Ribosome Profiling: Global Views of Translation

Nicholas T. Ingolia,[1] Jeffrey A. Hussmann,[2,3] and Jonathan S. Weissman[2,3]

[1]Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

[2]Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California 94158

[3]Howard Hughes Medical Institute, San Francisco, California 94158

Correspondence: ingolia@berkeley.edu; jonathan.weissman@ucsf.edu

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The translation of messenger RNA (mRNA) into protein and the folding of the resulting protein into an active form are prerequisites for virtually every cellular process and represent the single largest investment of energy by cells. Ribosome profiling-based approaches have revolutionized our ability to monitor every step of protein synthesis in vivo, allowing one to measure the rate of protein synthesis across the proteome, annotate the protein coding capacity of genomes, monitor localized protein synthesis, and explore cotranslational folding and targeting. The rich and quantitative nature of ribosome profiling data provides an unprecedented opportunity to explore and model complex cellular processes. New analytical techniques and improved experimental protocols will provide a deeper understanding of the factors controlling translation speed and its impact on protein function and cell physiology as well as the role of ribosomal RNA and mRNA modifications in regulating translation.

The translation of messenger RNA (mRNA) into protein and the folding of the resulting polypeptide into an active form connect genetic information to functional proteins—a prerequisite for virtually every cellular process. Translation is also a costly biosynthetic process that often comprises the single largest investment of energy by cells (Verduyn et al. 1991; Russell and Cook 1995). Translation is thus highly regulated to ensure that the right proteins are made in the right places within the cell. Ensuring that newly made proteins fold, assemble, and function properly is also a major challenge to the cell (Gloge et al. 2014). The biogenesis of functional proteins depends on cotranslational folding chaperones as well as the speed of translation itself, and quality control of aberrant translation suppresses the harmful effects of mutations and errors (Brandman and Hegde 2016).

The central role played by translation has motivated the development of experimental approaches to analyze both the proteins produced by the cell and the process of their synthesis. In particular, the advent of genomics and gene expression profiling has driven interest in extending such global analyses to the study of translation (Vogel and Marcotte 2012). Beyond taking a complete and quantitative inventory of proteins produced in the cell, there is also great interest in the dynamic, multistep process of synthesizing these proteins. The ribosome in-

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corporates amino acids with varying chemical properties by translating mRNAs that show biased use of synonymous codons, show secondary structures, and are decorated with chemical modifications. It seems natural to ask how these features affect the speed of the ribosome and what consequences result from varying the speed of elongation (Plotkin and Kudla 2011). These variations, encoded within the mRNA sequence, impact mRNA stability (Presnyak et al. 2015; Bazzini et al. 2016; Chan et al. 2017) and the rate of protein synthesis (Gingold et al. 2014), as well as the identity (Kawakami et al. 1993), folding (Kimchi-Sarfaty et al. 2007; Zhang et al. 2009), and localization (Pechmann et al. 2014) of the resulting protein.

Ribosome profiling, in which next-generation sequencing is used to identify ribosome-protected mRNA fragments, thereby revealing the positions of the full set of ribosomes engaged in translation, has emerged as a transformative technique for enabling global analyses of in vivo translation and coupled, cotranslational events. Historically, it has been challenging to measure these even in vitro; now, ribosome profiling provides a comprehensive view in living cells. It has been applied to address a remarkably broad diversity of mechanistic and physiological questions (Fig. 1). In this review, we present the historical context for ribosome profiling and highlight studies that exemplify the insights it can provide. We cannot at this point hope to cover all uses of this technique. It has been reviewed extensively (Michel and Baranov 2013; Ingolia 2014, 2016; Brar and Weissman 2015), and so we place emphasis here on recent developments.

RIBOSOME FOOTPRINT PROFILING OF IN VIVO TRANSLATION

Ribosome profiling relies on deep sequencing of ribosome footprints—the short (typically, ~30 nucleotide [nt]) fragments of mRNA that are physically enclosed by the ribosome and shielded from nuclease digestion (Fig. 2). These footprints are converted into a library of DNA fragments and analyzed by next-generation sequencing (Ingolia et al. 2012; McGlincy and Ingolia 2017). Each sequenced footprint reports on the position of one ribosome, revealing what transcript that ribosome was translating and where along the coding sequence it was captured during cell lysis, often with single-nucleotide resolution. Current deep-sequencing technologies analyze hundreds of millions of individual short reads in one experiment. When applied to libraries of ribosome footprints, this sequencing yields a comprehensive view of the translational landscape that can address many fundamental questions about translation (Fig. 1). Whereas there remain important experimental and analytical challenges to fully exploiting these data, especially in regard to the analysis of ribosome pause sites as discussed below, the presence of ribosome footprints indicates which sequences are being translated in the cell, and thus what protein is being produced. The overall density of ribosome footprints reflects the rate of translation occurring on different transcripts, allowing a direct and quantitative measure of how rapidly a cell is producing each of its proteins. These densities must be corrected for differences in the average elongation rate of each mRNA, which, when explored, have been

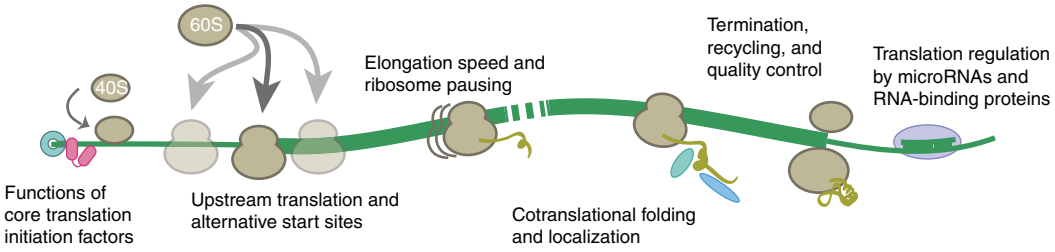

Figure 1. Insights from ribosome profiling. Ribosome profiling experiments have addressed many aspects of the mechanisms of protein synthesis and its regulation in the cell as well as related, cotranslational processes.

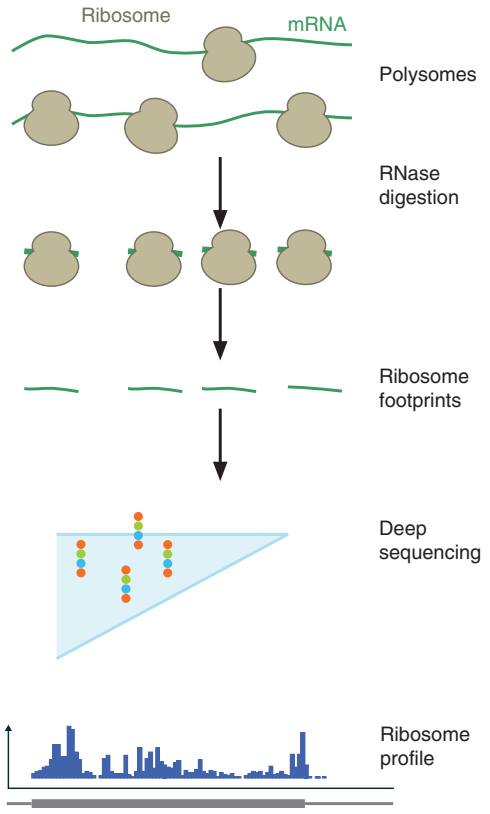

Figure 2. Ribosome footprint profiling. Steps in a typical ribosome profiling experiment are shown. Polysomes reflecting in vivo translation are isolated from cells and subjected to RNase digestion, which degrades unprotected messenger RNA (mRNA). The ribosome-protected footprints are analyzed by deep sequencing, schematized by the flowcell (light blue) with clusters of fluorescently labeled DNA attached to it (colored dots). Aligning these footprint sequences back to the transcriptome produces a quantitative profile of ribosome occupancy.

found to be relatively modest (Li et al. 2014). The detailed pattern of ribosome footprints within a coding sequence varies substantially, however, and reveals the relative speed of the ribosome along the transcript. As described below, these rich data can be augmented further, using drugs that modulate translation or targeted purification of interesting ribosomal subpopulations to address a wide array of biological questions.

QUANTIFYING GENE-SPECIFIC TRANSLATION

An important antecedent to the ribosome profiling approach was a study from Joan Steitz (1969), who mapped the sites of translation initiation in bacteriophage RNA by analyzing the RNase-resistant mRNA fragments protected by initiating ribosomes assembled in vitro. Subsequently, Wolin and Walter (1988, 1989) identified sites of in vitro translational pausing by mapping the relative density of ribosome-protected fragments using a primer extension assay. These studies made fundamental contributions to our understanding of translation through the analysis of ribosome footprints, but they were limited to the study of individual mRNAs translated in vitro.

Historically, studies of in vivo translation relied on analyzing polysomes recovered from cells and tissues (Mathews et al. 2000). These polysomes comprise multiple ribosomes translating a single mRNA template, and they can be fractionated according to the number of ribosomes they contain by ultracentrifugation through a sucrose density gradient. The distribution of an mRNA across these fractions reflects its translational status. Gene-specific and global polysome analyses (Arava et al. 2003) have provided a wealth of information about translation, but their quantitative resolution is limited by the poor separation of heavier polysomes and the difficulty of distinguishing ribosomes on different open reading frames (ORFs) in polycistronic mRNAs and transcripts with regulatory upstream translation. This challenge is exacerbated when comparing translation between different genes, as the number of ribosomes on a transcript scales with the length of the coding sequence as well as the translation level.

Ribosome profiling circumvents these limitations and precisely measures translation levels by counting discrete ribosome footprints (Ingolia et al. 2009). This quantitative precision revealed a principle of "proportional synthesis" that holds in bacterial and eukaryotic cells: protein subunits of multimeric complexes are synthesized in proportion to their stoichiometry in the assemblies (Li et al. 2014) thus reducing

waste of producing unneeded subunits and eliminating the need to dispose of such uncomplexed species (Fig. 3). In bacteria, proteins with differing stoichiometry are often translated from a single polycistronic transcript, and so these differences likely reflect differential translation of the individual reading frames within that RNA. Translation-driven proportional synthesis can even be seen in chloroplasts, in which plastid ribosome profiling revealed that this organelle produces photosystem components in a tight stoichiometric ratio not seen in mRNA abundance (Chotewutmontri and Barkan 2016). More broadly, the fine-tuning seen in proportional synthesis highlights the accuracy and precision of ribosome profiling measurements.

This fine-tuning of stoichiometry also emphasizes how protein abundance is typically the functionally relevant output of gene expression in the cell, and selective constraints arise on protein levels rather than mRNA levels. Protein abundance correlates better with ribosome profiling measurements than with mRNA levels (Liu et al. 2017b; Cheng et al. 2018), and so experiments capture additional, biologically relevant information about gene expression by incorporating profiling data. Combining ribosome profiling with transcriptomic and proteomic data promises the opportunity to learn more about the genetic determinants of protein levels that impact translation as well as transcription.

Ribosome profiling has enabled the study of interallelic (Muzzey et al. 2014), interstrain (Albert et al. 2014), and interspecies (Artieri and Fraser 2014b; McManus et al. 2014) translational differences in yeast. It has also been applied to study gene expression variation between individual humans (Battle et al. 2015; Cenik et al. 2015). In some cases, transcriptional divergence is buffered by translational changes to conserve protein levels, whereas in other cases transcriptional and translational changes reinforce each other. Future ribosome profiling studies promise further insight into the roles of translational changes as well as the nature of the polymorphisms that drive these changes.

During dynamic remodeling of cell physiology, global expression profiling has shown how genes are induced just in time to fulfill their functional roles (Brown and Botstein 1999). Ribosome profiling in meiotic yeast has revealed how this principle of just-in-time regulation extends to the translational as well as transcriptional control of the proteins synthesized for each stage of this highly ordered process (Brar et al. 2012). Ribosome profiling in mitosis, too, points to translational control of protein production in concert with cell cycle progression (Stumpf et al. 2013; Tanenbaum et al. 2015).

Subsequently, concerted programs of translational regulation have emerged in many other models, ranging from basal eukaryotic parasites

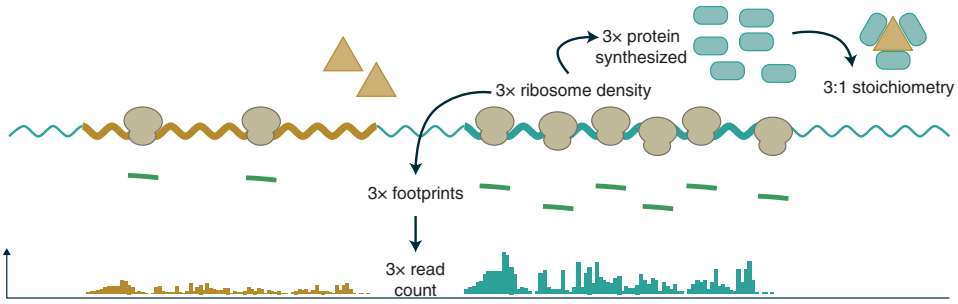

Figure 3. Quantifying protein synthesis. The number of ribosomes translating a reading frame determines the number of footprints generated in a profiling experiment, and so counting the footprint sequences derived from a reading frame indicates the amount of the encoded protein that is being synthesized. An exemplary polycistronic bacterial transcript is shown, with two open reading frames ([ORFs] A and B) encoding a pair of proteins that assemble with a 1:3 stoichiometric ratio. To achieve this stoichiometry, ORF B is translated threefold more heavily than ORF A, leading to threefold higher ribosome density and threefold more ribosome footprints.

Trypanosoma (Jensen et al. 2014; Vasquez et al. 2014) and *Plasmodium* (Caro et al. 2014) to circadian cycles in mammalian tissue (Janich et al. 2015). This principle even extends to the distinct mitochondrial translational apparatus, in which profiling of mitoribosomes points to coordinated synthesis of oxidative phosphorylation components translated in the mitochondrion with those produced in the cytosol (Couvillion et al. 2016).

One common theme arising in many studies is the coordinated regulation of ribosomal proteins and ribosome biogenesis factors linked to the rate of cell growth. In metazoa, ribosome production and protein synthesis levels are regulated in part by the protein kinase, mammalian target of rapamycin (mTOR), which serves as a master regulator of growth (Hindupur et al. 2015), in part through controlling the translation of mRNAs encoding ribosomal proteins, but affecting many other transcripts as well (Proud 2018). This mTOR-driven translation supports proliferating cells during normal development and malignant growth in cancer (Dowling et al. 2010; Alain et al. 2012; Robichaud et al. 2018). As mTOR activity drives cell growth downstream from well-known oncogenic signaling pathways, there is great interest in developing active-site mTOR inhibitors as clinically useful anticancer therapies (Bhat et al. 2015). Ribosome profiling studies of cells treated with these inhibitors has revealed a broad range of target transcripts beyond ribosomal proteins that seem to support the cancer cell phenotype (Hsieh et al. 2012; Thoreen et al. 2012). Intriguingly, many of the same genes are translationally repressed in mouse embryonic stem cells induced to differentiate into embryoid bodies rather than continue rapid proliferation (Ingolia et al. 2011).

MOLECULAR MECHANISMS OF TRANSLATIONAL CONTROL

Ribosome profiling has revealed the mechanism underlying the action of other anticancer drugs that target the translational apparatus (Chu and Pelletier 2018). Rocaglate drugs are a class of natural products that target eukaryotic initiation factor 4A (eIF4A), a prototypical DEAD-box RNA helicase, selectively killing cancer cells (Santagata et al. 2013). Ribosome profiling revealed that rocaglates inhibit translation of specific mRNAs, suggesting that this targeted inhibition could explain their selectivity for cancer cells (Wolfe et al. 2014). By measuring the relative sensitivity of different transcripts to rocaglate treatment—which differed greatly from their sensitivity to hippuristanol, a more conventional eIF4A inhibitor—ribosome profiling further elucidated the unique repressive mechanism of these drugs. Rather than mimicking the loss of eIF4A function, rocaglate drugs clamp eIF4A onto certain polypurine RNA sequences, where it serves as a roadblock to translation initiation (Iwasaki et al. 2016). Transcripts with polypurine-rich transcript leaders are thus particularly sensitive to rocaglate treatment.

Similar correspondences between translational changes measured by ribosome profiling and transcript features have provided new insights into the normal function of eIF4A as well as other translation initiation factors, often challenging our current understanding of their roles. Ribosome profiling measurements conducted after inactivation of eIF4A revealed profound but fairly uniform reduction in translation (Sen et al. 2015). Conditional inactivation of yeast Ded1, another DEAD-box translation initiation factor, argued that it is particularly important for translating mRNAs with longer and more structured 5′ untranslated regions (UTRs) (Sen et al. 2015). The scaffolding protein eIF4G recruits eIF4A and stimulates its ATPase activity (Merrick and Pavitt 2018; Sokabe and Fraser 2018). Although eIF4G is typically thought to be recruited to mRNAs through its interactions with the cap-binding protein eIF4E and poly(A)-binding protein, recent studies suggest that in yeast it preferentially binds and promotes the translation of mRNAs with oligo (U) tracts in their 5′UTRs (Zinshteyn et al. 2017). Transcripts that depend on eIF4G also show reduced translation in the absence of the ribosome-associated factor Asc1/RACK1 (Thompson et al. 2016). In plants, poly(A)-binding proteins interact with A-rich motifs

directly in the 5′UTR, in which they promote translation in pattern-triggered immune response (Xu et al. 2017).

Global ribosome profiling, combined with mRNA and protein abundance measurements, has also provided critical insights into the mechanism of microRNA-mediated repression (Duchaine and Fabian 2018), making it possible to disentangle the effects of mRNA destabilization from reduced translation. Early ribosome profiling studies measured concordant effects at both stages of expression, with translational repression contributing ~1/6th of the total reduction in protein abundance at steady state (Guo et al. 2010). Later work in developing zebrafish embryos showed that strong translational repression precedes mRNA decay during the activation of miR-430 in the maternal-to-zygotic transition (Bazzini et al. 2012). It remains unclear whether this reflects a general kinetic pathway for microRNA-mediated repression, or a shift in the mode of action between earlier or later stages of embryogenesis (Subtelny et al. 2014). Similarly, ribosome profiling has distinguished the translational effects of RNA modifications such as adenosine N^6 methylation, which seem to influence both translation and mRNA stability (Wang et al. 2015; Zhou et al. 2015; Coots et al. 2017; Slobodin et al. 2017; Vu et al. 2017; Peer et al. 2018).

Quantitative and comprehensive ribosome profiling measurements broadly offer the insights available from transcriptome profiling, augmented with information about translational regulation. Using these comprehensive measurements as input, modeling approaches have been used to systematically quantify the roles of various transcript features in determining the translational output of an mRNA (Weinberg et al. 2016; Hockenberry et al. 2017). This approach can also be applied to learn about cellular physiology by profiling natural biological processes and to learn about regulatory mechanisms by observing the effects of targeted molecular disruptions, as shown by the examples above. Ribosome profiling contains information about the exact positions of ribosomes as well; however, that goes beyond expression profiling.

DISCOVERY OF NONCANONICAL TRANSLATION EVENTS

The prevalence of ribosome footprints in 5′UTRs was one of the most striking features we observed in the first ribosome profiling data from yeast and mammalian cells (Fig. 4A) (Ingolia et al. 2009, 2011). Upstream translation itself can serve to repress expression of downstream protein-coding genes when scanning ribosomes initiate at upstream ORFs (uORFs) and

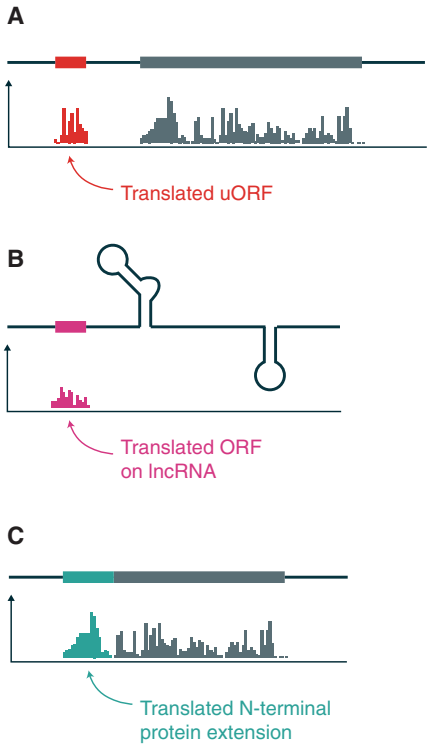

Figure 4. Annotating the proteome with ribosome profiling. The figure diagrams mRNAs (*top*) showing the frequency of ribosome footprints along them (*below*). (*A*) Ribosome footprint sequences mapping to the 5′ leader of a transcript indicates the translation of an upstream open reading frame (uORF, red segment) and downstream ORF (gray segment). (*B*) Likewise, ribosome footprint sequences on a noncoding RNA indicate the presence of a translated region, typically near the 5′ end of the transcript. (*C*) Alternative protein isoforms translated in addition to or in place of annotated reading frames also appear in ribosome profiling data.

translate them instead of proceeding on to the main reading frame (Sonenberg and Hinnebusch 2009). Ribosome profiling data are critical for understanding this mode of regulation, as not all uORFs are translated, and their repressive effects can be modulated by genetic variation between individuals, including polymorphisms that create or destroy uORFs (Calvo et al. 2009; Cenik et al. 2015). Indeed, in some cases, uORFs can promote the translation of the downstream coding sequence (Sonenberg and Hinnebusch 2009). Alternative transcript isoforms can also include or exclude upstream translated regions, thereby modulating translation. In the most extreme cases, totally unproductive transcript isoforms may result from the inclusion of long upstream regions replete with uORFs (Brar et al. 2012; Chen et al. 2017; Cheng et al. 2018).

Regulatory uORFs control the paradoxical induction of specific mRNAs such as *ATF4* and *CHOP* during stress-induced translational shutoff, mediated by the phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α) (Sonenberg and Hinnebusch 2009; Wek 2018). Ribosome profiling has now provided a comprehensive list of dozens of genes that show similar up-regulation (Andreev et al. 2015; Sidrauski et al. 2015). Surprisingly, although ribosome profiling confirms the translation of *ATF4* and *CHOP* uORFs, many other targets lack ribosome occupancy in their 5′UTRs, raising the question of how their translation is controlled. Furthermore, translated uORFs appear to be widespread across the transcriptome (Johnstone et al. 2016), not restricted to the small number of phospho-eIF2α-induced genes (Andreev et al. 2015; Sidrauski et al. 2015).

Much of the upstream translation seen in ribosome profiling cannot be attributed to AUG codons, and instead appears to initiate at a near-cognate, non-AUG codon. As the most common non-AUG initiation sites occur at codons that are quite similar to AUG (e.g., CUG), some of this translation results from mispairing of the initiator transfer RNA (tRNA) with the noncanonical start codon. There is also evidence for uORF peptide products that begin with amino acids other than methionine. These alternative start codon products are induced when eIF2 is inhibited by phosphorylation, and depend on the poorly understood initiation factor eIF2A, which is unrelated to the canonical eIF2 complex (Starck et al. 2016; Merrick and Pavitt 2018). Ribosome profiling recently uncovered a shift toward *EIF2A*-dependent, non-AUG initiation in a *SOX2*-driven mouse model of squamous cell carcinoma (Sendoel et al. 2017). Many oncogenic transcripts were induced in this translational program, and tumor progression depended on *EIF2A*, pointing toward a causal link between unconventional 5′UTR translation and cancer. The recent development of translation complex profile sequencing (TCP-Seq) promises a more direct view of the mechanism of translation initiation. TCP-Seq augments the standard ribosome profiling approach with formaldehyde cross-linking that stabilizes preinitiation complexes on mRNAs to profile 40S subunits scanning through 5′UTRs, providing insights into the molecular choreography of the scanning process (Archer et al. 2016).

Ribosome footprints were seen on many presumptively noncoding RNAs in addition to the 5′UTRs of coding genes (Fig. 4B) (Ingolia et al. 2011, 2014; Chew et al. 2013; Ji et al. 2015). The patterns of footprints on long noncoding RNAs (lncRNAs) (Chekulaeva and Rajewsky 2018) matched our expectations for those of translating ribosomes; they fell in AUG-initiated reading frames near the 5′ ends of transcripts and in aggregate showed three-nucleotide periodicity (Ingolia et al. 2011; Calviello et al. 2016). The lack of canonical features of protein-coding sequences in these translated regions motivated a variety of experiments aimed at validating the profiling results. lncRNA footprints copurified with affinity-tagged ribosomes and responded to drugs that targeted the ribosome, and thus represented ribosome-protected footprints rather than nonribosomal background (Ingolia et al. 2014). We have also reported evidence for protein products derived from lncRNA translation (see Chekulaeva and Rajewsky 2018). Viral infection leads to an immunological memory of epitopes derived from lncRNA translation (Stern-Ginossar et al. 2012), similar to the

epitopes produced by noncanonical upstream translation (Starck et al. 2012). Certain peptides have also been detected directly (Calviello et al. 2016), although in general these short products are challenging targets for proteomics, and ribosome profiling predictions can greatly aid in finding them (Menschaert et al. 2013).

ANNOTATING THE EXPANDED PROTEOME

The functional impact of pervasive alternative translation remains an important question. In a few cases, ribosome profiling has directly identified short, functional proteins such as Toddler (Pauli et al. 2014). Ribosome profiling points to a remarkable breadth of short, translated ORFs supported by conservation analysis (Menschaert et al. 2013; Aspden et al. 2014; Bazzini et al. 2014; Fields et al. 2015; Calviello et al. 2016) that could add to our growing catalog of functional micropeptides (Anderson et al. 2015; D'Lima et al. 2017). In many cases, however, this translation may be adventitious and subject principally to negative selection to avoid harmful effects from protein products or RNA destabilization (Ulitsky and Bartel 2013). Even nonfunctional proteins can serve as antigens, however (Ingolia 2014), and understanding this cryptic source of antigens (Starck et al. 2012) has implications for cancer immunotherapy (Schumacher and Schreiber 2015) and immunobiology more generally.

Ribosome profiling has also revealed alternative translation that extends or truncates classical protein-coding genes (Fig. 4C). This variation can add or remove entire domains, changing or reversing the function of the protein product. For instance, a truncated form of the innate immune signaling protein, mitochondrial antiviral signaling protein (MAVS), called miniMAVS, seems to antagonize the function of full-length MAVS in antiviral gene expression (Brubaker et al. 2014). Our data suggest that such alternative isoforms are widespread (Ingolia et al. 2011; Fields et al. 2015).

The diversity of translation products has spurred adaptations of ribosome profiling optimized for identifying translated regions of the transcriptome. We reported on the use of harringtonine, a drug that immobilizes initiating ribosomes, producing footprints that mark sites of translation initiation (Ingolia et al. 2011). Others showed, independently, that lactimidomycin or pateamine A could likewise trap initiating ribosome footprints (Lee et al. 2012; Gao et al. 2015; Popa et al. 2016), while high doses of the drug puromycin could drive rapid premature termination to produce a similar initiation-specific footprint profile (Fritsch et al. 2012). Joint analysis of lactimidomycin- and harringtonine-treated profiling data promises more robust identification of translational start sites appearing in both data sets by excluding possible artifacts resulting from either of these mechanistically distinct drugs (Stern-Ginossar et al. 2012; Arias et al. 2014). This combined analysis was used to define translated reading frames in human cytomegalovirus, a large herpesvirus with a complex life cycle that expresses a variety of alternative translation products, some of which seem to display specific molecular function (Stern-Ginossar et al. 2012). Novel translated ORFs upstream of, or within, known ORFs have also been detected in other viruses (Stern-Ginossar et al. 2018). More recently, a systematic, regression-based combination of ribosome profiling data generated with these different drugs revealed hundreds of novel coding sequences in mammalian cells along with a wealth of shorter, translated reading frames (Fields et al. 2015).

To draw accurate inferences about in vivo translation from deep-sequencing data, it is essential to know that the RNA fragments being sequenced are ribosome-protected footprints. We have provided several lines of evidence showing that this is true in general (Ingolia et al. 2014), and we and others have shown how straightforward computational approaches can distinguish signatures of translation from footprints left by nonribosomal RNA–protein interactions (Ingolia et al. 2014; Ji et al. 2016). The bulk of these nonribosomal reads derive from abundant structural RNAs, including tRNAs, spliceosomal small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs) (Ji et al. 2016). Fragments of these RNAs, along with other nonribosomal background, can be identified and excluded from ri-

bosome footprint analysis because they differ in length from ribosome footprints and lack triplet periodicity (Ingolia et al. 2014; Ji et al. 2016).

To equate ribosome footprint density with protein production, it is also important to know that these ribosomes are translating productively. We have followed run-off elongation after blocking new initiation with drugs and shown that coding sequences are quickly depleted of ribosomes (Ingolia et al. 2011), except under conditions in which elongation is arrested (Barry et al. 2017). These basic features seem to hold in most systems, although it does not obviate the need to evaluate them in unusual biological contexts.

TRACKING THE FOOTPRINTS OF TRANSLATION ELONGATION

Ribosome profiling has broad applications in annotating genes as well as measuring expression, but its most distinctive contributions may stem from insights into the activities of ribosomes in vivo. We know that the speed of translation elongation can vary across a coding sequence, presumably as a result of variations in the mRNA template and the protein product. Ribosomes will spend more time at positions of slow elongation, and so we will observe a higher density of footprints at these sites (Fig. 5).

Indeed, footprint counts vary substantially across genes and accumulate at specific "pause" sites. There is great interest in understanding the features that correlate with the speed of translation elongation and deconvolving this from biases in capturing and sequencing footprints (Stadler and Fire 2011; Dana and Tuller 2012; Qian et al. 2012; Charneski and Hurst 2013; Lareau et al. 2014; Pop et al. 2014; Liu and Song 2016; O'Connor et al. 2016; Weinberg et al. 2016; Dao Duc et al. 2017). Likewise, there is interest in understanding the factors that drive dramatic ribosome pausing at specific locations, which may reflect a qualitatively different process than the variation in translation speed seen across typical codons (Han et al. 2014; Li et al. 2014; Martens et al. 2015; Mohammad et al. 2016; Zhang et al. 2017). Various measures of codon usage bias correlate with footprint occupancy, suggesting that favored codons are decoded more quickly. However, consensus has not emerged on the exact basis of this effect, which seems to extend beyond the time required for decoding and tRNA recruitment. Elongation rates learned from ribosome profiling nonetheless provide an empirical basis for tuning the translation of a coding sequence, thereby controlling its expression (Tunney et al. 2017).

Translation of even a single codon is a complicated, multistep process (Dever et al. 2018; Rodnina 2018), and ribosome profiling has opened a new window into the operation

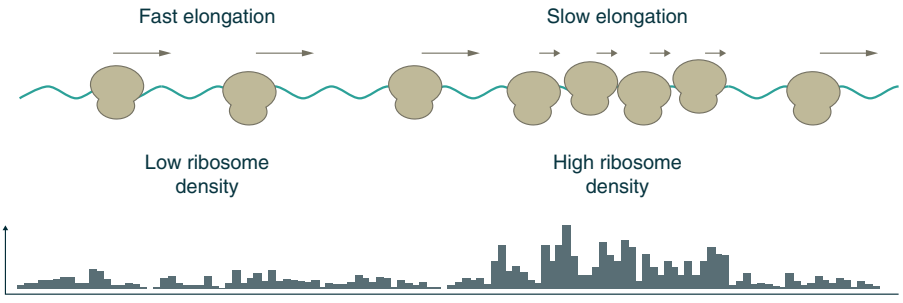

Figure 5. Inferring elongation speed from variations in ribosome footprint density. The *lower* part of the figure reports the frequency of ribosomal footprints along the mRNA. Regions of slow elongation will accumulate higher ribosome occupancy than regions of faster elongation on the same transcript. These differences in ribosome density are visible in profiling data, and they can be used to infer how codon usage, peptide sequence, and other features control the speed of translation.

of the translational machinery by reporting on normal elongation and on the effects of mutations. tRNAs in particular are heavily modified, and disrupting these modifications can change the speed of translation (Zinshteyn and Gilbert 2013) and thereby disrupt protein folding (Nedialkova and Leidel 2015). A broad survey of tRNA modifications revealed diverse effects on specific codons and on gene expression (Chou et al. 2017). In a similar fashion, base modifications on mRNA can affect decoding (Choi et al. 2016; Li et al. 2017), representing another factor that can contribute. In yeast, ribosome profiling provided in vivo confirmation that certain pairs of synonymous codons induce major ribosome pausing only when adjacent and in a particular order, suggesting structural cross talk between tRNAs in the A and P sites (Gamble et al. 2016). Ribosome profiling can distinguish between different phases of the translation elongation cycle, as different ribosome conformations protect footprints of differing length (Lareau et al. 2014). The longer (~28 nt) footprints, captured in most ribosome profiling experiments, probably reflect unrotated ribosomes. Cycloheximide treatment traps ribosomes in this long-footprint conformation, and yeast ribosome profiling performed without cycloheximide revealed a population of shorter (~21 nt) footprints that are attributed to rotated ribosomes. Although the abundance of long ribosome footprints correlates with codon usage and tRNA availability, short footprint density correlates with physicochemical amino acid properties instead, likely reflecting effects on translocation rather than decoding. Short footprints accumulate at certain tRNA-dependent stalls (Matsuo et al. 2017), suggesting that this cross talk affects translocation rather than tRNA recruitment (Lareau et al. 2014). Notably, these short footprints differ from the ~16 nt footprints reflecting a ribosome stalled at the end of a broken mRNA (Guydosh and Green 2014). More generally, these examples show the importance of identifying and quantifying all ribosome footprints regardless of their length (Mohammad et al. 2016).

Translation elongation also slows in response to amino acid limitation, leading to ribosome footprint accumulation on codons encoding the affected amino acids. Footprint density peaks induced by histidine deprivation were used to generate fiduciary marks in ribosome profiling data (Guydosh and Green 2014; Lareau et al. 2014), and inadvertent serine restriction likewise caused a buildup of footprints on serine codons in bacteria (Li et al. 2014). Systematic amino acid starvation coupled with ribosome profiling provided a spectrum of perturbed ribosome occupancy profiles that inform biophysical models of bacterial translation (Subramaniam et al. 2014). Remarkably, ribosome profiling likewise uncovered proline limitations in certain human tumors, based on slowed elongation when decoding proline codons (Loayza-Puch et al. 2016), and may serve more generally to probe for metabolic disruptions in cancer and other diseases.

Ribosome profiling has also facilitated the study of peptide-mediated translational pausing that occurs naturally in cells (Nakatogawa and Ito 2002). We observed ribosome footprint accumulation at certain tandem proline codons in mammalian cells (Ingolia et al. 2011), consistent with the slowed rate of elongation at these sites in vitro and the unfavorable conformation of polyprolyl nascent chains in the ribosome (Huter et al. 2017). The universally conserved elongation factor EF-P/eIF5A is implicated in translation of polyproline peptides (Doerfel et al. 2013; Gutierrez et al. 2013; Ude et al. 2013; Dever et al. 2018; Rodnina 2018) but ribosome profiling after eIF5A depletion reveals broader perturbation of ribosome footprint profiles, supporting a wider role for eIF5A in elongation through many unfavorable peptide sequences and in efficient translation termination (Schuller et al. 2017). Ribosome footprinting of these eIF5A stalls agreed with stalling sites identified by 5PSeq (Pelechano and Alepuz 2017), which focuses on in vivo RNA degradation intermediates whose 5′ terminus marks the trailing edge of the last translating ribosome (Pelechano et al. 2015). In contrast, ribosome profiling showed that *Legionella* toxins targeting elongation factor 1A (eEF1A) show no such specificity (Barry et al. 2017). Many peptide sequences can block bacterial translation (Woolstenhulme et al. 2013), and this effect is often

exploited for biological regulation. Bacterial ribosome profiling has also defined peptide-specific arrest caused by antibiotics targeting the translational machinery (Kannan et al. 2014; Marks et al. 2016). Stalling also occurs at programmed ribosomal frameshifting, which can stand out dramatically in footprint profiles (Michel et al. 2012; Napthine et al. 2017).

Detailed analyses of ribosome footprint occupancy patterns on individual mRNAs are particularly impacted by technical challenges. Studies in prokaryotes face a unique obstacle: ribonucleases do not degrade unprotected RNA precisely to the edge of the prokaryotic ribosome (Oh et al. 2011), making it challenging to precisely identify functionally relevant positions within each footprint (Woolstenhulme et al. 2013). More universally, all methods for converting ribosome footprints into a deep-sequencing library display biases that over- or underrepresent certain footprints, thereby distorting the apparent ribosome occupancy observed after sequencing (Artieri and Fraser 2014a; Bartholomaus et al. 2016; Lecanda et al. 2016; Tunney et al. 2017). Translation inhibitors used before cell lysis can distort ribosome occupancy profiles more directly (Gerashchenko and Gladyshev 2014; Hussmann et al. 2015). Eukaryotic cells are often treated with cycloheximide before ribosome profiling to immobilize and stabilize ribosomes. In our early studies, we reported that this treatment did not affect overall ribosome occupancy across a coding sequence but did change the pattern of footprints within that sequence (Ingolia et al. 2011). Subsequently, use of cycloheximide varied between studies. Later analysis showed that peaks of ribosome density appear to shift downstream in cycloheximide-treated samples relative to untreated ones (Gerashchenko and Gladyshev 2014; Hussmann et al. 2015).

Interest in a quantitative understanding of elongation has been heightened by recent studies that identified a potential role for elongation rates in dictating mRNA half-lives (Presnyak et al. 2015; Chan et al. 2017), either through direct surveillance of ribosome speed by mRNA decay machinery (Radhakrishnan et al. 2016) or indirectly by inducing ribosome collisions that then trigger decay pathways (Ferrin and Subramaniam 2017; Simms et al. 2017). Ribosome profiling will undoubtedly play a key role in unraveling the molecular mechanisms connecting elongation to decay and in quantifying the role of elongation in determining steady-state mRNA levels.

TRANSLATION TERMINATION AND BEYOND

In most ribosome profiling studies, 3′UTRs are devoid of footprints, in contrast to the surprising abundance of upstream initiation. Stop codon readthrough causes a specific accumulation of in-frame ribosome footprints in 3′UTRs, which are particularly prominent in *Drosophila* (Dunn et al. 2013). Defects in posttermination ribosome recycling (Hellen 2018) allow unrecycled ribosomes to enter 3′UTRs with no particular reading frame, and then reinitiate translation in some different reading frame, producing 3′UTR footprints out of frame from the coding DNA sequence (CDS) (Young et al. 2015). It appears that the ribosome rescue factors Dom34/Pelota and Hbs1 typically rescue many posttermination, unrecycled ribosomes, as the loss of these factors also causes an accumulation of vacant ribosomes past the stop codon (Guydosh and Green 2014). This distinctive accumulation of 3′UTR footprint patterns arose in reticulocytes and platelets, bringing to light a depletion in normal recycling factors and a disruption of ribosome homeostasis in both of these anucleate blood lineages (Mills et al. 2016). Indeed, translational regulation is pervasive in hematopoiesis (Alvarez-Dominguez et al. 2017), and altered ribosome recycling may underlie the particular sensitivity of red blood cells to defects in the translational machinery (Mills and Green 2017). It seems that the loss of ribosome rescue may serve a positive role in platelets, however. The rescue of ribosomes is linked to quality control processes that degrade aberrant protein products and mRNA templates (Brandman and Hegde 2016) and the loss of ribosome rescue factors seems to stabilize mRNAs that cannot be replaced by transcription (Mills et al. 2017).

PICKING THE RIGHT FOOTPRINTS

Footprinting of purified ribosome subpopulations enables profiling of cotranslational processes that act on proteins but, using a sequencing-based assay. Selective ribosome profiling by purifying ribosomes that are engaged by specific chaperones or targeting factors has revealed the in vivo substrates and engagement patterns in bacteria (Oh et al. 2011) and eukaryotes (Döring et al. 2017). Likewise, profiling of ribosome footprints engaged with the signal recognition particle (SRP) monitors cotranslational secretion and suggests that determinants beyond the classic signal sequence may aid SRP targeting (Chartron et al. 2016). Profiling has even been adapted to profile the folding state of nascent protein chains directly (Han et al. 2014), highlighting the fact that protein folding can be coupled directly to translation (Gloge et al. 2014). Indeed, selective ribosome profiling of ribosomes associated with different members of a multiprotein complex has suggested that complex assembly can begin cotranslationally (Shieh et al. 2015). Such cotranslational assembly could couple with the degradation of monomers that lack partner proteins for complex formation (Ishikawa et al. 2017) to complement proportional synthesis (Li et al. 2014) in maintaining proteome stoichiometry. This selective profiling strategy has been extended to study ribosomal subpopulations with varying composition. After identifying proteins RPL10A and RPL38 as substoichiometric in ribosomes, selective ribosome profiling of only those ribosomes containing these proteins revealed a potential role for heterogeneity between ribosomes in shaping overall translational output (Shi et al. 2017).

Recently, an approach termed proximity-specific ribosome profiling has enabled selective profiling of ribosomes at specific subcellular locations. Subcellular organization is inevitably disrupted by lysis and homogenization, but proximity labeling with a localized biotin ligase can mark ribosomes according to their in vivo localization for subsequent purification and footprinting. This approach was used first to identify the ribosomes that localize near the endoplasmic reticulum and the mitochondria in yeast, providing further insight into protein targeting (Jan et al. 2014; Williams et al. 2014). We have recently combined this method with rapid and specific depletion of SRP to comprehensively characterize the role of SRP in cotranslational localization. This approach uncovered an unexpected class of mRNAs encoding proteins that are normally secreted but become mistargeted to mitochondria in the absence of SRP (Costa et al. 2018). Recent results suggest that subcellular organization of protein synthesis may be widespread, especially in tissues in which cells polarize and form three-dimensional structures (Moor et al. 2017). Localized translational control is particularly prominent in neurons, in which it is implicated in fundamental neural processes such as long-term potentiation and depression (Glock et al. 2017; Biswas et al. 2018; Sossin and Costa-Mattioli 2018).

Ribosome affinity purification has also emerged as a tool for cell type–specific translational profiling in animals, by using translating ribosome affinity purification (TRAP) (Doyle et al. 2008; Heiman et al. 2008) and RiboTag (Sanz et al. 2009). These approaches seem quite complementary to ribosome profiling, and indeed, tissue-specific ribosome profiling was recently shown in *Drosophila* (Chen and Dickman 2017). TRAP has seen its broadest application in the nervous system, which is characterized by extreme cell type diversity as well as a prominent role for translational control in synaptic plasticity (Sossin and Costa-Mattioli 2018). Further integration of cell type–specific ribosome profiling seems particularly promising in understanding the molecular basis of neuronal functions.

PERSPECTIVE

The translation of mRNA into protein and the folding of the resulting protein into an active form are prerequisites for virtually every cellular process and represent the single largest investment of energy by cells. Ribosome profiling-based approaches have revolutionized our ability to monitor protein synthesis in vivo, making it possible to determine the start, stop, reading frame, chaperone engagement, subcellular tar-

geting, and rate of translation for virtually every mRNA and protein encoded in a cell. The rich and quantitative nature of ribosome profiling data provides an unprecedented opportunity to explore and model complex cellular processes. Finally, by virtue of the precise genomic positional information obtained by ribosome profiling, the protein coding capacity of genomes can now be explored experimentally.

Nonetheless, important technical and conceptual questions remain. For example, the function of the many novel, short, and alternate translated regions identified thus far by ribosome profiling remains an intriguing and largely open question and one whose answer could fundamentally change the way that we believe about information encoding in genomes. Newly available CRISPR-based methods now make it possible to shut down the expression of any transcript (Gilbert et al. 2013, 2014; Liu et al. 2017a) or introduce nonsense mutations into any ORF (Hess et al. 2017). These approaches provide a central tool for efforts to define the functional roles for this broad array of newly identified translation products.

We have already seen demonstrations of specialized alterations to ribosome profiling that will advance its utility in complex systems. These developments include the analysis of molecularly defined subsets of ribosomes, either associated with specific factors or protein modifications, or even specialized ribosomes missing a core ribosomal protein entirely. Similar approaches allow the analysis of localized ribosomes within increasingly specific cell types or subcellular locations. We also know little about how ribosomes are distributed across individual transcripts of the same gene: Is the spacing between ribosomes purely stochastic, or are initiation and elongation "metered" to shape the traffic of ribosomes and minimize collisions? Along this line, understanding the biological roles for the use of synonymous codons remains one of the oldest outstanding questions in the field. Ribosome profiling provides an unprecedented view of their impact by yielding position-specific densities of ribosomes along a message. However, better protocols are needed to ensure that in vivo ribosome positions are captured faithfully and turned into sequencing libraries free of biases or distortions. Finally, transformative advances are likely to emerge from progressively more sophisticated and creative analysis of the rich data sets generated from ribosome profiling experiments, enabling major surprises to be revealed, even in systems that were thought to be well characterized.

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