insertion for optimal cleavage)	pTYB2 (Stock 182) backbone, UpaZ from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pJS022	5913	T7 OEP	Bf UpfY-inteinCBD (CTD Ala insertion for optimal cleavage efficiency)	pTYB2 (Stock 182) backbone, UpfY from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pJS028	5917	T7 OEP	Bf UphY-inteinCBD (CTD Ala insertion for optimal cleavage efficiency)	pTYB2 (Stock 182) backbone, UphY from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pJS031	5920	T7 OEP	BfGre-inteinCBD (CTD Ala insertion for optimal cleavage efficiency)	pTYB2 (Stock 182) backbone, Gre from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp

pJS034	5922	T7 OEP	Bf UpbY-inteinCBD (CTD Ala insertion for optimal cleavage efficiency)	pTYB2 (Stock 182) backbone, UpbY from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pJS036	5924	T7 OEP	Bf UpeZ-inteinCBD (CTD Ala insertion for optimal cleavage efficiency)	pTYB2 (Stock 182) backbone, UpeZ from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pJS044	5932	T7 OEP	NusG-inteinCBD (CTD Ala insertion for optimal cleavage efficiency)	pTYB2 (Stock 182) backbone, NusG from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pJS045	5934	T7 OEP	NusA-inteinCBD (CTD Ala insertion for optimal cleavage efficiency)	pTYB2 (Stock 182) backbone, NusA from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pJS058	5943	T7 OEP	Bf UpeY(NTD)-inteinCBD (CTD Ala insertion for optimal cleavage efficiency)	pTYB2 (Stock 182) backbone, fragments from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pJS059	5944	T7 OEP	Bf UpfY(NTD)-inteinCBD (CTD Ala insertion for optimal cleavage efficiency)	pTYB2 (Stock 182) backbone, fragments from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pJS060	5945	T7 OEP	Avi-UpeY-inteinCBD (CTD Ala insertion for optimal cleavage efficiency)	pTYB2 (Stock 182) backbone, 2 oligos for Avi tag, fragments from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pJS063	5948	T7 OEP	Bf eY(NGN)-bY(KOW)-intein-CBD (CTD Ala insertion for optimal cleavage efficiency)	pTYB2 (Stock 182) backbone, eY NGN and bY KOW from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp

pJS064	5949	T7 OEP	Bf bY(NGN)-eY(KOW)-inteinCBD (CTD Ala insertion for optimal cleavage efficiency)	pTYB2 (Stock 182) backbone, bY NGN and eY KOW from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pJS073	5958	T7 OEP	Bf UpcY-intein CBD (CTD Ala insertion for optimal cleavage efficiency)	pTYB2 (Stock 182) backbone, UpcY from Bfragilis NCTC 9343	pTYB2 (Stock 182)	Amp
pKF32	5963	Test ops replacement effects on PS expression	[-10:-1]opsA in PSE = "opsE>opsE/A" in figure	See methods and Table S2		
pKF36	5966	epitope tag RNAP	rpoC-3xFLAG	See methods and Table S2		
pKF52		Test ops replacement effects on PS expression	[-43:-1]opsA in PSE = "opsE>opsA" in figure	See methods and Table S2		

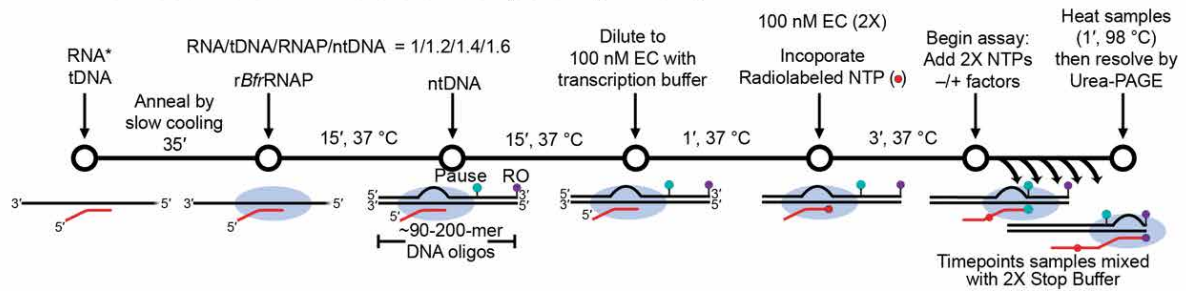
Table S4 – Strains used in this study

Strain	Notes	Source
<i>B. fragilis</i> NCTC 9343	wild type strain from which all mutants were derived	ATCC
<i>B. fragilis</i> 9343 Ω PSE	insertional mutant in PSE locus abrogating PSE synthesis	Ref ¹
<i>B. fragilis</i> 9343 Δ <i>mpi</i> M44	Promoter PSA and PSE locked on. (PSC constitutive)	Ref ²
<i>B. fragilis</i> 9343 Δ <i>mpi</i> M44 Δ <i>upaZ</i>	Promoter PSA and PSE locked on. (PSC constitutive). <i>upaZ</i> deletion	Ref ³
<i>B. fragilis</i> 9343 <i>mpi</i> M44 [-10:-1] <i>opsE</i> >[-10:-1] <i>opsA</i>	replacing <i>opsE</i> [-10:-1] with <i>opsA</i> [-10:-1]	This study
<i>B. fragilis</i> 9343 <i>mpi</i> M44 HP- <i>opsE</i> >HP- <i>opsA</i>	replacing <i>opsE</i> [-38:-1] with <i>opsA</i> [-38:-1]	This study
RL3569	<i>E. coli</i> B F ⁻ <i>ompT</i> gal [dcm ⁻] [lon ⁻] <i>hsdS</i> ⁻ (rB ⁻ mB ⁻) [λ DE3(lacUV5::T7 RNAP) <i>imm21</i> int ⁻ - Δ <i>nin5</i> Δ EcoRI(21226-26104) BamHI27972 ⁻] pRARE2 Rif-resistant expression strain (S522F) for expressing recombinant RNA polymerases. Made by transduction of RL1674 with P1 lysate of Gourse Lab strain (RLG3360). RLG3360 is stored in our database as RL3570.	This study
<i>B. fragilis</i> 9343 <i>rpoC</i> -FLAG	3x FLAG tag on C-terminus of b' subunit to enable immunoprecipitation	This study

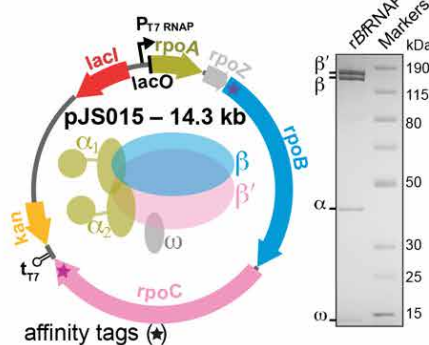


Supplementary Fig. 1. *ops_X* pauses found by NET-seq. a) Schematic depiction of NET-seq. **b)** CPS operon leaders aligned with mapped NET-seq reads (Genbank accession NC_003228.3). Genome coordinates are PSA 1634806:1634931(+); PSB 2211455:2211581 (+); PSC 1260676:1260910 (+); PSD 4361353:4361141(-); PSE 3032389:3032254(-); PSF 1807026:1807210(+); PSG 894725:894921(+); PSH 4091659:4091436(-). Red arrows indicate PH stems. Orange arrows indicate ntDNAhp stems. **(c)** Comparison of ntDNAhps in some CPS leader *ops_X* sites to the RfaH *ops* ntDNAhp⁴ and comparison of *ops_X* RNA hairpins to known examples. Base lettering follows IUPAC nomenclature. Blue colored nucleotides in *ops_X* make base-specific contacts to RfaH⁴⁻⁶. The model was generated in PyMol (see Methods and Reporting Summary).

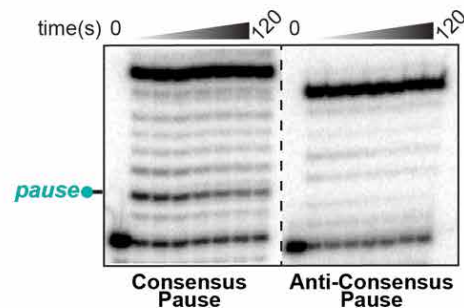
a Promoter-less in vitro transcription (PIVoT)



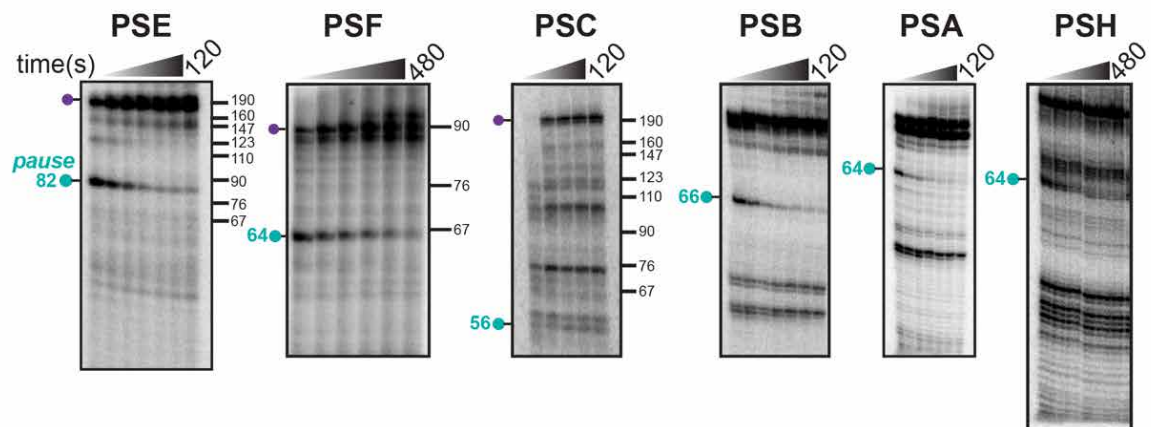
b recombinant *Bfr*RNAP



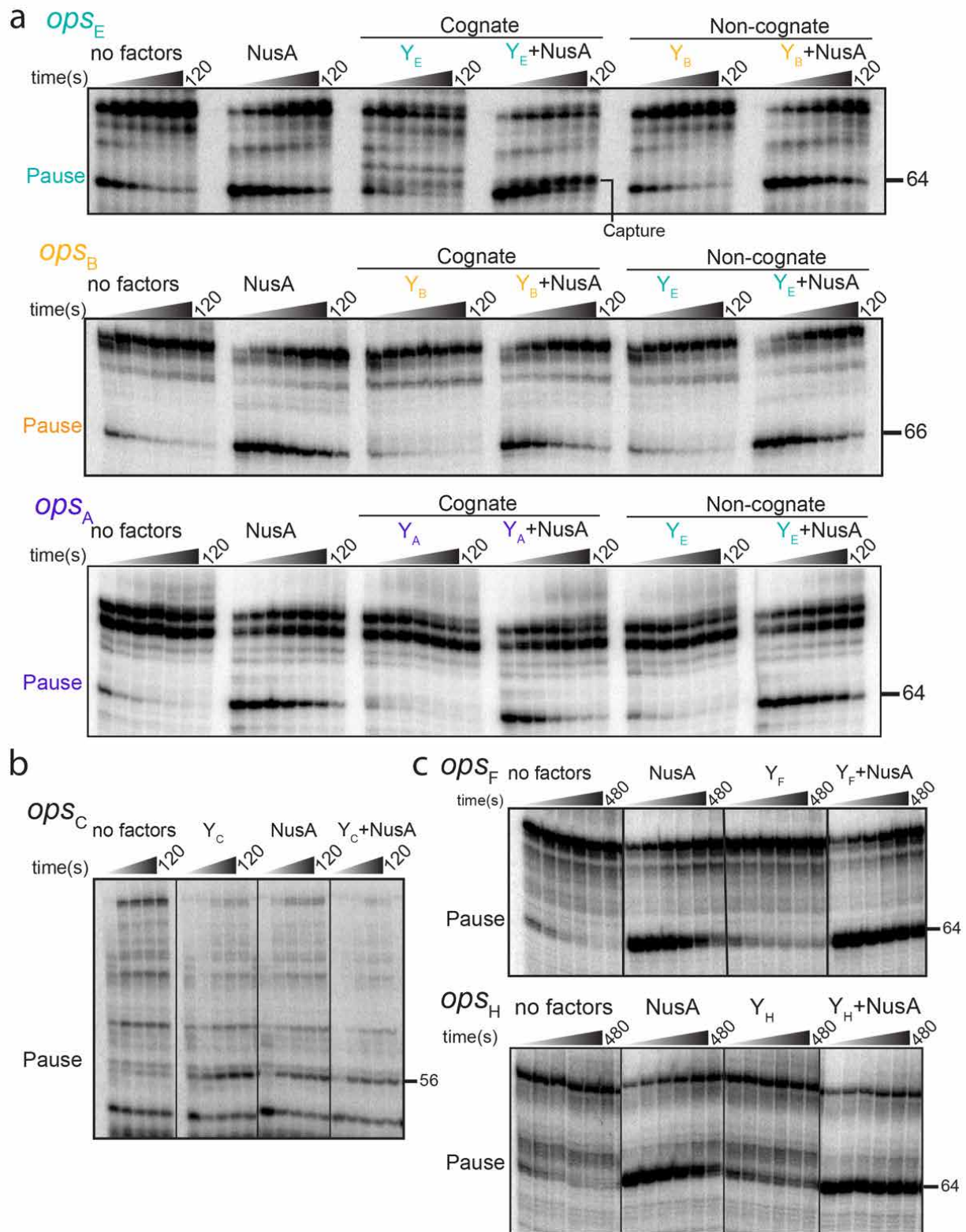
c *Bfr*RNAP recognizes *Eco* consensus pause



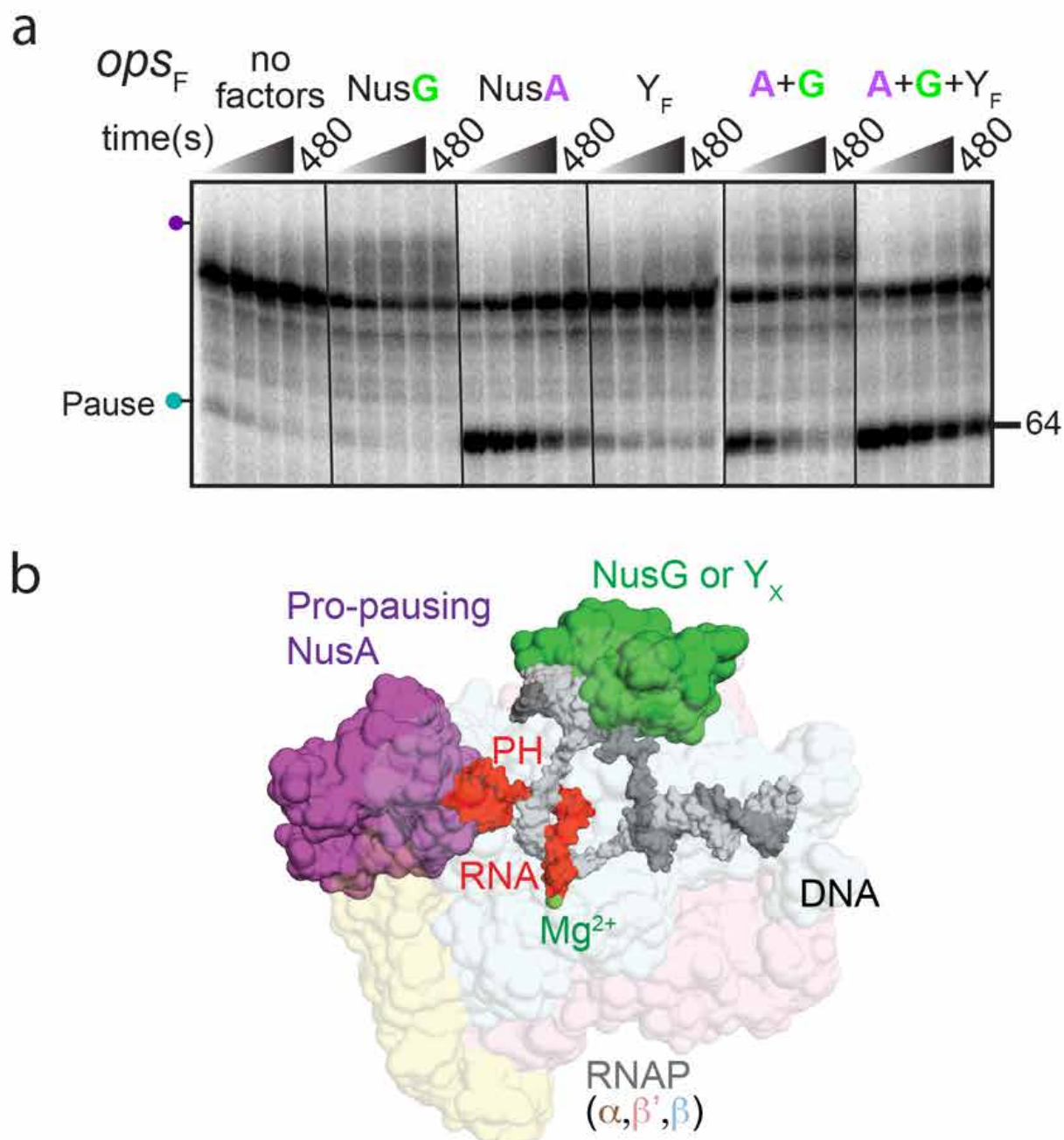
d PIVoT assays of *ops_x* pauses



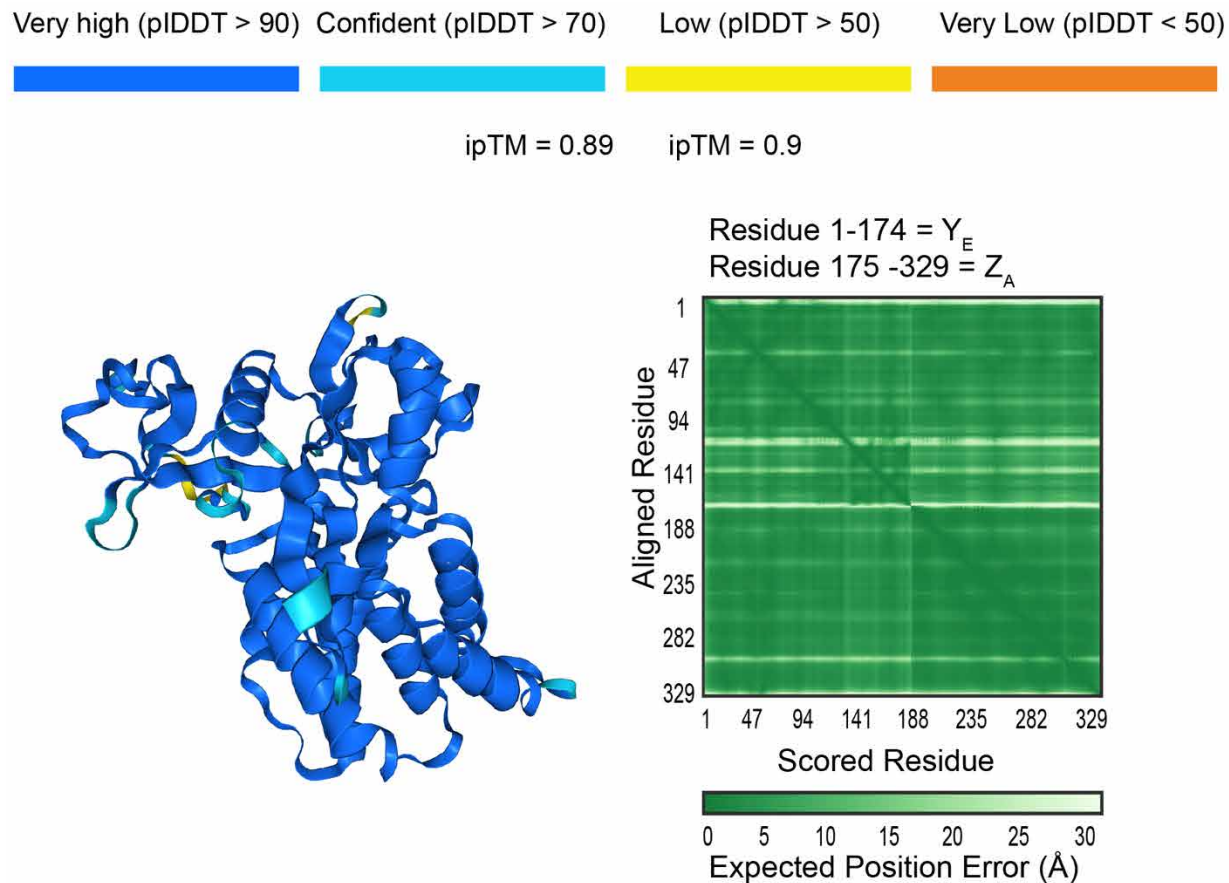
Supplementary Fig. 2. *ops_x* pause mapping by PIVoT. a) Schematic of PIVoT assays. *, source radiolabel (either 5'-³²P-labeled RNA or incorporation of [α -³²P]NMP at RNA 3' end, depending on the assay; see Methods). **b)** *rBfr*RNAP overexpression plasmid and final purified RNAP separated by SDS-PAGE and Coomassie-stained. Stars indicate terminal affinity tags. **c)** *rBfr*RNAP pauses on consensus but not anti-consensus pause sequences. **d)** Representative transcriptional pauses from distinct CPS operon leader regions mapped in vitro. PIVoT assay of relevant regions from CPS operon leader regions. Some pauses (e.g., PSH, are more prominent in the presence of NusA or cognate Y_X (see Supplementary Fig. 4). Pause quantitation is provided for representative Class 1 and 2 *ops_x* in Fig. 1d.



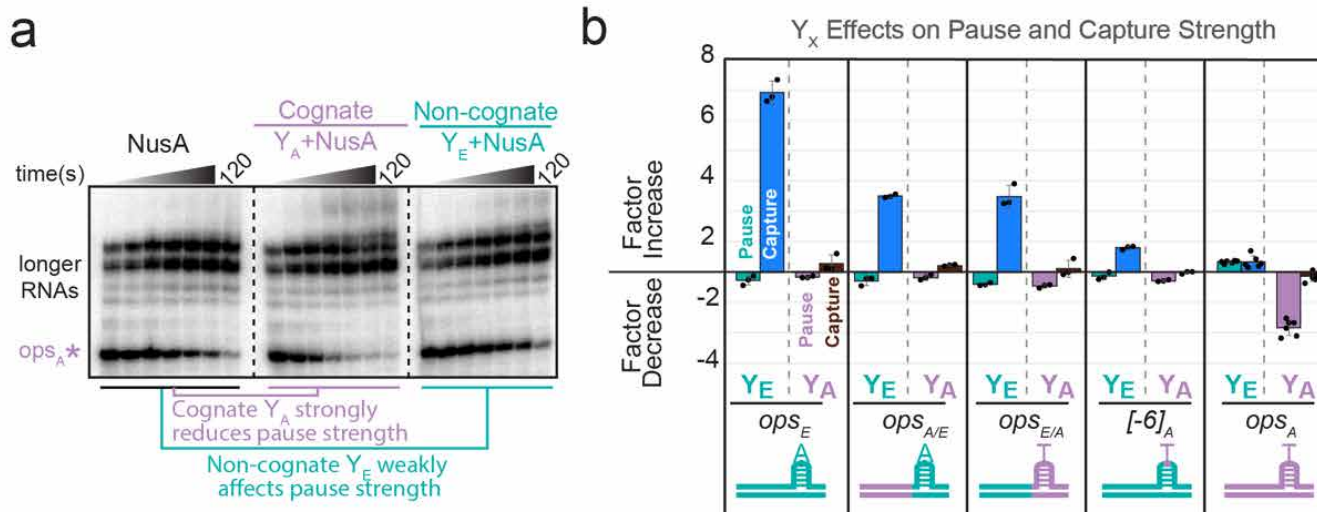
Supplementary Fig. 4. Y_X and NusA effects at 6 different ops_X sites. Pause strengths and factor effects are quantified for representative Class 1 and 2 ops_X sites in Fig 1d. **a)** PIVoT assays for Class 1 ops_E , ops_A , and ops_B (150 nM Y_X , 1 μ M NusA, and 200 μ M NTPs added concomitantly where indicated). **b)** PIVoT assay for ops_C (0.5 μ M Y_C , 0.5 μ M NusA, and 200 μ M NTPs added concomitantly where indicated). **c)** PIVoT assays for Class 2 ops_H and ops_F (1 μ M Y_X , 1 μ M NusA, and 500 μ M NTPs added concomitantly where indicated).



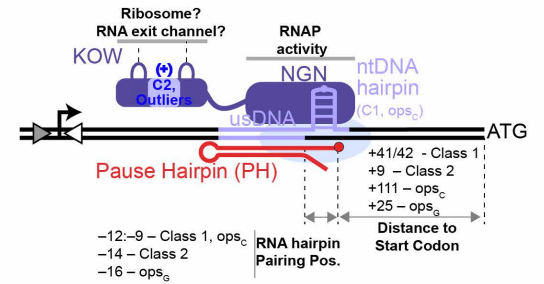
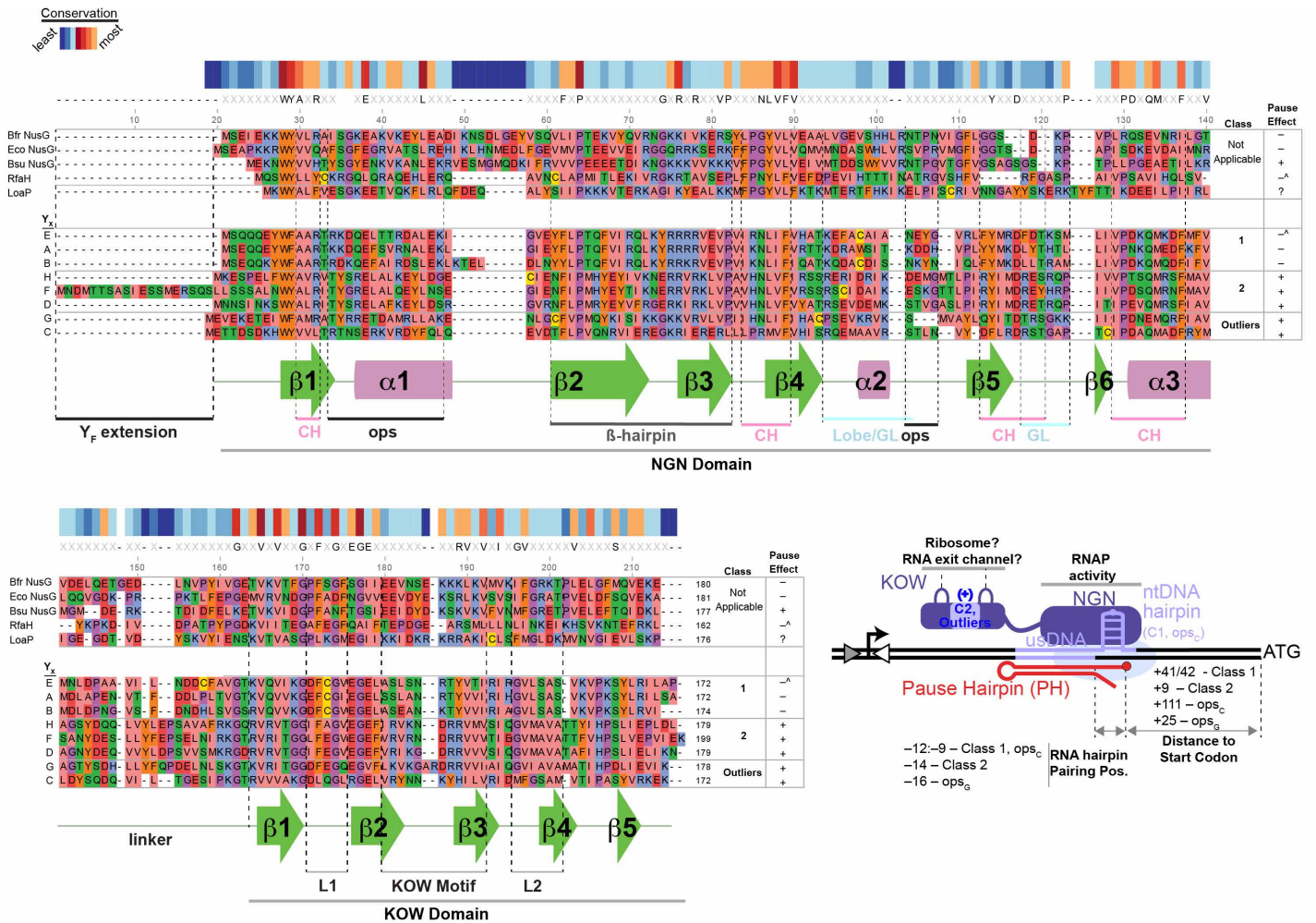
Supplementary Fig. 5. Y_F outcompetes NusG at *ops*_F. **a)** PIVoT assays performed with 1 μM Y_F, 1 μM NusG, 0.5 μM NusA, and 0.5 mM NTPs added concomitantly where indicated. **b)** Homology model of a *Bfi*-RNAP PEC bound by NusG NGN, and NusA (see Methods).



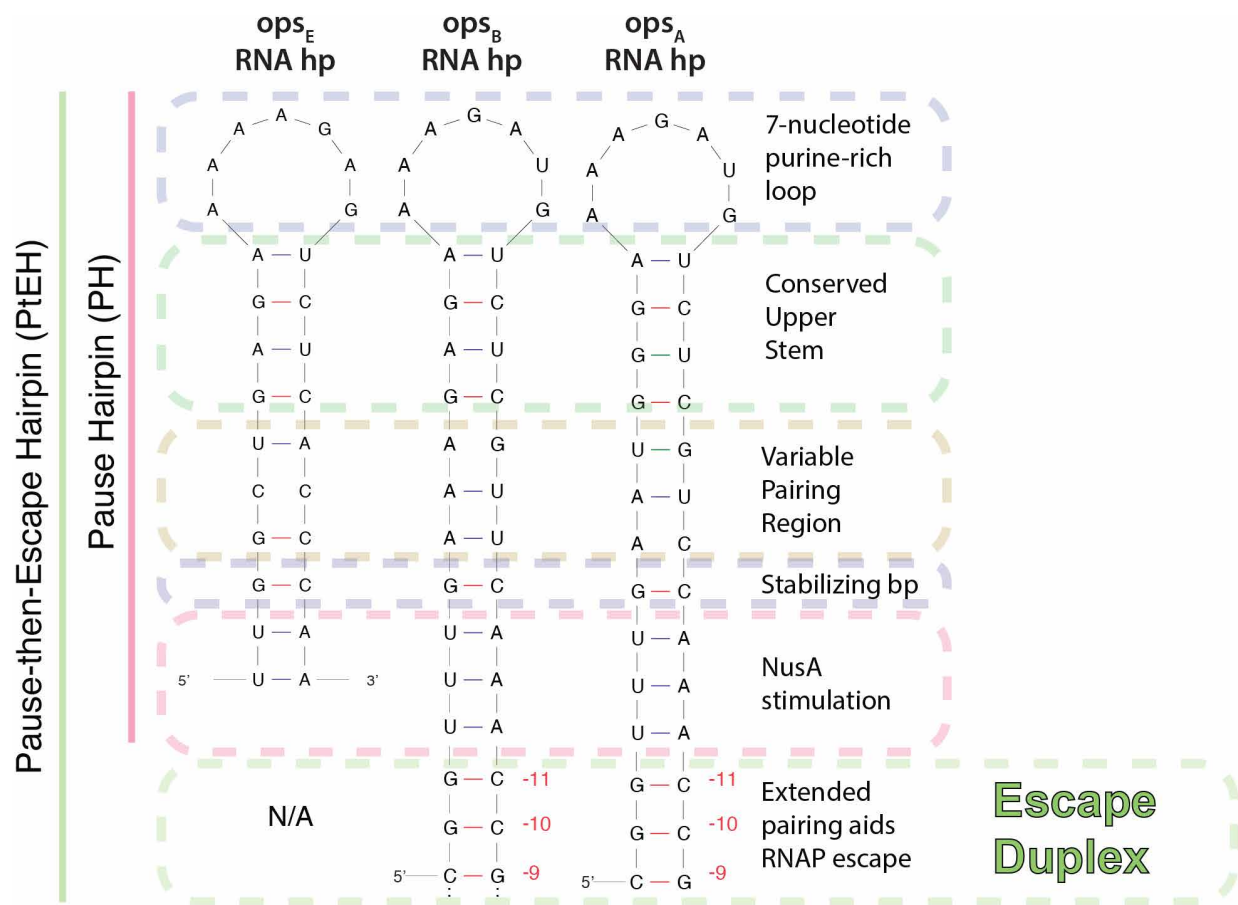
Supplementary Fig. 6. Confidence metrics from AlphaFold 3⁷ Y_E - Z_A complex structural prediction. The interface predicted template modeling (ipTM) score of 0.89 and predicted template modeling (pTM) score of 0.9 represent confident high-quality predictions (values greater than 0.8).



Supplementary Fig. 7. Effects of Y_E and Y_A on WT ops_X and hybrid ops_X sequences in PIVoT assays. **a)** Full time-course PIVoT assay illustrating cognate Y_A , but not non-cognate Y_E , modulates the strength of the ops_A pause. **b)** PIVoT assay quantitation of Y_A and Y_E activities on WT or hybrid scaffolds. Assays were performed in at least triplicate at a single timepoint (45 s) in the presence of 1 μ M NusA and 100 μ M NTPs, adding 150 nM Y_E or 150 nM Y_A . Fold effects are relative to the NusA-only condition. Y_E association on WT ops_E manifests primarily as capture activity, whereas Y_A association at ops_A manifests as anti-pausing activity (see Supplementary Fig. 4). Error bars represent standard deviations from three experiments.

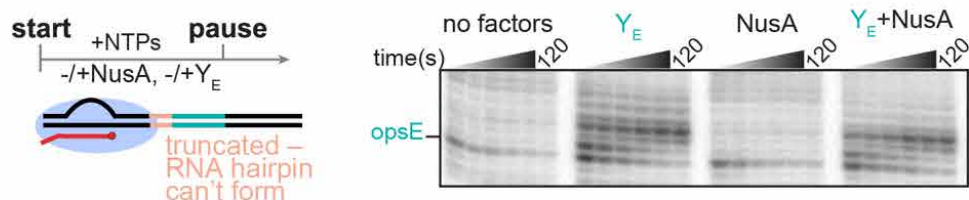


Supplementary Fig. 8. Y_X alignment compared to NusGs and orthologous factors. Sequences were aligned in SnapGene using the ClustalOmega algorithm. Amino acids are highlighted based on physico-chemical properties. ‘Pause effects’ indicate Y_X effects on pausing (‘^’ superscript indicates that RfaH⁶ or Y_E suppressed pausing at the pause site (*ops* or *ops_E*), but enhanced pausing a few nucleotides downstream). *Bfr*-NusG pause suppression is shown in Supplementary Fig. 5. *Eco*NusG pause suppression and *Bsu*NusG pause enhancement are documented⁸⁻¹¹. Features depict some NusG_{SP}-interacting modules of a PEC (lobe-gate loop [GL], clamp helices [CH]), *ops* ntDNAhp, and features of NusG_{SP} (NGN, KOW, β hairpin). Bottom right: a cartoon summary of some class-specific features. Blue (+) indicates a positively charged KOW motif is found in Class 2 Y_X and Outlier Y_X, similar to the positively charged KOW motif of LoaP¹².

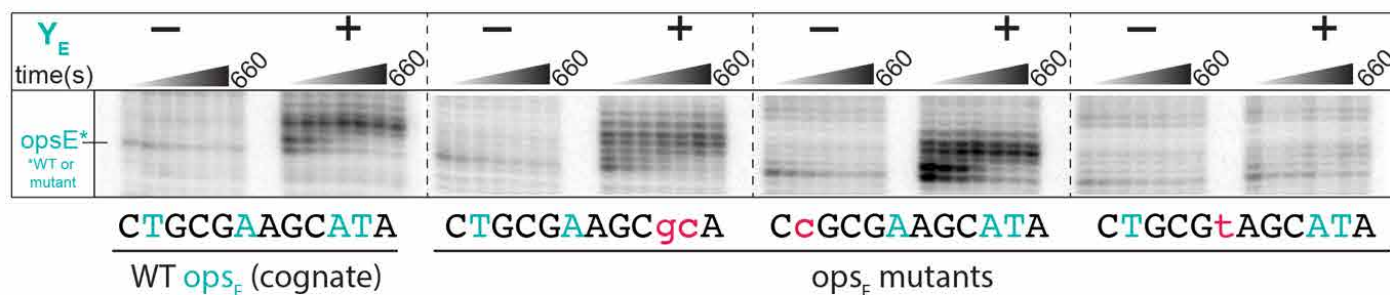


Supplementary Fig. 9. Predicted secondary structures of nascent RNA hairpins.

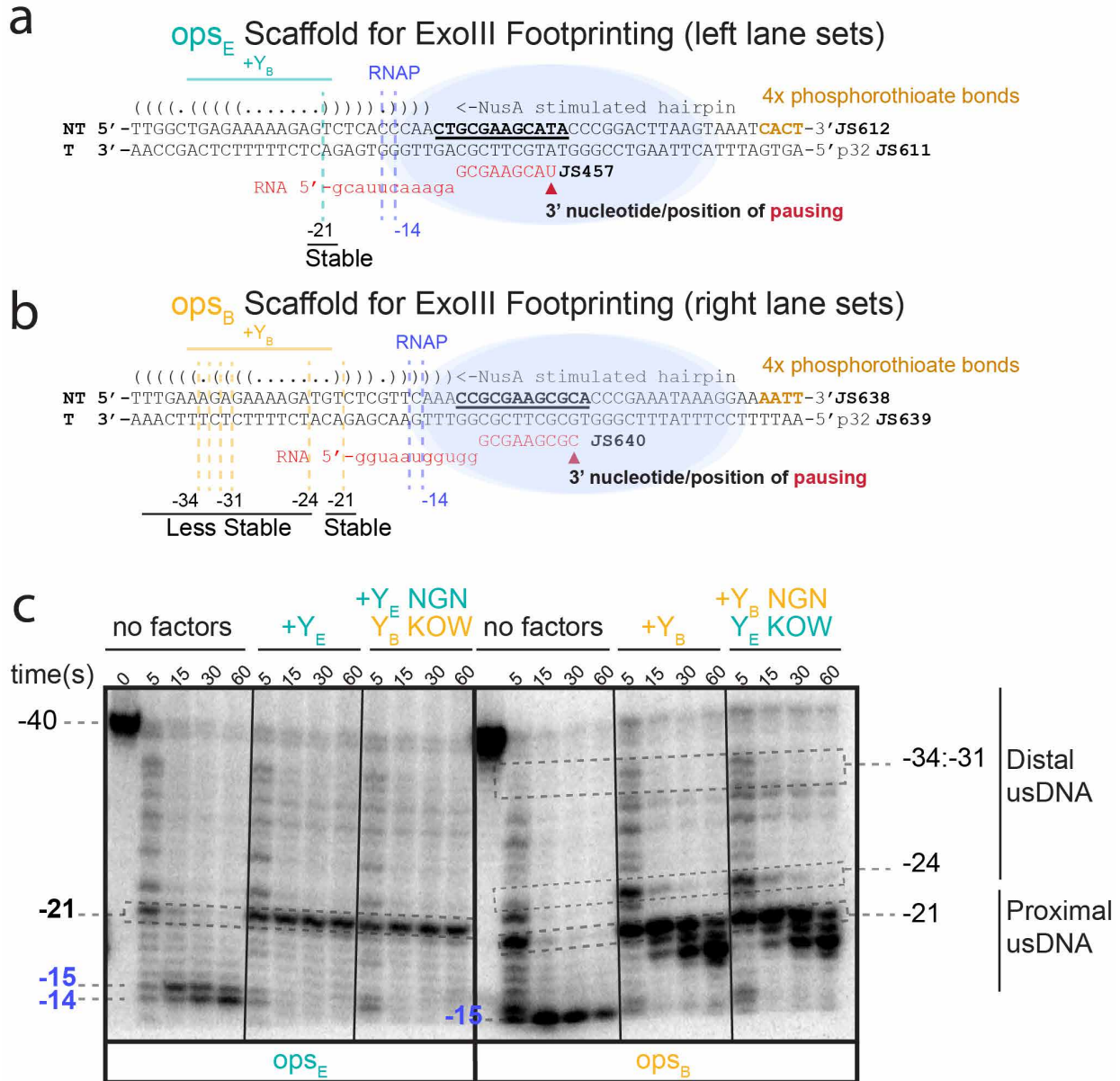
a



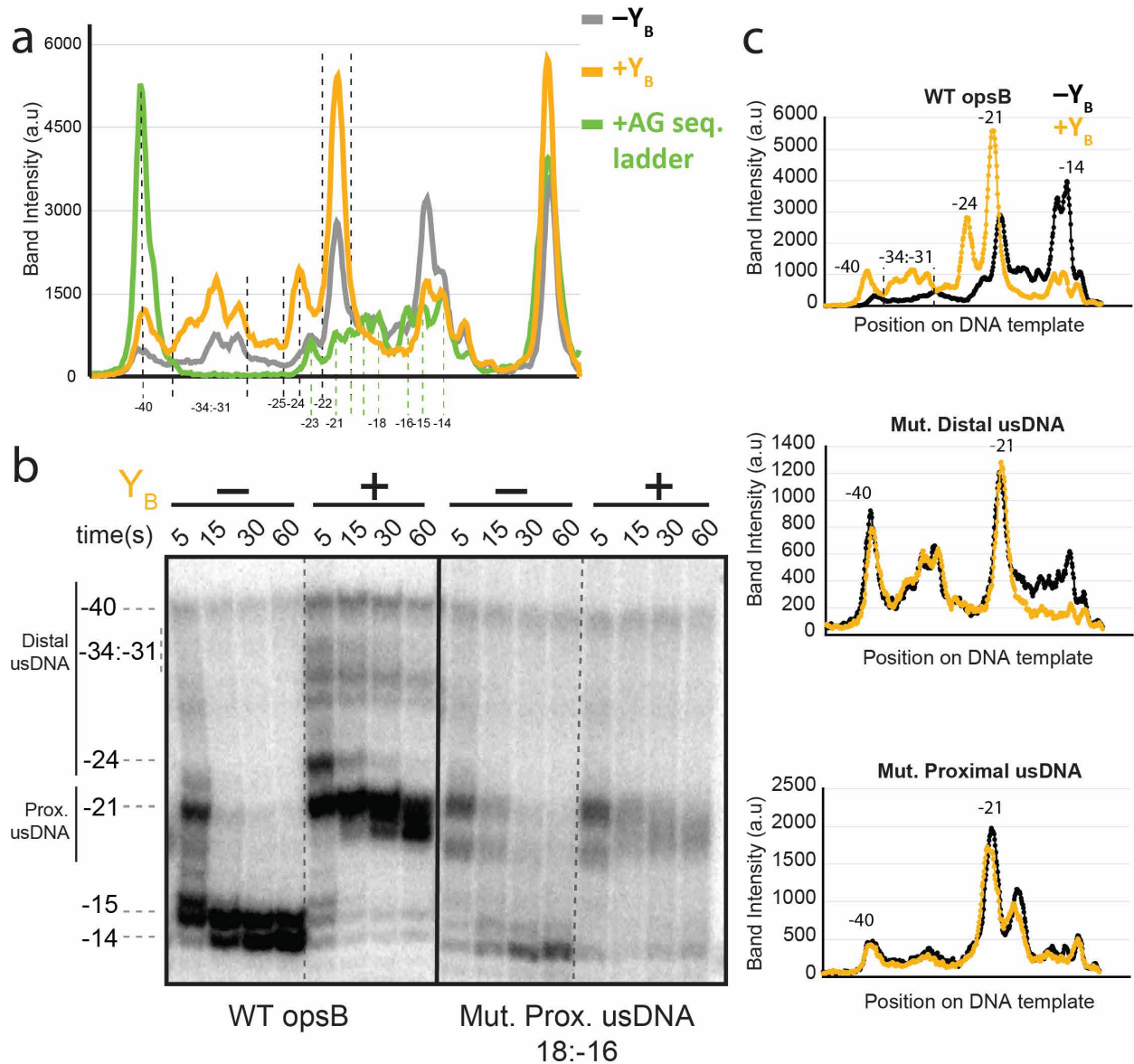
b



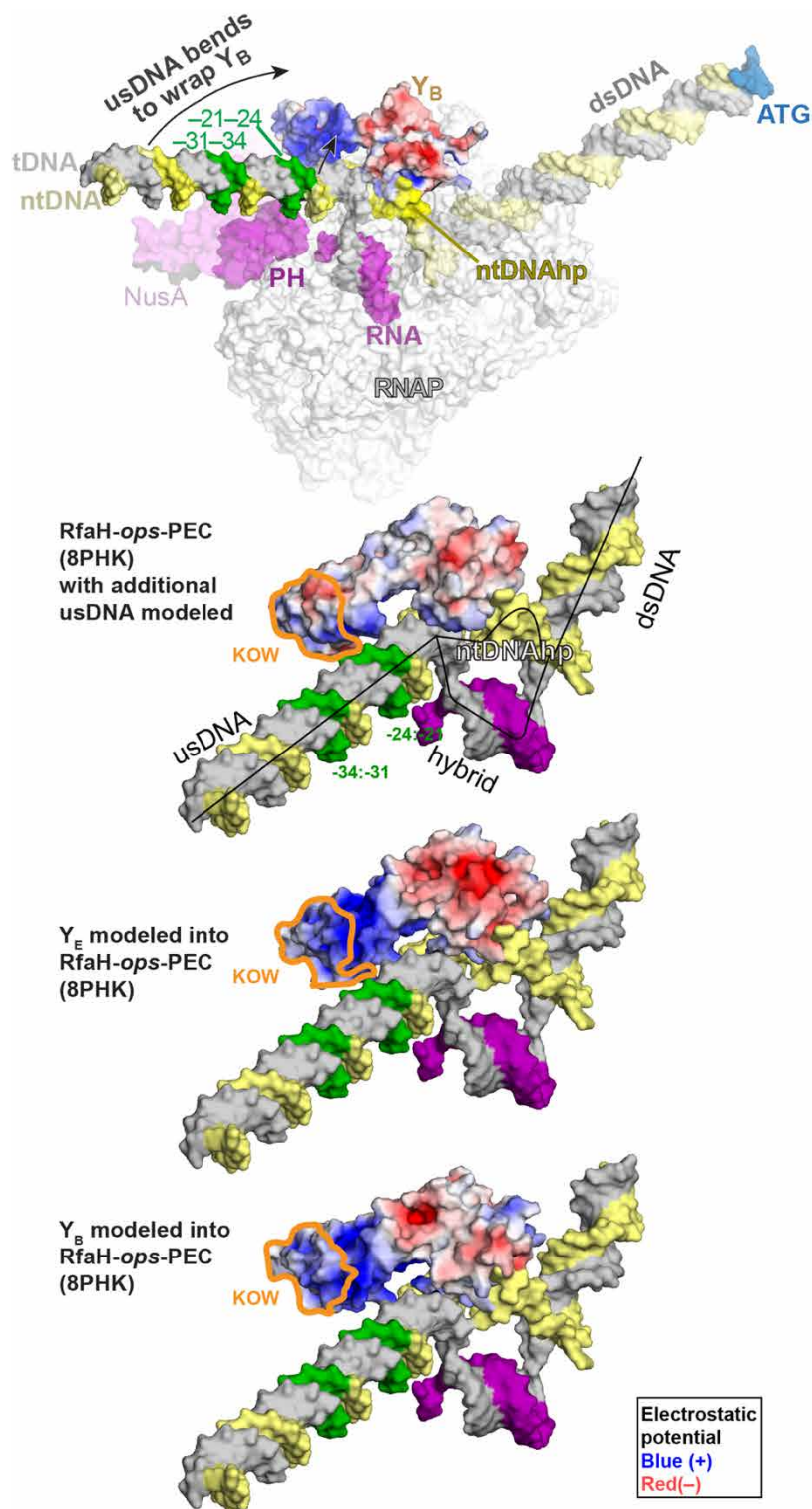
Supplementary Fig. 10. Effects of *opsE* mutants on Y_E or NusA activity. **a)** NusA does not noticeably enhance pausing at *opsE* in the absence of RNA hairpins. Experimental scheme testing the effect of NusA in the absence of upstream sequence enabling RNA hairpin formation. RNAP is reconstituted on a nucleic-acid scaffold with truncated upstream DNA. PIVoT assays performed with 150 nM Y_X, 1 μM NusA, and 100 μM NTPs added concomitantly where indicated. **b)** Effects of substitutions within the -10:-1 *opsE* window on Y_E activity. Results shown are representative of triplicate experiments.



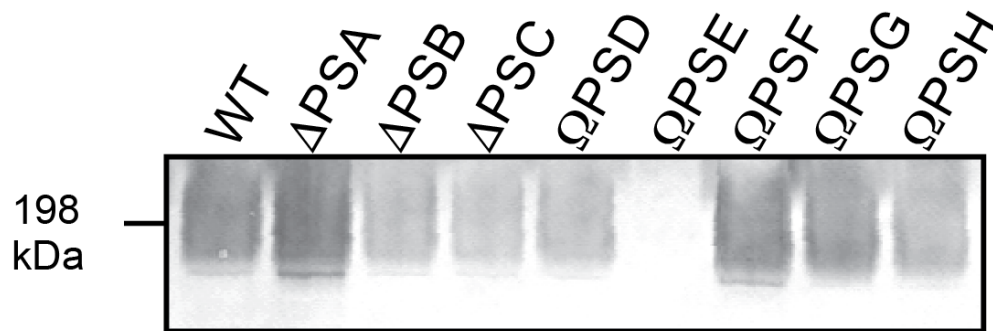
Supplementary Fig. 12. Primary data associated with Fig 6b. Representative of experiments performed in at least duplicate (see Methods). **a)** Nucleic acid scaffold used for exonuclease footprinting experiments mapping Y_E or hybrid Y_E-Y_B NGN-KOW footprints on *ops_E*. Footprints in the absence or presence of Y_B are indicated by dashed lines. 4x phosphorothioate bonds were incorporated at the 3' end of the NT strand to prevent its cleavage and associated artifacts during the assay. **b)** Nucleic acid scaffold used for exonuclease footprinting experiments mapping Y_B or hybrid Y_B-Y_E NGN-KOW footprints on *ops_B*. Footprints in the absence or presence of Y_E are indicated by dashed lines. 4x phosphorothioate bonds were incorporated at the 3' end of the NT strand to prevent its cleavage and associated artifacts during the assay. **c)** Representative exonuclease footprinting gel (n=2) illustrating that Y_B, but not Y_E, protects distal upstream DNA. These footprints were identical between WT and hybrid NGN-KOW proteins harboring an identical NGN domain, suggesting *ops_X* specificity determinants are created by the NGN domain.



Supplementary Fig. 13. Primary data associated with Fig 6c. **a)** Identification of Y_B protection footprints by comparison to AG sequencing ladder. **b)** Full ExoIII-footprinting time course associated with Fig 6c. Representative of experiment performed in at least duplicate. **c)** Pseudodensitometry plots of the 5-second timepoint lane associated with Fig 6c.



Supplementary Fig. 14. Modeling of Class 1 Y_X suggest that Y_X provides a larger positively charged surface for usDNA interaction relative to RfaH (*E. coli*). (top) Electrostatic surface potential model of Y_B recruited to *ops_X* using the RfaH-ops-PEC (8PHK)⁴ as template and modeling additional upstream DNA. Green highlighted regions in the upstream DNA indicate Y_B footprints. The usDNA must distort to interact with sequence-specifically with Y_B (black arrows). The RfaH, Y_E, and Y_B models below the full PEC model were created using the same structure but with RNAP and NusA are hidden for clarity. RfaH lacks the significant positive charge observed in models of Class 1 Y_E and Y_B, suggesting this charge is an evolved feature of Y_X facilitating readout of upstream DNA. Most of the positive charge is localized to the NGN domain (KOW outlined in orange).



Supplementary Fig. 15. The PSE monoclonal antibody (mAb 16) is specific to PSE. This Western immunoblot shows that the mAb 16 reacts with WT and each PSX mutant except for the PSE mutant.

Scaffolds in Supplementary Fig. 4

opsE+hp (Class 1) (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
(((((.(((.....)))))).)))<-NusA stimulated pause hairpin
NT 5'-GTCCGTTTCGTTTCCACGATCTTGCAGTTTGGCTGAGAAAAAGAGTCTCACCCAACTGCGGAAGCATAACCCGGACTTAAGTAAATCACTCAG-3' JS521
T 3'-CAGGCAAGCAAGGTGCTAGAACGTCAAACCGACTTTTTTCTCAGAGTGGGTGACGCTTCGTATGGGCTGAATTCATTTAGTGAGTC-5' JS522
RNA 5'-UUUUAACGUUCCAC JS146 (bolded RNA nucleotides pair with template DNA oligonucleotide)
```

opsB+hp (Class 1) (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
(((((.(((.(((.....)))))).)))<-Escape Hairpin (shared w/ opsA)
(((((.(((.....)))))).)))<-NusA stimulated hairpin
NT 5'-GTCCGTTTCGTTTCCACGAATTTGACGGTTTGAAAGAGAAAAGATGTCTCGTTCAAAACCGCGAAGCGCAACCCGAAATAAGGAAAATTTCC-3' JS551
T 3'-CAGGCAAGCAAGGTGCTTAAACTGCCAACTTTCTCTTTCTACAGAGCAAGTTTGGCGCTTCGCGTGGGCTTTATTTCTTTTAAAGG-5' JS552
RNA 5'-UUUUAACGUUCCAC JS146 (bolded RNA nucleotides pair with template DNA oligonucleotide)
```

opsA+hp (Class 1) (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
(((((.(((.(((.....)))))).)))<-Escape Hairpin
(((((.(((.....)))))).)))<-NusA stimulated hairpin
NT 5'-GTCCGTTTCGTTTCCACGTCTTACGGTTTGAATGGGAAAAGATGTCTCGTCCAAACCGCGTAGCGCAACCCGAAAGTAACCTCTCGACTTTG-3' JS553
T 3'-CAGGCAAGCAAGGTGCGAGAACTGCCAACTTACCTTTTCTACAGAGCAGGTTTGGCGCATCGCGTGGGCTTTCATTGGAGAGCTGAAAC-5' JS554
RNA 5'-UUUUAACGUUCCAC JS146 (bolded RNA nucleotides pair with template DNA oligonucleotide)
```

opsH+hp (Class 2) (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
(((((.(((.(((.....)))))).)))<-NusA-stimulated hairpin
NT 5'-GTCCGTTTCGTTTCCACGTACCTGACCGGGAGGTACTTTTCGTACTCCGGTGTCCCAAAGAACATCCTTTTGTGAAGGAATCCCCAGAGC-3' JS583
T 3'-CAGGCAAGCAAGGTGCGAGTGGACTGGCCCTCCATGAAAGCATGAGGCCACAGGGGTTTCTTGTAGGAAACACTTCCTTAGGGGTCTCG-5' JS584
RNA 5'-UUUUAACGUUCCAC JS146 (bolded RNA nucleotides pair with template DNA oligonucleotide)
```

opsF+hp (Class 2) (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
(((((.(((.(((.....)))))).)))<-NusA-stimulated hairpin
NT 5'-GTCCGTTTCGTTTCCACGTAACCTGACCGGGAGGTATGCTTCGTACTCCGGTGTCCCAAAGAACATCCTGACGTGAACGACATGACAAC-3' JS523
T 3'-CAGGCAAGCAAGGTGCAATTGAACTGGCCCTCCATACGAAGCGATGAGGCCACAGGGGTTTCTTGTAGGACTGCACTTGCTGTACTGTTG-5' JS524
RNA 5'-UUUUAACGUUCCAC JS146 (bolded RNA nucleotides pair with template DNA oligonucleotide)
```

PSC (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
Bubble Ultramer Scaffold # 1 from PSC 5'UTR (bold region not from UTR)
NT 5'-
GATCGAATCCGTTAACGATCTGTCAAAATCCGGTGCGAATACTTTTTCTGTACCCGATTATCAATAAACTTAATTTGTAAATTTGCTGAAAATAAGGCATGTTTTTGAATATTCCTGTTTTTAAACAAATTTTCATCCTTAGTCATTACTGAAAC
TTTTTCTTACGAACGTAGTCTTGGAGACAACAGATAGCGATAAAC-3' JS708
T 3'-
CTAGCTTAGGCAAGGTGCAAGTTTAGGCCACGCTTATGAAAAGACATGGGCTAATAGTATTTTGAATTAACATTTAACGACTTTTATCCGTACAAAAAACTTATAAGGACAAAAATTTGTTAAAGTAGGAATCAGTAATGACTTTG
AAAAAGAAATGCTTGCATCAGAACTCTGTTGTCTATCGCTATTTG-5' JS709
RNA 5'- UUUUAACGUUCCAC-3' JS146 (bolded RNA nucleotides pair with template DNA oligonucleotide)
```

Scaffolds in Fig 1d, Supplementary Fig. 5

opsF+hp (Class 2) (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
(((.....(((.....)))))) <-NusA-stimulated hairpin
NT 5'-GTCCGTTTCGTTTCCACGTAACCTGACCGGGAGGTATGCTTCGCTACTCCGGTGTCCCAAAGAACATCCTGACGTGAACGACATGACAAC-3' JS523
T 3'-CAGGCAAGCAAGGTGCTAGAACGTCAAACCGACTCTTTTCTCAGAGTGGGTTGACGCTTCGTATGGGCCTGAATTCATTTAGTGAGTC-5' JS524
RNA 5'-UUUUAACGUUCCAC JS146 (bolded RNA nucleotides pair with template DNA oligonucleotide)
```

Scaffolds in Fig 2d (Graph Z titration effect Y_E activity) – 2023-01-04 expt

opsE+hp (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
(((.....(((.....)))))) <-NusA stimulated pause hairpin
NT 5'-GTCCGTTTCGTTTCCACGATCTTGACGTTTGGCTGAGAAAAAGAGTCTCACCAACTGCGAAGCATACCCGGACTTAAGTAAATCACTCAG-3' JS521
T 3'-CAGGCAAGCAAGGTGCTAGAACGTCAAACCGACTCTTTTCTCAGAGTGGGTTGACGCTTCGTATGGGCCTGAATTCATTTAGTGAGTC-5' JS522
RNA 5'-UUUUAACGUUCCAC JS146 (bolded RNA nucleotides pair with template DNA oligonucleotide)
```

Scaffolds in Supplementary Fig. 7a (Cog vs Non-cog Y_X on ops_A) – 2022-03-23 expt

opsA+hp (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
(((.....(((.....)))))) <- Escape Hairpin
(((.....(((.....)))))) <-NusA stimulated hairpin
NT 5'-GTCCGTTTCGTTTCCACGTTTACGGTTTGAATGGGAAAAGATGTCTCGTCCAAACCGCGTAGCGCACCCGAAAGTAACCTCTCGACTTTG-3' JS553
T 3'-CAGGCAAGCAAGGTGCTAGAACGTCAAACCGACTCTTTTCTCAGAGCAGGTTTGGCGCATCGCGTGGGCTTCATTGGAGAGCTGAAAC-5' JS554
RNA 5'-UUUUAACGUUCCAC JS146 (bolded RNA nucleotides pair with template DNA oligonucleotide)
```

Scaffolds in Supplementary Fig. 7b (ops_E→_A mutants) (2022-05-12 and 2022-06-02 expts)

opsE+hp (Pause Cycling Observed) (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
...(((.....(((.....))))))..... < Does not form bc structure below
forms first
(((.....(((.....)))))) <-NusA stimulated hairpin
NT 5'-GTCCGTTTCGTTTCCACGATCTTGACGTTTGGCTGAGAAAAAGAGTCTCACCAACTGCGAAGCATACCCGGACTTAAGTAAATCACTCAG-3' JS521
T 3'-CAGGCAAGCAAGGTGCTAGAACGTCAAACCGACTCTTTTCTCAGAGTGGGTTGACGCTTCGTATGGGCCTGAATTCATTTAGTGAGTC-5' JS522
RNA 5'-UUUUAACGUUCCAC JS146 (bolded RNA nucleotides pair with template DNA oligonucleotide)
```

HP_A[-10:-1]_E (Pause Cycling Observed) (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
((.....(((.....)))))) <-Escape Hairpin Disrupted
(((.....(((.....)))))) <-NusA stimulated hairpin
NT 5'-GTCCGTTTCGTTTCCACGTTTACGGTTTGAATGGGAAAAGAGTCTCGTCCAAACCTGCGAAGCATACCCGAAAGTAACCTCTCGACTTTG-3' JS555
T 3'-CAGGCAAGCAAGGTGCTAGAACGTCAAACCGACTCTTTTCTCAGAGCAGGTTTACGCTTCGTATGGGCCTTCATTGGAGAGCTGAAAC-5' JS556
RNA 5'-UUUUAACGUUCCAC JS146 (bolded RNA nucleotides pair with template DNA oligonucleotide)
```

opsE+hp -6 mut (Pause Cycling Observed) (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
...(((.....(((.....))))))..... < Does not form bc structure below
forms first
(((.....(((.....)))))) <-NusA stimulated hairpin
NT 5'-GTCCGTTTCGTTTCCACGATCTTGACGTTTGGCTGAGAAAAAGAGTCTCACCAACTGCGTAGCATACCCGGACTTAAGTAAATCACTCAG-3' JS591
T 3'-CAGGCAAGCAAGGTGCTAGAACGTCAAACCGACTCTTTTCTCAGAGTGGGTTGACGCTTCGTATGGGCCTGAATTCATTTAGTGAGTC-5' JS592
RNA 5'-UUUUAACGUUCCAC JS146 (bolded RNA nucleotides pair with template DNA oligonucleotide)
```

HP_E[-10:-1]_A (Pause Cycling Observed) (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
...(((.....(((.....))))))..... <-NusA stimulated hairpin
NT 5'-GTCCGTTTCGTTTCCACGATCTTGACGTTTGGCTGAGAAAAAGAGTCTCACCAACCGCGTAGCGCACCCGGACTTAAGTAAATCACTCAG-3' JS557
T 3'-CAGGCAAGCAAGGTGCTAGAACGTCAAACCGACTCTTTTCTCAGAGTGGGTTGACGCTTCGTATGGGCCTGAATTCATTTAGTGAGTC-5' JS558
RNA 5'-UUUUAACGUUCCAC JS146 (bolded RNA nucleotides pair with template DNA oligonucleotide)
```

opsA+hp (NO Pause Cycling) (bold DNA is arbitrary (not from UTR) used for reconstitution)

```
(((.....(((.....)))))) <- Escape Hairpin
(((.....(((.....)))))) <-NusA stimulated hairpin
```

Scaffolds in Fig 4ab – Antisense DNA expts ops_E

(bold DNA is arbitrary (not from UTR) used for reconstitution)

(bold DNA is arbitrary (not from UTR) used for reconstitution)

Scaffolds in Supplementary Fig. 10a

Truncated scaffold for testing ops_E without pause hairpin

5' -GAGAAAAAGAGTCTCACCCAA**CTGCGAAGCATA**CCCGGACTTAAGTAAATCACTCAGGAA-3' **JS156**
3' -CTCTTTTCTCAGAGTGGGTTGACGCTTCGTATGGGCCTGAATTCATTTAGTGAGTCCTT-5' **JS157**
CUCACCCA **JS473**
RNA5' -UUUUUUA

Scaffolds in Supplementary Fig. 10b – ntDNA hp mutations (red)

opsE (WT)

5' -GAGAAAAAGAGTCTCACCCAA**CTGCGAAGCATA**CCCGGACTTAAGTAAATCACTCAGGAA-3' **JS156**
3' -CTCTTTTCTCAGAGTGGGTTGACGCTTCGTATGGGCCTGAATTCATTTAGTGAGTCCTT-5' **JS157**
CUCACCCA **JS473**
RNA5' -UUUUUUA

opsE_mut1(opsA/B-mimic-1(AT→GC))

5' -GAGAAAAAGAGTCTCACCCAA**CTGCGAAGCga**CCCGGACTTAAGTAAATCACTCAGGAA-3' **JS467**
3' -CTCTTTTCTCAGAGTGGGTTGACGCTTCG**cg**TGGGCCTGAATTCATTTAGTGAGTCCTT-5' **JS468**
CUCACCCA **JS473**
RNA5' -UUUUUUA

opsE_mut2(opsA/B-mimic-2(T→C))

5' -GAGAAAAAGAGTCTCACCCAA**CgGCGAAGCATA**CCCGGACTTAAGTAAATCACTCAGGAA-3' **JS469**
3' -CTCTTTTCTCAGAGTGGGTTG**g**CGCTTCGTATGGGCCTGAATTCATTTAGTGAGTCCTT-5' **JS470**
CUCACCCA **JS473**
RNA5' -UUUUUUA

opsE_mut3(opsA-mimic-3(A→T))

5' -GAGAAAAAGAGTCTCACCCAA**CTGCGtAGCATA**CCCGGACTTAAGTAAATCACTCAGGAA-3' **JS471**
3' -CTCTTTTCTCAGAGTGGGTTGACGCa**T**CGTATGGGCCTGAATTCATTTAGTGAGTCCTT-5' **JS472**
CUCACCCA **JS473**
RNA5' -UUUUUUA

Scaffolds in Fig 5c,d – opsB to E mutants (-PH only) 2023-04-05 expt

Bold-underlined regions represent upstream DNA regions of interest. Substitutions are red, bold, lowercase, underlined.

opsB-hp (reconstitute -3)

```
(((((.((((.....)))))).))))<-NusA stimulated hairpin
NT 5'-TTTGAAAGAGAAAAGATGTCTCGTTCAAAACCGCGAAGCGCACCCGAAATAAGGAAAAATTCCTCAAG-3' JS654
T 3'-AAACTTTCTCTTTCTACAGAGCAAGTTTGGCGCTTCGCGTGGGCTTTATTTCTTTTAAAGGGGTC-5' JS655
                                CCGCGAAGC JS666
RNA5'-UUUUUU
```

opsB -36-34 mut to opsE

```
(((((.((((.....)))))).))))<-NusA stimulated hairpin
NT 5'-TTTGggcGAGAAAAGATGTCTCGTTCAAAACCGCGAAGCGCACCCGAAATAAGGAAAAATTCCTCAAG-3' JS660
T 3'-AAACccgCTCTTTCTACAGAGCAAGTTTGGCGCTTCGCGTGGGCTTTATTTCTTTTAAAGGGGTC-5' JS661
                                CCGCGAAGC JS666
RNA5'-UUUUUU
```

opsB -26-24 mut to opsE

```
(((((.((((.....)))))).))))<-NusA stimulated hairpin
NT 5'-TTTGAAAGAGAAAAAgGTCTCGTTCAAAACCGCGAAGCGCACCCGAAATAAGGAAAAATTCCTCAAG-3' JS662
T 3'-AAACTTTCTCTTTTcCAGAGCAAGTTTGGCGCTTCGCGTGGGCTTTATTTCTTTTAAAGGGGTC-5' JS663
                                CCGCGAAGC JS666
RNA5'-UUUUUU
```

opsB -18-16 mut to opsE

```
(((((.((((.....)))))).))))<-NusA stimulated hairpin
NT 5'-TTTGAAAGAGAAAAgacGTCTCcaCAAAACCGCGAAGCGCACCCGAAATAAGGAAAAATTCCTCAAG-3' JS664
T 3'-AAACTTTCTCTTTCTACAGAGgtgGTTTGGCGCTTCGCGTGGGCTTTATTTCTTTTAAAGGGGTC-5' JS665
                                CCGCGAAGC JS666
RNA5'-UUUUUU
```

Scaffolds in Fig 5d (+PH only) – compares opsB-escape duplex to scaffold “-10:1, -14 ins opsB”

Bold DNA is arbitrary (not from UTR) used for reconstitution. Bolded RNA nucleotides pair with template DNA oligonucleotide.

opsE+hp > -10:1, -14 ins

```
(((((.((((.....)))))).))))<-NusA stimulated hairpin
NT 5'-GTCCGTTCGTTTCCACGATCTTGCAGTTTGGCTGAAGAAAGAGTCTCACCCAAACCGCGAAGCGCACCCGGACTTAAGTAAATCACTCA-3' JS597
T 3'-CAGGCAAGCAAGGTGCTAGAACGTCAAACCGACTCTTTTCTCAGAGTGGGTTTGGCGCTTCGCGTGGGCCTGAATTCATTAGTGAGT-5' JS598
RNA 5'-UUUUAACGUUCCAC JS146
```

opsB-escape duplex

```
(((((.((((.....)))))).))))<-NusA stimulated hairpin
NT 5'-GTCCGTTCGTTTCCACGATCTTGCAGTTTGAAAGAGAAAGATGTCTCGTTCAAACCGCGAAGCGCACCCGAAATAAGGAAAAATTCCTCAAG-3' JS674
T 3'-CAGGCAAGCAAGGTGCTAGAACGTCAAACCTTCTCTTTCTACAGAGCAAGTTGGCGCTTCGCGTGGGCTTTATTTCTTTTAAAGGG-5' JS675
RNA 5'-UUUUAACGUUCCAC JS146
```

Bold DNA is arbitrary (not from UTR) used for reconstitution. Bolded RNA nucleotides pair with template DNA oligonucleotide.

(((((((.(((((.)))))).))))))<-NusA stimulated hairpin

NT 5'-**GTCCGTTTCGTTTCCACG**ATCTTGCAGTTTGGCTGAGAAAAAGAGTCTCACCCAAACCGCGAAGCGCACCCGGACTTAAGTAAATCACTCA-3' **JS597**
T 3'-**CAGGCAAGCAAAGGTGCT**AGAACGTCAAACCGACTCTTTTCTCAGAGTGGGTTGGCGCTTCGCGTGGGCCTGAATTCATTTAGTGAGT-5' **JS598**
RNA 5'-UUUUAAACGUUCCAC **JS146**

opsB+hp

(((((((.(((((.)))))).))))))<-Escape Hairpin
 ((((((((((.(((((.)))))).)))))).))))<-NusA stimulated hairpin

NT 5'-**GTCCGTTTCGTTTCCACGA**ATTTGACGGTTTGAAAGAGAAAAGATGTCTCGTTCAAACCGCGAAGCGCACCCGAAATAAAGGAAAATTTCC-3' **JS551**
T 3'-**CAGGCAAGCAAAGGTGCTT**AAACTGCCAAACTTTCTCTTTCTACAGAGCAAGTTTGGCGCTTCGCGTGGGCTTTATTTCTTTTAAAGG-5' **JS552**
RNA 5'-UUUUAAACGUUCCAC **JS146**

Scaffolds in Fig 6, Supplementary Fig. 12, Supplementary Fig. 13 – Exo assay scaffolds

opsE+hp—for exo footprinting w/ phosphorothioated NT DNA

```
(((((.((((.....)))))).))))<-NusA stimulated hairpin
NT 5'-TTGGCTGAGAAAAAGAGTCTCACCCTGCGAAGCATACCCGGACTTAAGTAAAT*C*A*T-3' JS612 * = phosphorothioate
T 3'-AACCGACTCTTTTCTCAGAGTGGGTGACGCTTCGTATGGGCTGAATTCATTAGTGA-5' JS611
GCGAAGCAU JS457
RNA 5'-gcuaucuaaga
```

Below: Bolded upstream DNA are regions of interest. Bold, red, lowercase, and underlined are substitutions made.

opsB+hp—for exo footprinting w/ phosphorothioated NT DNA

```
(((((.((((.....)))))).))))<-NusA stimulated hairpin
NT 5'-TTTGAAAAGAGAAAAGATGTCTCGTTCAAACCGCGAAGCGCACCCGAAATAAGGAA*A*A*T-3' JS638 * = phosphorothioate
T 3'-AAACTTTCTCTTTTCTACAGAGCAAGTTTGGCGCTTCGCGTGGGCTTTATTTCCCTTTAA-5' JS639
GCGAAGCGC JS640
RNA 5'-gguaauggu
```

opsB+hp[-36:-34]+[-26:-24] mut to opsE—for exo footprinting

```
(((((.((((.....)))))).))))<-NusA stimulated hairpin
NT 5'-TTTGAggcGAGAAAAAgGTCTCGTTCAAACCGCGAAGCGCACCCGAAATAAGGAA*A*A*T-3' JS710 * = phosphorothioate
T 3'-AAACccgCTCTTTTtTcCAGAGCAAGTTTGGCGCTTCGCGTGGGCTTTATTTCCCTTTAA-5' JS711
GCGAAGCGC JS640
RNA 5'-gguaauggugg
```

opsB+hp[-18:-16] mut to opsE—for exo footprinting

```
(((((.((((.....)))))).))))<-NusA stimulated hairpin
NT 5'-TTTGAAAAGAGAAAAGATGTCTCcacCAACCGCGAAGCGCACCCGAAATAAGGAA*A*A*T-3' JS712 * = phosphorothioate
T 3'-AAACTTTCTCTTTTCTACAGAGgtgGTTTGGCGCTTCGCGTGGGCTTTATTTCCCTTTAA-5' JS713
GCGAAGCGC JS640
RNA 5'-gguaauggug
```

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