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In vitro reconstitution of translational arrest pathways

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ABSTRACT

Protein translation is tightly regulated to ensure high-fidelity expression of genetic information. Various conditions cause ribosomes to stall while synthesizing new proteins. Different types of translational arrest initiate specific mRNA surveillance, protein quality control, and stress response pathways that directly impact gene expression and protein homeostasis. Our understanding of these pathways is greatly enhanced by reconstituting these processes in cell-free systems. The high degree of biochemical manipulability of in vitro systems facilitates the identification of key machineries, mechanistic dissection of their functional roles, and structural analysis of intermediate complexes. Here, we describe principles and methods for reconstituting and analyzing translational arrest pathways in cell-free translation systems using rabbit reticulocyte lysate as an example. These approaches can be exploited to dissect various fundamental, regulatory, and quality control mechanisms of eukaryotic protein translation.

1. Introduction

Protein biosynthesis is the most energetically costly process in dividing cells, and is highly regulated to ensure efficient and faithful translation of genetic information. While the initiation step of translation has long been appreciated to be a point of regulation [1], research over the past decade has discovered that various steps during translation elongation are both monitored and regulated. Ribosomes may pause or stall during protein synthesis for numerous physiological and pathological reasons [2–4]. These include mutated or damaged mRNAs, tRNAs, or ribosomal components, as well as cellular insults, such as nutrient deprivation or pathogenic invasions. Different types of translational arrest elicit specific downstream effects, including mRNA surveillance, protein quality control, and cell-wide stress responses (Fig. 1). These translational arrest pathways directly affect gene expression and protein homeostasis.

For example, ribosomes stall on mRNAs that are aberrantly truncated ('no-go' mRNA) or that lack an in-frame stop codon ('nonstop' mRNA). These stalled translation complexes initiate specific mRNA surveillance pathways to degrade the faulty mRNAs [5,6], as well as the ribosome-associated quality control (RQC) pathway to degrade the partially-synthesized nascent protein [7,8]. In contrast, ribosomes that stall due to tRNA insufficiency or mutation appear to engage different cellular machinery to activate the integrated stress response, which inhibits bulk protein translation to promote cell survival [9,10]. We still lack a clear understanding of how cellular machinery distinguish different stalled ribosomes from each other and from translating ribosomes, as well as the mechanisms that activate and mediate the functional consequences downstream of specific types of translational arrest.

Understanding translational arrest pathways requires reconstituting these processes in biochemically-defined systems that permit mechanistic dissection. Studying these pathways in vitro requires three basic components: (i) methods to generate and stabilize translational arrest intermediates, (ii) methods to assay and isolate arrested products, and (iii) strategies to functionally reconstitute translational arrest pathways in progressively more purified systems. Once established, these core strategies can be combined in a variety of ways to address different mechanistic and structural questions.

Cell-free in vitro translation (IVT) systems are an attractive option for studying translational arrest pathways due to the ease of biochemically manipulating these systems for mechanistic analyses. In particular, rabbit reticulocyte lysate (RRL) has been the basis of the predominant mammalian IVT system for over four decades, primarily due to the ability to reproducibly obtain large amounts of RRL that is highly efficient in protein translation [11]. Reticulocytes are immature red blood cells at the penultimate stage of differentiation, in which the nucleus has already been lost, but protein synthesis (primarily of hemoglobin) continues robustly

Abbreviations: RRL, rabbit reticulocyte lysate; IVT, in vitro translation; RNC, ribosome-nascent protein complex; rpm, revolutions per minute.

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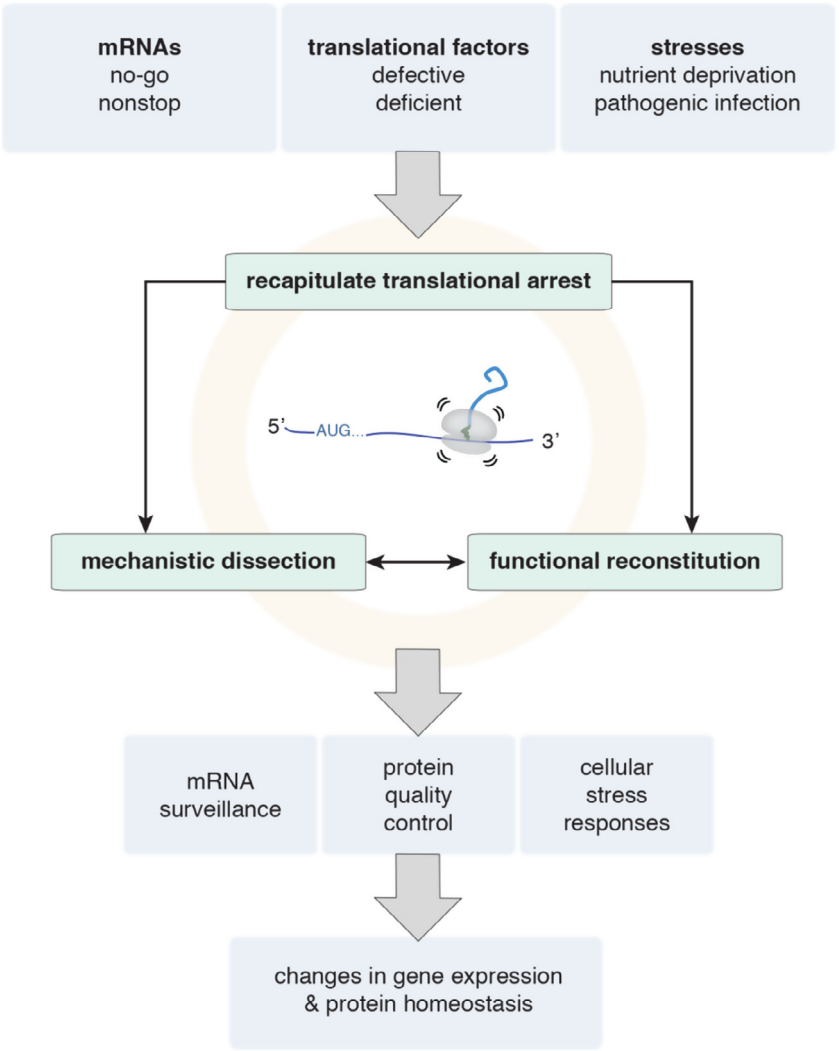

Fig. 1. Overview of translational arrest pathways. Various reasons (top; blue boxes) cause translational arrest and ribosome stalling (center), which induce cellular pathways (bottom; blue boxes) that influence gene expression and protein homeostasis. Recapitulating translational arrest in cell-free systems permits mechanistic dissection of these pathways and paves the way towards reconstituting these pathways with minimal components (green boxes and thin arrowheads). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

utilizing preexisting ribosomes, mRNAs, tRNAs, and translation factors [12]. Although reticulocytes normally account for only 1–2% of circulating blood cells, inducing anemia with phenylhydrazine drastically increases the production of reticulocytes such that they accumulate to 80–90% of the circulating blood cell population in an effort to replace damaged red blood cells. These "stress" reticulocytes are easily isolated and hypotonically lysed to generate highly concentrated cytosolic lysate that is directly amenable for reconstituting protein translation and posttranslational activities [11]. Treating RRL with a calcium-dependent nuclease degrades endogenous mRNAs, producing an IVT system that can be programmed with exogenous mRNA [11,13]. This IVT system has been used to study numerous protein biosynthesis and quality control pathways, including secretory and membrane protein targeting and insertion into the endoplasmic reticulum [14–17], protein folding and complex assembly pathways [18,19], and identifying protein quality control activities [20–24].

Reconstituting translational arrest using the RRL IVT system opens the door to a wide range of experimental analyses. Both the nascent protein and mRNA can be specifically labeled in IVT reactions to follow their respective fates. The biochemical manipulability of the RRL IVT system permits complicated processes to be precisely staged and broken down into individual steps for mechanistic studies. The ability to fractionate the biochemical components of the system allows for specific depletion and replenishment of individual factors (or mutant variants) to assay their functions, as well as the isolation of substrate complexes that can be used to identify interacting partners or for structure determination [23,25–28]. Here, we describe general strategies for using this RRL-based IVT system to reconstitute and generate functional components of translational arrest pathways for these types of mechanistic analyses. These approaches can be readily adapted to other cell-free translation systems derived from yeast, wheat germ, and mammalian cells.

2. Materials

Most equipment and consumables needed for these experiments are commonly found in typical cell biology, biochemistry, or molecular biology labs (Appendices A and B). Although commercial RRL-based in vitro translation (IVT) kits are available, we use a

system assembled in-house that is more economical and permits more facile and extensive biochemical manipulations. The basis for this IVT system was previously described in detail and consists of two mixes that carry out the sequential reactions of transcription (using the "T1" mix) and translation (using the "cT2" mix) [15]. The reagents needed to assemble these mixes are described in Appendices C and D. In this section, we summarize how to generate this linked in vitro transcription and translation system, which incorporates the following modifications that:

(i) replaces the reducing agent DTT with the physiological reducing agent glutathione in the translation (cT2) mix,
(ii) removes hemin from the IVT system. Hemin was originally included to repress the translational inhibition activity of heme-regulated kinase [12]. However, assays needing only a single round of translation are unaffected by eventual eIF2α phosphorylation, which now can also be inhibited by ISRIB if desired [29,30],
(iii) concentrates the translation (cT2) mix to permit more room for experimental manipulations and to reduce the reliance of the cT2 mix performance on components of the transcription (T1) mix, which facilitates the use of purified mRNA transcripts from other sources.

2.1. Preparation of transcription (T1) mix

The T1 mix makes up 76% of transcription reactions by volume (see Section 3.1), and contains the following components, whose final concentrations in the total transcription reactions are: 40 mM Hepes pH 7.6, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 0.5 mM ATP, 0.5 mM UTP, 0.5 mM CTP, 0.1 mM GTP, and 0.5 mM 7-methyl di-guanosine cap analog (CAP). To facilitate its preparation, we typically prepare concentrated stocks of the buffer components (10× RNA polymerase buffer), nucleotides (10× 4-NTP mix), and 5 mM CAP separately and store these in aliquots at −80C (see Appendix C). This simplifies the assembly of T1.

To prepare 760 μL of the T1 mix, which is sufficient for 20 mL of translation reactions, assemble the reagents on ice in the following order:

RNase-free H_2O	460 μL
10× RNA polymerase buffer	100 μL
10× 4-NTP mix	100 μL
5 mM CAP	100 μL
Total	760 μL

Mix well, aliquot the T1 mix into working stocks (100 or 200 μL), flash freeze in liquid nitrogen, and store the aliquots at −80 °C. When thawed quickly, kept on ice, and re-frozen in liquid nitrogen, the mix is stable for at least five freeze-thaw cycles, and can be kept for at least a year at −80 °C without noticeable loss of activity.

2.2. Preparation of translation (cT2) mix

The cT2 mix, which makes up 50% of translation reactions, contains the following: 70% RRL by volume, 40 mM Hepes, 20 mM KOH, 100 mM KOAc, 2 mM ATP, 2 mM GTP, 24 mM creatine phosphate, 0.2 mg/mL tRNA, 80 μg/mL creatine kinase, 4 mM $MgCl_2$, 2 mM reduced glutathione, 0.6 mM spermidine, and 80 μM of each of the 20 amino acids except for methionine. As with the T1 mix, assembly is substantially simplified by preparing all of the individual reagents ahead of time as concentrated stocks (see Appendix D) that are stored in aliquots and combined as needed to generate the cT2 mix. The following procedure describes how to generate 50 mL of cT2 mix from 35 mL of crude RRL, which can be linearly scaled to produce the desired amount.

The most labile constituent of the cT2 mix is the RRL. For this reason, we gather the appropriate amounts of all other reagents to generate the desired amount of cT2 and have them thawed and on ice before thawing the RRL. Thaw the RRL in a room temperature (~20–22 °C) water bath, gently mixing occasionally to minimize local warming of the RRL. Immediately transfer the RRL onto ice as soon as it is completely thawed. If the entire tube of RRL will not be used for this preparation of cT2, freeze the remainder (in aliquots as desired) in liquid nitrogen and store at −80 °C to avoid multiple freeze-thaw cycles.

We first treat the crude RRL with calcium-dependent micrococcal nuclease to digest endogenous mRNAs [9]. To do this, first prepare a 25 °C water bath that is sufficiently deep to immerse the tube containing the RRL. On ice, add 350 μL 0.1 M $CaCl_2$ (1 mM final concentration) and 350 μL of 15,000 U/mL Nuclease S7 to 35 mL of freshly thawed RRL, mix well, and incubate at 25 °C for 12 min. During the incubation, mix the RRL occasionally to ensure even warming. After the incubation, promptly add 350 μL 0.2 M EGTA to chelate the calcium and inactivate the nuclease, then transfer the RRL to ice for immediate assembly into cT2. It is possible to flash-freeze and store nucleased RRL at −80 °C for later assembly, but we find that it is less stable than crude RRL.

To generate the cT2 mix, add the following items in this order on ice, mixing intermittently:

Nucleased RRL	35 mL
1 M Hepes/0.5 M KOH	2 mL
5 M KOAc, pH 7.5	1 mL
100 mM ATP	1 mL
100 mM GTP	1 mL
1.2 M creatine phosphate	1 mL
10 mg/mL liver tRNA	1 mL
20 mg/mL creatine kinase	0.2 mL
2 M $MgCl_2$	0.1 mL
0.25 M reduced glutathione	0.4 mL
100 mM spermidine	0.3 mL
19 amino acid mix	4 mL
RNase-free H_2O	3 mL
Total	50 mL

Invert to mix well. For larger cT2 preps, aliquot the cT2 mix into working stocks (500 μL or 1 mL), flash freeze the aliquots in liquid nitrogen, and store at −80 °C.

Variations to this procedure can be incorporated for specific experimental purposes. For example, the cT2 translation mix can be generated with non-nucleased lysate to study the synthesis of endogenous mRNAs (primarily hemoglobin), or to avoid EGTA interference of calcium-dependent processes [31]. In addition, the RRL can be biochemically fractionated or otherwise modified in various ways before being incorporated into the cT2 translation mix [20], and the cT2 mix itself can withstand some amount of manipulations, such as immunodepletions [20,23]. In general, these modifications minimally impair translation efficiency as long as they do not: (i) deplete essential translation factors, (ii) substantially dilute the RRL, or (iii) significantly alter the small molecule concentrations of the final cT2 mix.

3. Cell-free transcription and translation reactions

For almost all experiments, we utilize the same general procedure for conducting in vitro transcription and translation reactions,

and incorporate minor modifications to achieve specific experimental goals (Section 5). Here, we describe the standard methodology for translating radiolabeled nascent proteins using the linked IVT system described in Section 2. Both the transcription and translation reactions can be scaled linearly up or down as needed.

3.1. Transcription reaction

Linear DNA templates are used for run-off transcription reactions. Historically, the template was produced by restriction digestion of a circular plasmid downstream of (or within) the open reading frame. While still a reasonable option, we find it is more convenient to produce templates by PCR amplification of the open reading frame from a vector (or synthetic DNA fragment, such as gBlocks from IDT) that contains either the SP6 or T7 promoter (Fig. 2A). We favor the SP6 promoter because it consistently gives higher final translation yields. For optimal translation, we have found that it is useful to have a minimum of ~15 nucleotides between the end of the SP6 promoter and start codon. An excessively long 5′ UTR often decreases translation, and should be avoided.

For PCR amplification of the template for transcription, we typically use a forward primer that starts annealing 13 nucleotides upstream of the SP6 promoter, which provides a more stable platform for SP6 polymerase engagement. The reverse primer should anneal within or after the open reading frame as desired. For full-length products, it is important to have at least ~12 nucleotides downstream of the stop codon to ensure efficient termination. Standard PCR conditions (Appendix E) are sufficient to amplify DNA targets.

We then purify the PCR products (e.g. with the QIAGEN PCR Purification kit) into RNase-free water for the transcription reactions. Elution of the products from 100 μL of PCR reaction into 50 μL H_2O usually gives a template concentration of approximately 30–50 ng/μL. The final elution buffer should not contain EDTA (e.g. as found in TE), as this can interfere with transcription. Purified PCR products can be stored at −20 °C and used repeatedly for in vitro translation reactions.

While we have almost always used cloned ORFs in the SP64 vector as the template, it is now feasible to obtain sequence-verified double-stranded DNAs at low cost from commercial sources (e.g. IDT gBlocks). These DNA templates can be used directly for in vitro transcription, or amplified by PCR and purified prior to transcription. Ordering double-stranded DNA templates affords the ability to customize the open reading frame (e.g., codon optimization, epitope tags, mutations) and avoid cloning.

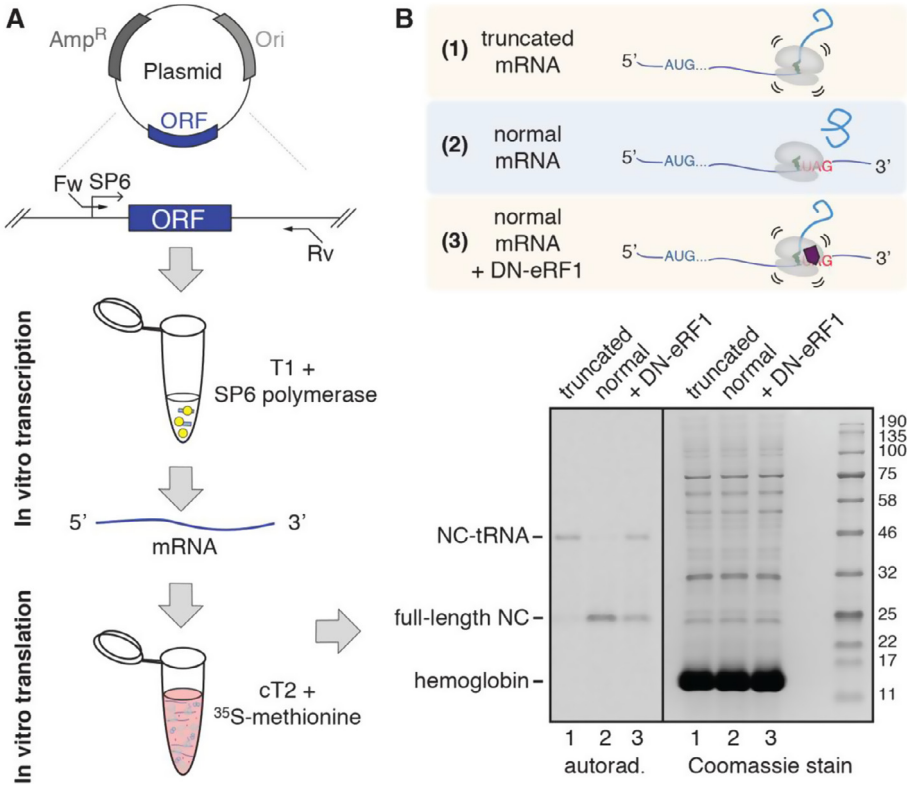

Fig. 2. Linked in vitro transcription and translation. (A) Workflow for linked in vitro transcription and translation reactions. An open reading frame (ORF, blue bar) is first cloned into a vector containing an upstream SP6 promoter (arrowhead). Linear DNA template is generated by PCR using primers flanking the SP6 promoter and the ORF. Purified PCR fragments are used directly as templates for in vitro transcription catalyzed by SP6 polymerase to generate mRNA substrates (T1 reaction). The transcription reaction can be used directly for in vitro translation (cT2 reaction). Here, ^{35}S-methionine is added to the translation reaction, allowing newly synthesized radiolabeled proteins to be monitored by SDS-PAGE and autoradiography. (B) In vitro translation (IVT) reactions of normal and translational arrest products. Upper panel, schematic representation of three IVT reactions of (1) an mRNA truncated within the open reading frame and a normal mRNA without (2) or with (3) dominant negative eukaryotic release factor (DN-eRF1). Lower panel, radiolabeled IVT products were analyzed by SDS-PAGE followed by autoradiography (left) and Coomassie staining (right). Note that the two panels show the same gel. Full-length radiolabeled nascent protein (NC) is indicated. Translational arrest is reflected in a nascent protein-tRNA adduct (NC-tRNA), which is shifted ~25 kD larger due to the size of the tRNA. Because these substrates stall at a valine, this nascent protein-tRNA adduct is stable through SDS-PAGE. The prominent hemoglobin band from RRL seen by Coomassie stain is indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

With purified linear DNA template in hand, assemble the following transcription reaction on ice:

T1 mix	7.6 μL
PCR product (30–50 ng/μL) or other linear DNA template	2 μL
rRNasin	0.2 μL
SP6 polymerase	0.2 μL
Total	10 μL

Incubate in a 37 °C water bath for 1 h.

The transcription reaction is then placed on ice and used directly in the translation reaction without further purification (Fig. 2A). A 10 μL transcription reaction is sufficient for a 200 μL translation reaction. The reaction can be kept on ice for at least one hour while the translation reactions are assembled. It is also possible to flash freeze the transcription reaction in liquid nitrogen and store it at −80 °C for several months without noticeable degradation.

Purified transcript or mRNA (e.g. with the QIAGEN RNeasy kit) eluted in RNase-free water can also be used for in vitro translation. This may be useful in applications in which further modification of the mRNA, such as radiolabeling or the addition of a poly(A) tail, is desired, or for translating mRNAs purified from native sources.

3.2. Translation reaction

For the in vitro translation reaction, assemble the following on ice:

cT2 mix	5 μL
RNase-free H_2O or additives to same volume	4 μL
T1 reaction or purified RNA transcript	0.5 μL
^{35}S-methionine	0.5 μL
Total	10 μL

Incubate in a 32 °C water bath for the desired amount of time. As a very rough guide, translation in this system proceeds ~0.5 residues per second (~10-fold slower than in vivo). Accounting for time needed for the sample to warm to temperature, and the time needed for successful initiation, we typically allow a minimum of 10 min for each 10 kD. Longer incubations may be needed to allow sufficient time for downstream processes, such as protein ubiquitination or other posttranslational modifications, to occur. Shorter incubations can be used in some instances to trap certain translational intermediates (see below).

On average, these IVT reactions synthesize approximately 1 μg of radiolabeled substrate protein per mL of translation reaction. In general, this reflects the yield of one, or perhaps two rounds of translation. Time courses have shown that as assembled, very little extra yield is obtained by longer incubations. Furthermore, the roughly equal protein yield from a truncated transcript lacking a stop codon and a matched terminated transcript further suggests one round of translation (Fig. 2B, lanes 1 and 2). Translations at other temperatures (from ~23 °C up to 37 °C) are also possible. Lower temperatures result in progressively slower translation, while higher temperatures result in rapid loss of translation activity. We have found empirically that 32 °C provides a reasonable tradeoff that permits most relevant biochemistry to occur.

The makeup of the cT2 mix (see Section 2.2) and of the IVT reaction allows for a high degree of flexibility that can be tailored to specific experimental purposes. If radiolabeled protein is not needed for downstream analysis, replace ^{35}S-methionine with unlabeled methionine to a final concentration of 40 μM in the IVT reaction. In addition, 40% of the translation reaction volume is available to incorporate additional components. These may include tagged ubiquitin to isolate and analyze ubiquitinated species (e.g. [20,23]), purified organelles or semi-permeabilized cells to investigate protein targeting (e.g. [22,31]), small molecule drugs (e.g. [26]), or other purified proteins (e.g. [28]). When incorporating such additives, it is important to consider the effects these may have on the performance of the translation system. For example, alterations that change the pH of the system (outside of the ~7.2 to 7.6 range), the salt composition, or the addition of more than 2% (v/v) of organic solvents to the IVT reaction will impair overall protein translation efficiency. Other additives such as small amounts of detergent may not directly impair protein translation, but will interfere with other cellular pathways that occur in RRL, such as chaperone binding to hydrophobic substrates. In our experience, the IVT reaction is tolerant to the addition of ~50 mM Na^+ or K^+, ~0.5 to 1 mM Mg^{2+}, and ~0.5 mM EDTA. Addition of Ca^{2+} in excess of ~0.2 mM risks re-activating the micrococcal nuclease in the RRL, thereby degrading the transcript.

3.3. Analyzing IVT products

We primarily use SDS-PAGE followed by autoradiography on X-ray film or phosphor screens to detect the translated radiolabeled protein (Fig. 2B). In our experience, 0.5 μL of an IVT reaction of an efficiently-translated ~10–40 kD protein is detected as a clear band within 1 h of exposure to film or 15 min of exposure to a phosphor screen. This high specific activity of radiolabeling permits sensitive detection of even minor populations, such as modifications, cleavage, crosslinking products, or ubiquitination, within a reasonable time frame. The translated protein can also be detected by immunoblotting, but this is both less sensitive and less quantitative.

An important limitation to the analysis of total IVT reactions by SDS-PAGE is the high concentration of hemoglobin (greater than 10 mg/ml in the IVT reaction). We find that loading any more than 1 μL of an IVT reaction in a single well of a 15-well, 0.75 mm thick SDS-PAGE mini-PROTEAN gel (Bio-Rad) results in unacceptable smearing and poor resolution. The problem is exacerbated for translation products that migrate near hemoglobin subunits (~14 kD) (Fig. 2B), further limiting the amount that can be analyzed without distortion. The iron and heme in hemoglobin in RRL can also limit other applications, such as assays based on fluorescence, absorbance, or light scattering, as well as certain chemical reactions, such as copper-catalyzed click chemistry or UV-activated crosslinking. These limitations can be bypassed using strategies that specifically enrich for IVT products (e.g. immunoprecipitations), or that deplete hemoglobin. For example, NiNTA resin binds hemoglobin efficiently and fairly specifically [32]. Passing IVT reactions over NiNTA permits the loading of up to ten-fold more volume of translation reaction on SDS-PAGE gels. Other fractionation approaches, such as ion exchange chromatography, can also separate hemoglobin from the products of interest [20,31]. All approaches should be verified for specificity and optimized to reduce experimental variation between individual samples.

4. Detecting and isolating translationally arrested products

In order to analyze and reconstitute translational arrest pathways in cell-free systems, we need to be able to detect and isolate

translationally-arrested products. In this section, we describe some general strategies for recognizing translationally-arrested products in IVT reactions and for isolating these ribosome-associated products.

4.1. Detecting translational arrest products by SDS-PAGE and autoradiography

The first hints of translational arrest can be detected by SDS-PAGE and autoradiography of total IVT products. Translationally-arrested products are usually represented in one of two forms, either as (i) radiolabeled products that are smaller than the expected size of the full-length protein (e.g. Fig. 4), or as (ii) radiolabeled nascent protein-tRNA adducts that are shifted up in size by the 25 kD molecular weight of tRNA (e.g. Fig. 2B). The reason for this is because during translational arrest, nascent proteins remain associated with stalled ribosomes as peptidyl-tRNAs (together referred to as a ribosome-nascent protein complex, or RNC). The stability of the ester bond of aminoacyl-tRNAs varies depending on the identity of the amino acid, with the valyl-tRNA bond being the strongest [33]. Although all of these peptidyl-tRNA bonds are stable in the physiological environments of IVT reactions, most get hydrolyzed in the basic pH and denaturing conditions of SDS-PAGE. Thus, translationally arrested products usually migrate at the size of shortened nascent protein after the tRNA ester bond is hydrolyzed, while those whose ester bonds survive the SDS-PAGE process are detected as nascent protein-tRNA adducts.

Several tricks can facilitate analysis of translationally-arrested products by SDS-PAGE and autoradiography. First, because of the different stabilities of peptidyl-tRNA bonds, we have found it useful, if possible, to engineer model substrates to stall at valine residues (see Section 5.1). This allows us to use the detection of the nascent protein-tRNA adduct as a direct proxy for translational arrest (e.g. Fig. 2B). Performing gel electrophoresis under more native conditions that avoid basic pH may also generally maintain the stability of peptidyl-tRNA products. Alternatively, treating IVT reactions with RNase A (e.g. 50 μg/mL RNase A at room temperature for 15 min) before SDS-PAGE and autoradiography will remove all of the tRNA-adducts and collapse the arrested radiolabeled nascent protein bands to the size of the shortened products. This reduces the heterogeneity of products within the gel lane and more accurately reflects the molecular weight of the arrested nascent protein.

Though these readouts are useful, observations of shortened nascent proteins and nascent protein-tRNA adducts in total IVT reactions are not sufficient to infer ribosome association and translational arrest. Various physiological and experimental conditions—some not related to any instance of translational arrest—can cause these products to become detached from ribosomes. For example, peptidyl-tRNAs can 'slip-out' of ribosomes upon ribosome splitting [23], and shortened products may be generated from alternative start sites or premature termination.

4.2. Isolating total ribosomes by size

In almost all cases when studying translational arrest, it will be necessary to verify that arrested products remain associated with ribosomes (Section 4.2.1) and to isolate these products for downstream assays (Sections 4.2.2 and 4.2.3). The large size of mammalian ribosomes (~3.3 MDa) is a useful molecular handle to accomplish this.

4.2.1. Analytical sucrose gradients

For analytical purposes, native size fractionation using 2 mL 10–50% sucrose gradients cleanly separates soluble proteins from ribosomes (Fig. 3). These gradients comprise five equal steps (400 μL, from the bottom of the gradient to the top) of 50%, 40%, 30%, 20% or 10% sucrose in a ribosome-stabilizing buffer [RNC buffer: 50 mM Hepes pH 7.4, 100 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT]. We build these gradients by pipetting 200 μL of sucrose solution at a time into polyallomer ultracentrifuge tubes (Seton 5011; Appendix A.2), and allow them to equilibrate on ice for at least 30 min. We then directly layer 200 μL translation reactions on top of the gradients and centrifuge the gradients in a TLS-55 swinging bucket rotor in a Beckman Coulter tabletop ultracentrifuge at 55,000 rpm for one hour at 4 °C with the slowest acceleration and deceleration settings. Afterwards, we collect eleven 200 μL fractions by carefully pipetting from the top of the gradient.

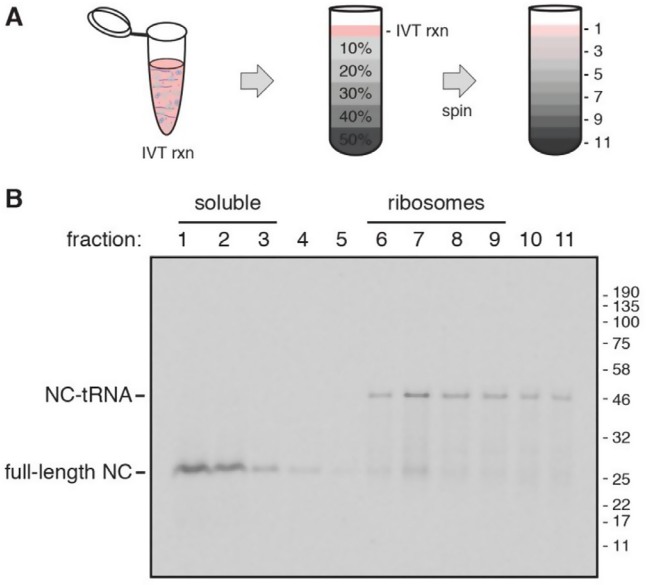

Fig. 3. Size fractionation of IVT reactions by sucrose gradients. (A) Schematic of a 10–50% sucrose gradient, which is built by layering equal volumes of 50%, 40%, 30%, 20%, and 10% sucrose solution from bottom to top in an ultracentrifuge tube. An IVT reaction is layered on top of the gradient, which is then spun in a swinging bucket rotor. After centrifugation, the contents of the IVT reaction will be size fractionated throughout the gradient, with smaller complexes towards the top, and larger complexes running towards the bottom. Eleven fractions are collected sequentially from the top of the gradient for downstream analyses. (B) 10–50% sucrose gradient size fractionation of an IVT reaction of a model mRNA stalled at a stop codon with dominant negative eRF1 (DN-eRF1, as in Fig. 2B). The eleven fractions (1 through 11, from top to bottom) were analyzed by SDS-PAGE and autoradiography. The full-length released radiolabeled nascent protein (NC) migrates in the soluble fractions at the top of the gradient, while translationally-arrested products containing a tRNA adduct (NC-tRNA) migrate in the ribosomal fractions (fractions 6–8).

With these conditions, soluble proteins reliably migrate in fractions 1–3, while single 80S monosomes migrate in fractions 6–8 (Fig. 3), providing a clear separation of released nascent proteins from ribosome-associated translationally arrested products. We can directly analyze these fractions by SDS-PAGE and autoradiography, or use them for downstream analytical or functional assays. If only small amounts of each fraction are required (e.g. only for SDS-PAGE analysis), we often scale down these gradients ten-fold using smaller polycarbonate ultracentrifuge tubes (Beckman Coulter 343775), specific adaptors for the TLS-55 rotor (Beckman Coulter 358615; see Appendix A.2), and adjusted centrifugation settings (55,000 rpm for 25 min with the slowest acceleration and deceleration settings).

Sucrose gradients are useful for resolving heterogeneous populations. For example, these gradients provided an initial hint

that ubiquitination of nascent proteins during the RQC pathway occurs on the large 60S ribosomal subunit instead of on intact 80S ribosomes [23]. Size fractionation gradients can also resolve ribosome-associated products from aggregated proteins, which may not be possible with simple pelleting assays. Sucrose gradient conditions are easily modified to resolve different size ranges of native complexes. The composition and size of the gradients, as well as the spin settings are all adjustable. We used larger 10–30% sucrose gradients spun in the MLS-50 rotor to separate small (40S) and large (60S) ribosomal subunits from 80S monosomes [23]. Continuous gradients spun in floor-model ultracentrifuges and collected with a gradient fractionator offer even higher resolutions, although the concern of radioactive contamination of common equipment makes this approach less compatible with standard IVT reactions.

4.2.2. One-step isolation of bulk ribosomes

While sucrose gradients are useful for analytical purposes, they limit the yields and concentrations of ribosomes recovered and are therefore less practical for generating ribosomal substrates for functional assays. To isolate concentrated ribosomes in bulk, we usually perform a one-step centrifugation of a 1 mL IVT reaction over a 1.6 mL 0.5 M sucrose cushion in RNC buffer in the TLA100.3 rotor at 100,000 rpm for one hour. Afterwards, we aspirate off the supernatant containing soluble proteins and the sucrose cushion. This should leave a glassy yellowish ribosome pellet at the bottom of the centrifuge tube, which we resuspend in an appropriate buffer for downstream applications. Resuspending ribosomal pellets in 50 μL of RNC buffer containing 10% sucrose per 1 mL of IVT reaction should yield a concentration of approximately 1 μM ribosomes (assuming 1 A_{260} unit = 20 nM). Including 10% sucrose in the resuspension buffer helps to reduce damage introduced from multiple freeze-thaws for long-term storage and functional assays. For other applications (e.g. freezing onto cryo-EM grids), the sucrose may be omitted.

Because these ribosomal pellets are quite "sticky", we perform this resuspension step by repetitive careful pipetting with a P-20 pipette: first using a wide-mouth tip (e.g. by cutting off the tip of normal P-20 tips) to crudely resuspend the ribosomal pellet, followed by resuspending with progressively finer pipette tips, such as uncut P-20 tips and ending with P-10 tips to produce a homogenous suspension. During this process, it is important to keep the pellet and buffers ice cold, and to avoid introducing bubbles during the resuspension. For larger amounts of ribosomes (resuspended in more than 200 μL), it may be convenient to gently homogenize the crude mixture with a Teflon pestle in an appropriately-sized glass dounce, or to perform a long incubation with gentle mixing at 4 °C to solubilize the ribosome pellet into buffer.

The final ribosomal mixture should produce an absorbance profile with a homogenous peak at the 260 nm wavelength due to the high rRNA content. Absorbance profiles that lack a strong peak at 260 nm, or that are not consistent between repeated measurements indicate the presence of contaminants. Recovery of a concentration significantly less than 1 μM indicates inefficient pelleting and/or resuspension. The integrity and quality of the isolated ribosomes can be further assessed by size fractionation of sucrose gradients to verify the expected native size, as well as in functional reconstitutions of translation or other ribosome-dependent processes [20,31].

If large amounts of ribosomes are needed, the throughput and purity of ribosomes preparations can be improved by including additional centrifugation steps. As an example, to isolate native ribosomes to reconstitute translation with fractionated lysates, we first spin crude RRL at 100,000 rpm for 40 min at 4 °C, and then spin the resuspended ribosomes from this initial spin on a 1 M sucrose cushion at 100,000 rpm for 1 h at 4 °C as a second clean-up step [20]. This can more than double the throughput of ribosome isolation, since the initial RRL does not need to be loaded onto a sucrose cushion, and is more effective at eliminating contamination, particularly of hemoglobin.

Gel filtration is an alternative strategy for quickly and gently separating bulk ribosomes from soluble proteins. Using a resin that excludes ribosomes, such as Sephacryl S-300, can yield a simple one-step isolation of ribosomes in the void fraction, while smaller protein complexes are retained [34,35]. Although this strategy is less stringent than centrifugation, gel filtration may be preferred to enrich ribosomal complexes that are unstable during centrifugation.

4.2.3. Stripping off peripheral factors with high salt

Sucrose gradients (Section 4.2.1) and one-step centrifugations (Section 4.2.2) in physiological buffer and salt conditions as described above isolate native ribosomal complexes containing peripherally-binding proteins. These include translation initiation, elongation, and termination factors, mRNA-binding proteins, and other factors that associate with ribosomes, mRNAs, or nascent proteins. In some instances, especially when reconstituting translational pathways (see Section 6.2), it may be useful to remove these factors that may interfere or complicate the interpretations of functional assays.

We typically strip off these factors with a high salt wash while isolating ribosomes. Our standard modification is to add 650 mM KOAc to IVT reactions, and to adjust the sucrose solutions (in a gradient or cushion) to a final concentration of 50 mM Hepes pH 7.4, 750 mM KOAc, 15 mM $Mg(OAc)_2$, 1 mM DTT, before performing the isolation procedures as described (Sections 4.2.1 and 4.2.2). For downstream applications, it is important to resuspend the salt-stripped ribosomal pellet back into a physiological buffer. The effectiveness of salt stripping can be analyzed by running equal amounts of ribosomes isolated under native conditions next to salt-stripped ribosomes on SDS-PAGE gels and assaying for peripherally-associated proteins by staining or immunoblotting for specific factors.

4.3. Affinity purification of ribosome-nascent protein complexes

The methods described above separate bulk ribosomes from soluble factors, but it is often desirable to specifically isolate stalled RNCs that comprise only a small fraction (usually 5–10%) of ribosomes in an IVT reaction. One way to accomplish this is to affinity purify RNCs via the arrested nascent protein. To do this in the RRL system, we incorporate an epitope tag at the N-terminus of the substrate protein. Translationally-arrested proteins will remain tethered on ribosomes with the N-terminal epitope tag exposed, permitting selective purification of these RNCs. The 3X-FLAG tag is particularly useful for efficient and clean one-step affinity purifications of RNCs using resin conjugated to the M2 monoclonal anti-Flag antibody (Sigma).

Affinity purifications can be performed directly from IVT reactions (which will isolate both soluble and ribosome-associated tagged products), or from resuspended ribosomes after a size fractionation step (see Section 4.2) to obtain only ribosomal complexes. We typically use 25 μL packed Flag slurry per 1.5 mL translation reaction (or equivalent amount of resuspended ribosomes), and allow the binding reaction to proceed for at least 1 h at 4 °C with slow rotation or gentle mixing. We prefer to perform these in plastic spin columns (Appendix A.1) of an appropriate volume to facilitate subsequent washing steps.

After the binding period, allow the unbound material to flow through the column by gravity flow (or by centrifugation with a

spin column). This flow-through can be saved for analytical purposes. Next, wash the affinity slurry three sequential times, each with at least 30-times the packed resin volume of buffer. Because ribosomes are large with many potential interaction interfaces that may retain them nonspecifically on the resin, we often include additional components in the wash buffers to reduce background recovery of ribosomes that do not contain the arrested nascent protein. If possible, include a small amount of detergent (e.g. 0.1% −0.5% Triton X-100) in the first two washes. Another modification that reduces background is to increase the salt concentration to 250 mM KOAc (or higher). We then generally use the third wash to equilibrate the resin into a physiological elution buffer (e.g. RNC buffer) that lacks these additives to avoid potential interference with downstream applications. As an example, we typically wash affinity purification columns containing 100 μL packed M2 resin with:

(i) 6 mL RNC buffer + 0.1% Triton X-100
(ii) 6 mL RNC buffer + an additional 150 mM KOAc (250 mM final concentration) + 0.5% Triton-X100
(iii) 6 mL RNC buffer (which will also be used for the elution step)

To elute the bound RNCs, incubate the resin with one column volume of 0.1–0.2 mg/mL 3X Flag peptide in RNC buffer. Apply this elution buffer to the top of the resin inside the column, let it fully enter the bed volume of the resin by gravity flow, and then leave the resin in this buffer at room temperature for at least 30 min. The higher temperature and incubation time are both important for increasing the elution efficiency. If dilution is not a concern, the total elution yield can be improved by performing two sequential elutions in this manner.

Compared to a soluble protein containing a 3X Flag tag, both the binding and elution of RNCs is significantly (∼50%) less efficient, probably due to steric issues caused by the large size of ribosomes. If needed, eluted ribosomal complexes can be further concentrated (and the Flag peptide in the elution buffer removed) by centrifugation and resuspending the ribosomal pellet in a smaller volume. This can be accomplished using the settings for bulk ribosomes described in Section 4.2.2. Smaller volumes of eluted ribosomal complexes can be directly pelleted without a sucrose cushion in a TLA120.2 rotor at 100,000 rpm for 40 min at 4 °C. However, concentrating diluted RNC elutions by centrifugation is fairly inefficient (e.g. only ∼ half of the starting ribosomes will be recovered after resuspending the pellet. The rest appears to stick to the sides of the ultracentrifuge tube.). This should be taken into account when determining how much buffer the pellet should be resuspended in, and deciding the overall scale of the purifications.

Similar affinity purification strategies can be employed to isolate ribosomes associated with specific epitope-tagged factors, or engaged with mRNAs that contain aptamers amenable to purification. RNCs purified under native conditions can be directly analyzed for interacting partners by mass spectrometry, or used for structural studies. Purified RNCs, either with endogenous associating factors, or with these factors stripped off with high salt, can also be used as starting substrates for downstream functional assays (see Section 6).

5. Inducing translational arrest in vitro

Several features of the RRL IVT system greatly facilitate its use for studying intermediates of translation, termination, and accompanying arrest pathways. First, single-round translation initiation results primarily in monosome complexes, substantially increasing the homogeneity of RNCs. Second, the slowness of most reactions in cell-free systems relative to in vivo conditions increases the window of time available to trap intermediates. Third, the amount of nascent protein that can be synthesized, together with the scalability of translation reactions to over 10 mls, allows the production of sufficient substrate for a wide range of biochemical and structural studies. The first step to studying a translation-related process of interest is to recapitulate the phenomenon. Below, we describe some basic strategies for inducing specific types of translational arrest in RRL IVT reactions.

5.1. mRNA stalling substrates

Mutated, damaged, and alternatively or aberrantly processed mRNAs are major causes of translation arrest. Programming the RRL IVT system with such stall-inducing mRNA substrates reconstitutes translational arrest and many physiological downstream events [23]. Three predominant types of aberrant mRNAs have been used to study translation stalling. The most extensively studied are so-called 'no-go' mRNAs that are truncated within the protein-coding region. Ribosomes reaching the end of such mRNAs cannot translate further and, at least for a time, remain assembled as an intact ribosome-mRNA-nascent chain complex. This property has been used for many years to assemble and analyze defined intermediates during cotranslational processes such as protein folding, organelle targeting, translocation, and membrane protein insertion [14–16,36]. A second type of aberrant mRNAs is also 'no-go' substrates caused by mRNA secondary structure. The third are 'non-stop' mRNAs that lack a stop codon, as in the case of mutation or premature polyadenylation, which cause ribosomes to stall once they translate into the poly(A) tail.

In cells, ribosomes stalled on these types of mRNAs initiate specific mRNA surveillance pathways to degrade the aberrant mRNAs, as well as the RQC pathway to degrade the truncated nascent protein trapped within the stalled ribosome. Ubiquitination of nascent proteins via the RQC pathway is recapitulated in the RRL IVT system [23]. We have therefore used these model substrates in the RRL IVT system to biochemically reconstitute the early steps of no-go mRNA recognition and the RQC pathway for functional and structural analyses [23,26].

Introducing these mRNA features into the IVT system is straightforward. One can directly incorporate sequences that induce stable RNA secondary structure into the DNA used for in vitro transcription (Fig. 2A, Section 3.1). Truncated mRNA products can be generated by using a reverse primer that anneals within the open reading frame in the PCR reaction that generates the linear template used for the transcription reaction. 'Non-stop' mRNAs can either be genetically encoded or generated by tailing a truncated mRNA with poly(A) polymerase.

5.2. Exogenous translational inhibitors

Translational inhibitors, such as small molecule compounds or dominant negative proteins, also effectively induce general translational arrest in the RRL IVT system. This is useful to elucidate new mechanisms of translational arrest, or to trap specific intermediates of protein translation. To facilitate mechanistic analyses, we usually program IVT reactions with a long model substrate to test general inhibitors, or with a specific model substrate to test dominant negative proteins. This provides a functional readout and potential molecular handle to study translational arrest products (e.g. via an epitope tag on the nascent protein).

The timing of adding translational inhibitors is critical. In almost all cases, introducing translational elongation inhibitors at

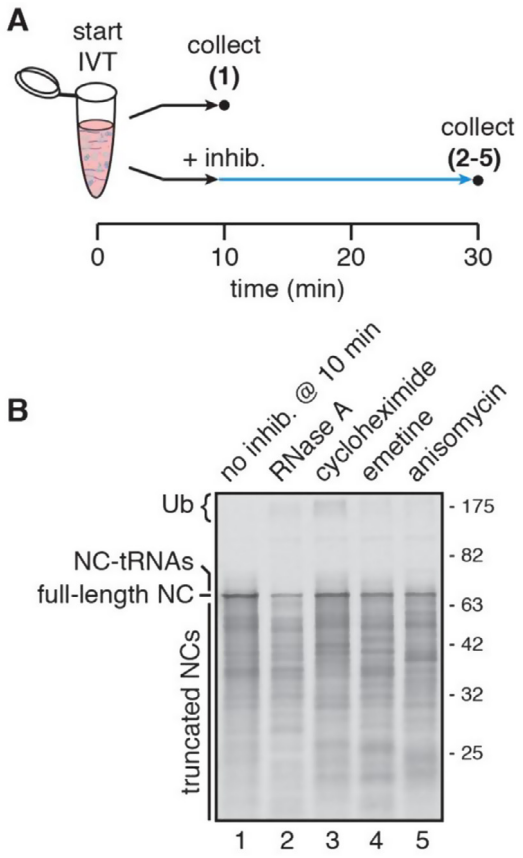

Fig. 4. Translational arrest induced by small molecule translational inhibitors. (A) Schematic and timeline of the experimental procedure for arresting IVT reactions with small molecule inhibitors. IVT reactions are initiated at time 0. After 10 min, a sample of the IVT reaction is collected for analysis (no inhib.), and small molecule inhibitors (50 μg/mL RNase A, 50 μg/mL cycloheximide, 200 μM emetine and 10 μM anisomycin) are added individually to separate aliquots of the IVT reaction. Samples of IVT reactions with each inhibitor are collected at 30 min for analysis. (B) SDS-PAGE and autoradiography analysis of radiolabeled IVT products from reactions with or without addition of inhibitors as in (A). Note that different small molecule translation inhibitors generate different patterns of heterogeneous truncated products. NC – nascent chain; Ub – ubiquitinated nascent chain.

the beginning of the translation reaction will result in no detectable translation product, presumably because the inhibitor inhibits ribosome run-off and recycling needed to initiate translation on the exogenous mRNA. Thus, we usually initiate translation reactions and add in inhibitors at an intermediate stage of translation (5–10 min into the reaction), shortly before full-length products are first observed (Fig. 4A).

Adding different general translation elongation inhibitors, such as cycloheximide or anisomycin, to IVT reactions of the same model substrate traps a heterogeneous mixture of translational arrest products (Fig. 4B), as individual ribosomes translate to different parts of the model mRNA at the time of inhibitor addition. Also, each individual inhibitor generates a distinct pattern of truncated products. This suggests that the mechanism of a given inhibitor is more effective on ribosomes in specific contexts. This is supported by structural, mechanistic, and bioinformatic studies demonstrating that these inhibitors trap ribosomes in different conformational states (for example, preferentially in a non-rotated state with canonical tRNAs, or in a rotated state with hybrid tRNAs) [37]. More work is required to understand how different mRNA sequences affect the transitions between ribosomal conformations, and how these dynamics determine the outcomes of different translational inhibitors.

Because the mechanisms of many common translational inhibitors on mammalian translation remain incompletely understood, caution is warranted in interpreting experiments that employ such inhibitors as general tools to 'freeze' protein translation due to potential unknown off-target effects. However, given sufficient mechanistic information, translational inhibitors are powerful analytical and methodical tools that can be used in conjunction with specific mRNA substrates to biochemically trap distinct ribosomal complexes for downstream mechanistic analyses. We have used small molecules to trap ribosomes at a specific stage of translation elongation [26], as well as dominant negative proteins on defined mRNA substrates to trap endogenous translation termination complexes [28], and to isolate specific functional translational intermediates to biochemically reconstitute downstream pathways [25–27] (see Section 6.2).

5.3. Modifying the IVT system

The biological content of the IVT system itself is another source for recapitulating translational arrest. For example, the specialized nature of rabbit reticulocytes confers a specific endogenous translational arrest activity. In reticulocytes, the translational machinery, particular the tRNA content, is optimized to produce hemoglobin, which accounts for 90% of protein synthesis in these cells [12]. Early comparisons of individual tRNA levels in reticulocytes with the mRNA codon content of hemoglobin revealed that leucyl tRNAs may be limiting [38]. Indeed, it has long been known that exogenous tRNAs are needed to translate certain substrates in RRL [13], forming the basis for generally supplementing RRL IVT systems with tRNA purified from mammalian liver tissue (see Section 2.2), which is not specialized for the translation of a specific protein.

Three out of the six possible leucine codons (UUA, CUU, and CUA) are completely absent from both rabbit hemoglobin subunits, while the UUG codon is only used once (Fig. 5A). To test whether these leucine codons induce translational arrest without exogenous tRNAs, we generated twelve model substrates that each had a test sequence of three sequential leucine codons inserted at amino acid position 127 of a 155-codon substrate (Fig. 5B). Using the most abundant leucine codon (CUG) as a placeholder, we inserted one, two, or three consecutive rare leucine codons into this test sequence. Importantly, all of these model substrates have an identical protein sequence, with the only differences residing in the mRNA sequences. We translated each of these model substrates in the RRL IVT system generated without or with liver tRNA included in the cT2 mix (Fig. 5C, also see Section 2.2).

Compared to size markers for 127-mer and full-length protein substrate (lanes 1–2), this showed that two or more sequential UUA leucine codons causes translational arrest in the absence, but not the presence, of exogenous tRNA (lanes 7 and 10). Using sucrose gradient fractionation, we can verify that these products are ribosome-associated (Fig. 5D). UUG codons also appear to be limiting, as three sequential UUG codons induced translational arrest at specific locations within the model substrate (faint lower bands in Fig. 5C, lane 13) that correspond to where UUG codons are present (Fig. 5B). This implicates that at these transcript levels, all of the available cognate tRNAs to these leucine codons are completely sequestered on ribosomes to induce translational arrest.

This paves the way towards using analyzing and reconstituting downstream events of translational arrest at rare codons (see below), and highlights how the content of IVT systems can be used to recapitulate specific translational activity that can be extended to numerous other purposes. For example, instead of omitting

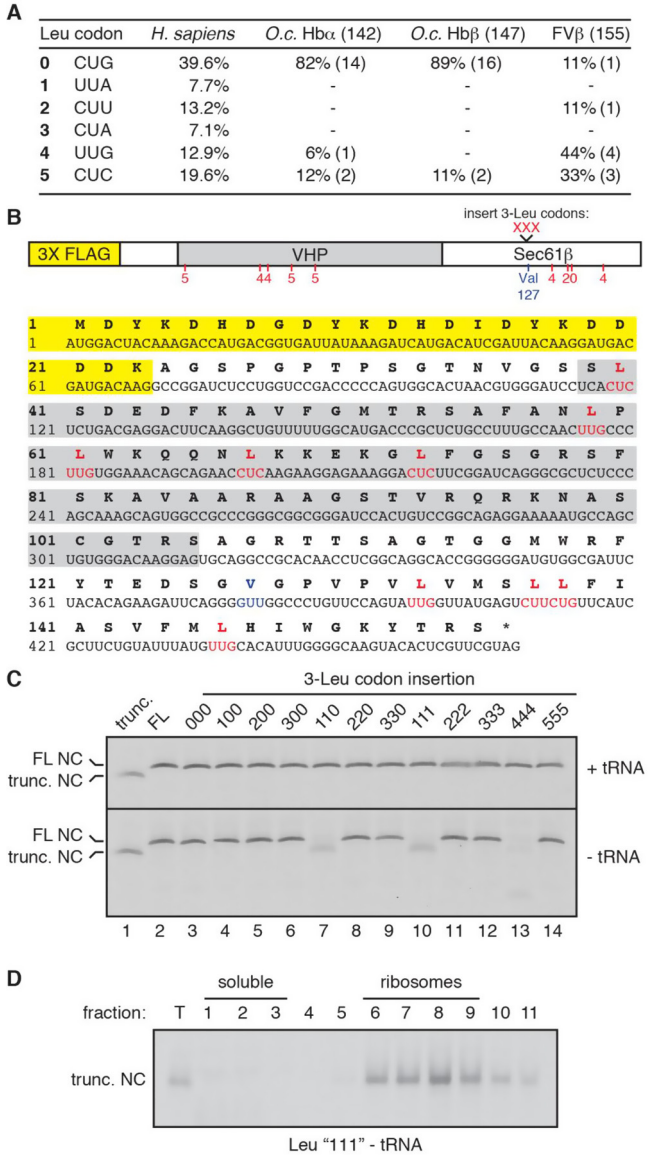

Fig. 5. Translational arrest at rare leucine codons. (A) Leucine usage in hemoglobin. Usage, in percentages, of the six eukaryotic leucine codons (labeled 0 through 5 in bold) in all annotated human cDNAs (*H. sapiens*), rabbit hemoglobin subunits (*O.c.* Hbα and *O.c.* Hbβ), and a model IVT substrate that encodes a 3X-FLAG tag, followed by Sec61beta containing the villin headpiece domain (FVβ). Length of the three proteins and the number of each leucine codon in that protein are indicated in parentheses. (B) Line diagram (upper), amino acid, and mRNA sequence of the model substrate. A series of different 3-Leu codon combinations were individually inserted in front of a valine residue at amino acid position 127 (blue) on the original full-length substrate, which contains an N-terminus 3X-FLAG tag (yellow), the folded villin headpiece (VHP) domain (grey), and Sec61β. (C) IVT reactions of the model substrates with (+ tRNA) or without (− tRNA) exogenous tRNAs included in the cT2 translation mix. IVT products of 14 model substrates as described above were analyzed by SDS-PAGE and autoradiography of ^{35}S-labeled full-length nascent protein (FL NC) and translationally arrested products (trunc. NC). IVT reactions of the original full-length substrate (lane 2) and of the same substrate containing a stop codon after Val127 (lane 1) indicate the size of expected products. The 3-Leu codon combinations inserted before valine 127 are indicated by the numeric codes depicted in panel (A), where 000 stands for three CUG codons in tandem, and 100 means replacing the first CUG with a UUA codon. Note that two or three consecutive UUA codons induce stalling at the test sequence. Arrested products appear as shortened nascent proteins after SDS-PAGE because the leucyl-tRNA ester bond does not survive SDS-PAGE. (D) An IVT reaction of the "111" 3-Leu construct conducted without tRNA was size fractionated on a 10–50% sucrose gradient. Eleven fractions were collected from the top and analyzed by SDS-PAGE and autoradiography. T refers to total IVT reaction. Note that all of the truncated nascent protein product migrates in fractions 6–9, corresponding to where ribosomes migrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tRNA from cT2, RRL can be specifically depleted of certain proteins [23], biochemically fractionated [20], or treated with small molecules or enzymes before being incorporated into cT2 in order to confer specific translational arrest activity. Alternatively, this approach may be especially useful for IVT systems generated from genetically modifiable sources, such as yeast or mammalian cells. In this case, translational arrest activity may be introduced and investigated by knocking out or mutating specific factors in these cells before generating lysates to incorporate into IVT systems. These strategies of modifying the IVT system can be used independently or in combination with defined model mRNAs to investigate specific types of translational arrest.

6. Reconstituting translational arrest pathways

The ability to generate (Section 5) and isolate specific translational arrest substrates (Section 4) opens the door to functionally reconstituting downstream processes in progressively more purified systems. To accomplish this, we first design experimental assays and set benchmark activity levels using the most complete system possible, and then gradually fractionate the components and attempt to rebuild the process while maintaining the same starting activity level. We discuss some considerations for these steps in this section.

6.1. Initial benchmarks and controls

For initial benchmark experiments, we usually first characterize the functional roles of candidate machinery in the complete RRL IVT system by depleting these components and specifically replacing them with purified factors to reconstitute an observed endpoint activity. In the simple example of leucine-stalling (Fig. 5), we can recapitulate translation beyond the stalling sequence by directly adding exogenous tRNA back into IVT reactions (Fig. 6A).

Restarting occurs even if tRNA is added 15 min or 30 min after initiating the IVT reaction (Fig. 6A), although the efficiency of synthesizing the full-length substrate gradually decreases over time. This observation indicates that the restarting activity can be temporally uncoupled from the initial stalling. This implicates that ribosomes stalled at the rare leucine codons can be isolated at an intermediate timepoint (e.g. 15 min into the IVT reaction) and should still be amenable to restarting translation in more minimal systems.

To test if this restarting activity can be physically uncoupled from the IVT reaction, our next benchmark experiment uses the simplest fractionation scheme of separating bulk ribosomes and soluble factors to reconstitute the target activity. For the leucine-stalling example, we isolate bulk ribosomes as described in Section 4.2.2 after 15 min of an IVT reaction without tRNA, and attempt to reconstitute the target activity of restarting translation with ribosome-free cytosolic supernatant (S100) from different sources (Fig. 6B). S100 fractions can be generated from any lysate, which permits the incorporation of genetic modifiable lysates obtained from cultured cells or from yeast. To generate S100 fractions, directly centrifuge the cT2 mix or clarified cell lysate in a TLA100.3 rotor at 100,000 rpm for 1 h at 4 °C to pellet the ribosomes, while saving the supernatant.

The S100 fraction of cT2 could reconstitute the 'restarting' of protein translation on ribosomes stalled at rare leucine codons isolated under both native and high salt conditions (Fig. 6B). Since high salt strips RNCs of peripherally associated proteins (see Section 4.2.3), this demonstrates that all of the factors required to restart translation reside as soluble factors within the cT2 mix, and do not need to be preassembled on the ribosomal complexes. With this starting activity in S100

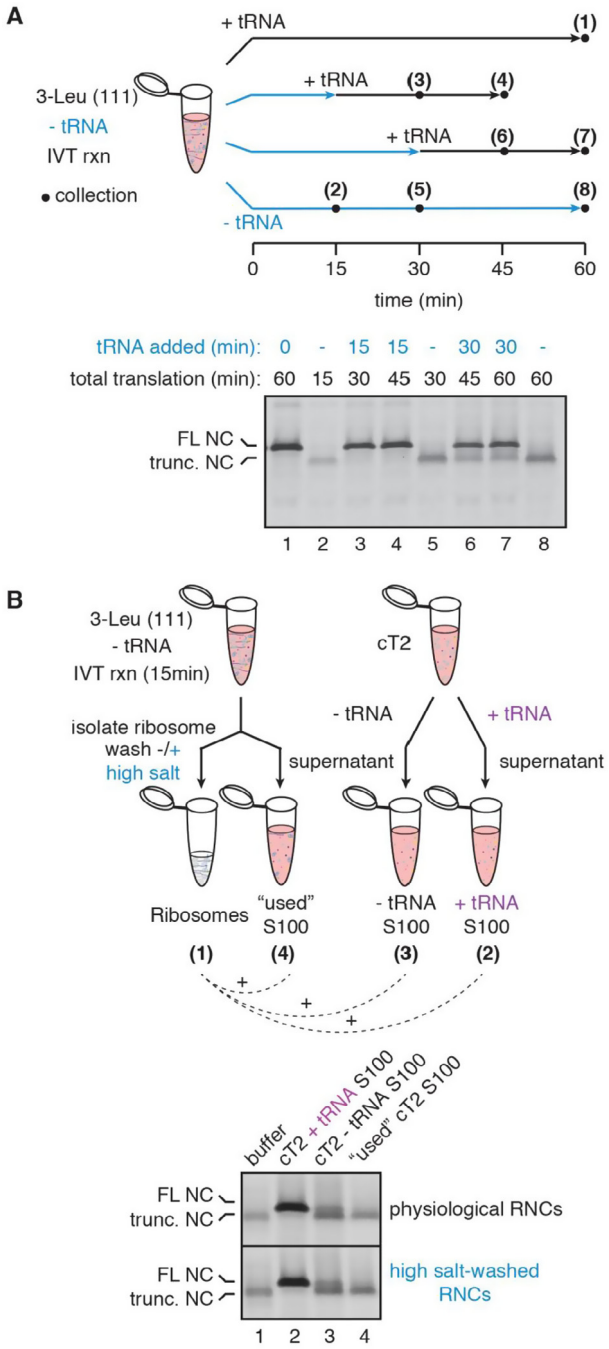

Fig. 6. Restarting translation at rare leucine codons. (A) Rescuing ribosome stalling at rare leucine codons in total IVT reactions. IVT reactions of the 3-Leu "111" construct lacking exogenous tRNAs (cyan arrows) were initiated, and exogenous tRNAs added 0 (lane 1), 15 (lanes 3 and 4), or 30 min (lanes 6 and 7) afterwards. Samples were collected at various time points (black dots, collection points) for SDS-PAGE and autoradiography analyses. Note that adding tRNA at 0 or 15 min fully rescues translation of the full-length protein, whereas adding tRNA at 30 min only partially rescues restarting of translation. This indicates that translational stalling and restarting can be temporally uncoupled. (B) Rescuing ribosome stalling at rare leucine codons with fractionated components. An IVT reaction of the 3-Leu "111" construct lacking exogenous tRNAs was stopped after 15 min. The reaction was subjected to centrifugation to isolate bulk ribosomes under physiological or high salt conditions, and the soluble cytosolic fraction ("used" S100, 2). In parallel, S100 supernatant fractions were isolated from cT2 mix without (3) or with (4) exogenous tRNAs. The isolated RNCs were incubated with buffer (1) or each of the S100 fractions for 15 min at 32 °C. Note that the S100 from the cT2 containing exogenous tRNAs could restart translation on RNCs isolated under both physiological and high salt conditions (lanes 2), whereas none of the other conditions were sufficient to reconstitute restarting. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

serving as a baseline comparison, we can now further fractionate the S100 to identify the necessary components needed to recapitulate this level of efficiency of restarting, and move to progressively more purified in vitro systems. These basic steps were followed to identify the functional roles of the ubiquitin ligase and ribosome splitting factors in the RQC pathway [23,25,27].

6.2. Considerations for isolating starting substrates

A first step towards reconstituting translational arrest pathways with purified factors is to specifically isolate RNCs away from bulk ribosomes. Heterogeneous substrate RNCs is one of the main sources for inefficient reconstitutions. To isolate RNCs that are as homogenous as possible, several sources of potential heterogeneity should be considered.

First, the method of inducing translational arrest (Section 5) determines at what position on mRNA ribosome stalling occurs. For example, truncated mRNAs and the use of dominant negative termination factors specifically stall ribosomes at a single distinct position on mRNA—either at the 3′ end of the mRNA or with a stop codon in the ribosomal A site, respectively [23,25–28]. Ribosome stalling on poly(A) segments is more heterogeneous, as some ribosomes may translate further into the poly (A) segment than others [23], while translation inhibitors generate even more heterogeneous products (Fig. 4). This directly impacts how clear downstream analyses using SDS-PAGE and autoradiography are.

Second, ribosomal conformation is another source of heterogeneity. The two subunits of ribosomes can exist in various rotational conformations relative to each other, with some being more stable than others, or more likely to engage specific translation factors [4,37]. For example, the GTP-dependent elongation factor eEF1A delivers aminoacylated tRNAs to the ribosomal A-site and occupies the universal GTPase center on canonical, or non-rotated, ribosomes [4,37]. In contrast, eEF2 is another GTP-dependent elongation factor that preferentially binds to the same A-site and GTPase center of ribosomes in the 'rotated' conformation to mediate the translocation of hybrid tRNAs [4,37]. In this case, translational inhibitors and dominant negative proteins are more likely to homogenously trap RNCs in a specific conformational state than mRNA-induced stalling.

For example, we recently used the translational inhibitor didemnin B to trap, isolate, and determine the structure of eEF1A-bound RNCs [26]. Even though didemnin B stalls ribosomes at different positions on the mRNA substrate, these ribosomes are all stalled in the same conformation, so that we could average RNC particles from an affinity purification of an IVT reaction into a consensus cryo-EM structure. In contrast, even though truncated mRNA stalls ribosomes at a single position on mRNA, these RNCs then undergo the RQC pathway in the RRL system to various extents, resulting in a heterogeneous set of conformations that represent different steps of this pathway [23].

Stalled RNCs that are not engaged with a translational factor or trapped in a specific conformation by an inhibitor probably dynamically sample multiple conformations. This provides opportunities for other factors to non-physiologically engage with unoccupied RNCs during the time it takes to perform biochemical manipulations and purifications from IVT reactions. Nonphysiological factor binding may lock RNCs into a 'dead-end' conformation that is not compatible with downstream functional reactions. In particular, eEF2 is very abundant in RRL, has high affinity for ribosomes in the 'rotated' state, and is a common nonphysiological contaminant in RNC preparations.

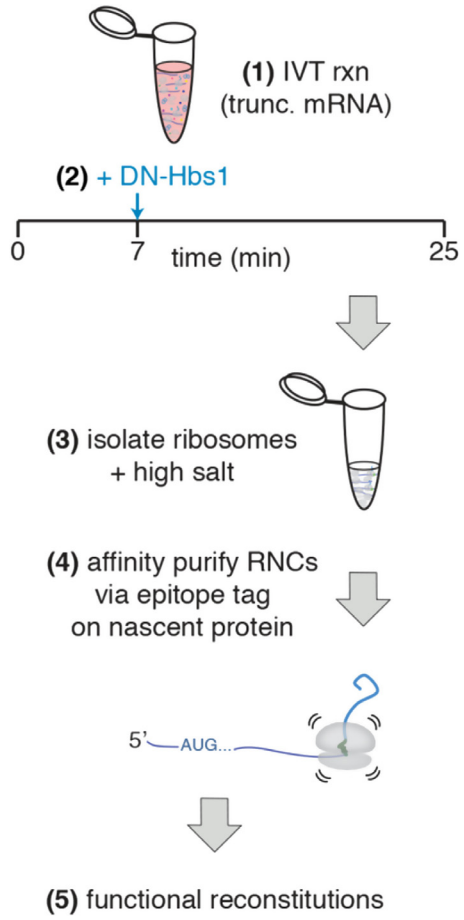

Fig. 7. Isolating starting RNCs for reconstitutions with purified factors. Strategy for isolating starting RNC substrates to reconstitute the ribosome-associated quality control pathway with purified factors [25,27]. IVT reactions of a truncated (trunc.) mRNA were initiated (1), an excess of dominant negative Hbs1L (DN-Hbs1) was added seven minutes afterwards (2), and the IVT reaction was allowed to proceed for a total of 25 min. DN-Hbs1L was added to trap substrate RNCs in a homogenous population in a canonical unrotated state. Afterwards, the IVT reactions were spun on a sucrose cushion in high salt conditions to isolate bulk ribosomes and remove peripherally-associated factors (3). The bulk ribosomes were resuspended and subjected to affinity purification against an N-terminal epitope tag encoded in the nascent protein (4). The resulting RNCs were used as starting substrates for downstream functional reconstitutions with fractionated cell lysates and purified factors.

Certain biochemical manipulations can counteract these potential sources of heterogeneity when isolating RNCs to use as starting substrates for functional reconstitutions. As an example, when reconstituting the RQC pathway, we used several specific steps to generate a homogenous set of purified RNCs for functional reconstitutions (Fig. 7). First, we started IVT reactions on truncated mRNA coding for an N-terminal 3X-Flag tag and ending with a valine codon. The homogenous nascent protein-tRNA adduct that this mRNA generates (Fig. 2B) directly facilitated our ability to follow the fate of radiolabeled nascent protein by SDS-PAGE and autoradiography. Second, we added GTPase-deficient dominant negative Hbs1L, another translational GTPase homologous to eEF1A that binds unrotated ribosomes, 7 min after initiating an IVT reaction [25–27]. This permits the dominant negative Hbs1L to engage the stalled RNCs without interfering with translation in general. Upon binding, dominant negative Hbs1L traps RNCs in the canonical unrotated conformation. This prevents the stalled RNCs from proceeding down the RQC pathway, as well as 'dead-end' eEF2 engagement in the RRL. This step was crucial for isolating RNCs that were amenable for functionally reconstituting downstream processes.

Subsequently, we stripped off dominant negative Hbs1L, as well as other peripherally ribosome-binding proteins such as eEF2, by isolating bulk ribosomes under high salt conditions (Section 4.2). Finally, we affinity purified the specific RNCs via the epitope-tagged nascent protein encoded by the truncated mRNA to serve as starting substrates for functional analyses. These general considerations can be applied using different translational inhibitors and other dominant negative proteins to isolate RNCs in different conformations for trapping and assembling various intermediates of translation elongation, termination, and splitting for mechanistic and structural studies (e.g. [26]).

6.3. Towards reconstituting translational arrest pathways with purified factors

To test the efficiency of isolated RNCs, we typically start by reconstituting the desired activity using an S100 extract or from the components of a ribosomal high salt wash [23]. Isolated RNCs should reconstitute the target activity, such as ubiquitination or restarting of translation, at least as efficiently as the activity seen from bulk ribosomes normalized for nascent protein level, as the bulk ribosomes should still contain a heterogeneous mixture of RNCs. Less efficient activity indicates that the RNC may be in a conformation that is not conducive for reconstituting the activity, or that other factors are missing from the reconstitution.

Once the S100 reconstitutes the desired activity on isolated RNCs with a sufficiently high signal-to-noise ratio for further dissection, we typically start biochemically fractionating the S100 to narrow down potential machinery in a top-down approach. In parallel, if candidate machinery involved in the process is known, we will also start reconstituting the process in a bottom-up approach using purified components.

For reliable physiological interpretations of experimental results in reconstituted systems, it is important to consider the specific activity generated by the machinery added back into the reactions. Identifying the minimal requirements for a cellular pathway ideally requires using endogenous concentrations of purified factors to reconstitute at least the full level of activity observed in more 'native' situations, such as S100, in terms of both overall efficiency and kinetics. This requires keeping a close eye on the activity levels recovered from the purified systems and comparing them with the original benchmarks representing more 'native' situations. This is especially critical for identifying factors that may play important physiological roles in enhancing a given activity, but whose roles may be obscured by an excess of a central enzyme or functional factor.

Obtaining interpretable physiologically-relevant results in reconstituted systems relies heavily on generating high-quality biochemical reagents. This includes not only isolating well-validated starting substrates, but also obtaining specific antibodies for detecting and potentially depleting specific factors, maintaining high specific activity through biochemical fractionations, and producing functional protein factors for the reconstitutions. A main technical source for losing activity arises from manipulations of soluble factors. When fractionating crude lysates to narrow down functional factors, diluting the concentration of individual factors and gross changes to the small molecule content, such as salts and nucleotides, of the fractions should be avoided to permit direct comparisons of different conditions and to prevent potential experimental artifacts. Individual fractions should be added back at normalized volumes, and when combined, all fractions should recapitulate the specific

activity seen in the total starting material. Where possible, the amounts of candidate factors should be followed throughout fractionations by immunoblotting or other methods to understand where individual activities are distributed.

Other inefficiencies may arise from the quality of purified factors, which may vary based on the conditions used for purifications, the incorporation of posttranslational modifications, the stability of purified proteins, etc. The most rigorous way to test the quality of purified factors is to add each back at endogenous levels to a more complete mixture (e.g. S100) that has been specifically depleted of that factor to test if full activity is recapitulated. If the purified factor in isolation cannot recapitulate full activity, then additional factors are probably needed, or the purified protein is not wholly active.

The causes of inefficiencies in functional reconstitutions can sometimes be difficult to diagnose and troubleshoot until all of the correct components and conditions are present. Discovering the underlying experimental issues and incorporating the necessary experimental modifications often requires some amount of previous mechanistic knowledge and biochemical trial-and-error. For this, simultaneously pursuing a top-down and bottom-up approach for reconstituting functional pathways is useful for generating reagents that afford the most flexibility in obtaining the requisite experimental insights to identify missing components and troubleshoot sources of inefficient activities.

7. Caveats and limitations

All in vitro systems are prone to limitations that arise primarily from how the reagents are generated. Special care should be taken when designing and interpreting experiments in cell-free systems to ensure that the conclusions are physiologically meaningful. Where possible, functional results should be validated using multiple assays and in different systems. The general principles and considerations described here are directly applicable to other cell-free systems, including IVT systems derived from bacteria, yeast, wheat germ, or cultured mammalian cells. Each system offers unique advantages and limitations. Both wheat germ and RRL IVT systems were established to optimize yield of eukaryotic proteins in single-round translation cycles. Translation efficiency in RRL is enhanced by, but less dependent than wheat germ, on the presence of a 5′ CAP on substrate mRNAs, while a 3′ poly(A) tail can enhance translation efficiency 1–5-fold [39,40]. In comparison, IVT systems derived from yeast or cultured human cells generally retain more regulatory mechanisms at the expense of overall protein output. Thus, choosing an appropriate cell-free system is a critical consideration when studying a given cellular process.

Below, we outline some specific caveats of using the RRL IVT system for studying translational arrest. First, the specialization of reticulocytes may limit the potential applications of the IVT system. In particular, because reticulocytes and most other common IVT systems lack nuclei, the study of downstream pathways that affect transcription are not possible. In addition, as illustrated by the phenomenon of leucine-stalling (Fig. 5), the preexisting cellular machinery in reticulocytes is highly specialized. Though initial steps of the RQC pathway and some aspects of mRNA decay have been reconstituted in RRL [23,41–43], how well RRL recapitulates downstream events of translational arrest, such as specific mRNA degradation, and the regulation of translation initiation, is not clear. The availability of essential factors that mediate these pathways, and their functions, may need to be verified in RRL (and other cell-free systems) on a case-by-case basis.

Second, the high efficiency of protein synthesis in the RRL IVT system may saturate endogenous cellular pathways dedicated for dealing with translational arrest. This was the case for the RQC pathway, in which only 5–10% of the arrested nascent proteins produced by translating truncated mRNA in the RRL IVT system were observed to be ubiquitinated [23]. This was due to saturation of RQC machinery, as titrating down the amount of mRNA resulted in higher proportions of ubiquitinated nascent proteins. For this reason, this modification was not appreciated for many years even though numerous studies utilized truncated mRNAs in the RRL IVT system to investigate cotranslational pathways. The study of other regulatory and quality control pathways, which typically employ low-abundance machinery, may face similar challenges.

Finally, a consideration of many in vitro systems is the slower kinetics of most cellular processes and the loss of certain activities. Slower kinetics is often useful for providing a wider window for biochemical adjustments and assays. However, if linked reactions are not slowed proportionally in a cell-free system, then this may limit how robustly certain processes can be reconstituted in vitro, or introduce experimental artifacts in the cell-free system that do not occur in cells. In addition, more nuanced cellular activities, such as signaling cascades that feed back to each other, may be lost in the process of generating a cell-free system, potentially affecting the physiological relevance of observations made in a cell-free system.

By keeping these potential caveats in mind and designing experiments to investigate and control for them, cell-free systems remain essential tools for mechanistically dissecting translational arrest pathways. Even in instances where entire pathways cannot be recapitulated in RRL, the high efficiency and manipulability of the RRL IVT system is particularly useful for generating individual reagents to study these processes in vitro, possibly in combination with other reagents from other systems, such as cultured cells, that contribute distinct advantages.

8. Discussion

Cell-free systems provide possibilities to dissect the mechanisms of various cellular pathways that are not possible in cultured cells or model organisms. These systems permit direct depletion of specific factors while minimizing potential indirect effects that may arise from knocking out, knocking down, or overexpressing (especially essential) cellular machinery. The biochemical manipulability of cell-free systems also allows complicated cellular pathways to be uncoupled and broken down into individual steps for mechanistic analysis. The well-characterized RRL IVT system offers many of these advantages for reconstituting and generating biochemical reagents to study protein translational arrest pathways. The methodologies we describe here and new biochemical manipulations to harness the power of in vitro systems will likely continue to play essential roles in revealing the molecular mechanisms and consequences of different types of translational arrest.

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Appendix A. Equipment and consumables

A.1. In vitro transcription, translation, and affinity purifications

Item	Source	Notes
Shallow 2 L water baths	General	Set at 37 °C and 32 °C for transcription and translation reactions, respectively.
Open racks for Eppendorf tubes	General	For water baths
SDS-PAGE equipment	General	We prefer the Bio-Rad mini-PROTEAN system with handcast 10% or 12% Tris-tricine gels
Mini spin columns (3.3 cm)	Bio-Rad 7326207	Store at room temperature
Spin columns (5 cm)	Bio-Rad 7326008	Store at room temperature
Gravity flow columns (9 cm)	Bio-Rad 7311550	Store at room temperature

A.2. Ultracentrifuge components for RNC isolation

Item	Source	Notes
Tabletop ultracentrifuge	Beckman Coulter Optima Max (or similar)	Only newer models are compatible with the MLS-50 rotor
Rotors		
TLS-55	Beckman Coulter 346936	For 0.2 mL and 2 mL sucrose gradients
TLA100.3	Beckman Coulter 349490	For bulk isolation of ribosomes
TLA120.2	Beckman Coulter 357656	For small-scale pelleting of ribosomes
MLS-50	Beckman Coulter 367280	For 4.8 mL sucrose gradients
Tubes		
For TLS-55	Seton 5011	For 2 mL sucrose gradients
230 μL tubes for TLS-55	Beckman Coulter 343775	For 200 μL sucrose gradients. Must use with adaptors below.
Adaptors for TLS-55	Beckman Coulter 358615	To use with 230 μL tubes in TLS55 rotor
For TLA100.3	Beckman Coulter 349622	For bulk isolation of ribosomes
For TLA120.2	Beckman Coulter 343778	For small-scale pelleting of ribosomes
For MLS-50	Beckman Coulter 326819	For 4.8 mL sucrose gradients

Appendix B. General reagents

B.1. Transcription

Item	Source	Notes
PCR reagents	General	e.g. Phusion kit (NEB E0553S)
RNasin	Promega N2511	Store at −20 °C
SP6 polymerase	NEB M0207S	Also possible to purify
H_2O for molecular biology	Millipore H20MB0124	Most sources of sterile Millipore water also work fine for IVT reactions
PCR purification kit	QIAGEN 28104	Store at room temperature
RNeasy RNA purification kit	QIAGEN 74104	Store at room temperature

B.2. Translation and after

Item	Source	Notes
^{35}S-methionine	Perkin Elmer NEG709A500UC	Store in a radioactive-safe container at 4 °C
M2 Flag agarose	Sigma A2220	Store at −20 °C
3X Flag peptide	Sigma F4799	To prepare a stock solution, dissolve in a physiological salt buffer [e.g. 50 mM Hepes pH 7.4, 100 mM KOAc, 2.5 mM $Mg(OAc)_2$] at a concentration of 5 mg/ml. Aliquot and store at −20 °C

B.3. Stock solutions

These stock solutions are sufficient to generate all of the buffers discussed in the text.

Item	Source	Notes
1 M Hepes/0.5 M KOH	General	Filter and store at 4 °C
5 M KOAc	General	Store at room temperature
1 M $Mg(OAc)_2$	General	Store at room temperature
1 M DTT	General	Make up fresh
70% sucrose	General	Dissolve in sterile water, and store at 4 °C
2 M sucrose	General	Dissolve in sterile water, and store at 4 °C
20% Triton X-100	General	Filter and store at 4 °C

Appendix C. Transcription mix reagents

Note: prepare all stock reagents with RNase-free water.

C.1. Stock solutions

Item	Source	Notes
1 M Hepes/0.5 M KOH	General vendor	Filter, and store at 4 °C
2 M $MgCl_2$	General vendor	Store at room temperature
100 mM spermidine	Sigma S0266	Aliquot & freeze in Nitrogen. Store at −20 °C
1 M DTT	Sigma/Roche 10708984001	1.54 g DTT dissolved in 10 mL water. Aliquot & freeze in Nitrogen. Store at −80 °C
100 mM ATP	Sigma/Roche 10519979001	Prepare 100 mM stocks in water, pH to ~7 with NaOH, freeze in nitrogen and store at −80 °C
100 mM GTP	Sigma/Roche 10106399001	Prepare 100 mM stocks in water, pH to ~7 with NaOH, freeze in nitrogen and store at −80 °C
100 mM UTP	Sigma U6875	Prepare 100 mM stocks in water, pH to ~7 with NaOH, freeze in nitrogen and store at −80 °C
100 mM CTP	Sigma C1506	Prepare 100 mM stocks in water, pH to ~7 with NaOH, freeze in nitrogen and store at −80 °C
5 mM CAP	NEB S1404L	Stock contains 25 A260 units per vial. 5 mM stock is prepared by adding 300 μL water

C.2. 10× RNA Polymerase buffer:

1 M Hepes/0.5 M KOH	400 μL
2 M $MgCl_2$	30 μL
100 mM spermidine	200 μL
1 M DTT	100 μL
RNase-free H_2O	270 μL
Total	1 mL

C.3. 4-NTP stock

100 mM ATP	50 μL
100 mM UTP	50 μL
100 mM CTP	50 μL
100 mM GTP	10 μL
RNase-free H_2O	840 μL
Total	1 mL

Appendix D. Translation mix reagents

Note: prepare all stock reagents with RNase-free water.

D.1. Nuclease treatment

Item	Source	Notes
Rabbit reticulocyte lysate (RRL)	Green Hectares	• Store at −80°. Limit freeze-thaws (make smaller aliquots if needed) • Thaw in a room-temperature water bath, mixing occasionally to ensure even warming • Immediately place on ice once thawed
0.1 M $CaCl_2$	General vendor	Store at room temperature
0.2 M EGTA	General vendor	Store at room temperature
Nuclease S7	Sigma/Calbiochem 492899	Dissolve in 50 mM Hepes buffer pH 7.4. Aliquot & freeze in liquid nitrogen. Store at −20 °C

D.2. cT2 generation

Item	Source	Notes
Nucleased RRL		See main text
1 M Hepes/0.5 M KOH	General vendor	Filter and store at 4 °C
5 M KOAc, pH 7.5	General vendor	Filter and store at room temperature
100 mM ATP	Sigma/Roche 10519979001	Prepare 100 mM stocks in water, pH to ~7 with NaOH, freeze in nitrogen and store at −80 °C
100 mM GTP	Sigma/Roche 10106399001	Prepare 100 mM stocks in water, pH to ~7 with NaOH, freeze in nitrogen and store at −80 °C
1.2 M creatine phosphate	Sigma/Roche 10621714001	Dissolve in water, freeze in liquid nitrogen, and store at −80 °C
10 mg/mL liver tRNA	Sigma R4752 or purified from mammalian liver	Freeze in liquid nitrogen and store at −80 °C
20 mg/mL creatine kinase	Sigma/Roche 10127566001	Dissolve in 10 mM Hepes pH 7.5, 50% glycerol, and store at −20 °C. Do not freeze in liquid nitrogen.
2 M $MgCl_2$	General vendor	Store at −80 °C
0.25 M reduced glutathione	General vendor	Store at −80 °C
100 mM spermidine	Sigma S0266	Aliquot & freeze in liquid nitrogen. Store at −80 °C
19 amino acid mix	Promega L9961	Store at −80 °C

Appendix E. PCR conditions

Standard PCR conditions for Phusion polymerase from NEB

Reagent	Volume	Final Concentration
5× HF buffer	10 μL	1×
10 mM dNTPs	1 μL	0.2 mM each dNTP
10 ng/μL plasmid	2 μL	20 ng total
50 μM forward primer	0.5 μL	0.5 μM
50 μM reverse primer	0.5 μL	0.5 μM
Phusion polymerase	0.5 μL	1 unit
H_2O	35.5 μL	
Total	50 μL	

Cycling conditions:

Step 1	98 °C	30 sec
Step 2	98 °C	5 sec
Step 3	55 °C*	30 sec
Step 4	72 °C	30 s/kb of amplified region
Repeat steps 2–4 a 24 times		
Step 5	72 °C	5 min
Step 6	4 °C	hold
*annealing temperature may need to be adjusted for individual templates		

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