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Supplemental Data

Coupling of Ribosomal L1 Stalk and tRNA Dynamics during Translation Elongation

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Supplemental Experimental Procedures

Preparation of translation factors, tRNAs, and mRNA Translation factors were purified as previously described (Blanchard et al., 2004). OH-tRNA^{Phe} was labeled with Cy3-NHS ester at the 3-(3-amino-3-carboxy-propyl) uridine 47 position. tRNA^{fMet}, (Cy3)tRNA^{Phe}, and tRNA^{Lys} were aminoacylated with the corresponding amino acids, and Met-tRNA^{fMet} was formylated, following previously published procedures (Blanchard et al., 2004). A T4 gene product 32-derived mRNA was chemically synthesized (Dharmacon, Inc) to contain a 5'-biotin followed by an 18 nucleotide spacer, a strong Shine-Dalgarno (AAAGGA) sequence, nucleotides encoding fMet, Phe, and Lys as the first three amino acids, and nucleotides encoding an additional six amino acids.

L1 deletion strain of E. coli Ribosomal protein L1 is not an essential gene in *E. coli*. L1 deletion strains, however, do display a slow-growth phenotype (Subramanian and Dabbs, 1980). Using the one-step technique described by Datsenko and Wanner (Datsenko and Wanner, 2000), we have successfully deleted the L1 gene from a

wild-type strain of *E. coli* (Supplementary Figure S1a) and replicated the previously reported slow-growth phenotype (Supplementary Figure S1b).

Purification of L1(-) ribosomes L1(-) ribosomes from the ΔL1 strain of *E. coli* were purified by sucrose density gradient ultracentrifugation as previously described for MRE600-derived wild-type ribosomes (Blanchard et al., 2004). The SDS-PAGE gel shown in Figure S1a verifies the absence of the L1 ribosomal protein from purified L1(-) ribosomes.

Design and construction of fluorescently-labeled L1 mutant Ribosomal protein L1 was cloned from *E. coli* C600 genomic DNA into a plasmid system containing a six-histidine (6xHis) affinity purification tag and a tobacco etch virus (Tev) protease cleavage site as previously described (Blanchard et al., 2004). Wild-type *E. coli* L1 contains no cysteine residues and, as such, we designed a single-cysteine mutant whose thiol group can be easily reacted, using well established maleimide chemistry, with commercially available maleimide-conjugated fluorophores. Using multiple sequence alignments of L1 from a variety of bacterial strains and guided by the available X-ray crystallographic structures (Nikulin et al., 2003; Yusupov et al., 2001), we identified a non-conserved amino acid position (T202) within L1 that is likely to be in close proximity to the P-site tRNA. The T202C mutation was made using the QuikChange Mutagenesis System (Stratagene, Inc.) and verified by DNA sequencing. $L1_{T202C}$ was overexpressed, purified, fluorescently-labeled with a maleimide-conjugated Cy5 dye (GE Biosciences) and tested for its ability to be reconstituted into ribosomes and support wild-type levels of ribosome activity (see

below). Based on a comparison of spectrophotometrically-determined L1 and Cy5 concentrations, we estimate an ~60% labeling efficiency of $L1_{T202C}$.

Reconstitution of L1(-) ribosomes with L1(Cy5) L1(Cy5) was reconstituted into L1(-) 70S ribosomes purified from the ΔL1 *E. coli* strain following previously described protocols (Odom et al., 1990). Reconstituted 70S ribosomes were separated from free, unincorporated L1(Cy5) by sucrose density gradient ultracentrifugation. Purified reconstituted 70S ribosomes were dissociated into 30S and 50S subunits by buffer exchange into low (1 mM) Mg^{2+} buffer and the subunits were purified by sucrose density gradient ultracentrifugation. Ribosomal proteins were extracted from the purified subunits (Hardy et al., 1969) and analyzed by SDS-PAGE to confirm the presence of L1(Cy5) in the reconstituted 50S subunits (Figure S2). Based on spectrophotometrically-determined ribosome and Cy5 concentrations, we estimate an ~60% labeling efficiency of ribosomes. Given the ~60% labeling efficiency of $L1_{T202C}$ (see above), this result indicates ~100% overall reconstitution of L1 into L1(-) ribosomes.

Biochemical characterization of reconstituted ribosomes A standard primer-extension inhibition, or toeprinting, assay (Blanchard et al., 2004) was used to test the ability of our L1(Cy5) 70S ribosomes and fMet-$tRNA^{fMet}$ to properly initiate on a defined mRNA in the presence of initiation factors. A straightforward extension of this assay was used to test the ability of these initiated ribosomes to undergo both peptide-bond formation and translocation for at least two rounds of elongation. The

results demonstrate that L1(Cy5) ribosomes can undergo all of these reactions at near wild-type levels (Figure S3).

Selection of single-molecule FRET traces Raw intensity data were analyzed with the Metamorph software suite (Molecular Devices). The Cy3 and Cy5 fields-of-view were aligned and single ribosomes were selected based on their characteristic fluorescence intensities and single-step photobleaching properties. Each trace is composed of a pair of corresponding Cy3 and Cy5 intensity *vs.* time trajectories from a single ribosome. All the acquired traces were subjected to three selection algorithms to elicit traces representing relevant single molecule data. The first algorithm averages the traces over three data points, differentiates, and then calculates the correlation coefficient of each Cy3/Cy5 pair. Traces with a negative correlation coefficient (anti-correlated) are kept for further analyses. The next algorithm allows the user to visually inspect the traces and discard multiple-dye traces. The final algorithm detects the Cy3 photobleaching event in each trace and discards traces in which Cy3 photobleaches within the first second of data collection. Cy3 and Cy5 signals in each trace were then baseline corrected individually by subtracting the average intensity of the last ten data points after photobleaching. Finally, due to imperfect performance of emission filters, we experimentally determined the Cy3 signal bleed-through into the Cy5 channel to be ~14% in our experimental system. The Cy5 intensity of each trace was therefore corrected using this bleed-through coefficient. smFRET values for each Cy3/Cy5 data point in each trace were then calculated using $I_{Cy5}/(I_{Cy3}+I_{Cy5})$, where I_{Cy3} and I_{Cy5} are the intensities of the Cy3 and Cy5 channels, respectively. Negative

smFRET values in the plots are a mathematical artifact arising from the baseline correction of the post-photobleached Cy3 and Cy5 intensities. While the average post-photobleached Cy3 and Cy5 intensities are zeroed, experimental noise causes individual data points to appear either above or below zero. The consequence of this is that zero FRET states (or low FRET states close to zero) may occasionally yield negative smFRET values (Blanchard et al., 2004).

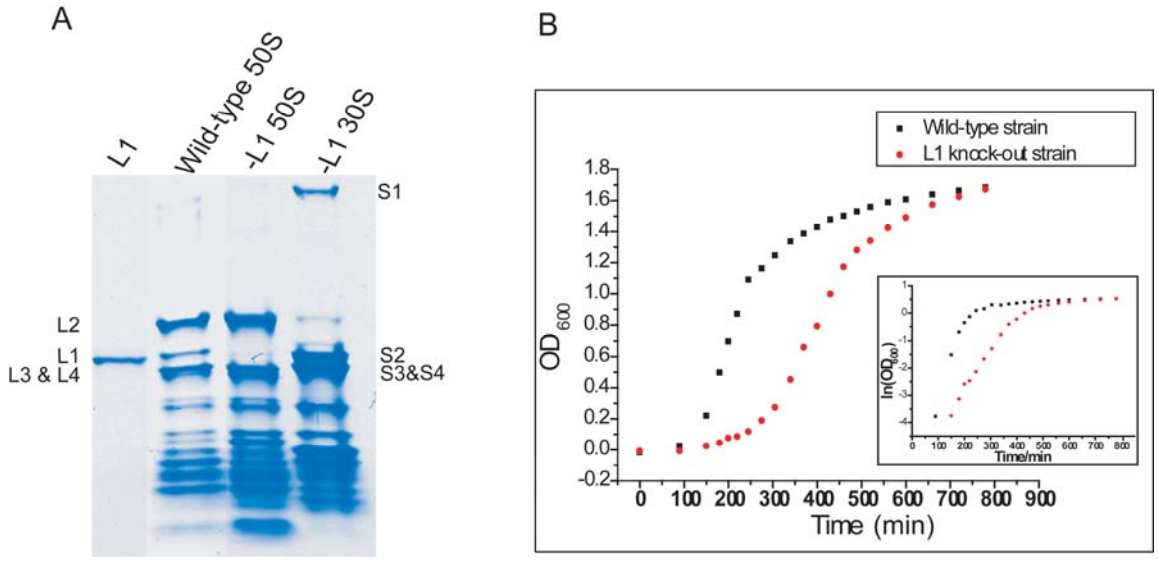

Figure S1. Verification of L1 deletion from *Escherichia coli*

(a) SDS-PAGE analysis of ribosomal proteins. Ribosomal proteins were extracted from purified 50S (L-proteins) and 30S (S-proteins) subunits under high Mg^{2+} concentration and low pH conditions as previously described (Hardy et al., 1969). Purified ribosomal protein L1 and 30S subunit proteins were used as size markers. While the wild-type 50S subunits contain a band for the L1 protein, 50S subunits purified from the ΔL1 strain do not. (b) Growth curves for wild-type and ΔL1 strains of *E. coli*. Deletion of ribosomal protein L1 results in a slow-growth phenotype of *E. coli* as previously reported (Subramanian and Dabbs, 1980). The inset is the plot of the natural logarithm of the OD_{600} *vs.* time, from which we can estimate the doubling time of the wild-type strain (39 minutes) as well as the L1 deletion strain (79 minutes).

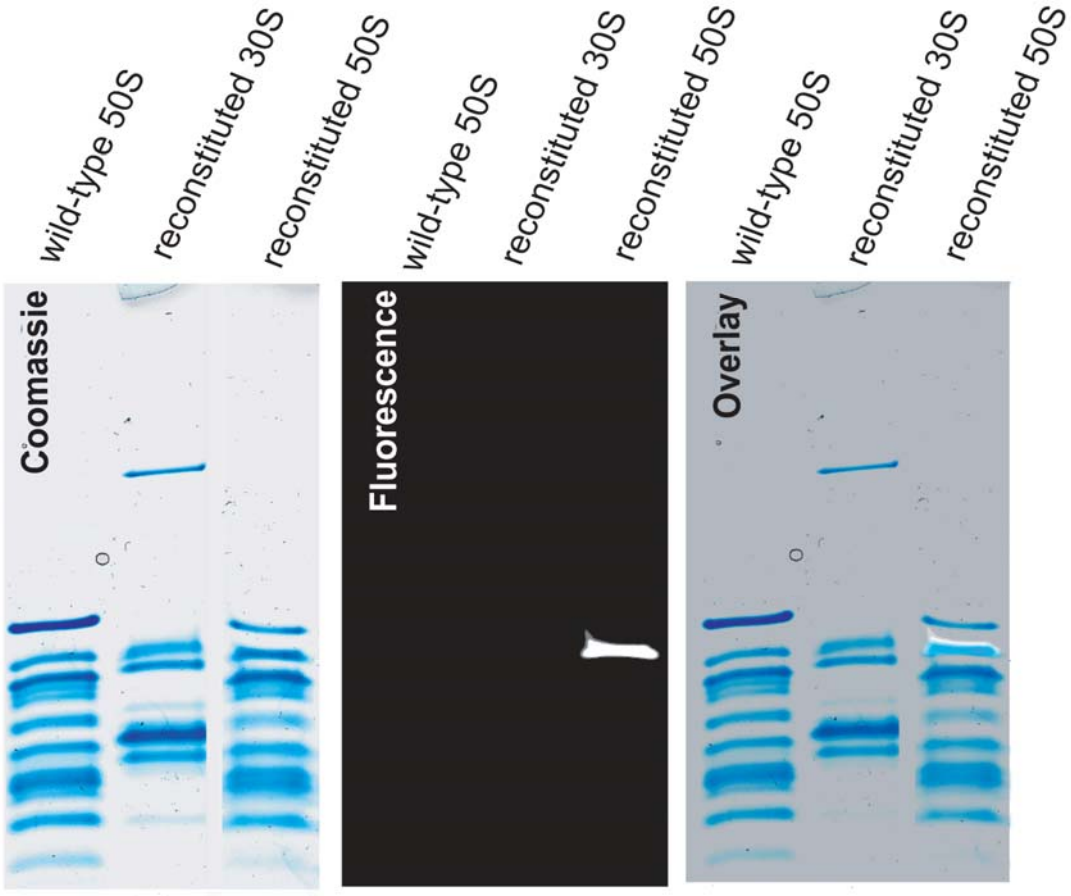

Figure S2. Incorporation of L1(Cy5) into L1(-) ribosomes

Coomassie staining (left), fluorescence scanning (middle), and overlay (right) of an SDS-PAGE analysis of ribosomal proteins derived from reconstituted ribosomes prepared as previously described (Odom et al., 1990). The purified, reconstituted 70S ribosomes were first dissociated into 30S and 50S subunits by buffer exchange into low (1 mM) Mg^{2+} buffer, and the subunits were separated and purified by sucrose density gradient ultracentrifugation. Ribosomal proteins were then extracted from the purified, reconstituted ribosomal subunits (Hardy et al., 1969) and analyzed by SDS-PAGE followed by fluorescence scanning and Coomassie staining.

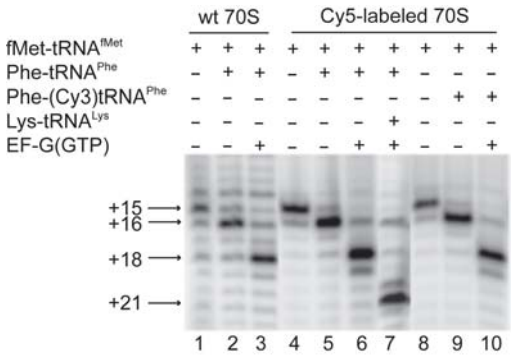

Figure S3. Primer-extension inhibition, or toeprinting, assay

The activity of the reconstituted ribosomes was tested by primer-extension inhibition, or toeprinting (Hartz et al., 1988; Joseph and Noller, 1998). Briefly, translation reactions were performed using purified components and an mRNA that was pre-annealed with a ^{32}P-labeled DNA primer. The position of the initiated 70S complex on the mRNA was toeprinted by monitoring the inhibition of a subsequent reverse transcription reaction. The reverse transcriptase typically encounters the initiated ribosome at a position +15 from the A of the AUG start codon. EF-Tu-catalyzed binding of an A-site tRNA (Phe-tRNA^{Phe} here) to an initiated 70S complex shifts the inhibition to position +16. Subsequent EF-G-catalyzed translocation shifts it further to position +18. Finally, a second round of A-site tRNA (Lys-tRNA^{Lys} here) binding and translocation shifts reverse transcriptase inhibition to position +21. Comparison of the bands at +21, +18, and +15 demonstrates that initiated L1(Cy5) ribosomes are ~95% competent for participation in the first round of elongation with a Phe-(Cy3)tRNA^{Phe} substrate and 80-85% competent for participation in the second round of elongation with a Lys-tRNA^{Lys} substrate. These activities are indistinguishable from those of wild-type ribosomes.

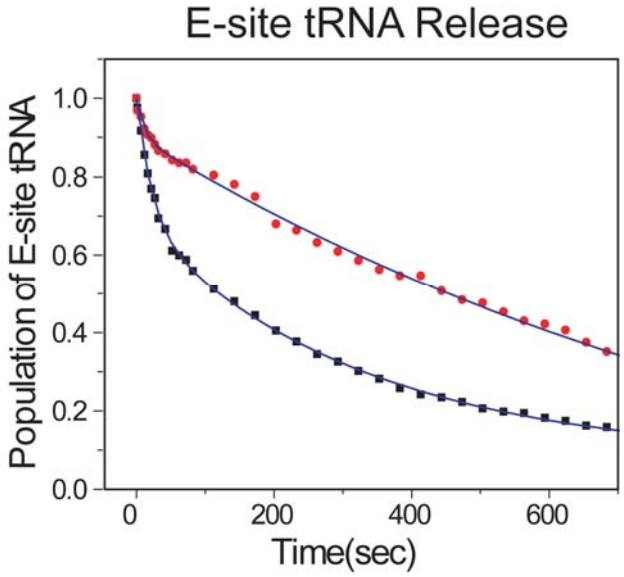

Figure S4. Single-molecule E-site release assay

Complex POST-1, carrying fMetPhe-(Cy3)tRNA^{Phe} at the P site, was immobilized *via* a biotinylated mRNA onto the surface of a streptavidin-derivatized microscope slide. Spatially localized Cy3 fluorescence from individual surface-immobilized complexes was recorded as a function of time. Stopped-flow delivery of 100 nM EF-Tu(GTP)LystRNA^{Lys} in the presence of 1 μM EF-G(GTP) to the complexes resulted in peptide bond formation and translocation of the mRNA-tRNA complex. The translocation event placed the newly deacylated OH-(Cy3)tRNA^{Phe} at the E site. Single OH-(Cy3)tRNA^{Phe} dissociation events from the E site were followed in real-time by monitoring the loss of the spatially localized Cy3 signals (black squares). To extend the lifetime of Cy3 fluorescence, but maintain the ability to resolve both fast and slow processes, the shuttering rate of the excitation source was decreased throughout the experiment such that the first five frames were recorded at 12 frames/min, followed by 5 frames at 6 frames/min and 30 frames at 2 frames/min. As a control, the intrinsic loss of Cy3 signals due to photobleaching and ribosome dissociation from the surface was

recorded by stopped-flow delivering buffer only (red circles) with identical shuttering parameters. The E-site tRNA release data was best described by a double exponential decay (blue curve) of the form A_1*exp(-t/t_1)+A_2*exp(-t/t_2)+y_0. The parameters obtained from the fit were: A_1=0.32, t_1=22 ± 1 sec, A_2=0.62, t_2=330 ± 10 sec with an R^2=0.999. Due to the variation in shuttering rates throughout data collection, and corresponding variation in photobleaching rates, the control curve was best described by a double exponential decay (blue curve) with the following fitting parameters: A_1=0.09, t_1=11 ± 3 sec, A_2=1.16, t_2=1050 ± 80 sec, with an R^2=0.998. The fast component describes the rate of photobleaching during the early timepoints of the experiment while the slow component describes the rate of photobleaching during the later timepoints. Setting t_2 in the E-site release curve to 1050 sec (i.e. the rate of photobleaching during later timepoints) yielded t_1=51 ± 4 sec and R^2=0.994, demonstrating that the slow population in the E-site release curve most likely represents the intrinsic loss of Cy3 signals due to photobleaching and ribosome dissociation from the surface.

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