

Homogeneous stalled ribosome nascent chain complexes produced *in vivo* or *in vitro*

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Cotranslational protein maturation is often studied in cell-free translation mixtures, using stalled ribosome–nascent chain complexes produced by translating truncated mRNA. This approach has two limitations: (i) it can be technically challenging, and (ii) it only works *in vitro*, where the concentrations of cellular components differ from concentrations *in vivo*. We have developed a method to produce stalled ribosomes bearing nascent chains of a specified length by using a ‘stall sequence’, derived from the *Escherichia coli* SecM protein, which interacts with residues in the ribosomal exit tunnel to stall SecM translation. When the stall sequence is expressed at the end of nascent chains, stable translation-arrested ribosome complexes accumulate in intact cells or cell-free extracts. SecM-directed stalling is efficient, with negligible effects on viability. This method is straightforward and suitable for producing stalled ribosome complexes *in vivo*, permitting study of the length-dependent maturation of nascent chains in the cellular milieu.

Newly synthesized polypeptide chains first pass through the ribosomal exit tunnel, which spans ~ 100 Å between the peptidyl transferase site and the surface of the ribosome^{1,2} (Fig. 1a). The tunnel protects 30–40 amino acids (aa) of the nascent chain from contacts with other cellular components and restricts the accessible conformational space^{3,4}. As the nascent chain lengthens, its amino terminus emerges into the cytosol. At this point, the chain has access to additional conformational space and may also interact with other cellular components. The rate of nascent chain synthesis (~ 20 aa/s in *E. coli*) is considerably slower than many folding events, some of which occur on the microsecond timescale. The difference between these rates implies that protein folding can begin during chain synthesis, and cotranslational folding has indeed been detected experimentally^{5–9}. Cotranslational folding therefore represents a fundamentally different starting ensemble for folding than dilution of a full length chain out of a chemical denaturant¹⁰, and conformations populated by nascent chains *in vivo* can be populated quite differently (or not at all) during refolding *in vitro*^{6,9}. For a given protein, cotranslational folding might therefore modify the dominant folding pathway, potentially influencing aggregation propensity. Yet even though recent studies have made some progress in tracking and observing the folding of

proteins within intact cells^{11–13}, the earliest steps of folding *in vivo* (while chain synthesis is underway) remain unclear, in part because of a lack of tractable experimental methods to dissect these early folding steps.

Translation mixtures (and intact cells) include ribosomes with nascent chains of all lengths; this heterogeneity means there now are no biophysical techniques available to assess nascent chain conformation ‘on the fly’, as translation occurs. Uncoupling chain elongation and folding, typically by increasing the population of ribosomes bearing nascent chains of a discrete length, can, however, provide ‘snapshots’ of nascent chain conformations during synthesis. Yet producing stalled ribosomes bearing nascent chains of a uniform length represents a substantial technical hurdle for measuring ribosome-bound nascent chain conformations.

For example, present methods to create stalled *E. coli* ribosome–nascent chain complexes are technically quite challenging—particularly for longer nascent chain lengths—yet *E. coli* is commonly used for protein expression. Here we describe a new method for producing stalled nascent chains of virtually any length *in vivo* or *in vitro* on *E. coli* ribosomes using simple molecular biology techniques. This method builds on recent results demonstrating that residues 150–166 of the SecM protein are sufficient to cause arrest of *secM* translation in *E. coli*¹⁴. Because mutation of residues lining the ribosome exit tunnel were sufficient to eliminate *secM* translation stalling, interactions between these amino acids and SecM residues 150–166 are presumably responsible for translation stalling¹⁴ (Fig. 1a). We demonstrate here that these residues, when expressed at the carboxy terminus of a nascent chain, induce prolonged translation arrest at a discrete point corresponding to the location of the SecM stall sequence. Ribosome-associated nascent chains produced in this manner are easily detected, and the quantities produced are sufficient for detailed biophysical measurements.

RESULTS

SecM-directed production of stalled ribosome complexes

To use the SecM 17-aa stall sequence as a general tool to create stalled ribosome complexes bearing nascent chains of any sequence *in vivo*, we first created three modified expression plasmids to insert the SecM stall sequence at three different places in the sequence of

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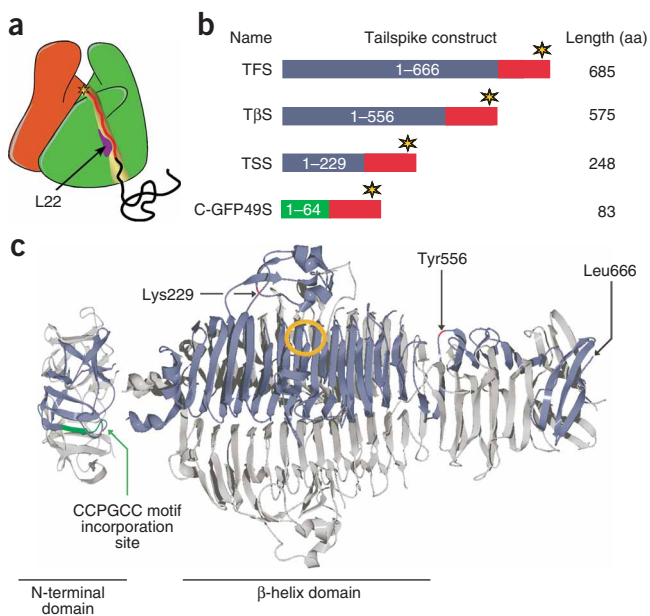


Figure 1 | Design of stalled ribosome-nascent chain complexes using the SecM stall sequence. **(a)** Nascent chain of interest (black) proceeds through the exit tunnel in the 50S subunit (green). Portions of the 17-aa SecM stall sequence (red) interact with components of the ribosomal exit tunnel, in particular ribosomal protein L22 (purple), to induce translation stalling. The 30S subunit is shown in orange, and the star represents the point of translation arrest. **(b)** Diagrams of the tailspike- (blue) and GFP- (green) SecM stall sequence (red) fusions used in this study. Length represents the distance from the N terminus to the SecM stall point: the sum of tailspike or GFP residues, glutamine and arginine from the *SacI* restriction site linker, and the 17 aa of the SecM stall sequence, FSTPVWISQAQGIRAGP. The yellow star indicates the translation arrest point, corresponding to the final proline of the stall sequence, which remains at the peptidyl transferase center of the 50S subunit¹⁴. **(c)** Crystal structure of the native tailspike homotrimer³². One tailspike monomer chain is colored blue. The SecM stall sequence insertions were made after each of the indicated amino acids to produce the constructs shown in **b**. For fluorescence experiments, the CCPGCC FAsH binding site motif²⁰ was incorporated in the N-terminal domain of tailspike, replacing residues 102–107 (green). For C-GFP49S, the CCPGCC motif was incorporated at the extreme N terminus of the polypeptide. The approximate location of the mAb 236 binding epitope¹⁷ is indicated by the orange circle.

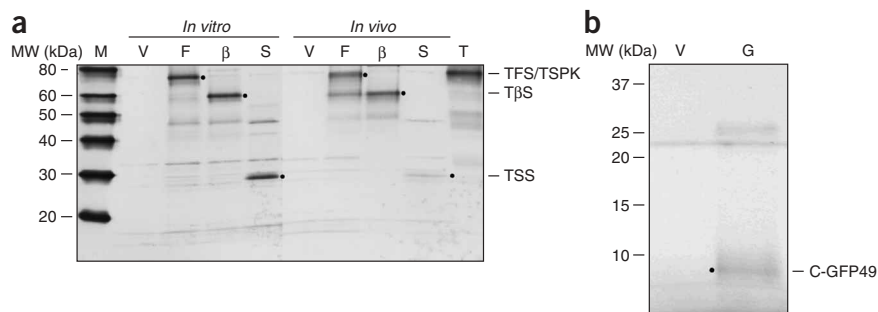
phage P22 tailspike (**Fig. 1b**; see Methods). Tailspike is a homotrimeric parallel β -helix protein previously shown to form some native-like structure while nascent chains are still attached to the ribosome^{8,15}. We designed the longest tailspike nascent chain construct (TFS) so that the almost all of the monomer would be exposed from the ribosome, with only the C-terminal ~ 20 aa occluded in the ribosomal exit tunnel. The mid-length (T β S) and shortest (TSS) nascent chain constructs were designed to expose the entire β -helix domain or the first three rungs of the β -helix domain, respectively, outside of the ribosome tunnel.

We transformed *E. coli* cells with one of three plasmids encoding tailspike-SecM stall fusion constructs, induced protein expression with IPTG, and lysed cells using a combination of lysozyme treatment and freeze-thaw cycles. We separated ribosomes from other components of the cell lysate supernatant by centrifugation through a sucrose cushion. We resolved tailspike nascent chains by SDS-PAGE and western blotting using two tailspike monoclonal antibodies with epitopes in the tailspike N-terminal domain (residues 1–109; **Fig. 1c**)^{16,17}. In the ribosome preparations we observed discrete bands corresponding to each of the tailspike

nascent chain lengths (**Fig. 2a**). We also detected faint bands corresponding to shorter tailspike fragments; in many cases these chain lengths correlated with endogenous translation pause sites (T.F. Clarke IV and P.L.C., unpublished data). We detected other bands, particularly those at ~ 33 kDa and < 20 kDa, in empty-vector lysates, and these are most likely due to nonspecific antibody binding. Other faint bands might arise from nascent chain degradation fragments and/or nascent chains still attached to tRNAs. To ensure that the truncated tailspike bands observed did not arise from chains released from the ribosome, we spiked a lysate from *E. coli* cells transformed with the empty vector with purified native tailspike and isolated the ribosomes as before. No tailspike was detectable by western blotting in the resuspended ribosomes (data not shown) indicating that released TFS, T β S or TSS chains, which are all smaller than native tailspike, were unlikely to sediment.

We also assayed SecM-directed production of stalled ribosome-nascent chain complexes in commercial *E. coli* coupled transcription-translation lysates, each programmed with a plasmid encoding one of the three tailspike-SecM stall fusions. In all reactions, we included an oligonucleotide complimentary to the *SsrA* RNA

Figure 2 | Visualization of stalled, truncated tailspike and GFP nascent chains. **(a,b)** SDS-PAGE separation of stalled ribosome-nascent chain complexes, detected by western blotting using anti-tailspike monoclonal antibodies **(a)**, or the Lumio in-gel detection system **(b)**. **(a)** TFS, T β S and TSS stalled nascent chains were detected on ribosomes derived from *E. coli* cultures (*in vivo*) or from coupled transcription-translation reactions (*in vitro*). M, molecular weight markers. T, full-length purified tailspike monomer. Other lanes depict nascent chains detected on ribosomes derived from cells (or *in vitro* translation reactions) containing V, empty vector; F, plasmid encoding TFS; β , plasmid encoding T β S; or S, plasmid encoding TSS. This figure is a composite of two western blots produced under identical conditions, one for *in vitro* samples, and one for *in vivo* samples. Bands for *in vitro* samples appear darker than *in vivo* samples only because *in vitro* reaction ribosomes were resuspended in a smaller volume of buffer. **(b)** C-GFP49S nascent chains were detected on ribosomes derived from *E. coli* cultures as described for tailspike nascent chains. V, empty vector; G, C-GFP49S. Dots indicate the expected sizes of tailspike and GFP nascent chains.



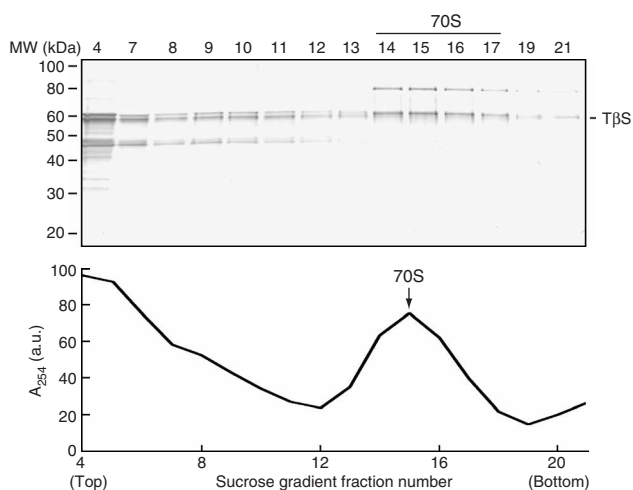


Figure 3 | T β S nascent chains are ribosome-associated. Top, lysates of *E. coli* cultures expressing T β S were separated by sucrose density gradient centrifugation, and the gradient was fractionated. T β S was detected by western blotting as in **Figure 2**. T β S is present in top gradient fractions (released chains) and 70S fractions. Bottom, A_{254} profile of gradient fractions, showing accumulation of 70S ribosomes in fractions 14–17.

sequence to suppress the release of stalled nascent chains¹⁸. We isolated ribosomes and analyzed them as described above. As with *in vivo* translation, we detected each of the tailspike nascent chain lengths by western blotting, with minimal background from other tailspike translation products (**Fig. 2a**).

SecM-directed stalling of short nascent chains

We also tested SecM-directed translation stalling using a completely unrelated sequence, C-GFP49S, which consists of a tetracycline fluorescein-based biarsenical dye (FLAsH) binding motif (CCPGCC) followed by the N-terminal portion of green fluorescent protein (GFP)¹⁹ (residues 1–64), fused to the SecM stall sequence (**Fig. 1b**). FLAsH binds specifically to this optimized tetracycline motif²⁰, and upon binding FLAsH fluorescence emission intensity increases dramatically²¹. We detected stalled C-GFP49S and bands corresponding to two larger species (~23 and 27 kDa) by fluorescence emission after denaturing gel electrophoresis of ribosomes prepared from *E. coli* cultures (**Fig. 2b**). The 23-kDa band was present in both the empty-vector control and C-GFP49S lanes, and it is most likely SlyD, an endogenous *E. coli* metal binding protein with high affinity for the FLAsH dye. The 27-kDa band may represent C-GFP49S still attached to tRNA or multimeric C-GFP49S nascent chains. Multimerization of C-GFP49S chains at the tetracycline motif via disulfide bond formation is known to occur during sample processing²⁰. Nevertheless, the major band is the expected 9.4-kDa monomeric C-GFP49S.

Separation of stalled ribosomes from released chains

To verify that tailspike nascent chains were ribosome-associated, we separated a lysate of *E. coli* expressing T β S on a 10–30% sucrose gradient (**Fig. 3**). We detected an intense band at 60 kDa, corresponding to released T β S, in top gradient fractions. We expected some release of chains given the relatively long (30 min) induction time. The T β S band decreased in intensity until 70S

Table 1 | SecM-directed translation stalling efficiency *in vivo* and *in vitro*

Nascent chain	Length to stall point (aa)	mAb 236 K_d (nM)	Percent stalled <i>in vitro</i> ^a	Percent stalled <i>in vivo</i> ^a
TFS	685	3 ± 1	21 ± 1	26 ± 4
T β S	575	6 ± 4	23 ± 1	52 ± 16
TSS	248	0	34 ± 3	13 ± 4

^aValues reported are the average of three determinations. Error is given as the standard deviation from the mean.

fractions, in which the intensity increased sharply. A band at 80 kDa also appeared in 70S fractions, which may represent T β S nascent chains still attached to tRNA. The 47-kDa band in fractions 4–12 is most likely the result of proteolytic degradation of released T β S.

Nascent chain stalling efficiency

To determine the efficiency of SecM-directed stalling *in vivo* and *in vitro*, we calculated the percentage of ribosomes bearing a stalled nascent chain. We determined the amount of stalled tailspike nascent chain detected by western blotting by comparison with a purified tailspike dilution series, and calculated the total concentration of ribosomes using the relationship 1 A_{260} unit = 23 pmol 70S ribosomes²². The percentage of ribosomes bearing a stalled tailspike nascent chain is listed in **Table 1**. In general, stalling was more efficient *in vivo* than *in vitro*.

Nascent chain detection by FLAsH fluorescence

As an alternative method to detect nascent chains on stalled ribosomes, we introduced the tetracycline FLAsH binding motif into the tailspike sequence, replacing residues 102–107 at the proteolytically labile junction between the N-terminal domain and the central β -helix domain²³, producing constructs C-TFS, C-T β S and C-TSS (**Fig. 1c**). We labeled each lysate with FLAsH, and prepared stalled ribosome–nascent chain complexes by centrifuging cell lysate supernatants through sucrose gradients. We observed a fluorescence emission maximum at ~528 nm for all fractions (data not shown), as previously described for FLAsH²¹. Fluorescence emission intensity was maximal in top gradient fractions (corresponding to excess free FLAsH) and decreased along the gradient, with the exception of an emission peak in 70S ribosome-containing fractions (**Fig. 4a**). We also separated FLAsH-labeled C-GFP49S lysates on sucrose gradients and measured the fluorescence emission of gradient fractions. As for stalled tailspike chains, there was a distinct peak of FLAsH fluorescence in ribosome-containing fractions (**Fig. 4b**).

Measuring cotranslational tailspike β -helix folding

One advantage of producing stalled ribosome–nascent chain complexes *in vivo* is the ability to easily and inexpensively produce large amounts of stalled nascent chains for biophysical assays. For example, we and others have previously characterized a panel of tailspike monoclonal antibodies that are sensitive to changes in tailspike conformation during *in vitro* refolding^{15,17}. One such antibody, monoclonal antibody 236 (mAb 236), recognizes native trimeric tailspike with subnanomolar affinity, yet has no affinity for early *in vitro* refolding intermediates¹⁷. The mAb 236 binding epitope is located in the center of the tailspike β -helix domain (**Fig. 1c**)¹⁷, and is therefore present in the sequence of the TFS and

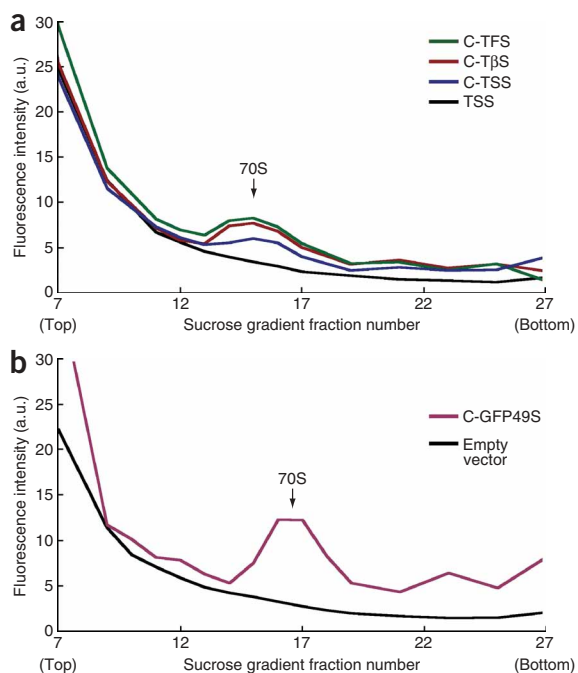


Figure 4 | Stalled ribosome-nascent chain complexes detected by FLAsH binding. (a,b) Lylysates from cells expressing C-TFS ('C' denotes incorporation of the CCPGCC FLAsH binding motif as shown in Fig. 1c), C-TβS, C-TSS, TSS, C-GFP49S or empty vector were separated by sucrose density gradient fractionation, and the fluorescence of the gradient fractions was measured. Fractions containing ribosomes, as judged by their absorbance at 254 nm (A_{254}) and sedimentation position (70S), showed a peak in FLAsH fluorescence emission intensity. Cells expressing TSS chains, lacking the FLAsH binding motif, or empty vector, emitted considerably less fluorescence in ribosome-containing fractions. Tailspike nascent chains (a); GFP nascent chain (b).

TβS constructs. To determine a dissociation constant (K_d) for the binding of mAb 236 to each of the tailspike nascent chain lengths, we prepared serial dilutions of ribosomes bearing each construct and measured mAb 236 binding using the competition enzyme-linked immunosorbent assay (ELISA)^{24,25}. Not surprisingly, mAb 236 had no affinity for the shortest construct (TSS), which lacks the mAb 236 epitope (Fig. 5). mAb 236 bound tightly to TβS and TFS tailspike nascent chains ($K_d = 6 \pm 4$ and 3 ± 1 nM, respectively), in contrast to the complete lack of mAb 236 binding to early tailspike *in vitro* refolding intermediates¹⁷.

DISCUSSION

We have shown that ribosome translation of four constructs stalled dramatically when we fused these constructs to the 17-residue SecM stall sequence. Stalling occurred on ribosomes derived from intact *E. coli* cells and *in vitro* translation reactions. *In vivo* and *in vitro*, stalled ribosomes accounted for 13–52% of the total ribosome pool. The percentage of stalled ribosomes might be even higher inside intact cells, as it is unknown if some ribosome complexes are disrupted during cell lysis. Regardless, it is expected that some stalling inefficiency (that is, free ribosomes plus released chains) is required in order to maintain cell viability. Indeed, negative effects on cell growth have been observed during expression of very short SecM stall fusions (ref. 14 and M.S.E., K.G.U., M.-A.F. and P.L.C., unpublished observations). Stalling was less

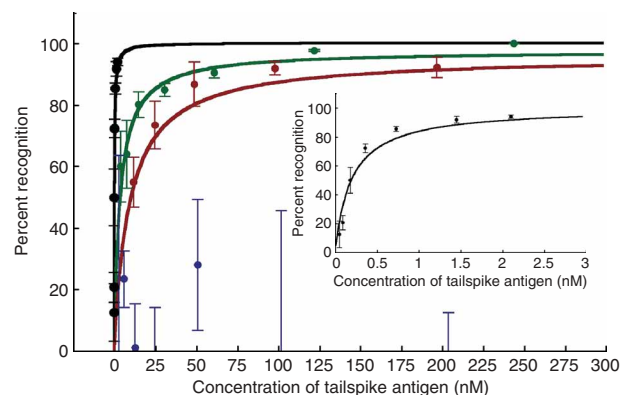


Figure 5 | Measurements of mAb 236 binding to stalled tailspike nascent chains and native tailspike trimer. Green, TFS; red, TβS; blue, TSS; black, native tailspike. Inset, mAb 236 binding to native tailspike trimer, using an expanded x-axis scale. In both, the concentration of native tailspike is expressed as the concentration of trimer, whereas the concentration of nascent chains is expressed as the concentration of monomer. Data shown are from one representative competition ELISA experiment; measurements for each concentration were performed in triplicate. Error bars represent s.d.; $n = 3$. Owing to the large error inherent to measuring very low or zero amounts of binding, some TSS data points lie beneath the x intercept. Lines represent the best fit to the data using the equation described in Methods.

efficient for nascent chain lengths > 800 aa (data not shown). Potentially, the increased frictional drag exerted on very long nascent chains is sufficient to 'pull' the nascent chain past the stall point. Moderate-length constructs, however, produce stalled ribosome-associated nascent chain complexes that accumulate to levels well within the detection limits of the assays used here. Notably, despite the tenfold difference in length between the longest (TFS) and shortest (C-GFP49S) nascent chains, both stalled efficiently, demonstrating that SecM-directed stalling is a robust method suitable for studying nascent chains of widely varied lengths.

The stalling of the TβS construct *in vivo* was considerably more efficient than the stalling of the other constructs. The enhanced stalling could be due to an association between the ribosome and the nascent chain, as newly synthesized tailspike chains were previously shown to interact with the ribosome post-translationally⁸. In the TβS construct, we expected the entire tailspike β-helix domain to be exposed outside the ribosome exit tunnel, where it might interact with the ribosome surface, possibly even after chain release. Thus, the high TβS stalling percentage could be a sum of SecM-stalled TβS chains and released TβS chains still associated with the ribosome surface. Synthesis of additional downstream tailspike residues, followed by the SecM stall sequence, could weaken this interaction, accounting for the lower stalling efficiency of TFS. Likewise, the absence of the β-helix domain in the TSS construct might explain its lower stalling efficiency.

For TβS, SecM-directed stalling produces a much higher percentage of stalled ribosomes *in vivo* than *in vitro* (52% versus 23%). Although *in vitro* translation lysates contain all factors necessary for translation, they are nevertheless more dilute and lack the complexity of the cellular environment. The translation apparatus used *in vitro* may thus not fully replicate all aspects of *in vivo*

translation, and these aspects might influence translation stalling. For example, macromolecular crowding might promote a ribosome–nascent chain interaction *in vivo*, as described above for T β S. The altered translation environment might explain the less efficient and more equivalent stalling of the three tailspike nascent chain constructs *in vitro*.

SecM-derived translation arrest provides a very simple method for producing stalled, ribosome-associated nascent chain complexes of virtually any defined length and sequence *in vivo*, opening up the possibility of a wide variety of biophysical measurements of nascent chain conformations and interactions, including experiments performed inside intact cells. For *in vivo* experiments, it would be necessary to remove background signals arising from released nascent chains, possibly by use of a C-terminal *SsrA* degradation tag encoded after the SecM stall sequence. Also, the SecM-directed nascent chain stalling method described here might be optimized to modify the interaction with the residues in the *E. coli* ribosomal exit tunnel. For example, a recent report described the stalling of nascent chains using only residues 161–166 (GIRAGP) of the SecM stall sequence²⁶, suggesting stall positions 150–160 could be mutated to promote super-stalling during translation *in vitro*. Such dramatic stalling might, however, be incompatible with expression *in vivo*, given that some ribosomes must unstick in order to translate cellular proteins¹⁴.

The nascent chain antibody binding results are exciting for several reasons. K_d measurements permit quantitative (rather than qualitative) comparisons of the binding of conformation-sensitive monoclonal antibodies to tailspike nascent chains of different lengths, allowing for a better description of tailspike cotranslational folding. For mAb 236, the binding to ribosome-associated nascent chains is in good agreement with previous results, which showed that other native tailspike antibodies can recognize conformations adopted by full-length, newly synthesized, ribosome-bound tailspike polypeptide chains, even though these conformations are not populated early during refolding *in vitro*⁸. mAb 236 binds tightly to both native tailspike trimer and longer tailspike nascent chains. Perhaps most exciting is the similar affinity of mAb 236 for TFS and T β S nascent chains, indicating that the tailspike β -helix domain can not only fold cotranslationally, but can fold prior to the completion of chain synthesis, concomitant with the appearance of the β -helix domain sequence at the ribosome surface. Wild-type tailspike is prone to aggregation during refolding *in vitro*²⁷, and previous studies have shown that the correct formation of the β -helix structure is a crucial junction for tailspike folding^{28,29}. Folding of the β -helix domain prior to chain release from the ribosome may therefore represent a mechanism to suppress tailspike aggregation *in vivo*.

Some biophysical measurements, such as NMR, require large quantities and concentrations of stalled ribosome–nascent chain complexes; these large samples would be technically impossible or prohibitively expensive to produce using traditional ribosome stalling methods based on *in vitro* translation of truncated mRNAs. SecM-directed translation stalling facilitates the production of large quantities of stalled ribosome–nascent chain complexes in conventional *E. coli* cultures. This opens up the possibility of a wide range of nascent chain conformation and interaction studies, including chain length– and sequence-dependent binding of molecular chaperones and/or membrane targeting processes.

METHODS

Plasmids. We obtained the gene encoding SecM from plasmid pNH21¹⁴. Preparation of plasmids encoding SecM stall fusion constructs is described in detail in **Supplementary Methods** online.

Isolation of ribosomes. We transformed *E. coli* strain BL21(DE3)-pLysS with one of the SecM stall construct plasmids, and grew the cells overnight at 37 °C in 25 ml of Luria-Bertani (LB) broth supplemented with 100 μ g/ml ampicillin. We inoculated 100 ml of LB broth supplemented with 100 μ g/ml ampicillin with 2 ml of the overnight culture and grew for 3.5 h at 30 °C. We then induced the cultures with 500 μ M isopropyl- β -D-thiogalactopyranoside (Fisher) and grew as before for an additional 30 min. We then added two 8-ml R-buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 150 mM KCl) ice cubes to each culture flask and placed the flasks on ice to quickly cool the cultures to <4 °C. Next, we pelleted cell cultures from each flask (typically an equal volume of empty vector and tailspike-stall fusion cultures) by centrifugation at 5,000g for 10 min. We then resuspended pellets from each culture in 200 μ l of cold R buffer and transferred to a microcentrifuge tube. We froze the resuspended cells at –80 °C for 30 min. To lyse the cells, we thawed them briefly in a 15–25 °C water bath, added 75 μ l of 10 mg/ml lysozyme (Promega), and incubated on ice for 15 min. We performed a second freeze-thaw cycle, which resulted in efficient lysis. To reduce the viscosity of the lysates, we added 50 μ l of RNase-free DNase (Ambion) and 50 μ l of 1 M MgSO₄ and incubated the lysates for 15 min on ice. We then spun the lysates at maximum speed for 5–8 min in a microcentrifuge at 15–25 °C until debris was well pelleted. We isolated the ribosomes from the cleared lysate in a procedure modified from⁶: we layered a 300- μ l volume of cleared lysate on top of a 2.5 ml 35% sucrose cushion prepared in R buffer. We then centrifuged the samples for 15 min at a maximum RCF of 438,000g in a Beckman Optima MAX-E benchtop ultracentrifuge, using a TLA 100.3 rotor. Next, we rinsed the ribosomes with cold R buffer, and then resuspended them in R buffer by gently swirling for 15 min at 4 °C. For fluorescence measurements, we labeled the cell lysates with 19 μ M FIASH dye (synthesized as described²³) on ice for 1 h. Then, we loaded the lysate supernatants onto 10–30% linear sucrose gradients prepared in R buffer. Next, we centrifuged the gradients at 4 °C in a Beckman Optima-L 90K ultracentrifuge to reach an accumulated centrifugal effect value of 2.3×10^{11} rad²/s. We then fractionated the gradients at 4 °C using a density gradient fractionation system (Teledyne ISCO).

***In vitro* transcription-translation reactions.** We used a T7 coupled transcription-translation system for circular DNA (Eco-Pro, Novagen) to generate stalled ribosome complexes *in vitro*. We prepared the reactions according to the manufacturer's directions, using 5 μ g of plasmid DNA supplemented with 1 μ l of RNasin (Promega) and 3 μ g of the anti-*SsrA* oligonucleotide (5'-TTAA GCTGCTAAAGCGTAGTTTTTCGTCGTTTGCG-3'). We incubated the reactions for 1 h at 30 °C and quenched with 250 μ l of ice-cold R buffer. We then isolated the ribosomes by centrifugation through a 35% sucrose cushion as described above.

Western blotting. We performed western blotting according to a procedure modified from³⁰. A detailed procedure is included in **Supplementary Methods**.

In-gel detection of fluorescently labeled nascent chains. We used the Lumio Green Detection Kit (Invitrogen) to visualize the C-GFP49S nascent chain in a denaturing polyacrylamide gel. We prepared and visualized samples according to the manufacturer's protocol. Briefly, we mixed resuspended ribosomes with Lumio gel sample buffer and labeled by mixing with 20 μ M FIAsh. We followed this with incubation at 70 °C for 10 min. We then cooled the samples to 15–25 °C, mixed them with the in-gel detection enhancer, incubated 5 min at 15–25 °C and separated the constituent proteins by denaturing polyacrylamide gel electrophoresis³¹ using a 14% acrylamide gel (Bio-Rad). We visualized bands using a UV trans-illuminator equipped with a digital camera (Kodak) and digitized the gel image using an ethidium bromide filter with a 10-s exposure.

Fluorescence measurements. We diluted sucrose gradient fractions 1:5 in R buffer and measured fluorescence excitation and emission spectra using a PTI QM-6 fluorescence spectrometer. We measured fluorescence emission from 520–600 nm with a constant excitation wavelength of 505 nm. Excitation and emission slit widths were 5 nm. We measured the samples in quartz cuvettes and used a circulating water bath to maintain the temperature at 25 °C. We recorded the fluorescence intensity at the wavelength of maximum emission (528 nm) for each fraction.

Antibody binding measurements. We prepared a dilution series of ribosomes bearing TFS, TBS or TSS nascent chains and measured the binding of mAb 236 using the competition ELISA^{24,25}. We determined concentrations of nascent chains as described for calculations of stalling efficiency and determined mAb 236 binding to native tailspike trimer as described previously¹⁷. We fit the data by nonlinear regression to the equation:

$$f = \frac{x}{x + K_d}$$

where f represents the fraction of bound antibody and x , the nascent chain concentration.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

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