Supplementary Information for:

The ABC-F protein EttA gates ribosome entry into the translation elongation cycle

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Keywords: Protein synthesis, translational regulation, ABC-F protein family, YjjK, ATP/ADP ratio, stationary phase fitness, X-ray crystallography.

SP/POA9W3/E	ttA ECOLI/1-555
TR Q8U559 E	ttA AGRT5/1-549
SP/053204/E	ttA MYCTU/1-558
SP/006476/Y	FMR BACSU/1-629
SP P43672 U	UP_ECOLI/1-635
SP/Q9LV93/A	B5F_ARATH/92-692
<i>SP Q9FIB4 A</i>	B2F_ARATH/78-678
SP POA9U3 Y	BIT_ECOLI/1-530
SP/Q9M1H3/A	B4F_ARATH/157-723
SP/Q8NE71/A	BCF1_HUMAN/298-845
SP/Q9FJH6/A	B1F_ARATH/63-595
SP/P40024/A	RB1_YEAST/76-610
SP/Q8H0V6/A	B3F_ARATH/169-715
SP P43535 G	CN20_YEAST/193-752
SP Q9UG63 A	BCF2_HUMAN/80-623
SP Q9NUQ8 A	BCF3_HUMAN/172-709
SP P63389 Y	HES_ECOLI/1-637
SP/P16521/E	F3A_YEAST/425-1044
SP/Q03195/R	LI1 YEAST/72-608

SP/P0A9W3/EttA ECOLI/1-555
TR/Q8U559/EttA AGRT5/1-549
SP/053204/EttA MYCTU/1-558
SP/006476/YFMR BACSU/1-629
SP/P43672/UUP ECOLI/1-635
SP/09LV93/AB5F ARATH/92-692
SP/09FIB4/AB2F ARATH/78-678
SP/P0A9U3/YBIT ECOLI/1-530
SP/09M1H3/AB4F ARATH/157-723
SP/08NE71/ABCF1 HUMAN/298-845
SP/09FJH6/AB1F ARATH/63-595
SP/P40024/ARB1 YEAST/76-610
SP 08H0V6 AB3F ARATH/169-715
SP/P43535/GCN20 YEAST/193-752
SP 09UG63 ABCF2 HUMAN/80-623
SP 09NU08 ABCF3 HUMAN / 172-709
SP/P63389/YHES ECOLI/1-637
SP/P16521/EF3A YEAST/425-1044

SP/POA9W3	EttA_ECOLI/1-555	259 ASQEA
TR Q8U559	EttA AGRT5/1-549	253 AREDA
SP/053204	EttA MYCTU/1-558	256 GRKDA
SP/006476	YFMR BACSU/1-629	255 EQKET
SP/P43672	UUP ECOLI/1-635	253 ELQNA
SP/Q9LV93	AB5F ARATH/92-692	356 NAAWE
SP/Q9FIB4	AB2F ARATH/78-678	342 YAAWE
SP/POA9U3	YBIT ECOLI/1-530	252 LADNA
SP/Q9M1H3	AB4F_ARATH/157-723	416 NKKFD
SP/Q8NE71	ABCF1 HUMAN/298-845	548 LKQYE
SP/Q9FJH6	AB1F_ARATH/63-595	309 MKQYR
SP/P40024	ARB1 YEAST/76-610	323 MKQYN
SP/Q8H0V6	AB3F ARATH/169-715	436 QKAFE
SP P43535	GCN20 YEAST/193-752	465 QREYDI
SP/Q9UG63	ABCF2 HUMAN/80-623	325 MKRFH
SP Q9NUQ8	ABCF3 HUMAN/172-709	424 QREVE
SP P63389	YHES ECOLI/1-637	246 QAMYE
SP/P16521	EF3A YEAST/425-1044	636
SP/Q03195	RLI1_YEAST/72-608	314 INIFL

	ABCB1-6	21	ABCR1-R2	COBEL	-R1	COBE1-	a1 AB	CR1-R3	COBE1-82	r (Q 100p)-	ABCa1-a1
	ABOPT		Abopt pz	OONET	pi	-		optpo	ooner pz	_	1 Dour ur
	10		20	30	40	50		60	70	80	90
1 -1	AQFVYTMHRV	GKVVPPK	H-ILKNISLS	FFPGAKIGVL	JINGA	GKSTLLRI	AGIDKDI	EGEARPQ-	PDIKIGYLPQ	POLNPE-H	TVRESIE 91
1 M7	ARQFIYHMAGL	NKSYG-A	KK-VLENVHLS	FYPDAKIGIL	<mark>GPNGA</mark>	GKSTVLKI	MAGLDKEY	TGEAWLA-	-EGATLGYLEQI	EPKLDEN-H	TVMENVM 91
1-1	AEFI TMKKV	RKAHG-D	KV-ILDDVTLS	FYPGAKIGVV	JPNGA	GKSSVLRI	MAGLDKPN	NGDAFLA-	TGATVGILQQI	EPPLNED-H	TVRGNVE 90
1	MSILKAENL	YK TYG -D	T-LFDHISFH	IEENERIGLI	SPNGT	GKSTLLKV	LAGLESIE	EGEITKS-	GSVQVEFLHQ	DPELPAG-Q	TVLEHIY 88
1	-MSLISMHGA	WLSFS-D	AP-LLDNAELH	IEDNERVCLV	GRNGA	GKSTLMKI	NREQGLD	DGRIIYE-	-QDLIVARLQQI	DPPRNVE-C	SVYDFVA 88
92 SC	JISSGVKLENI	RKSYK-G	VT-VLKDVTWE	VKRGEKVGLV	SVNGA	GKTTQLRI	TGQEEPD	SGNVIKAP	(PNMKVAFLSQ	FEVSMS-P	TVREEFM 183
1	MUCCNU	TMORE-S	P-I PENT CUK	FCCCNPYCLI	CANCE	CKOTEMET	COLEPT	I CHUCTD-	PNERTEKIPO	OFAFFF-I	TVLEEPM 109
157 DZ	NVKDTTTESE	SVSAR-G	E-LLKNASVR	TSHCKRYCLT	PNGM	CKSTLLKL	AWRKT	PVP-	KNTDVLLVEO	EVVGDEF	SALNAVY 240
298 LE	ENASDIKLEKE	SISAH-G	KE-LEVNADLY	IVAGRAYGLV	GPNGK	GKTTLLKH	ANRAL	SIP-	PNIDVLLCEO	EVVADE	PAVOAVL 381
63 H	OSRDIRIESL	SVTFH-G	YD-LIVDSMLE	LNYGRRYGLL	GLNGC	GKSTLLTA	GRREI	PIPD	OMDI YHLSHE	IEATDN	SSLEAVV 146
76 LE	TSRDIKLSSV	SLLFH-G	V-LIQDSGLE	LNYGRRYGLL	SENGC	GKSTFLKA	ATREY	<mark>PIP</mark> -	EHIDIYLLDE	PAEPSEI	SALDYVV 159
169 G	SAIRD IHMONE	NVSVG-G	RDL-IVDGSIT	LSFGRHYGLV	GRNGT	GKTTFLRY	AMHAI	E <mark>GIP</mark> -	-TNCQILHVEQ	EVVGDK1	TALOCVL 253
193 A	KSKDIHIDTE	DLYVG-D	GQRILSNAQLT	LSFGH <mark>RY</mark> GLV	GONGI	GKSTLLRA	LSRREL	NVP-	-KHVSILHVEQ	ELRGDD1	rkal <mark>o</mark> svl 277
80 H	PNSTDVHIINL	SLTFH-G	QE-LLSDTKLE	LNSGRRYGLI	JLNGI	GKSMLLSA	GKREV	<mark>PIP</mark> E	HIDI YHLTRE	MPPSDB	TPLHCVM 163
172 KM	NKSYDVRIENF	DVSFG-D	R-VLLAGADVN	LAWGRRYGLV	GRNGL	GKTTLLKM	LATRSL	RVP-	AHISLLHVEQ	EVAGDD1	PALOSVL 255
1	MIVFSSL	QIRRG-V	R-VLLDNATAT	INPGQKVGLV	GKNGC	GKSTLLAL	KNEISAD	GGSYTFP-	GSWQLAWVNQI	ETPALP(AALEYVI 85
425 EL	DEGEDICNCEF	SLAYG-A	LI-LINKTQLR	LKRARRYGIC	SPNGC	GKSTLMRA	LANGOVDG	FPTQ-	-EECRTVYVE	HDIDGTHSI	TSVLDEV 512
/211	INLETNLEAH	THRIS-A	NS-EKLARLET	PRPGQVLGLV	STNGT	GRSTAL	AGRORPH	L <mark>9</mark> +321	INALINPU	I VDNI PRA	INGPVQKV 187
	Armo	11			Ar	rma2			ABCa1-a2	S	Sig. Motif-1
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	100		110			120	120	140	150		
00.00		T DRUN	TTO DE		-	120			150		167
92 54	UACKTA TUDD	VNETM	ALIA-DP	D	-ADFD	FTADECAK	COMTREC	NT WDT PNC	TERAADALRL	PDWDAK	TTCL SCCE 161
92 E	MODINIKI	FNEUA	ET MA - TD			FIMEEMCD	OFFLDHA	DAWDLDAC	TEOAMDALRC		TIGLIGGE 161
89 50	ESAVMETTRE	YEKAL-Y	ELGK-DP		ENE	OROKHLLA	OAKMDAN	NAWDANTI	AKTVLSKLGV	NDVTKP	NELSGGO 163
89 E	IEEOAEYLKR	YHDIS-R	LVMN-DP		SE	KNLNELAK	VOEOLDHH	NLWOLEN	INEVLAOLGL	PNVAI	SSLSGGW 161
184 TZ	AFKEEMEITEK	LEKVQ-K	A	IEGSVD-D	LDLMG	RLLDEFDL	LORRAQAV	NLDSVDA	ISKLMPELGF	APEDADRL	ASFSGGW 264
170 CT	FKEEMEIARK	LENLQ-K	A	IEEAVD-D	LELMG	KLLDEFDL	LQRRAQEV	DLDSIHA	ISKLMSELGF	VSEDADRL	ASFSSGW 250
87 M	HKELWEVKQE	RDRIY-A	LPEMSEE	DGYKVA-D	LEV		<mark>Kyg</mark> em	DGYSAEA	RAGELLLGVGI	PVEQHYGP	ISEVAPGW 160
241 SZ	AN-EELVKLRE	EAEALQK	SSS <mark>GADG</mark>	ENVD	SEDDD	DT <mark>G</mark> EKLAE	LYDRLQIL	GSDAAEAC	ASKILAGLGF	TKDMQVRA	QSFSGGW 324
382 R	AD-TKRLKLLE	EERRLQG	QL]	EQGDD	TAAERLEK	YEELRAT	GAAAAEA	ARRILAGLEF	DPEMQNRP	QKFSGGW 456
147 SC	D-EERLRLEK	EVEIL			VQQDD	GGGERLQS	IYERLDAM	DAETAEK	RAAEILFGLGFI	DKEMQAKK	KDFSGGW 217
254 10	SAQHELKRIED	LVERT		AND CHEMPER -D	TLEDG	PESELLEP	INCOMPANY	DPDTFESE	AATILIGLEF	NKKTILKK	TRDMSGGW 231
279 DZ	DUERTKLLEE	EIGITAN	OKETEEPT	REDGIEVENT	IVEGD	DIDNHITO		PEDKARAF	AASILAGLSE	TPENQLA	NTESGGW 344
164 EV	D-TERAMLEK	EAERL			AH-ED	AECEKIME	VERLEEL	DADKAEM	ASETTHELE	TPAMORKK	KDESCOW 233
256 ES	SDSVREDLLRR	ERELTAO	IAAGR		AEG	SEAAELAE	IYAKLEEI	EADKAPAR	ASVILAGLEF	TPKMOOOP	REFSGGW 332
86 D	DREYROLEAO	L		н	DANER	NDGHAIAT	IHGKLDAI	DAWSIRS	AASLLHGLGF	SNEOLERP	SDFSGGW 154
513 F	SG							VGTKE	IKDKLIEFGF	TDEMIAMP	SALSGGW 548
188 <mark>G</mark> E	ELLKLRMEKSP								DVKRYIKILQI	LENVLKRD	EKLSGGE 226
								CORER1	-5		
	ABCa1-a3	CORE	1-β3	CORE1-a2	CC	DRE1-β3	CORE1-0	14	CORE1-B	6 PtIN	1-α1
		-						-			
	170	180	190	200		210	220	230	240	2	50
										-	

								-1	
	170	180	190	200	210	220	230	240	250
SP/P0A9W3/EttA ECOLI/1-555	168 RREVALCELL	LEKPOMLI	LDEPTNHLDA	SVAWLERF	-LHDFEGTVVAITHDE	YFLONVA	GWILELDRGE	I PWEG-NY	SSWLEOKDORLACE 258
TR 08U559 Etta AGRT5/1-549	162 RREVALCELL	LSOPDLLI	LDEPTNHLDA	TIAWLEKH	-LRDYPGAVMMITHDE	YFLONVT	GWILELDRGRO	IPYEG-NY	SATLOAKAKRMOOE 252
SP/053204/EttA MYCTU/1-558	165 RREVALCELL	LSKPDLLI	LDEPTNHLDA	ESVOWLEOH	-LASYPGAILAVTHDE	YFLONVA	EWILELDRGRA	YPYEG-NY	STYLEKKAERLAVO 255
SP/006476/YFMR BACSU/1-629	164 KKRVAIAKNL	IOPADLLI	LDEPTNHLDN	TIEWLEGY	-LSOYPGAVMLVTHDF	YFLNRVT	NRIYELERGSI	YTYKG-NY	EVFLEKRAEREAOA 254
SP/P43672/UUP ECOLI/1-635	162 LRKAALGRAL	VSNPRVLI	LDEPTNHLDI	ETIDWLEGF	-LKTFNGTIIFISHDF	SFIRNMA	TRIVDLDRGKI	VTYPG-NY	DOYLLEKEEALRVE 252
SP/Q9LV93/AB5F ARATH/92-692	265 OMRMSLGKIL	LQDPDLLI	LDEPTNHLDL	DTIEWLEGY	-LQKQDVPMVIISHDF	AFLDQLC	TKIVETEMGVS	RTFEG-NY	SQYVISKAEWIETQ 355
SP/Q9FIB4/AB2F ARATH/78-678	251 OMRMSLGKIL	LQNPDLLI	LDEPTNHLDL	DTIEWLEGY	-LIKQDVPMVIISHDF	AFLDQLC	TKIVE TEMGVS	RTFDG-NY	SQYVISKAELVEAQ 341
SP P0A9U3 YBIT_ECOLI/1-530	161 KLRVLLAQAL	FAD <mark>PD</mark> ILI	LDEPTNNLDI	DTIRWLEQV	-LNERDSTMIIISHDF	HFLNMVC	THMADLDYGEI	RVYPG-NY	DEYMTAATQARERL 251
SP/Q9M1H3/AB4F ARATH/157-723	325 RMRISLARAL	FVQPTLLI	LDEPTNHLDL	RAVLWLEEY	-LCRWKKTLVVVSHDF	DFLNTVC	TEIIHLHDQNI	HFYRG-NF	DGFESGYEORRKEM 415
SP/Q8NE71/ABCF1_HUMAN/298-845	457 RMRVSLARAL	FMEPTLLN	ILDEPTNHLDL	NAVIWLNNY	-LQGWRKTLLIVSHDC	GFLDDVC	TDIIHLDAQRI	HYYR <mark>G-NY</mark>	MTFKKMYQQKQKEL 547
SP/Q9FJH6/AB1F_ARATH/63-595	218 RMRIALARAL	FIMPTILI	LDEPTNHLDL	EACVWLEES	-LKNFDRILVVVSHSC	DFLNGVC	TNIIHMQSKQI	KYYT <mark>G-NF</mark>	DQYCQTRSELEENQ 308
SP P40024 ARB1_YEAST/76-610	232 KMRVALAKAL	FVKPTLLI	LDDPTAHLDL	EACVWLEE Y	-LKRFDRTLVLVSHSC	DFLNGVC	TNMIDMRAQKI	TAYGG-NY	DS <mark>YH</mark> KT <mark>R</mark> SELETNO 322
SP Q8H0V6 AB3F_ARATH/169-715	345 RMRIALARAL	FIEPDLLI	LDEPTNHLDL	HAVLWLETY	-LTKWPKTFIVVSHAF	EFLNTVV	TDIIHLQNQKI	STYKG-NY	DIFERTREEQVKNQ 435
SP P43535 GCN20_YEAST/193-752	373 RMRLSLARAL	FCQPDLLI	LDEPSNMLDV	PSIAYLAEY	-LKTYPNTVLTVSHDF	AFLNEVA	TDIIYQHNERI	DYYRGQDF	DT <mark>FY</mark> TT <mark>K</mark> EERRKNA 464
SP Q9UG63 ABCF2_HUMAN/80-623	234 RMRVALARAL	FIRPFMLI	LDEPTNHLDL	DACVWLEEE	-LKTFKRILVLVSHSC	DFLNGVC	TNIIHMHNKKI	KYYT <mark>G-NY</mark>	DQ <mark>YVKTRLELEENQ</mark> 324
SP Q9NUQ8 ABCF3_HUMAN/172-709	333 RMRLALARAL	FARPDLLI	LDEPTNMLDV	RAILWLENY	-LQTWPSTILVVSHDF	NFLNAIA	TDIIHLHSQRI	D <mark>GY</mark> R <mark>G</mark> -DF	et <mark>fi</mark> ks <mark>k</mark> qerllnq 423
<i>SPP63389YHES_ECOLI1–637</i>	155 RMRLNLAQAL	ICRSDLLI	LDEPTNHLDL	DAVIWLEKW	-LKSYQGTLILISHDF	DFLDPIV	DKIIHIEQQSN	FE <mark>Y</mark> T <mark>G-NY</mark>	ssfevo <mark>r</mark> atrlao <mark>o</mark> 245
SP P16521 EF3A_YEAST/425-1044	549 KMKLALARAV	LRNADILI	LDEPTNHLDT	VNVAWLVNY	-LNTCGITSITISHDS	VFLDNVC	EYIINYEGLKI	RKYKG-NF	TEFVKKCPAA 635
SP/Q03195/RLI1_YEAST/72-608	227 LQRFAIGMSC	VQEADVYN	IFDEPS SYLDV	KQR <mark>LNA</mark> AQ <mark>I</mark> IF	SLLAPTKYVICVEHDI	SVLDYLS	DFVCIIYGV-F	SVYGV	VTL <mark>P</mark> ASVRE <mark>G</mark> 313

	Ptilv	/Ι-α1			Ptilv	1-a2a	Ptilvi-a20)		ABC	B5-B1	
										-		
	270	260	280		290	30	0	310	3.	20	330	
259 A	SOEAARRK	STEKELEWVR	0-GT	KGROSK-G	KARLARF	EELNSTE	YOKRN	ETNELFIPP-	GPRLG	KVLEV	SNLRKS 3	332
253 A	REDASROK	AISREOEWIA	S-SP	KAROTK-S	KARIRAY	DELVEAA	ENRRP	GDAOIVIPV-		VVIEA	ENLTKS 3	326
256 G	RKDAKLOK	RLTEELAWVR	S-GA	KAROAK-S	KARLORY	EEMAAEA	EKTRKL	DFEEIOIPV-	GPRLG	VVVEV	DHLDKG 3	330
255 E	OKETKRON	LLRRELAWLR	R-GA	KARSTK-Q	KARIDRV	ETLKEQT	GPQSS	G-SLDFAIG-	SHRLG		ENVMIA 3	327
253 E	LONAEFDR	KLAQEEVWIR	Q- <mark>GI</mark>	KARRTR-N	EGRVRAL	KAMRRER	GERR-EVM	GTAKMQVEE-	ASRSG	IVFEM	EDVCYQ 3	328
356 N	AAWEKQQK	DIDSTKDLIA	RLGA	GANSGRAS	TA-EKKL	EKLQEQE	LIEKPFOR	KOMKIRFPE-	RGTSG	SVVNV	KNIDFG 4	133
342 Y	aa <mark>w</mark> ekqqk	EIEATKDLIS	RLSA	GANSGRAS	SA-EKKL	EKLQEEE	LIEK <mark>P</mark> FQR	KQMKIRFPE-	CGLSG	RSVVTV	KNLVFG 4	119
252 L	ADNAKKKA	Q <mark>I</mark> AELQS <mark>FV</mark> S	RFSA	NASKSRQA	TSRARQI	DKIKLEE	VKASSR	QNPFIRFEQ-	DKKLF	RNALEV	e <mark>glt</mark> k <mark>g</mark> 3	328
416 N	KKFDVYDK	Q <mark>M</mark> KAAKR <mark>TG</mark> N	RGQQEKVKDR	AKFTAAKEASKS	KS <mark>KGK</mark> TV	DEEGPAP	EAPRKWRD	YS <mark>VVFHFP</mark> E-	PTELT	PPLLQL	IEVSFS 5	504
548 L	KQ <mark>Y</mark> EKQ <mark>E</mark> K	KLKELKAGG K	STKQAEKQTK	EALTRKQQKCR-	RKNQD	EE SQEAP	ELLKR <mark>P</mark> KE	YTVRFTFPD-	PPPLS	PPVLGL	H <mark>GVTFG</mark> 6	533
309 M	KQYRWEQE	QISHMKEYIA	RF <mark>GHG</mark>	SAKLARQA	QS <mark>KE</mark> KTL	AKMERGG	LTEKVARD	SVLVFRFAD-	VGKLP	PPVLQF	vevsfg 3	388
323 M	KQYNKQQE	EIQHIKKFIA	SAGT	YANLVKQA	KS <mark>RQ</mark> KIL	DKMEADG	LVQPVVPD	KVFSFRFPQ-	VERLP	PPVLAF	DDISFH 4	101
436 QI	KAFESSER	SRSHMQAFID	KFRY	NAKRASLV	QS <mark>RIKAL</mark>	DRLA	HVDQVIND	PDYKFEFPTP	DDKPG	PPIISF	SDASFG 5	512
465 Q	REYDNQMV	YRKHLQEFID	KYRY	NAAKSQEA	QS <mark>RIKKL</mark>	EKLP	VLEPPEQD	KTIDFKFPE-	CDKLS	PPIIQL	QD <mark>VSFG</mark> 5	540
325 M	KRFHWEQD	QIAHMKNYIA	RFGHG	SAKLARQA	QS <mark>KE</mark> KTL	QKMMASG	LTERVVSD	KTLSFYFPP-	CGKIP	PPVIMV	QNVSFK 4	104
424 Q	REYEAQQQ	YRQHIQVFID	RFRY	NANRASQV	QS <mark>KLKML</mark>	EKLP	ELKPVDKE	SEVVMKFPDG	FEKFS	PPILQL	DEVDFY 5	500
246 Q	AMYESQQE	RVAHLQSYID	RFRA	KATKAKQA	QSRIKML	ERME	LIAPAHVD	NPFRFSFRA-	PESLP	PLLKM	ekvsag 3	321
636 -					KAY	EE	LSN	TDEFKFPEP	GYLEGVKTKQ	AIVKV	INMEFQ 6	\$75
314 TI	NTRLD-	CHT DAENT	FR	FALOFRIA		EDLO			NIDG7	RAFSY	DOLKKU 3	162

LD <mark>G</mark> HIPAENI	L <mark>R</mark> FRT	EALQFRIA-
	WalkerA(F	-loop)-2
10000 00	0000004	000000

MLERMF	LIA <mark>P</mark> AHVDN <mark>P</mark> FRF	'SFRA KFPEPGYLEG	PESLPNPL VKTKQKAI	LKMEKVSAG VKVTNMEFQ	3
ATEDLQ			NDSASRA	FSYPSLKKT	3
	γPi switch (Q lo	op)-2			
		A .		TOF 04	

				vvalkerA	(P-100p)-2		YPI SWITCH (Q 100)))-2
			ABCβ2-β2	CORE2-B1	CORE2-a1	ABCβ2-β3	CORE2-B2	ABCa2-a1 TOE-B1
			340	350	360 370	380	390	400 410
SP	POA9W3	EttA_ECOLI/1-555	333 YGDRLLIDDLSFS	IPKGAIVGIIGPN	GAGKSTLFRMISGO	EQPDSGTITLG	TVKLA-SVDQFRD-	SMDNSKTVWEEVSGGLDIMKI-GN 419
TR	Q8U559	EttA AGRT5/1-549	327 YGDRVLIENLTFK	LPPGGIVGVIGPN	GAGKTTLFRMITGO	EQPDSGSVTVG	TVHLG-YVDQSRD-	FLAGDKTVWEEISGGNDIIKL-GK 413
SP	053204	EttA MYCTU/1-558	331 YDGRALIKDLSFS	LPRNGIVGVIGPN	GVGKTTLFKTIVGI	ETPDSGSVKVG	TVKLS-YVDQARA-	JIDPRKTVWEVVSDGLDYIQV-GQ 417
SP	006476	YFMR BACSU/1-629	328 YDGRMLVDRFNEL	VIPGERIGIIGPN	GIGKTTLLNALAG F	HTPDGGDITIG(TVRIG-YYTODHS-	EMNGELKVIDYIKETAEVVKTADG 415
SP	P43672	UUP ECOLI/1-635	329 VNGKQLVKDFSAQ	VLRGDKIALIGPN	GCGKTTLLKLMLGC	LOADSGRIHVG	KLEVA-YFDOHRA-	ELDPDKTVMDNLAEGKQEVMV-NG 415
SP	Q9LV93	AB5F ARATH/92-692	434 FEDKMLFKKANLS	IERGEKIAILGPN	GCGKSTLLKLIMGI	EKPVKGEVILGE	HNVLPNYFEQNQAE	LDLDKTVLETVCEAAEDWRS 520
SP	Q9FIB4	AB2F ARATH/78-678	420 FDDKMLFNKANLA	IERGEKVAIIGPN	GCGKSTLLKLIMGI	EKPMRGEVILG	HNVLPNYFEQNQAE	AQDLDKTVIETVVEAAVDWRI 506
SP	POA9U3	YBIT ECOLI/1-530	329 FDNGPLFKNLNLL	LEVGEKLAVLGTN	GVGKSTLLKTLVGD	LOPDSGTVKWSH	NARIG-YYAQDHEY	EFENDLTVFEWMSQWKQEGD 413
SP	Q9M1H3	AB4F ARATH/157-723	505 YPNRP-DFRLSNVDVG	IDMGTRVAIVGPN	GAGKSTLLNLLAGE	LVPTEGEMRRS	KLRIG-RYSOHFVD	LLTMGETPVQYLLRLHPDQEG-FS 594
SP	Q8NE71	ABCF1 HUMAN/298-845	634 YOGOK-P-LFKNLDFG	IDMDSRICIVGPN	GVGKSTLLLLLTGK	LTPTHGEMRKNH	IRLKIG-FFNQQYAE	LRMEETPTEYLORGFNL 717
SP	Q9FJH6	ABIF ARATH/63-595	389 YTPDYLIYKNIDFG	VDLDSRVALVGPN	GAGKSTLLKLMTGE	LHPTEGMVRRH	HIKIA-QYHOHLAE	KLDLELPALLYMMREFPGTE 474
SP	P40024	ARB1 YEAST/76-610	402 YESNPSENLYEHLNFG	VDMDSRIALVGPN	GVGKSTLLK IMTGE	LTPOSGRVSRH	HVKLG-VYSOHSOD	DLDLTKSALEFVRDKYSNISQ 490
SP	08H0V6	AB3F ARATH/169-715	513 YPGGPLLFRNLNFG	IDLDSRIAMVGPN	GIGKSTILKLISGE	LOPSSGTVFRS	KVRVA-VFSOHHVD	SLDLSSNPLLYMMRCYPGVPE 599
SP	P43535	GCN20 YEAST/193-752	541 YDENNLLLKDVNLD	VOMDSRIALVGAN	GCGKTTLLKIMMEQ	LRPLKGFVSRN	RLRIG-YFTOHHVD	SMDLTTSAVDWMSKSFPGKTD 627
SP	090G63	ABCF2 HUMAN/80-623	405 TKD-GPCIYNNLEFG	IDLDTRVALVGPN	GAGKSTLLKLLTGE	LLPTOGMIRKHS	HVKIG-RYHOHLOE	DLDLDLSPLEYMMKCYPEIKE 492
SP	09NU08	ABCF3 HUMAN/172-709	501 YDPKHVIFSRLSVS	ADLESRICVVGEN	GAGKSTMLKLLLGD	LAPVRGIRHAH	NLKIG-YFSOHHVE	DLDLNVSAVELLARKFPGRPE 587
SP	P63389	YHES ECOLI/1-637	322 YGDRIILDSIKLN	LVPGSRIGLLGRN	GAGKSTLIKLLAGE	LAPVSGEIGLA	GIKLG-YFAQHQLE	YLRADESPIOHLARLAPQELE 407
SP	P16521	EF3A YEAST/425-1044	676 YPGTS-KPOITDINFO	CSLSSRIAVIGPN	GAGKSTLINVLTGE	LLPTSGEVYTHE	NCRIA-YIKOHAFA	HIESHLOKTPSEYIQWRFQTGED+10 768
SP	Q03195	RLI1_YEAST/72-608	363 QGDFVLNVEEGE	SDSEILVMMGEN	GTGKTTLIKLLAGA	LKPDEGQDIPKI	NVSMKPOKIAP	KFPGTVRQLFFKKIRGQFLNP 445

			Sig. Motif-2	WalkerB-	2	
	TOE-B2 ABCa2-c	12 340 340	ABCa2-a3	CORE2-B3	CORE2-a2	CORE2-B4 CORE2-a3
		010 010				
	420 43		450	460 470	480 4	500
an/nanalatta anos 1/1 555	420 43		4.50	100 170	100 1	500
SP/PUA9W3/EttA_ECUL1/1-555	420 TEMPSRATVGRE	NEKGV-DQGKRVGE	LSGGERGRLHLAKL	LQVGGNMLLLDEPTNI	DIDIETLKALENALLEF	PGCAMVISHDRWFLDRIA- 508
CD/052204/E++3_MYC771/1-549	414 FEVNSRATCGAF	NEKGG-DQQQKVGN	LSGGORNRVHLARM	LKAGGNVLLLDEPTNI	T DUPET COL FNALLEAF	AGCAVIISHDRMFLDRLA- 502
SP/055204/EttA_MICTU/1-558	418 TEVPSRATVSAF	GERGE-DQQRPAGV	LSGGERNRLNLALT	LKQGGNLILLDEPTNI	DEDVETEGSLENALENF	PGCAVVISHDRWFLDRTC- 506
SP/000470/ITMR_BACS0/1-029	416 DMITAEQMLERF	LEPRS-MQQTIIRK	LSGGERKRLILLQV	ENGEPNVLFLDEPTNI	DIDIETLSVLEDITDQF.	PGVVITVSHDRIFLDRVV- 504
SP P45072 00F_ECOL171=055	521 DDTECLICEC	NEKAD-MIDEKUSI	LOCCERAPIAECKE	MUT DOTI I VI DE DINI	T DT DEKEMT FEATNEY.	
SP/09FTB4/AB2F APATH/78-678	507 DDTRALLCRC	NEKAD-MIDRIVSI	LSCCERAPIAECKE	MUK DOTT I VI DE DTNI	I DT DSKEMLFEATNEY	KGTVITVSHDPYFTKOTV- 593
SP/P0A9U3/VBTT ECOLT/1-530	414 DECAVESTICET	LESOD-DIKKPAKV	LSCCEKCEMI FCKI	MMOK PNTT. TMDE PTNI	TOMESTESTNMALELY	OCTLIEVSHOPEEVSSIA- 502
SP/09M1H3/AB4F ARATH/157-723	595 KOFAVRAKLOKE	GT.PSH-NHT.SPTAK	LSCCOKARVVETST	SMSKPHTLLLDEPTN	ILDMOSTDATADALDEF	TGCVVLVSHDSBLTSBVCA 684
SP/08NE71/ABCE1 HIMAN/298-845	718 PYODARKCIGRE	GLESH-AHTTOTCK	LSCCOKARVVFAEL	ACREPOVITIOEPTN	IDTESTDALGEATNEY	KGAVTVVSHDABLTTE 804
SP/09F.TH6/AB1F ARATH/63-595	475 -EEKMBAATGRE	GT.TGK-AOVMPMKN	LSDGORSBYTFAWL	AYKOPNMLLLDEPTNE	LDTETTOSLAEALNEW	DGGLVLVSHDFBLTNOV 561
SP/P40024 ARB1 YEAST/76-610	491 DEDEWEGOLGEY	GLTGE-GOTVOMAT	LSEGORSEVVFALL	ALEOPNVLLLDEPTN	LDIPTIDSLADAINEF	NGGVVVVSHDFRLLDKT 578
SP/08H0V6 AB3F ARATH/169-715	600 OKLESHLGSL	GVTGN-LALOPMYT	LSGGOKSEVAFAKT	TEKKPHLLLLDEPSN	LDLDAVEALIOGLVLE	OGGICMVSHDEHLISGSV- 686
SP/P43535/GCN20 YEAST/193-752	628 EEYRRHLGSE	GITGT-LGLOKMOL	LSGGOKSEVAFAAL	CLNNPHILVLDEPSN	ILDTTGLDALVEALKNF	NGGVIMVSHDISVIDSVC- 714
SP/09UG63/ABCF2_HUMAN/80-623	493 -KEEMRKIIGRY	GLTGK-OOVSPIRN	LSDGOKCRVCLAWL	AWONPHMLFLDEPTN	IDIETIDALADAINEF	EGGMMLVSHDFRLIOOV 579
SP/09NU08/ABCF3 HUMAN/172-709	588 EEYRHOLGRY	GISGE-LAMPLAS	LSGGOKSRVAFAOM	TMPCPNFYILDEPTN	ILDMETIEALGRALNNF	RGGVILVSHDERFIRLVC- 674
SP/P63389/YHES ECOLI/1-637	408 OKLEDYLGGE	GFQGD-KVTEETRR	FSGGEKARLVLALI	VWORPNLLLLDEPTN	ILDLOMRQALTEALIEF	EGALVVVSHDRHLLRSTT- 494
SP/P16521/EF3A YEAST/425-1044	869 0+KEIEEHCSML	GLDPEIVSHSRIRG	LSGGOKVKLVLAAG	TWORPHLIVLDEPTNY	LDRDSLGALSKALKEF	EGGVIIITHSAEFTKNLT- 958
SP/Q03195/RLI1 YEAST/72-608	446 OFOTDVVKPL	RID-D-IIDQEVQH	LSGGELQRVAIVLA	LGIPADIYLIDEPSA	LDSEQRIICSKVIRRE	ILHNKKTAFIVEHDFIMATYLA- 535
	00050.0	00000000	00050 -4 0			
	CORE2-p	5 CORE2-pe	CORE2-04 B	asic C-terminal Ta	aii	
	510	520	530 5	40 550		
SP/P0A9W3/EttA ECOLI/1-555	509THILDYOD	EGKVEFFEGN	FTEYEEYKKRTLGA	DALEPKRIKYKRIAK		
TR/08U559/EttA_AGRT5/1-549	503THILAFEG	DSHVEWFEGN	FEDYEKDKVRRLGP	ESIKPGRVSYKRLTR		
SP/053204/EttA MYCTU/1-558	507THILAWEG	DDDNEAKWFWFEGN	FGAYEENKVERLGV	DAARPHRVTHRKLTR		
SP/006476/YFMR BACSU/1-629	505DRLIVFEG	NGVISRFOGS	SDYMEESKAKKAA	PKPAAEEKTAEAEPKI	K+77	
SP/P43672/UUP ECOLI/1-635	505TECWIFEG	GGKIGRYVGG	THDARGOOEQYVAL	KOPAVKKTEEAAAAKA	+83	
SP/Q9LV93/AB5F ARATH/92-692	608NRVIEVED	GCLEDYAGD	YNYYLEKNLDARTK	ELEREAELEEKAPKVH	K+38	
SP/Q9FIB4/AB2F ARATH/78-678	594NRVIEVRD	GGLMDYAGD	YNYFLEKNVEARAR	ELEREAELEEKA <mark>P</mark> KVH	K+38	
SP/P0A9U3/YBIT_ECOLI/1-530	503TRILEITP	ERVIDFSGN	YEDYLRSKGIE			
SP Q9M1H3 AB4F ARATH/157-723	685 EEEKSQIWVVED	GTVNFFPGT	FEEYKEDLQREIKA	EVDE		
SP/Q8NE71 ABCF1_HUMAN/298-845	805 TNCQLWVVE	QSVSQIDGD	FE <mark>DY</mark> KREVLEALGE	VMVSRPRE		
SP/Q9FJH6/AB1F_ARATH/63-595	562 AHE IWVCEK	QCITKWNGD	IM <mark>DF</mark> KRHLKAK <mark>AG</mark> L	ED		
SP P40024 ARB1_YEAST/76-610	579 AQDIFVVEN	KTATRWDGS	ILQYKNKLAKNVVL			
SP Q8H0V6 AB3F_ARATH/169-715	687DELWVVSD	GRIAPFHGT	FHDYKKLLQSST	-		
SP P43535 GCN20_YEAST/193-752	715KEIWVSEQ	GTVKRFEGT	IY DY RDYILQSADA	AGVVKKH		
SP Q9UG63 ABCF2_HUMAN/80-623	580 AQE IWVCEK	QTITKWPGD	ILAYKEHLKSKLVD	EE PQLTKRTHNV		
SP Q9NUQ8 ABCF3_HUMAN/172-709	675RELWVCEG	GGVTRVEGG	FDQYRALLQEQFRR	EGFL		
SP P63389 YHES_ECOLI/1-637	495DDLYLVHDI	RKVEPFDGD	LEDYQQWLSDVQKQ	ENQTDEAPKENANSA	2+96	
SP P16521 EF3A_YEAST/425-1044	859EEVWAVKD	GRMTPSGHNWVSGQ	GAGPRIEKKEDEED	KFDAMGNKIA <mark>GG</mark> KKKE	K+32	
SP/Q03195/RLI1_YEAST/72-608	536DKVIVFEG	IPSKNA-HARAPES	LLTGCNRFLKNLNV	TFRRD <mark>P</mark> NSFR <mark>P</mark> RINKI	+22	

Supplementary Figure 1: Structure-based ABC-F sequence alignment. The secondary structural elements observed in the crystal structure of E. coli EttA are shown above the sequence alignment, with α -helices represented by cylinders, β -strands by arrows, and 3_{10} helices by circles. Secondary structure elements are colored as in Fig. 2a in the main text. The disordered protein segments are represented by dotted lines. The alignment shows EttA orthologues from E. coli (ECOLI), A. tumefaciens (AGRT5), M. tuberculosis (MYCTU) and B. subtillis (BACSU), as well as the three additional ABC-F proteins from E. coli (YheS, Uup, and YbiT), the three ABC-F in humans (ABCF1, 2 and 3), the two ABC-F (ARB1 and GCN20) and two other non-ABC-F soluble ABC (EF3A and ABCE1/RNaseLI (RLI1)) from S. cerevisiae (yeast). The alignment was initially generated using CLUSTAL- Ω^{-1} via the Uniprot website (http://www.uniprot.org/) and then manually edited using Jalview² to correctly align the Q loop motifs in all sequences and to accurately represent the structural alignment of EttA to the other ABC domains of known structure. The alignment is colored according to the default CLUSTAL coloring code. Large insertions compared to EttA were truncated, and the number of residues removed at each such site is indicated in red and flanked by "+" signs in the text of the alignment.



Supplementary Figure 2: *Topology diagram of EttA*. Secondary structure elements are colored as in Fig. 2a, with β -strands represented by arrows, α -helices represented by cylinders, and turns of 3₁₀ helix represented by circles. The locations of the Walker A, Walker B, and ABC Signature (LSGGQ/E) motifs involved in ATP binding are indicated by thick dashed lines. The locations of disordered segments in the crystal structure are also indicated.



Supplementary Figure 3: Variations in ABC α subdomain alignment in the crystal structure of the E. coli EttA dimer. (a) Stereopair showing the molecular surface of a model for the ATPbound conformation of ABC1 and ABC2 in EttA, which was produced by applying a rigid-body rotation to one protomer in the crystallographically observed EttA dimer (as described in the legend for Fig. 2b in the main text). The structure is colored according to subdomain in an equivalent manner to Fig. 2 in the main text and Supplemental Fig. 1-2 above. The ATP molecules shown in gray space-filling representation are from the Na-ATP-bound MJ0796 dimer, which was used to model the ATP-bound EttA conformation as described above. This surface representation demonstrates that the ATP-binding site on the right, which is at the interface of the ABCa subdomain in ABC1 and the ABCB and F1-like core subdomains in ABC2, is more open that the ATP-binding site on the left, which is formed by the ABC α subdomain in ABC2 and the ABCβ and F1-like core subdomains in ABC1. This asymmetry is attributable primarily to the different alignments of the ABCa subdomain relative to the F1-like core subdomain in ABC1 vs. ABC2, as shown in panels b-c below in this figure. (b,c) Stereo ribbon diagrams showing ABC1 (panel b) or ABC2 (panel c) from EttA superimposed on a protomer from the Na-ATP-bound dimer of the E171Q mutant of MJ0796 (PDB id 1L2T), based on least-squares alignment of the ABCB and F1-like core subdomains. The EttA subdomains are colored like panel a above, while MJ076 is colored pink. These images show that, in the nucleotide-free crystal structure of EttA, the ABCa and F1-like core subdomains within each ABC domain are rotated relative to one another by 18-20° compared to their alignment in the canonical ATP-binding conformation. Similar reorientations of these subdomains have been

observed in other ABC domain structures that do not have the γ -phosphate group of ATP bound in the active site ^{3,4}. Formation of the catalytically active complex upon ATP binding to EttA presumably involves relative rotation of the ABC α and F1-like core subdomains within both the ABC1 and ABC2 domains as well as a mutual rotation of these domains (as modeled in **Fig. 2c**) to bring them into the canonical ATP-sandwich conformation observed in the ATP-bound structures of other ABC proteins ⁵⁻⁷. **(d)** Observed *apo* structure and modeled ATP-bound conformation of the *E. coli* EttA dimer. Stereo ribbon diagrams of a model for the ATP-bound (solid colors) conformation superimposed on the crystallographically observed nucleotide-free conformation by aligning the ABC β and F1-like core subdomains in ABC1 and ABC2 in different EttA protomers to the protomers in the Na-ATP-bound dimer of the E171Q mutant of MJ0796 (PDB id 1L2T). ATP molecules from the MJ0796 dimer are represented in gray spacefilling representation. The structures are colored as in **Fig. 2** in the main text and oriented such that the interface of one of the two ABC1-ABC2 interfaces in the EttA dimer is visible in each of the two views, which are related by a 90° rotation around a vertical axis.



Supplementary Figure 4: Conformational change in the PtIM in the EttA monomer vs. dimer and the packing interactions of the C-terminus of PtIM. These stereopairs show the molecular surface of ABC2 and a ribbon representation of ABC1 and the PtIM (i.e., the interdomain linker between ABC1 and ABC2), colored like Fig. 2 in the main text. (a) Crystallographically observed apo conformation of one ABC1-ABC2 domain pair in the EttA dimer (Supplementary Fig. 3 and Table 1). This image was generated as described for Fig. 2b in the main text, *i.e.*, by deletion of all the residues prior to the Lys 286 in the protomer A and deletion of all the residues after the Gln 278 in the protomer B. (b) ATP-bound conformation of the EttA monomer modeled from the cryo-EM structure of EttA-EQ₂ bound to 70S ribosomes, as presented in the accompanying paper⁸. Two α -helices from the C-terminal half of the PtIM that pack onto the surface of ABC2 in the dimer structure (panel a) must undergo a conformational change to enable ABC1 and ABC2 in a single protomer to interact with one another in proper geometry in the EttA monomer (panel b). The C-terminal segment of the PtIM (*i.e.*, the segment after that undergoing the conformational change but before the start of ABC2) packs into a crevice at the interface between the ABC β subdomain and the F1-like core subdomain in ABC2. The crystallographically observed conformation of these C-terminal residues in the PtIM could modulate the relative rotation of ABC α and F1-like core subdomains required to adopt the catalytically active nucleotide-sandwich conformation (described in Supplemental Fig. S3b-c). The cryo-EM structure of ribosome-bound EttA⁸ shows that the latter two α -helices in the PtIM refold compared to their crystallographically observed conformation to form a long α -helical hairpin in conjunction with the first α -helix in the PtIM (panel **a** vs. **b**), which remains intact. This conformational change in the PtIM is likely to be coupled to dissociation of the EttA dimer that is observed in its crystal structure.



Supplementary Figure 5: Further characterization of EttA in vitro and in vivo. (a) Gel filtration analysis of purified E. coli EttA shows a slow but reversible monomer-dimer equilibrium. Analytical gel-filtration chromatography on a system equipped with in-line UV, refractive index (RI), and static light-scattering detectors (SLS) detectors was used to analyze two samples of WT EttA at different concentrations. A protein fraction from the leading edge of the peak from a preparative gel-filtration column, representing the final step in the purification of EttA, was either injected directly onto an analytical gel-filtration column (red trace) or diluted 1:1 in the elution buffer and heated for 2 hours at 37 °C prior to injection onto the same analytical gel-filtration column (blue trace). The insert shows equivalently color-coded plots of the RI (left axis and dotted lines) and calculated molar mass (right axis and solid lines) vs. volume for these two chromatograms. The same volume of protein solution (100 µl) was injected for both of the analytical gel filtration runs displayed here. Debye analyses of the SLS/RI data indicate that the single peak in the black trace and the earlier peak in the gray trace have a mass-averaged molecular weight of ~126 kDa, consistent with the presence of an EttA dimerA (based on the 62.4 kDa predicted molecular weight of the protomer). Debye analysis of the second peak in the gray trace indicates a mass-averaged molecular weights of ~64 kDa, consistent with the presence of an EttA monomer. Concentration of this monomer peak and reinjection on an analytical gel-filtration column shows a mixture of monomer and dimer. Analytical gel filtration chromatography was conduced on a Shodex 804 column running at 4 °C with a 0.5 ml/min flow-rate in 150 mM NaCl, 5% glycerol, 20mM Tris-HCL, pH 7.2. SLS and RI detectors were from Wyatt Technology. (b) E. coli EttA protein co-fractionates with polysomes. An extract of mid-log phase cultures of E. coli was fractionated using sucrose density

gradient sedimentation either without (black trace and upper gel) or with (red trace and lower gel) RNaseA treatment. The optical density of the gradients at 254 nm is shown above immunoblots of the corresponding fractions developed using a polyclonal anti-EttA antiserum. RNaseA treatment cleaves the mRNA holding the polysomes together to yield single fully assembled ribosomes (containing both large and small subunits) that sediment at 70S. (c) Analysis of variations in EttA expression level during the E. coli life cycle. WT or $\Delta ettA$ MG1655 cells were inoculated into fresh LB medium, and the cultures were monitored during 24 hours of growth at 37 °C. The graph is showing the OD₆₀₀ of the MG1655 culture in gray (right axis) and the intensity of the signal on the immunoblot in green (left axis). The near-IR fluorescence recorded from the immunoblot was quantified using ImageJ⁹ software. Insert, Western blot analysis of total cells extracts using a polyclonal anti-EttA antibody, after growth for the number of hours indicated at the top of the blot. (d) Stimulation of EttA ATPase activity by 70S ribosome. Initial velocity of ATP hydrolysis (vertical axis) was measured as a function of ATP concentration (horizontal axis) for WT EttA alone or in the presence of 70S E. coli ribosomes. Assays were performed in polymix buffer (5 mM Tris-acetate (pH 7.5), 95 mM KCl, 5 mM NH₄Cl, 5 mM Mg(OAc)₂, 0.5 mM CaCl₂, 2 mM 2-mercaptoethanol, 5 mM putrescine, and 1 mM spermidine).



Supplementary Figure 6: EttA-EQ₂ inhibits protein translation in vitro in complete and *minimum translation assays.* (a) Luminescence assays were used to quantify the level of *in* vitro translation of luciferase from T7-luc mRNA using a complete in vitro translation system, specifically the E. coli S30 Extract System for Linear Templates (Promega). Briefly, reactions containing the indicated EttA variant (or an equivalent volume of buffer) were started by adding T7-luc mRNA and incubated at 37 °C for 2 hours prior to conducting luciferase activity assays using a luminometer. Background from a reaction without T7-luc mRNA was subtracted from the values reported here. See the Methods section for details. (b) Equivalent luminescence assay were used to quantify the level of in vitro translation of luciferase from the same mRNA template using a minimal in vitro translation system, specifically the PURExpress System (New England Biolabs). These reactions were conducted in triplicate in 96-well plates, which were incubated at 37 °C for 2 hours prior to conducting luminescence assays using a microplate reader. (c) Autoradiography was used to assay *in vitro* translation of the bacteriophage T4 pg32 protein using the PURExpress system (New England Biolabs). EttA variants were added to a final concentration of 5 µM, and reactions programmed with pT7gp32.1-224 mRNA were incubated at 37 °C for 2 hours prior to autoradiography of samples run a 12.5% (w/v) SDS-PAGE gel. Each EttA variant was added either before the mRNA (labeled "1^{st,}"), immediately after the mRNA (labeled "2nd"), or 15 min after addition of the mRNA (labeled "at 15"").



Supplementary Figure 7: Order-of-addition experiments demonstrate that EttA-EQ₂ acts after 70S IC formation to trap ribosomes following dipeptide formation and that it can stimulate peptide-bond formation on the ribosome. Minimum in vitro translation assays were conducted as in Fig. 4 in the main text in the presence of 0.5 mM Mg-ATP but with variations in the timing of addition of some components, as illustrated in the schematic diagrams on the left in panels a-Ribosomally synthesized peptides, up to 4 amino acids in length, were separated and C. quantified by autoradiography of eTLC plates, as shown on the right in panels **a-c**. (a) Addition of EttA-EQ₂ before or after formation of the 70S IC produces an equivalent inhibition of translation following dipeptide formation. The tripeptide synthesis experiment shown here, in which EttA was added before 70S IC formation, gives equivalent results to the experiment shown in Fig. 4a in the main text, in which EttA was added after 70S IC formation. Translation reactions were started by adding the elongation factors and Phe-tRNA^{Phe} and Lys-tRNA^{Lys}. **(b)** Addition of EF-G prior to EttA-EQ₂ reduces the extent of inhibition of protein translation but does not lead to strong accumulation of tripeptide in a tetrapeptide synthesis assay. Translation reactions containing EF-G were set up to stop after dipeptide synthesis due to lack of a cognate tRNA for the third codon in the model mRNA. Inclusion of EF-G in these reactions should result in translocation of the dipeptide-bearing tRNA to the P site in a substantial fraction of ribosomes. Subsequent addition of EttA-EQ₂ inhibits dipeptide elongation in these assays much less effectively than in assays in which translocation of the dipeptide-bearing tRNA has been prevented by omission of EF-G prior to the addition of EttA (Fig. 4b in main), showing that it acts preferentially on the pretranslocation complex with deacylated tRNA^{fmet} in the A site of the

ribosome. Moreover, tetrapeptide is formed in higher yield than tripeptide in these assays in which EF-G is added to translation reactions before EttA-EQ₂, demonstrating that it inhibits elongation of dipeptides more strongly than that of tripeptides, again consistent with it acting preferentially on ribosomes bearing tRNA^{fmet} in the P site. (c) Addition of EttA-EQ₂ after tripeptide synthesis on the ribosome complex modestly stimulates tetrapeptide synthesis rather than inhibiting it. Translation reactions were set up in presence of EF-G with cognate tRNAs for the first three but not the fourth codon in the mRNA, thereby stopping translation after tripeptide synthesis. EttA-EQ₂ was subsequently added prior to addition of the cognate tRNA for the fourth codon. The modest stimulation of tetrapeptide synthesis in this experiment provides evidence that interaction of the ATP-bound conformation of EttA with ribosomes promotes peptide-bond formation in the peptidyl-transferase center. The results of this experiment also support the conclusion that EttA-EQ₂ does not inhibit post-translocation ribosomal complexes because it specifically blocks the translocation step in the ribosomal elongation cycle. (d) Filterbinding assay showing that the ribosomal E site does not stably retain deacylated tRNAs at the Mg^{2+} concentration used in standard translation assays. The retention of 0.4 μ M deacylated ³²P]tRNA^{Phe} by 70S ribosomes was assayed in the presence of different concentrations of $Mg(OAc)_2$ (3.5, 10, and 20 mM). Assays were conducted using a 0.2 μ M concentration of 70S ribosomes in 0.1 M NH₄Cl, 20 mM Tris-HCl, pH 7.4. At 3.5 mM Mg²⁺, which is the concentration used in the minimum in vitro translation reactions, the deacylated tRNA is not retained on the E site of the ribosome, even through it is retained at the higher Mg^{2+} concentrations. This observation indicates that EttA-EQ₂ should have access to the E site in the post-translocation complex prepared in the experiment shown in panel c, consistent with the interpretation that binding of EttA-EQ₂ at this site stimulates peptide bond formation in the peptidyl-transferase center on the ribosome.



Supplementary Figure 8: Characterization of EttA interactions with the 70S IC using the smFRET_{L1-L9} signal. (a) Cartoon diagram of the 70S IC used in these experiments. The 30S and 50S subunits of the ribosome are shown in tan and blue, respectively. The fMet-tRNA^{fMet} is represented by a green ribbon, while the mRNA is represented by a black curve. The Cy3 donor and Cy5 acceptor fluorophores are represented by green and red circles, respectively. (**b-d**) Data from smFRET_{L1-L9} experiments recorded in the presence of either 2 mM Mg-ATP (top panels) or 2 mM Mg-ADP (bottom panels) and either in the absence of EttA (panel b), in the presence of 6 μ M WT-EttA (panel c), or in the presence of 6 μ M EttA-EQ₂ (panel d). These surface contour plots show the time evolution of the FRET efficiency (E_{FRET}) distribution in an ensemble of individually observed 70S ICs. The plots were generated by superimposing a large set of individual E_{FRET} versus time trajectories as previously described¹⁰; the variable N shown on each plot indicates the number of superimposed trajectories. The contours are color-coded as calibrated by the color bars shown on the right, with white and red representing the lowest and highest populated E_{FRET} levels, respectively. (e) Vertical column scatter plots showing the mean E_{FRET} values measured in a series of independent smFRET_{L1-L9} experiments conducted in the presence of either Mg-ATP (top panel, three independent experiments) or Mg-ADP (bottom panel, five independent experiments). A plot encompassing the full E_{FRET} range (0.0–1.0) is shown on the left side of each panel, while an expanded view of the relevant E_{FRET} range (0.5-0.6) is shown on the right side of each panel. Independent experiments in the absence or presence of WT-EttA were recorded in a paired fashion (i.e., with data collected from the same flowcell before and after adding WT-EttA). The mean E_{FRET} values from experiments conducted in the same flowcell are shown in the same color. Independent experiments in the absence or presence of EttA-EQ₂ were recorded in an unpaired fashion (*i.e.*, with data collected from different flowcells). The back horizontal lines on the graphs represent the mean E_{FRET} value for each experimental condition, while the p-values for the observed differences between the experimental conditions are shown at the top of each scatter plot. The p-values were calculated in version 5 of the program PRISM (Graphpad Inc.) using a paired t-test for WT-EttA and an unpaired t-test for EttA-EQ₂.



Supplementary Figure 9: Full view of the cropped eTLC plates and agarose gels presented in Figures 4 and 6 in the main text. (a) Miniature cropped eTLC data presented in Figure 4a (left) and the corresponding uncropped eTLC plate (left), with the red rectangle delimiting the area presented on the left. (b) Miniature cropped eTLC data presented in Figure 4b (left) and the corresponding uncropped eTLC plate (left), with the red rectangles delimiting the two areas (1 and 2) presented on the left. (c) Miniature cropped eTLC data presented in Figure 4c (left) and the corresponding uncropped eTLC plate (left), with the red rectangles delimiting the two areas (1 and 2) presented on the left. (d) Miniature cropped DNA agarose gels presented in Figure 6 (top) and the corresponding uncropped agarose gels (center and bottom); the red rectangles delimit the 6 areas (1 to 6 in red) presented in the miniature of Figure 6 (top).

SUPPLEMENTARY TABLES

			ABCβ+Core 1					ABCa 1				
Protein	PDB	Rank	Z-score	rmsd (Å)	% id	length	Rank	Z-score	rmsd (Å)	% id	length	
EttA-ABC2		1	19.4	2.0	36	138	23	6.1	2.5	26	50	
MalE	2R6G(B)	2	18.8	1.9	27	140	5	7.2	1.7	34	56	
MJ0796	1L2T(B)	3	18.6	1.5	31	138	27	5.6	2.2	24	58	
CFTR ^b	2PZE(B)	4	18.6	2.2	24	139	26	5.7	1.8	23	52	
ArtP	2OLK(B)	5	18.3	2.1	28	141	21	6.4	1.9	29	56	
LolD	2PCJ(A)	6	17.9	1.8	30	139	17	6.4	1.6	31	51	
BtuD	4DBL(D)	9	17.7	1.8	29	135	1	8.1	1.8	27	55	
RLI	1YQT(A)	12	17.4	1.9	28	138	12	7.0	1.5	39	49	
EF3	2IX3(B)	24	15.9	2.2	31	134	19	6.5	1.6	22	50	

Supplementary Table 1A. Closest structural homologs to ABC1 of E. coli EttA as identified by DALI.^a

^a The ABC β subdomain and F1-like core subdomain were submitted to the DALI server¹¹ for analysis together, but the ABC α subdomain was submitted separately because of its variable alignment in different structures was determined without the γ -phosphate of ATP bound in the ATPase active site (Supplementary Fig. 3a-c). The letter in parentheses in the PDB id is the chain identifier of the aligned subunit. The Rank represents the highest position of the aligned protein molecule when the list of structures with significantly structural similarity is sorted by decreasing Z-score (*i.e.*, ignoring structures giving lower Z-scores containing the same protein). The Z-score is a standard measure of statistical significance (given by the ratio of the difference between the structural similarity score and the mean score when scanning the database to the standard deviation in that score). The abbreviation rmsd stands for root-mean-square deviation. The % id represents the percent of identical amino acids in the aligned regions of the structures, while length is the number of residues included in these regions.

^b This structure represents the first nucleotide-binding domain of human CFTR with the regulatory insertion and extension excised.

			ABCβ+Core 2					ABCa 2					
Protein	PDB ^b	Rank	Z-score	rmsd (Å)	% id	length	R	ank	Z-score	rmsd (Å)	% id	length	
EttA-ABC1		1	19.4	2.0	36	138		3	6.1	2.5	26	50	
HI1470	2NQ2(C)	2	18.6	2.1	28	150		20	4.9	1.8	18	50	
CFTR ^b	2PZE(B)	3	18.2	2.3	21	146		23	3.8	2.8	19	53	
RLI	3BK7(A)	4	17.8	2.2	27	150		21	4.7	2.9	23	53	
ArtP	2OLK(A)	5	17.8	2.8	24	154		12	5.3	1.9	17	52	
FbpC	3FVG(A)	6	17.8	2.5	31	150		14	5.0	2.4	20	54	
TM0544	1VPL(A)	7	17.4	2.8	30	153		21	4.8	2.1	18	50	
EF3	2IX3(B)	10	17.1	2.8	32	148		2	6.2	2.3	23	53	
BtuD	2QI9(B)	20	16.0	2.4	28	145		1	6.2	2.1	25	52	

Supplementary Table 1B. Closest structural homologs to ABC2 of E. coli EttA as identified by DALI.^a

^a The analyses were performed and the parameters are defined identically to Table S1A.

^b This structure represents the first nucleotide-binding domain of human CFTR with the regulatory insertion and extension excised.

SUPPLEMENTARY NOTES

Possible models for the different activity of EttA in presence of ATP vs. ADP

Additional research will be needed to clarify many facets of EttA's mechanism and physiological function. Important mechanistic questions include how the two ATP-binding sites in EttA interact in the course of its functional reaction cycle and how ADP mediates EttAdependent inhibition of synthesis of the first peptide bond in the nascent protein Our data show that WT-EttA has a qualitatively different effect on translation reactions conducted in the presence of ADP compared to either WT-EttA or EttA-EQ₂ in the presence of ATP (Fig. 5 and Fig. 4). In the presence of ADP, WT-EttA inhibits synthesis of the first peptide bond by the 70S IC rather than promoting this reaction or trapping it following this reaction, as observed for WT-EttA and EttA-EQ₂, respectively, in the presence of ATP. Several models (see Supplementary Note) can be envisioned to explain this alternative activity in the presence of ADP compared to ATP. One possibility is that ADP interacts directly with the ribosome to alter its mode of interaction with EttA. This possibility is suggested by the small shift in the FRET efficiency distribution observed in the smFRET data recorded from the L1-L9 reporter pair on the 70S IC in the presence of ADP but not in the presence of ATP (Supplementary Fig. 8b top vs. bottom). An alternative possibility is that ADP binds directly to one or both of the ATPase active sites in EttA, resulting in an altered conformation that still binds to the 70S IC but stabilizes it in a conformation that inhibits formation of the first peptide bond rather than promoting it. In this case, there are several possible explanations for the different behavior of EttA upon directly binding ADP compared to the post-hydrolysis complex with ADP formed following the binding of ATP. One explanation is that peptide-bond synthesis on the ribosome while EttA is in the ATP-bound state pushes the ribosome into a conformation in which it is no longer sensitive to the ADP-bound conformation of EttA, which results in release of EttA from the ribosome following ATP hydrolysis. Another possibility is that transient electrostatic forces generated during the hydrolysis reaction induce the dissociation of EttA prior to adoption of its equilibrium ADP-bound conformation. A related mechanistic issue concerns whether there is functional asymmetry between the two ATPase active sites in EttA. Such asymmetry has been documented in ABCE1¹² and other members of the ABC superfamily having two ABC domains that differ in primary sequence. In this context, it is possible that one of the ATPase active sites in EttA plays a regulatory role and that binding of ADP to this site prevents adoption of the conformation promoting peptide-bond synthesis on the 70S IC even if ATP is bound to the other site. It also remains to be established whether ADP can be exchanged for ATP while EttA remains bound to the ribosomal E site or whether exchange can only occur after dissociation of EttA from the ribosome. Additional biochemical and biophysical studies will be needed to address these unresolved issues concerning the location of the ADP binding site influencing the activity of EttA and its mechanism of action.

Supplementary Methods

Protein expression and purification. All proteins expressed in a pBAD vector were grown in MG1655-*ettA::Tn5* strain in LB media (3 liter for all the constructs excepted EttA-EQ₂ which has very low expression and therefore was grown in 12 liter) to an OD₆₀₀ of 0.6 and induced with 0.1 % of L-arabinose and grown for an extra three hours. Cells were centrifuged at 4,000rpm for 30 min (JS-4.2 rotor in Beckman J6-B), washed with Phosphate Buffered Saline (137 mM NaCl, 2.7 mM KCl, 10 mM, Na₂HPO₄, 2 mM KH₂PO₄) and then centrifuged for 10 min at 6,000 rpm in a GSA rotor (Sorvall).

To purify hexahistidine-tagged constructs (His₆-EttA-EQ₂, His₆-EttA-Δarm and His₆-EttA), pellets were weighed, stored at -80 °C, and resuspended in 5 ml per gram of cells of lysis buffer (150 mM NaCl, 10 mM imidazole, 2 mM 2-mercaptoethanol, 10% glycerol, 20 mM Tris-HCl, pH 7.5) supplemented with EDTA-free Complete Protease Inhibitor Cocktail Tablets (Roche). They were incubated 10 min on ice with 2 mg/ml of lysozyme (Sigma-Aldrich) and lysed using 3 passages through an Emulsiflex C3 (Avestin) at 16,000 psi. Lysates were centrifuged at 30,000 g for 30 min and loaded on Ni-NTA column (Qiagen). They were washed with 10 columns volume of buffer A (same as lysis buffer but without protease inhibitor), then with 10 columns volume of buffer B (same as A but with imidazole at 30 mM), and finally the protein was eluted with buffer C (same as A but with imidazole at 500 mM). The fractions containing the protein were pooled, concentrated and buffer exchanged (same as buffer A without imadazole) on a Amicon Ultra 30 kDa (Millipore). The concentrated proteins were then brought to 1M (NH₄)₂SO₄ prior to purification on a butyl-Sepharose FF column eluted with a 1000-0 mM gradient of (NH₄)₂SO₄ in the standard buffer (20 mM Tris-HCl pH 7.5, NaCl 150 mM, 10 mM imidazole, 2 mM 2-mercaptoethanol, 10% glycerol). The fraction containing EttA were pooled and buffer exchanged the same way as described previously.

For purification of untagged WT EttA, washed cell pellets were stored at -80 °C were thawed and resuspended in 5 ml per gram of cell with 10% glycerol, 5 mM DTT, Complete Protease Inhibitor Cocktail Tablets (Roche), 25 mM Tris-Cl, pH 8.0. Cells were lysed using 3 passages through an Emulsiflex C3 (Avestin) at 16,000 psi, and the extract was clarified by centrifugation 30 min at 30,000 g prior to purification on a DEAE-Sepharose FF column eluted with a 0-500 mM gradient of NaCl in the standard buffer (*i.e.*, the extraction buffer without protease inhibitor). EttA-containing fractions were then brought to 1M (NH₄)₂SO₄ prior to purification on a butyl-Sepharose FF column eluted with a 1,000-0 mM gradient of (NH₄)₂SO₄ in the standard buffer. EttA-containing fractions were pooled and purified on a Sephacryl S300HR gel filtration column (GE Healthcare) in the standard buffer plus 100 mM NaCl. The final protein pool was exchanged into the standard buffer. For the WT-EttA dimer monomer study the peaks corresponding to the monomer and the one corresponding for the dimer were concentrated separately. The dimer fraction was concentrated ~150 μ M and was used for the static light-scattering experiment.

Selenomethionine labeled protein was expressed using the method of Le Master ¹³ in strain B834(λ DE3). A 2 liter culture containing 30 µg/ml kanamycin sulfate and 50 mg/ml DL-selenomethionine was grown at 37°C to an OD₆₀₀ of 0.6. At this point, 0.4 mM isopropyl 1-thio-

β-D-galactopyranoside was added and the cells were grown a further 2 hours with constant shaking. Cells were harvested by centrifugation and stored at -80°C. All subsequent steps were carried out at 4°C. For purification, harvested cells were mixed with 100ml buffer A (25mM Tris-HCl pH 8.0, 10% (w/v) glycerol, 2 mM phenylmethylsulfonyl fluoride, and 5 mM DTT) and homogenized using a French pressure cell at 20,000 psi. This cell lysate was clarified by centrifugation at 30,000 g for 30 min. Streptomycin sulfate was added to the resulting supernatant to a final concentration of 4% (w/v). The resulting mixture was incubated for 30 min followed by centrifugation at 30,000g for 30 min. After filtration through a 0.22 µm filter, the supernatant was loaded onto a 15 ml column of DEAE-Sepharose (GE Healthcare) equilibrated in Buffer A. Protein was eluted using a continuous gradient of 1M NaCl in Buffer A with EttA (as monitored by SDS-PAGE) eluting at ~250 mM NaCl. EttA containing fractions were then brought to 1M (NH₄)₂SO₄, filtered, and added onto a 50 ml column of Butyl-Sepharose (GE Healthcare) equilibrated in buffer A containing 1M (NH₄)₂SO₄. Proteins were eluted from the column using successively lower concentrations of (NH₄)₂SO₄. EttA containing fractions were then pooled, concentrated to 5 ml volume, and loaded on an S300HR size exclusion column (GE Healthcare) equilibrated in buffer A with 100 mM NaCl. EttA-containing fractions were pooled and desalted into buffer B (10 mM Tris-HCl pH 8.0, 10% glycerol, and 10 mM dithiothreitol), concentrated to \sim 240 μ M, frozen in liquid nitrogen and stored at -80 °C.

Synthesis of mRNA for in vitro translation. A T7-luc mRNA was generated using the pBEST plasmid (Promega) as a template to amplify the gene of the luciferase (*luc*). The PCR amplification was done first by using primer flanking the *luc* open reading frame; the forward primer contained the sequence compatible with the universal primer PURExpress (New England Biolab) kit which included a Ribosome Binding Site. Secondly, this PCR product was PCR amplified with the universal primer, which introduced the T7 promoter. This PCR product was gel purified using the gel extraction kit (Qiagen) and used as a template to generate a RNA with the T7 RiboMAX kit (Promega). The RNA was DNase treated and purified by Trizol extraction (invitrogen) and resuspended in the RNA buffer (Ambion). For the pT7gp32 mRNA, the RNA was produced using the same procedure, except without PCR amplification. The pT7gp32.1-224 plasmid ¹⁴ was linearized by restriction with BamHI and used as a template for the RiboMAX kit. The resulting RNA was purified as described above. The pT7gp32.1-224, except that it used the pT7gp32.1-20 plasmid as a template. This plasmid has a stop codon after the residue 20 and the 4 first codons are AUG-UUU-AAA-GAA (Met-Phe-Lys-Glu).

In vitro translation using an E. coli S30 extract. T7-luc mRNA was used as a template for translation reactions conducted according to the manufacturer's recommendations using an extract purchased from Promega. In brief, the reaction contained 2.5 μ l AA mix, 10 μ l S30 premix and 7.5 μ l S30 extract. Four μ l of buffer or protein were assembled to test at the final indicated concentration on the graph. The reactions were started by adding 1 μ l of T7-*luc* mRNA at 5 μ g/ μ l and incubating at 37°C for 2 hours. A 2 μ l volume of each reaction was mixed with 50 μ l of Luceferase Assay Reagent (Promega). Light emission was quantified with a luminometer for 10 seconds. Background was determined on a reaction without T7-*luc* mRNA and subtracted for the other measurements.

In vitro translation using purified components. The PURExpress system was purchased from New England Biolabs. Each reaction was carried out in a 5 µl total volume (2.5 µl of solution A, 1 μ l of solution B, 0.5 μ l of T7-luc mRNA at 2 μ g/ μ l and 1 μ l of buffer or protein to test at the final concentration indicated on the graph). Reactions were done in a 96 wells plate and in triplicate. After 2 hours of incubation at 37°C, 1 µl of the reaction was mixed with 50 µl of Luciferase Assay Reagent (Promega). Light emission was quantified in a microplate reader in luminescence setup with 10 second read by well (Tecan Infinite 200 PRO). In vitro translation of pT7gp32.1-224 mRNA was performed with the PURExpress system (New England Biolabs) using $[^{35}S]$ methionine In vitro Translation Grade (MPbio). The reaction contained 4 µl of solution A, 3 µl of solution B, and 1 µl of buffer or protein to test (EttA, EttA-EQ₂ and EttA EQ₂- Δ arm) at the final concentration of 5 μ M and 1 μ l of [³⁵S]methionine (10 μ Ci). The protein was added before the mRNA (1st) or after the mRNA (2nd) or 15 min after addition of the mRNA (15'). The reactions were started by adding 2 µl of T7-luc mRNA at 0.5 µg/µl and incubating at 37°C for 2 hours. The reactions were stopped by adding 20 µl of 2X Laemmli and heating for 2 min at 60°C. Then 10 µl of each reaction were run on a 12.5% SDS-PAGE. The gel was stained and dried on Whathman as well as subjected to autoradiography, which is presented on this figure.

E site binding tRNA binding assays. For synthesis of deacylated $[^{32}P]$ tRNA^{Phe 15,16}, 34 µM of tRNA^{Phe} (Sigma-Aldrich) was incubated with 50 mM of glycine pH 9, 10 mM MgCl₂, 360 Ci/mmol $\left[\alpha^{-32}P\right]$ ATP (Perkin Elmer), 0.05 mM sodium pyrophosphate and 0.03 mg/ml of nucleotidyl tranferase. The reaction was incubated for 5 min at 37°C. Labeled tRNA was purified by phenol/chloroform extraction followed by ethanol precipitation, resuspension in milliQ water and filtration through a P6 column (Bio-Rad) pre-equilibrated with milliQ water in order to remove unincorporated radioactivity. Assessment of the stability of the deacylated tRNA bound in the E site as a function of $Mg(OAc)_2$ were done using an E site filter-binding assay based on the original assay of Grajevskaja *et al.*¹⁷. The 70S ribosomes used for the assay were prepared as for the minimal purified translation assay¹⁴. Reactions containing 70S ribosomes $(0.2 \mu M)$ were incubated in presence of deacylated 0.4 µM [³²P]tRNA^{Phe} and increasing concentrations of Mg(OAc)₂ (3.5, 10, or 20 mM) for 1 min at 4 °C in a 20 µl reaction in 100 mM NH₄Cl, 20 mM Tris-HCl, pH 7.4. A 5 µl volume of each reaction was spotted on a nitrocellulose filter (25 mm, 0.45µm, nitrocellulose, disc filters, Millipore) installed on a Sampling Manifold (Millipore) under constant vacuum. After 3 washes with 2 ml of 20 mM Mg(OAc)₂, 100 mM NH₄Cl, 1mM EDTA, 20 mM Tris-HCl, pH7.4, the filters were transferred to scintillation fluid (Ultima Gold, PerkinElmer) and counted on a scintillation counter (Beckman LS6500). A 5 µl volume of each reaction was also counted. The radioactivity retained on the filter was divided by the total radioactivity and multiplied by the total concentration of radiolabeled tRNA to yield an estimate of the concentration of radiolabeled tRNA bound to ribosomes, which was divided by the total ribosome concentration for graphing as fractional occupancy of the E site.

ATPase Assays. ATP hydrolysis assays were performed in polymix buffer (5 mM Tris-OAc (pH 7.5), 95 mM KCl, 5 mM NH₄Cl, 5 mM Mg(OAc)₂, 0.5 mM CaCl₂, 2 mM 2-

mercaptoethanol, 5 mM putrescine, and 1 mM spermidine). To convert all ADP present in the reaction to ATP, solutions were pre-incubated at 37 °C for 15 min with pyruvate kinase (PK) and phosphoenolpyruvate (PEP). Solution I consisted of $[\gamma^{-32}P]ATP$ (100-200 dpm per pmol), 0.25 $\mu g/\mu l$ PK, and 3 mM PEP in polymix buffer; solution II consisted of His₆-EttA, 0.25 $\mu g/\mu l$ PK, and 3 mM PEP in polymix buffer. Reactions were carried out in 60 μl assays, with a final His₆-EttA and 70S concentration of 1 μ M, respectively. ATP concentrations were varied as indicated (final concentrations). Background hydrolysis by ribosomal particles was also examined and subtracted if applicable; ribosome preparations typically did not have significant ATPase activity in these assays. Reactions were stopped with 50 μ l of quenching solution (2 M perchloric acid with 3 mM KH₂PO₄). Then, 300 μ l of 20 mM Na₂MoO₄ solution and 400 μ l of isopropyl acetate were added and thoroughly mixed for 5 min. The organic phase was separated by centrifugation and the amount of liberated inorganic phosphate was quantified by scintillation counting (Perkin Elmer TriCarb 2800TR).

L1-L9 single molecule fluorescence energy transfer (smFRET_{L1-L9}) experiments. Populations of 70S ICs harboring a donor fluorophore (Cy3) on ribosomal protein L9 and an acceptor fluorophore (Cy5) on ribosomal protein L1 were imaged using total internal reflection fluorescence microscopy as previously described¹⁸⁻²⁰. These experiments were performed in Tris-Polymix Buffer (50 mM Tris acetate (pH 7.0 at 25 °C), 100 mM KCl, 5 mM NH₄OAc, 0.5 mM Ca(OAc)₂, 0.1 mM EDTA, 5 mM putrescine dihydrochloride, and 1 mM spermidine free base) with 15 mM Mg(OAc)₂ and 10 mM 2-mercaptoethanol, supplemented with an oxygenscavenging system (2.5mM 3.4-dihydroxybenzoic acid (PCA), 25nM protocatechuate 3.4dioxygenase (PCD) and 1% (w/v) β -D-glucose). The small (30S) and large (50S) ribosomal subunits used for the formation of the 70S IC complex in smFRET_{L1-L9} experiments were prepared as previously described²⁰. The initiation factors, mRNA, and fMet-tRNA^{fMet} were prepared as previously described¹⁴. The 70S IC was formed in two steps. First, 30S subunits and L9(Cv3)-L1(Cv5)-labeled 50S subunits were incubated at 37 °C for 10 min in Initiation Polymix Buffer (Tris-Polymix Buffer in which the Mg(OAc) has been reduced to 5 mM and 2mercaptoethanol has been reduced to 6 mM) at a final concentration of 1.2 µM, in the presence of 1.6 µM IF1, 1.2 µM IF2, 1.7 µM IF3, and 1 mM GTP. Second, 5'-biotinylated mRNA and fMet-tRNA^{fMet} were added to the above reaction to a finial concentration of 2.0 and 1.5 μ M, respectively, prior to incubation for another 20 min at 37 °C. Once formed, the 70S IC was placed on ice for 15 min and purified using sucrose density gradient ultracentrifugation as previously described¹⁴. Independent trials recorded in the absence or presence of WT-EttA were recorded in a paired fashion. For these paired experiments, 100 pM 70S IC was pipetted into the flowcell in 15 mM Mg(OAc)₂ Tris-Polymix Buffer. After binding of the 5'-biotinylated mRNA 70S IC complex to the streptavidin-coated flowcell, the same buffer with Mg-ATP (2 mM) or Mg-ADP (2 mM) was added, and the smFRET data were recorded. Subsequently, 6 µM EttA in the same buffer with 2mM Mg-ATP or Mg-ADP was pipetted into the same flowcell, and the smFRET data were recorded. Independent experiments conducted in the in the absence or presence of 6 μ M EttA-EQ₂ were performed in an unpaired fashion (*i.e.*, the data in the absence of EttA-EO₂ were recorded from one flowcell, while the data in the presence of EttA-EO₂ were recorded separately from a second flowcell).

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