

Supplementary information for manuscript

Identification of a Fusobacterial RNA-binding protein involved in host small RNA-mediated growth inhibition

Pu-Ting Dong¹, Mengdi Yang^{2,#}, Jie Hu^{3,4,#}, Lujia Cen¹, Peng Zhou⁵, Difei Xu^{3,4}, Peng Xiong^{3,4,*}, Jiahe Li^{6,*}, and Xuesong He^{1,*}

¹Department of Microbiology, The American Dental Association Forsyth Institute, Cambridge, MA 02142, USA.

²Department of Bioengineering, Northeastern University, Boston, MA 02115, USA.

³University of Science and Technology of China, Hefei 230026, China.

⁴Department of Biomedical Engineering, Suzhou Institute for Advanced Research, University of Science and Technology of China, Suzhou 215123, China.

⁵Department of Microbiology and Molecular Genetics, University of Texas McGovern Medical School, Houston, TX 77030, USA.

⁶Department of Biomedical Engineering, College of Engineering and School of Medicine, University of Michigan, Ann Arbor, MI 48109, USA.

#: These authors contribute equally.

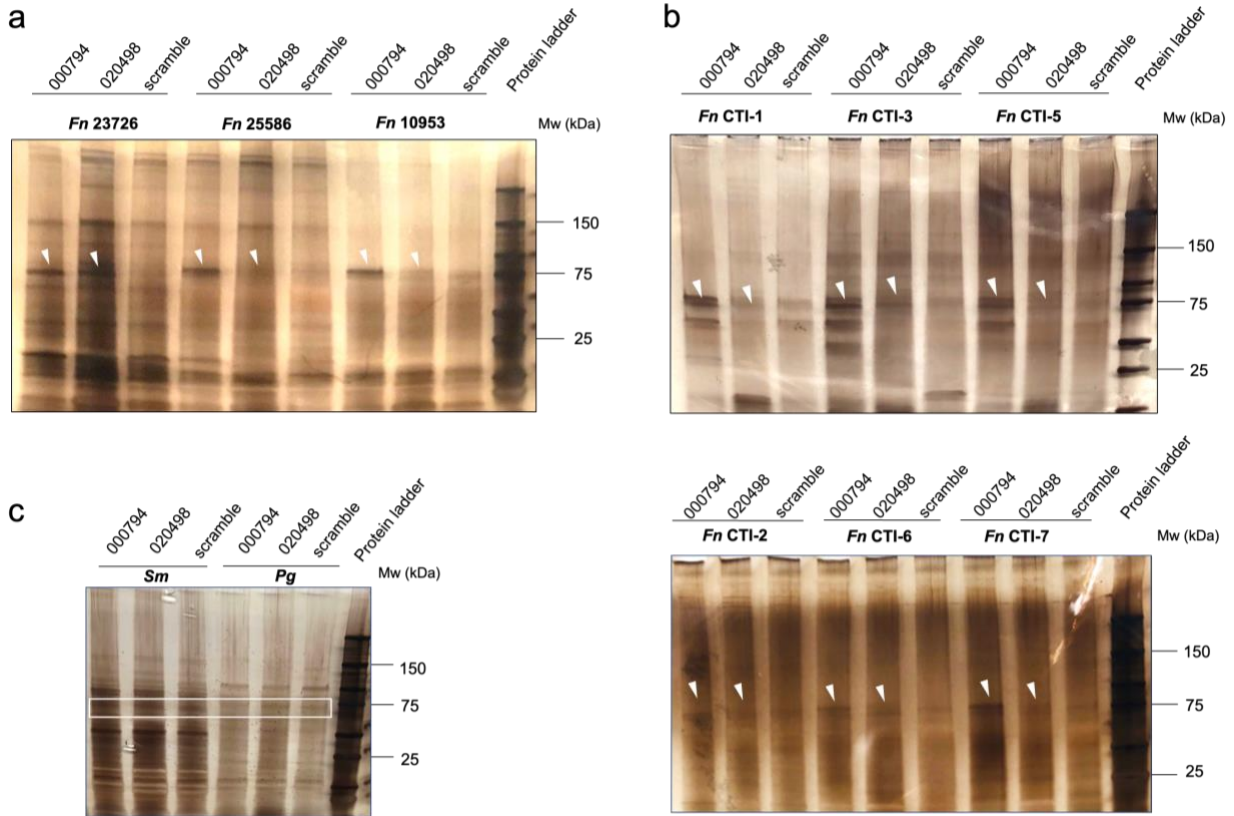
*: Corresponding authors: Dr. Xuesong He (xhe@forsyth.org), Dr. Jiahe Li (jiaheli@umich.edu), Dr. Peng Xiong (xiongxp@ustc.edu.cn).

This document includes:

Figure S1-S10

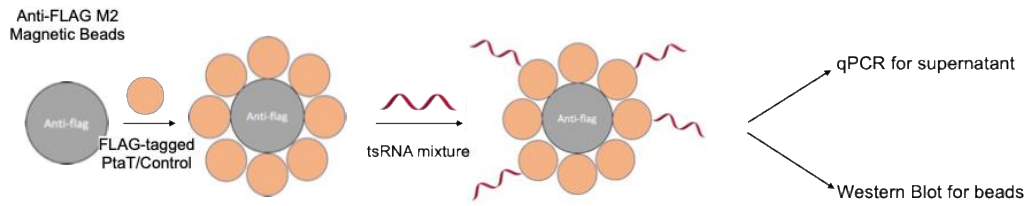
Table S1 for PtaT sequence alignment is provided in a separate file

Table S2-S3

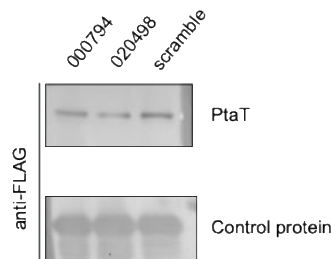


Supplementary Figure 1. Identification of PtaT by tsRNA-mediated affinity pulldown assay. **a.** Silver staining of denaturing SDS-PAGE gel for biotinylated tsRNA pulldown samples in three different ATCC *Fn* strains. Arrowheads indicate the gel bands, which were excised for protein identification by Mass Spectrometry analysis. **b.** Silver staining of denaturing SDS-PAGE gel for biotinylated tsRNA pulldown samples in six *Fn* clinical tumor isolates (CTIs). Arrowheads indicate the gel bands, which were excised for protein identification by Mass Spectrometry analysis. CTI-1: *F. nucleatum. ssp. animalis*; CTI-3: *F. nucleatum. ssp. animalis*; CTI-5: *F. nucleatum. ssp. animalis*; CTI-2: *F. nucleatum, ssp. nucleatum*; CTI-6: *F. nucleatum. ssp. polymorphum*; CTI-7: *F. nucleatum. ssp. vincentii*. **c.** Silver staining of denaturing SDS-PAGE gel for biotinylated tsRNA pulldown samples in *Streptococcus mitis* ATCC 6249 (*Sm*) and *Porphyromonas gingivalis* ATCC 33277 (*Pg*).

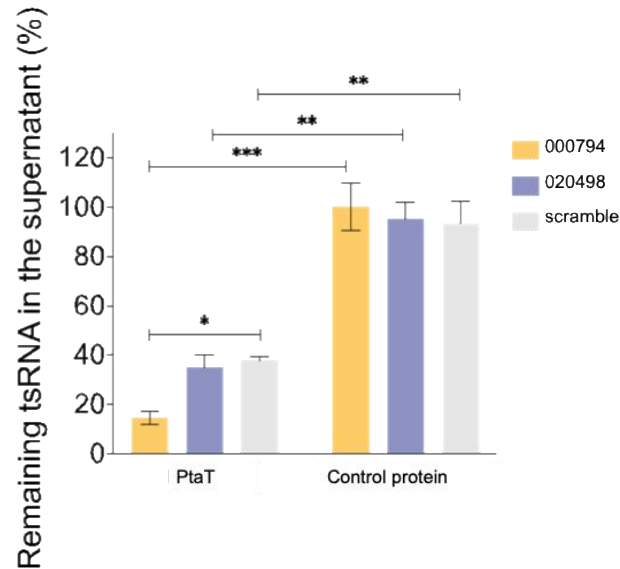
a



b



c

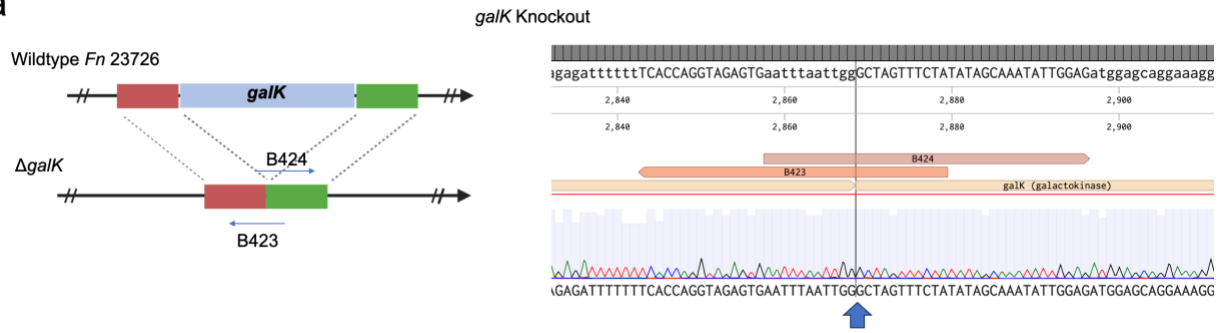


Supplementary Figure 2. Validation of the binding interaction between PtaT and tsRNA. **a.** Schematic of using anti-FLAG M2 magnetic beads and FLAG-tagged recombinant proteins to validate tsRNA binding *in vitro*. **b.** Western blotting of FLAG-tagged PtaT and STING (a negative control known to bind cyclic dinucleotides but not RNA oligos). **c.** Pull-down of naturally occurring tsRNA-000794, tsRNA-020498 or scramble control by purified FLAG-tagged PtaT and anti-FLAG antibody-conjugated magnetic beads. A FLAG-tagged irrelevant protein (STING) was used as a negative control for nonspecific binding. Unbound RNAs were quantified by stem-loop qPCR and normalized to the initial concentration. Results = Mean $\pm$ SEM (N=4) and are representative of two biological replicates. Statistical analyses were performed by the two-way ANOVA followed by Dunnett's Bonferroni multiple comparison tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

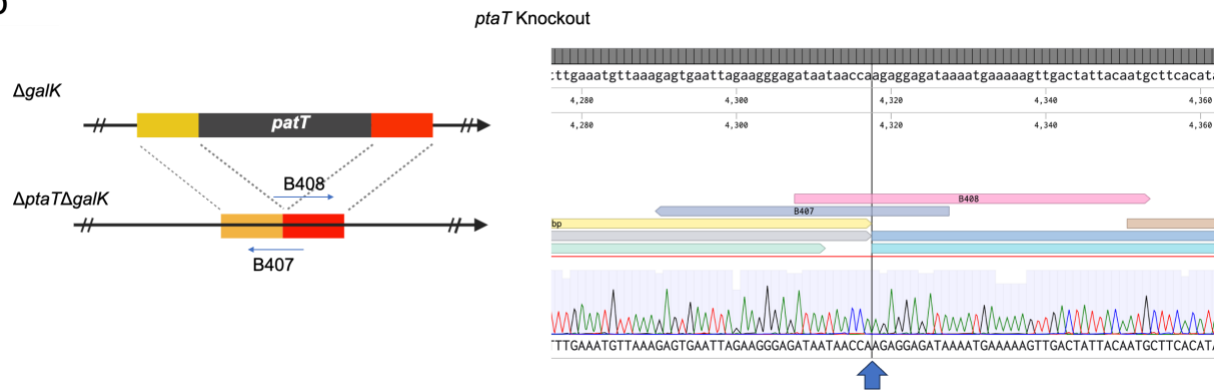
Classification	Protein	Localization	Uniport	Groups
RNA metabolism	Polyribonucleotide nucleotidyltransferase	Cytoplasmic	D5RFI6	tsRNA000794
Metal ion transport	Heavy metal translocating P-type ATPase	Membrane	D5RD38	
	Uncharacterized		D5RA61	
Metabolic process	S-methyl-5-thioribose-1-phosphate isomerase	Membrane	D5RDB1	tsDNA000794
Metabolic process	Signal peptide peptidase SppA		D5REW1	
Transport	ABC transporter, substrate-binding protein		D5RBV3	
	Uncharacterized		D5RA61	
RNA metabolism	Ribonuclease J	Cytoplasmic	D5RD58	piRNA_016792
RNA metabolism	Polyribonucleotide nucleotidyltransferase	Cytoplasmic	D5RFI6	
	Uncharacterized		D5RA61	
RNA metabolism	Polyribonucleotide nucleotidyltransferase	Cytoplasmic	D5RFI6	piRNA_006465
RNA metabolism	Ribonuclease R	Cytoplasmic	D5RAL6	
	Uncharacterized		D5RA61	
Metabolic process	S-methyl-5-thioribose-1-phosphate isomerase		D5RDB1	Beads only

Supplementary Figure 3. Identification of RNA-binding proteins from *Fn* ATCC 23726 total lysate by RNA pulldown and Mass Spectrometry. P-type ATPase was highlighted by red in the table.

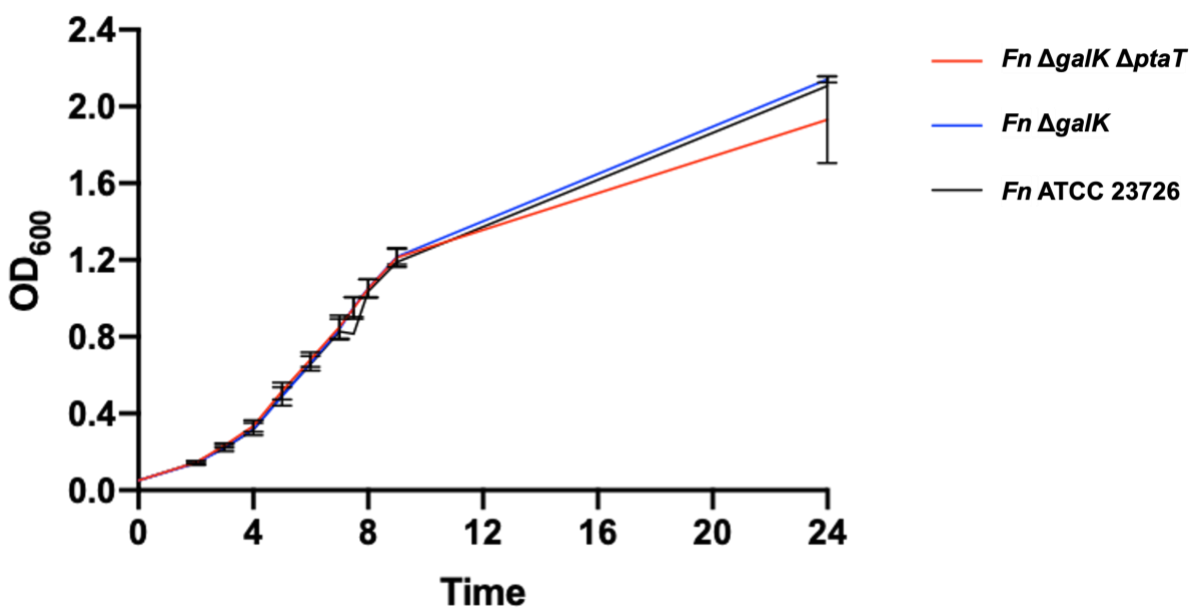
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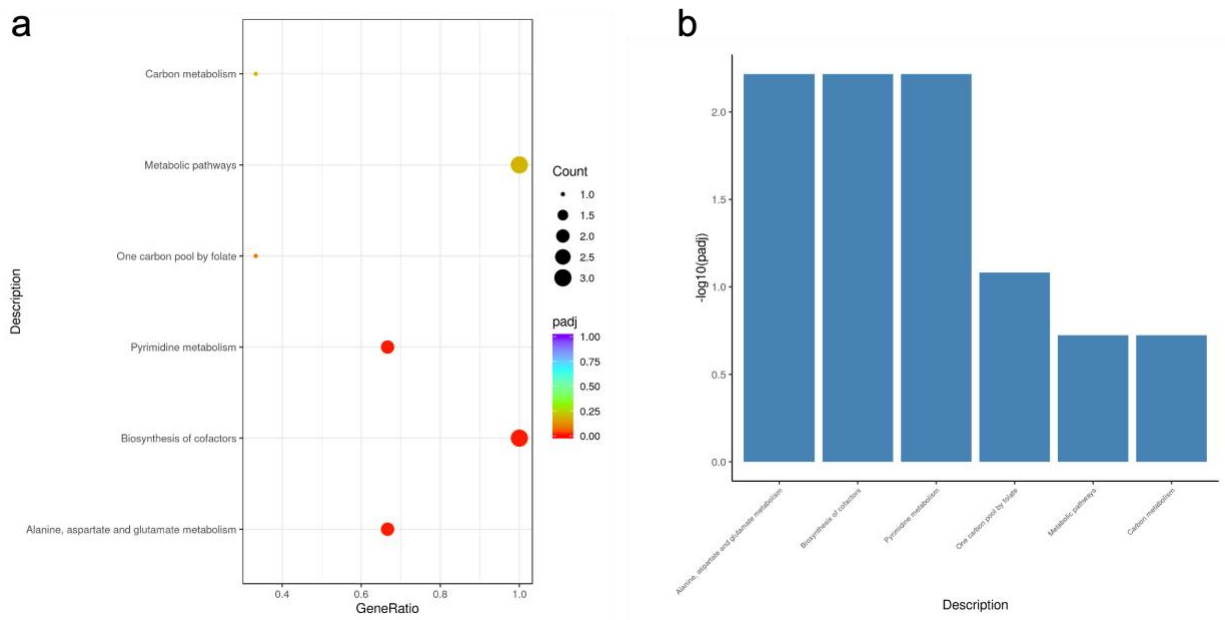
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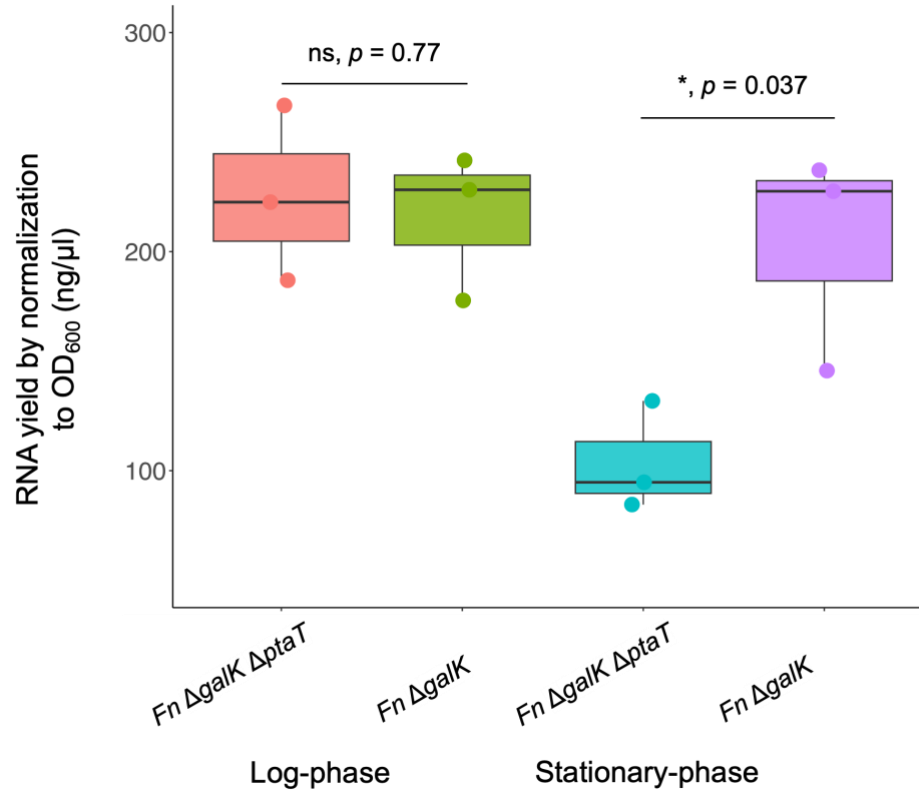
Supplementary Figure 4. A double crossover-mediated knockout of the full-length *ptaT* in *Fn* $\Delta galK$.
a. Generation of insertional mutagenesis via a double crossover at a 1kb central region of *galk* in *Fn* ATCC 23726. **b.** Double crossover-mediated complete knockout of the full-length *ptaT* in *Fn* ATCC 23726 $\Delta galK$.



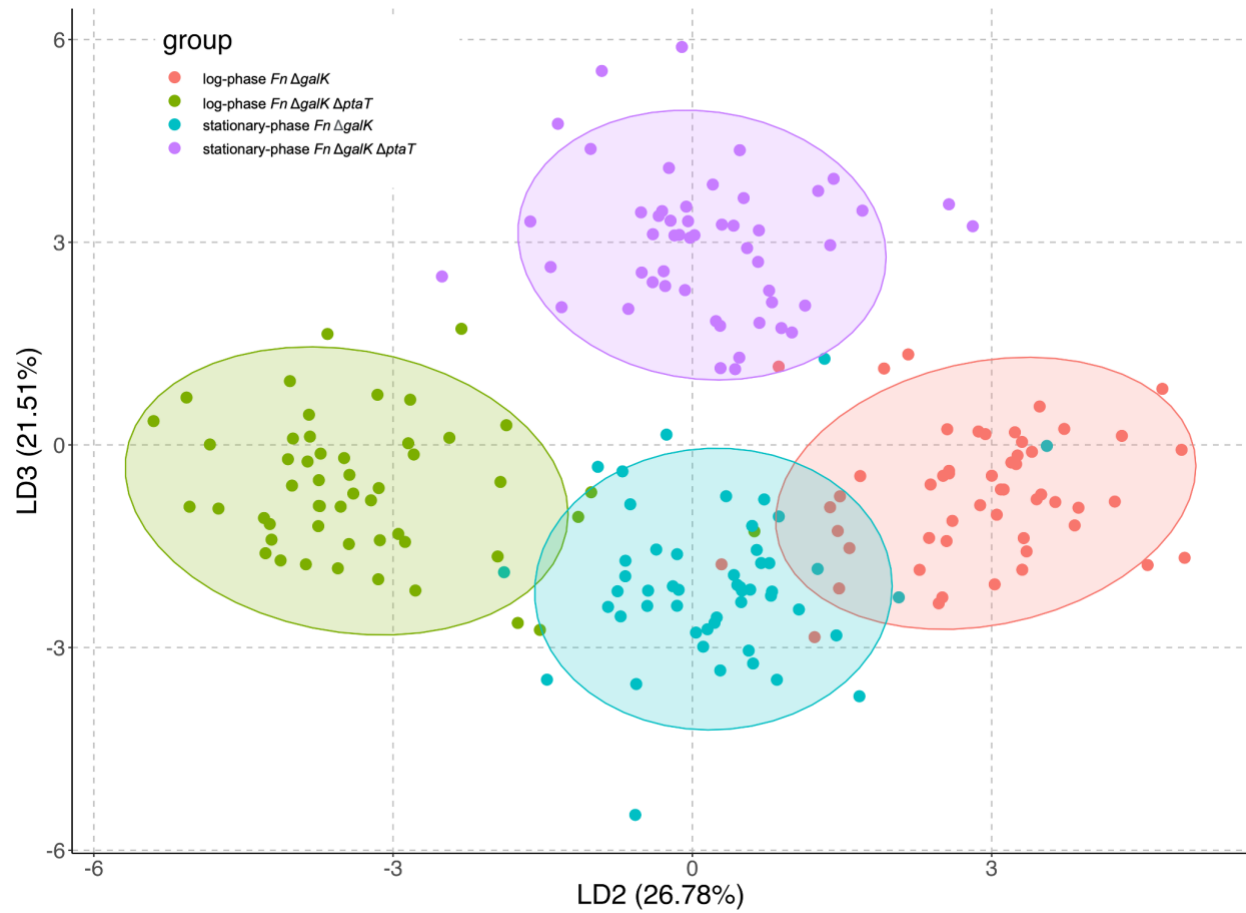
Supplementary Figure 5. The time-course growth kinetic monitored by optical density at 600 nm (OD_{600}) from *Fn* ATCC 23726, *Fn* $\Delta galK$ and *Fn* $\Delta galK$ $\Delta ptaT$. N = 3.



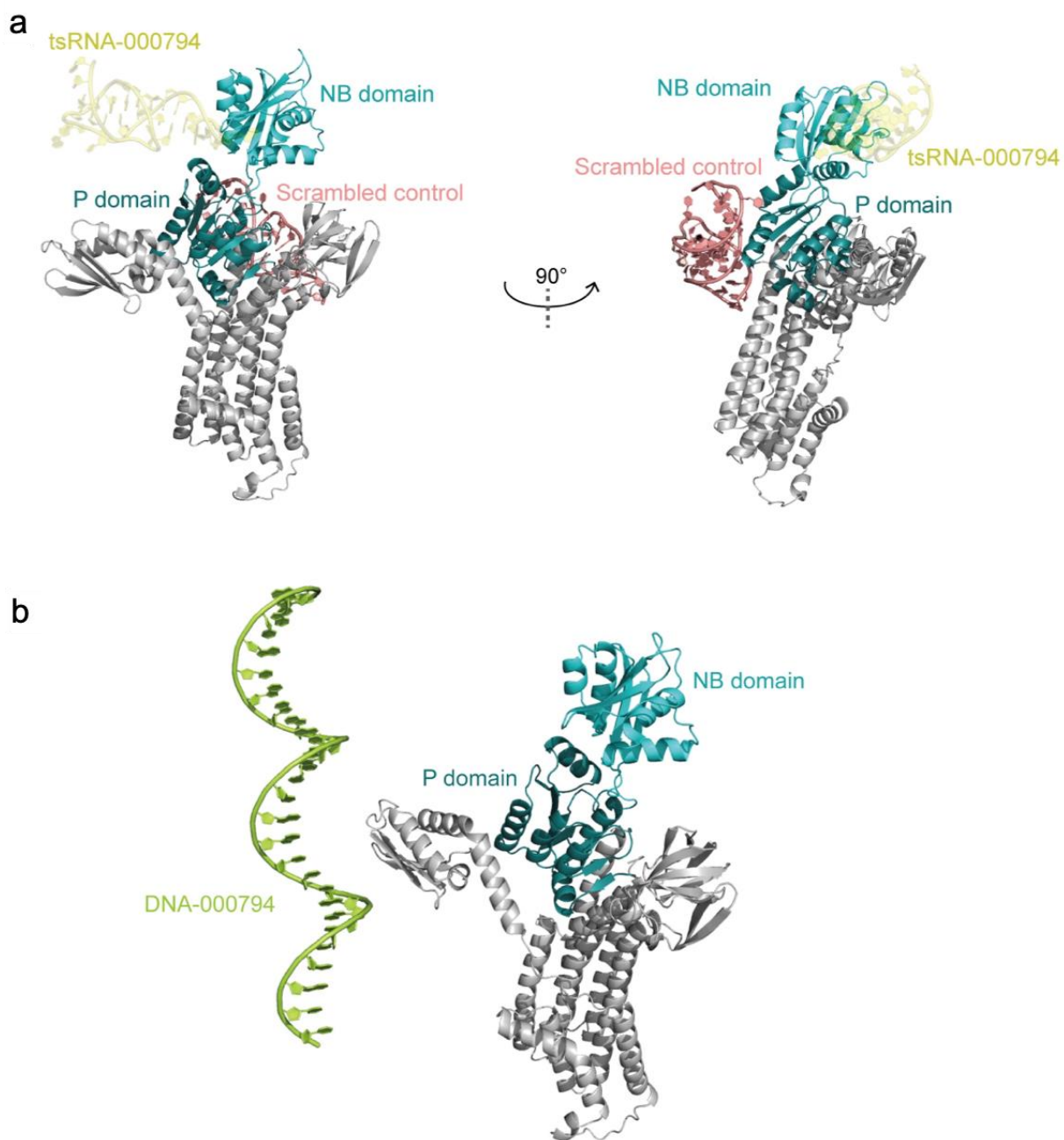
Supplementary Figure 6. Clusters of orthologous groups (COG, a) and quantification of differentially expressed genes (b) from stationary-phase *Fn ΔgalK ΔptaT* relative to *Fn ΔgalK*.



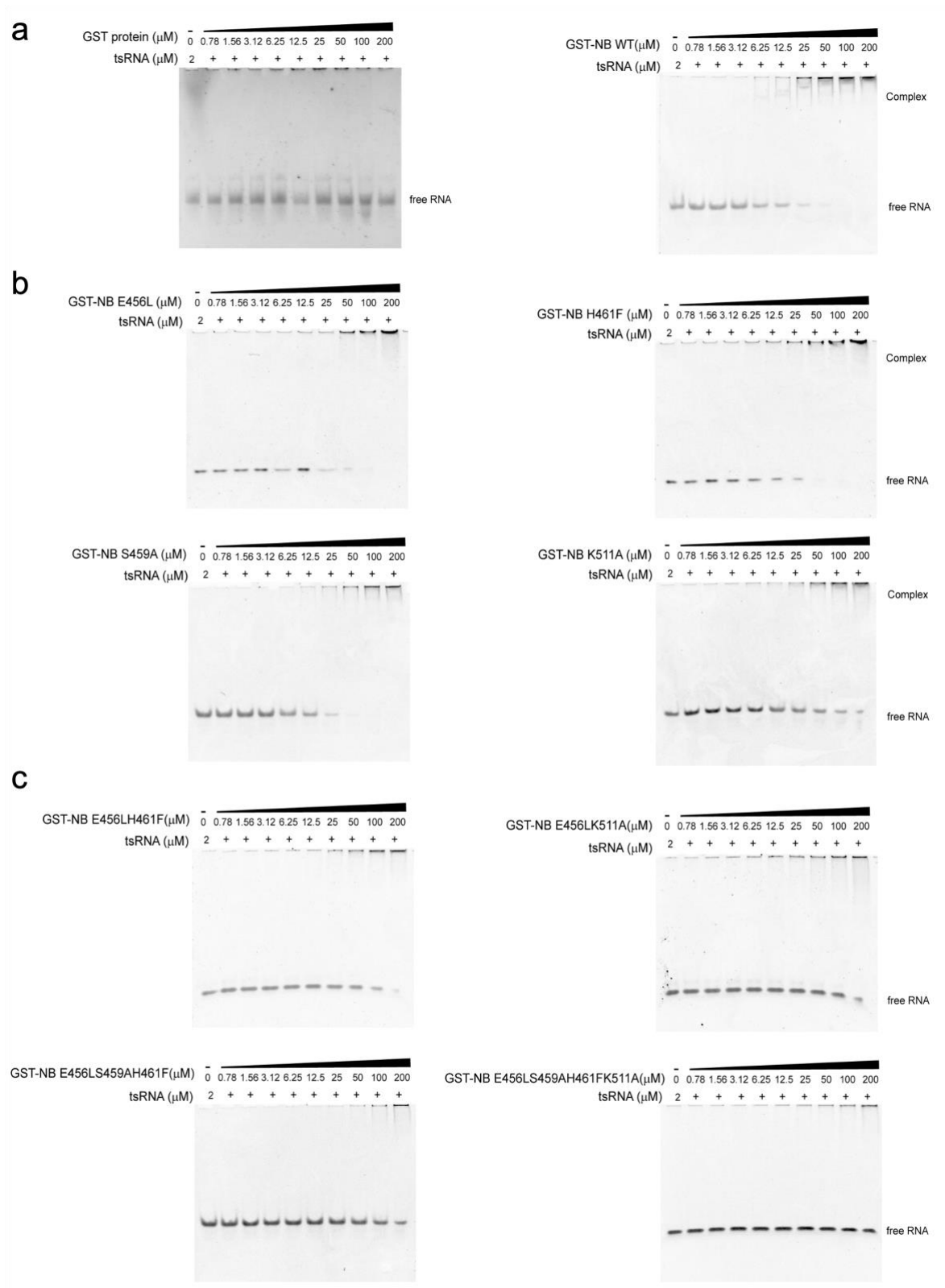
Supplementary Figure 7. The yield of total extracted RNA of both *Fn ΔgalK* and *Fn ΔgalK ΔptaT* from three biological replicates. Significant difference was determined through two-tailed unpaired Student's *t*-test.



Supplementary Figure 8. LDA analysis (LD3 versus LD2) of 200 Raman spectra from log-phase and stationary-phase *Fn ΔgalK* and *Fn ΔgalK ΔptaT*.



Supplementary Figure 9. Structural analysis of the predicted complex model between scrambled control (a), tsDNA-000794 (b) and PtaT by AlphaFold 3.



Supplementary Figure 10. EMSA results of alanine scanning mutations. a. GST negative control and wild-type control; **b.** Single-site mutations; **c.** Double and multi-sites mutations.

Table S2 Bacterial strains and plasmids used in this paper

Bacterial Species	Strains	Characteristics	Source
<i>Porphyromonas gingivalis</i>	ATCC 33277	WT	ATCC
<i>Streptococcus mitis</i>	ATCC 6249	WT	ATCC
<i>F. nucleatum</i>	ATCC 23726	<i>ssp. nucleatum</i> WT	ATCC
	ATCC 25586	<i>ssp. nucleatum</i> WT	ATCC
	ATCC 10953	<i>ssp. nucleatum</i> WT	ATCC
	<i>Fn_ΔgalK</i>	<i>galK</i> markerless deletion	this study
	<i>Fn_ΔgalK ΔptaT</i>	<i>ptaT</i> markerless deletion	this study
	CTI-1 (<i>ssp. animalis</i>)	clinical tumor isolate	Previous study ¹
	CTI-2 (<i>ssp. nucleatum</i>)	clinical tumor isolate	Previous study ¹
	CTI-3 (<i>ssp. animalis</i>)	clinical tumor isolate	Previous study ¹
	CTI-5 (<i>ssp. animalis</i>)	clinical tumor isolate	Previous study ¹
	CTI-6 (<i>ssp. polymorphum</i>)	clinical tumor isolate	Previous study ¹
	CTI-7 (<i>ssp. vincentii</i>)	clinical tumor isolate	Previous study ¹

	CTI-2 $\Delta ptaT$	deletion of <i>ptaT</i> in CTI-2	this study
<i>Escherichia coli</i>	NEB5 α		NEB
	Rosetta (DE3)		NEB
	C2987	cloning host	NEB
Plasmids	Purpose	Characteristics	Source
pSH200-PtaT	PtaT purification	amp	this study
pHS31_FLAG_ <i>galK</i>	In-frame deletion of <i>galK</i>	<i>catP</i>	this study
pHS31_FLAG- <i>galK</i> - $\Delta ptaT$	In-frame deletion of <i>ptaT</i>	<i>catP</i>	this study
pBCG02	<i>E. coli</i> / <i>Fusobacterium</i> shuttle vector	<i>cm^R/thia^R</i>	ref ²
pBCG02-CTI2-1787updn	Derivative of pBCG02 lacking <i>repA</i> and <i>ori_{Fn}</i> , deletion plasmid of <i>ptaT</i>		this study

Table S3 Primers used for strain construction.

Primer Name	Sequence (5' to 3')	Description
B267	cgaaaacctgtactccagggatccAAAAATGATAATT TACTCGCTT	Fwd primer to clone <i>ptaT</i> from <i>Fn</i> genome.
B268	tggtggtggtgctcgagtgcggccgcTTATCATTTGTC ATCGTCGTCTTTGTAGTCATTAGTTTTTAT ATCATATTTTAATAAT	Rev primer to clone <i>ptaT</i> from <i>Fn</i> genome.
B406	tttaaaagcggccggtacGTTATTAATAATTTTA AAATTATTCAAAGTCTTGGAAG	Fwd primer to clone 750bp upstream gene of <i>ptaT</i>
B407	tatctccttggtaTTATCTCCCTTCTAATTCAC C	Rev primer to clone 750bp upstream gene of <i>ptaT</i>
B408	ATAAaaccaagaggagataaaATGAAAAAGTTG ACTATTACAATG	Fwd primer to clone 750bp downstream gene of <i>ptaT</i>
B409	agggacacttttcactcgaAGTTACATATTTTTCAT TAAATTGACAATAGTCAC	Rev primer to clone 750bp downstream gene of <i>ptaT</i>
B422	aaagaaaaactgccgggtacTGTTAATCCATTTGCT ACTGTTATTGC	Fwd primer to clone 1kb upstream gene of <i>galK</i>
B423	tagaaactagcCCAATTAAATTCACCTCTACCTG GTGA	Fwd primer to clone 1kb upstream gene of <i>galK</i>
B424	aatttaattggGCTAGTTTCTATATAGCAAATAT TGGAG	Fwd primer to clone 1kb downstream gene of <i>galK</i>
B425	cggggatcgatcccgggtacCTCTCCAAGCTTTTAA AGTTTTATCTGC	Fwd primer to clone 1kb downstream gene of <i>galK</i>
B459	tttaaaagcggccggtacCAACTTGGAATATTC AAGAACAAT	Fwd primer to verify <i>ptaT</i> deletion
B462	agggacacttttcactcgaTATTCGTTCCATAAAAT TTTCCTTAATAATT	Rev primer to verify <i>ptaT</i> deletion

CTI2-1787upF	GAGTGTGATATGTATGGAAGCTACGAAA GATGAAAGATAAAAACG	pBCG02-CTI2-1787updn
CTI2-1787upR	CCATAGCAAAATTTTCTTTTATCTCAATT TCAGATTTTAAAG	pBCG02-CTI2-1787updn
CTI2-1787dnF	CTTTAAAATCTGAAATTGAGATAAAAGA AAATTTTGCTATGG	pBCG02-CTI2-1787updn
CTI2-1787dnR	CGGAAATTTCACTAGTTCTAGGACGCTTG ATTTTCTTATATAG	pBCG02-CTI2-1787updn
Det-1787-F	GAAAGATGAAAGATAAAAACG	Confirms <i>ptaT</i> deletion
Det-1787-R	GCTTGATTTTTCTTATATAG	Confirms <i>ptaT</i> deletion
pCWU6-F	GTCCTAGAACTAGTGAAATTTCCG	Removes repA and oriFn from pBCG02
pCWU6-R	GTAGCTTCCATACATATCACACTC	Removes repA and oriFn from pBCG02
Beginning of sequencing primers for plasmids confirmation		
S99	AACCCCTCAAGACCCGTTTA	T7 terminator Rev; sequencing pHS200
S101	TAATACGACTCACTATAGGG	T7 promoter Fwd; sequencing pHS200
S124	CCCAGTCACGACGTTGTAAAACG	M13
S133	CAGAAGATAATGTAAAAAGAGTTC	<i>ptaT</i> internal primer for <i>Fn</i> 23726
S136	CCACTTCGACTGCACTCCCGAC	pHS31 digestion site Fwd
S137	GTCCCTAGCGCCTACGGGGAAT	pHS31 digestion site Rev
S139	TTAGGACGGCAATCAATCAA	<i>CatP</i> Fwd on pHS31
S140	AAACGGCAAATGTGAAATCC	<i>CatP</i> Rev on pHS31
S150	AGGTGCTAATATTCCAGTTGCTC	Sequencing KO <i>ptat</i> strain

*The lower letters are the overlap region. Fwd, forward; Rev, reverse. Nucleotides with upper case indicate sequences complementary to the genome; nucleotides with lower case indicate sequences matched to digested plasmid pHS31 for genetic knockout. Underlined are primer sequences specific to the ends of the corresponding assembly.

References:

- 1 Abed, J. *et al.* Fap2 mediates *Fusobacterium nucleatum* colorectal adenocarcinoma enrichment by binding to tumor-expressed Gal-GalNAc. *Cell Host & Microbe* **20**, 215-225 (2016).
- 2 Peluso, E. A., Scheible, M., Ton-That, H. & Wu, C. Genetic manipulation and virulence assessment of *Fusobacterium nucleatum*. *Current Protocols in Microbiology* **57**, e104 (2020).

Table S1

CLUSTAL W (1.81) multiple sequence alignment

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*****:*****.****:*.** :***:*****:*****:*****

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23726
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23726
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10953
SQDEKIIIGLIGVSDPPRENIKKAINRLRNYGVDDIVLLTGDLRQQAETIASRMSIDRYES
CTI-6
AQDEKIIIGLIGVSDPPRENIKKAINRLRNYGVDDIVLLTGDLRQQAETIASRMSIDRYES
CTI-7

CTI-3

AQNEKIIGLIGVSDPPRENIKKAINRLRNYGVDDIVLLTGDLRQQAETIASRMSIDRYES
CTI-1
AQNEKIIGLIGVSDPPRENIKKAINRLRNYGVDDIVLLTGDLRQQAETIASRMSIDRYES
CTI-5
AQNEKIIGLIGVSDPPRENIKKAINRLRNYGVDDIVLLTGDLRQQAETIASRMSIDRYES

23726
ELLPEDKAKNILKFQSKGSNVIMIGDGVNDAPALSYANVGVALGSTRTDVAMEAADITIT
CTI-2
ELLPEDKAKNILKFQSKGSNVIMIGDGVNDAPALSYANVGVALGSTRTDVAMEAADITIT
25586
ELLPEDKAKNILKFQSKGSNVIMIGDGVNDAPALSYANVGVALGSTRTDVAMEAADITIT
10953
ELLPEDKAKNILKFQSKGSNVIMIGDGVNDAPALSYANVGVALGSTRTDVAMEAADITIT
CTI-6
ELLPEDKAKNILKFQSKGSNVIMIGDGVNDAPALSYANVGVALGSTRTDVAMEAADITIT
CTI-7

CTI-3
ELLPEDKAKNILKFQSKGSNVIMIGDGVNDAPALSYANVGVALGSTRTDVAMEAADITIK
CTI-1
ELLPEDKAKNILKFQSKGSNVIMIGDGVNDAPALSYANVGVALGSTRTDVAMEAADITIT
CTI-5
ELLPEDKAKNILKFQSKGSNVIMIGDGVNDAPALSYANVGVALGSTRTDVAMEAADITIT

23726
QDNPLLPGVIGLSKNTVKTIKENFAMVIGLNTFALVLGATGILAPIYASVLHNSTTILV
CTI-2
QDNPLLPGVIGLSKNTVKTIKENFAMVIGLNTFALVLGATGILAPIYASVLHNSTTILV
25586
QDNPLLPGVIGLSKNTVKTIKENFAMVIGLNTFALVLGATGILAPIYASVLHNSTTILV
10953
QDNPLLPGVIGLSKNTVKTIKENFAMVIGLNTFALVLGATGILAPIYASVLHNSTTILV
CTI-6
QDNPLLPGVIGLSKSTVKTIKENFAMVIGLNTFALVLGATGILAPIYASVLHNSTTILV
CTI-7

CTI-3
QDNPLLPGVIGLSKNTVKTIKENFAMVIGLNTFALVLGATGILAPIYASVLHNSTTILV
CTI-1
QDNPLLPGVIGLSKNTVKTIKENFAMVIGLNTFALVLGATGILAPIYASVLHNSTTILV
CTI-5
QDNPLLPGVIGLSKNTVKTIKENFAMVIGLNTFALVLGATGILAPIYASVLHNSTTILV

23726 VMNSLKLLKYDIKTN
CTI-2 VMNSLKLLKYDIKTN
25586 VLNSLKLLKYDIKTN

10953	VLNSLKLLKYDIKTN
CTI-6	VMNSLKLLKYDIKTN
CTI-7	-----
CTI-3	VMNSLKLLKYDIKTN
CTI-1	VMNSLKLLKYDIKTN
CTI-5	VMNSLKLLKYDIKTN