Functional Impact of Readthroughs & Ribosomal Stalling

A literature review submitted

by

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Introduction

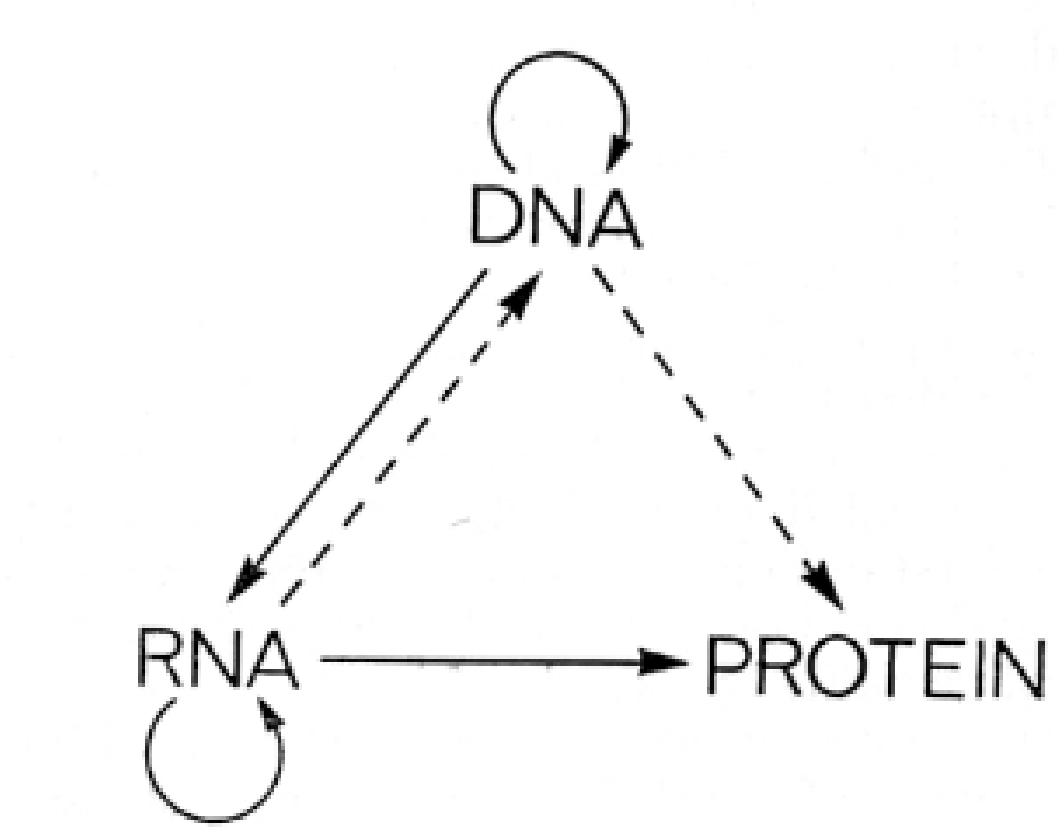

Figure 1.1: Central Dogma of Molecular Biology. [1]

Adenine, Guanine, Cytocine, & Thymine (A, G, C & T) are biomolecular alphabets which are the building blocks of DNA. The development and maintenance of any biological life form is driven by a system very similar to the one used in

linguistics. This core system of the central dogma of molecular biology states *"DNA->RNA->Protein"* [Fig 1.1]. The transition from DNA to protein involves a biochemical language in the form of nucleotide bases (*DNA*), context-sensitive grammar (*RNA*− >non-terminal symbols analogous to *introns* and terminal symbols to *exons*) leading to meaning and expression(*protein*). DNA is a double stranded chain of nucleic acid biomolecules and it tends to be a mode of "code preservation" due to it's stability, whereas mRNA, a single stranded chain of nucleic acids, is the translatable component. mRNA is encoded from DNA with same A,G & C bases, but T is replaced with U. RNA is translated by microscopic machines (called ribosomes) into proteins [Fig 1.2]. All the biochemical structures involved in transcription & translation(*protein synthesis*) are prone to mutations or substitutions, therefore it is always varying. The focus of the review is on canonical translation. Translation is a biological function through which genes are expressed. Ribosomes read the mRNA bases in groups of 3(*each group is a codon*) and uses it as a template to synthesize a protein(*chain of amino acids*) until a stop codon is reached. Stop codons are special triplet sequences which terminate translation . The event of a ribosome reading past a stop codon is called as a read-through (*RT*).

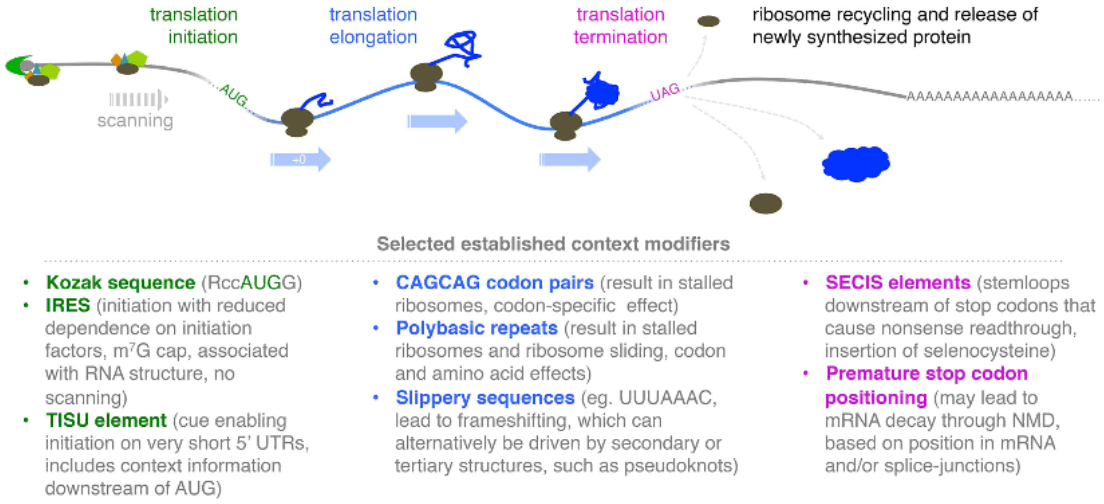

Figure 1.2: Canonical translation [2].

Read-through is a phenomenon which has been observed and its purpose has been interrogated for quite some time. This invokes a debate on whether the translational capability of the ribosome or the efficiency of the stop codon used in the mRNA being translated should be questioned. Observations on both the translational efficacy of the ribosomes and the stopping ability of the stop codons (in different domains of life) shows that this phenomenon is an interplay between

multiple pathways such as gene-regulation, protein-synthesis, mRNA surveillance and ribosomal-quality control. The significance of readthroughs in different domains of life can provide a clear picture of the evolutionary pressure acting on the stop codons (*based on both normal termination and read-through contexts*) and the level of conservation in different domains of life.

1.1 Readthroughs

1.1.1 Stop Codon Efficacy

Stop codons have variable efficacies ($UAA > UAG > UGA$) but their efficiency only plays a small part in the execution of a read-through [11]. One of the factors which promote readthroughs are the nucleotides surrounding the silenced stop codon (because it is read through, hence suppressed/silenced) at the 3' and 5'. "UGA" is reported as the "leakiest" codon, and the influence of the +4 nt is established as C>U>G>A, on the contrary, mammals rarely use UGA-C and UAG-C. Elevated and low read-through levels are observed with a A and U in the position upstream -1 of the stop codon UAG respectively [12]. This 5' & 3' contextual dependency for read-through initiation is conserved in both prokaryotes & eukaryotes [5] and the efficacies are selected through evolution [2].

1.1.2 Readthrough effects in different domains of life

In viruses, there are 3 types of known common readthroughs which often express polymerase or coat proteins [13]. Zooming out from the scope of viruses to model organisms, *Tobias von der Haar et al.* [14] pinned down the epigenetic consequence of misfolded protein (prion) from eRF3 gene (non-functional termination factor in yeast [PSI+] subtype) which caused in-efficient termination and allowed read-throughs in yeast. The [PSI+] subtype of yeast therefore has a mechanism for regulating translation termination efficiency. These strains of yeast show good tolerance to heat [15] and its absence in wild type [PSI-] yeast cells is not only astonishing but also infers that selective evolution occurs only when it confers advantages.

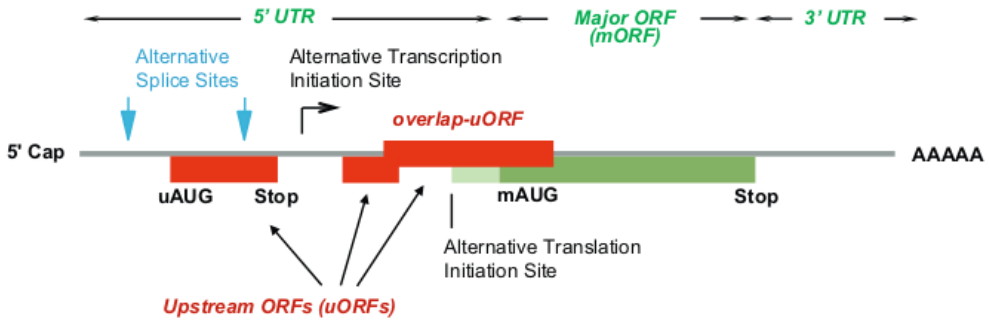

Figure 1.3: mRNA with uORF [3].

In 2011, *Jungreis et al.* [16], listed about 300 candidate read-through genes in Drosophila and related species. It also resulted in overruling alternate splicing and

substitution of stop codons as plausible explanations for a read-through. The paper narrows down on possible conserved read-through events in humans and found a fitting candidate: SACM1L [17] [16], an integral membrane protein. Similar incidents were observed in E.Coli which read through UGA stop codon in the membrane protein coding gene which produced β particles necessary for infection [18]. This raises questions on the correlation between readthroughs and membrane protein signatures (*C terminus extensions*) which is beyond the scope of this review. Some functional read-through products observed in mammals include *Peroxisomal lactate dehydrogenase* [19] and vitamin D receptor with reduced calcitriol response [20]. In 2014, Four genes (*SACM1L,OPRL1,OPRK1,BRI3BP*) which were candidates of efficient readthroughs with highly conserved motifs (*UGACUAG*) were isolated in mammals [17]. Molecular consequences of any proximal mutations which induce readthroughs are C-terminally extended non functional proteins, increased mRNA turnover rate & ribosomal stalling . *Arribere et al.* [21] provides an outlook on mechanisms which diminish this effect. The authors simulated readthroughs on sequences from *C. elegans* and found that when an unexpected RT occurred, translation of the 3' UTR region and consequetive post-translational modification (*PTM*) is adequate to reduce the protein levels in circulation. Figure 1.3 illustrates putative uORFs (*upstream Open Reading Frames*) in an arbitrary mRNA and why a read-through context in uORF stop codon is necessary for proper protein synthesis. A functional derivative consisting of short amino acid coding uORFs which have been conserved for more than 550 million years and which play a crucial role in calcium transport and muscle contraction in the heart of *D. Melanogaster* has been discovered by *Magny et al.* [22]. ORFs are not expected to show conserved protein coding ability nor do they show an explicitly mutated protein when their function is disrupted, which makes their study difficult [7]. This proves that they are critical in the synthesis, of some if not all, proteins and must be thoroughly interrogated.

1.1.3 Translation and Termination Contexts

By examining the read-throughs in different domains of life, protein synthesis seems to be most perturbed function. For a read-through to occur the stop codons have to be translated, but that does not follow the common model of stop codons terminating translation. In the *normal termination* context, on reading a stop codon, the ribosome waits for the tRNA with the correct anti-codon loop and a release factor to bind to the ribosome so that the translation can terminate. But in *read-through* context, suppressor tRNA molecules(*instead of the release factors*) bind to the ribosome and continue translation [23] [Fig 1.4]. In *S. cerevisiae*, *Blanchet et al.* [24] report that during readthroughs in some protein coding genes, the stop codons UAA and UAG are translated into *glutamine, tyrosine and*

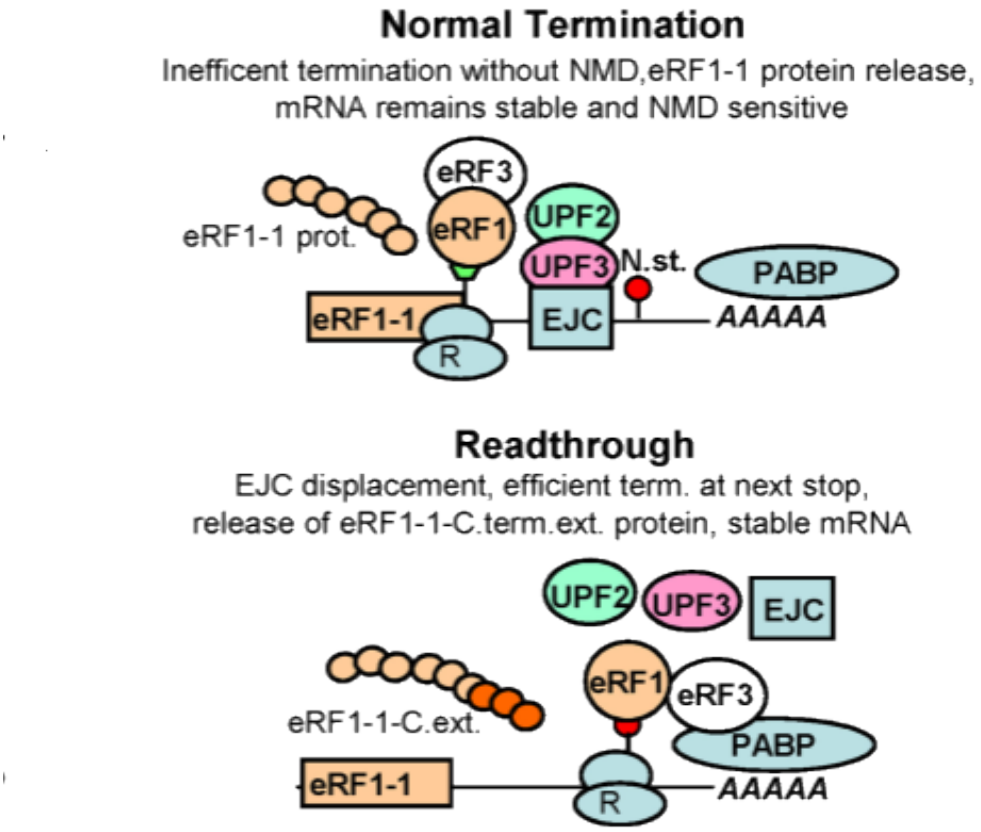

Figure 1.4: Normal vs Readthrough contexts [4].

lysine and UGA is translated into *tryptophan, cysteine and arginine* by suppressor tRNA. *Ng et al.* [25] propose a new experimental assay to uncover the identities of such stop codon suppressor tRNAs. Recent studies unlock implementation of such engineered suppressor tRNAs as putative solutions for premature stop codons [26]. A premature implementation of the same concept has been carried out by *Bordeira-Carrico et al.* to rescue wild type E-cadherin(Inter-cell adhesion protein) from cancer cells with premature stop codons [27].

1.1.4 Functional Translational Readthrough

Readthroughs were thought only to have some major implications such as malformed proteins with abnormally longer C-terminus which cannot play its func-

tional role, but it is a surprise to see that this is used as a gene regulatory mechanism, not only in viruses and bacteria, but also in mammals [20]. The new insight creates the addition of a new category of regulatory mechanism called "Functional Translational Readthrough" (*FTR*) [28]. FTRs are known to produce extended polypeptide chain by translating the stop codon which is commonly used to terminate translation. The extensions are not pathological in nature unless a protein coding sequence did not formerly have a contextual FTR and it occurred due to a mutation. The existence of FTR was first confirmed in *E. coli* and viruses.

1.1.5 Role of release factors in FTRs

Release factors are proteins which actively participate in translation termination. Generally in eukaryotes, when the frame of nucleotides read by a ribosome is a stop codon, eRF1 (eukaryotic release factor 1) mediates translation termination and releases the newly synthesized protein [29]. The RT & termination contexts would raise the question of the role and the importance of the release factors. Such a query can be answered by the study of eRF1 expression in 2017 [4].

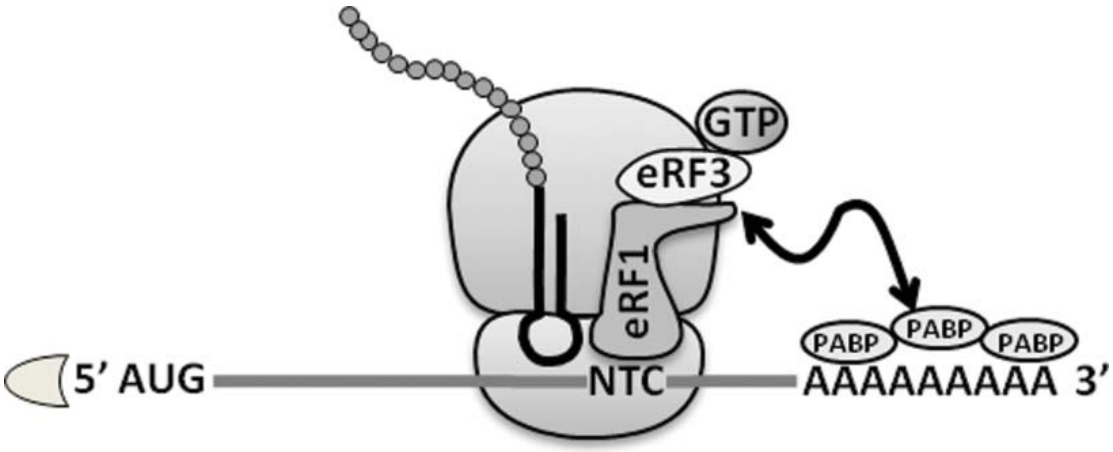

Figure 1.5: Translation termination in eukaryotes mediated by eRF1 Factor [5].

Nyiko et al. illustrate that eRF1 expression is controlled by a negative feedback loop wherein, higher concentrations of eRF1 leads to nonsense-mediated decay (*NMD*) and lower concentrations of the factor induced read-throughs due to the absence of cognate eRF1 factors to terminate translation. Conversely, the gene which encodes the factor is constrained by 2 auto-regulatory mechanisms where it's nonsense codon encourages a read-through and it's 3' UTR promotes NMD. Thus, if the expression of eRF1 is high, read-through is inhibited and the mRNA degrades and lower expression promotes read-through and protects the mRNA from degradation. The authors also postulate that NMD is prevented because the

translating ribosome suppresses the signal from the 3' UTR region which stimulate a quicker NMD, a concept which will be discussed further below. This observation in yeast and plants further implicates the tightly linked regulatory roles in read-throughs and terminations and how they are visible throughout nature.

1.2 Ribosomal Stalling

1.2.1 Dynamics of Ribosomes

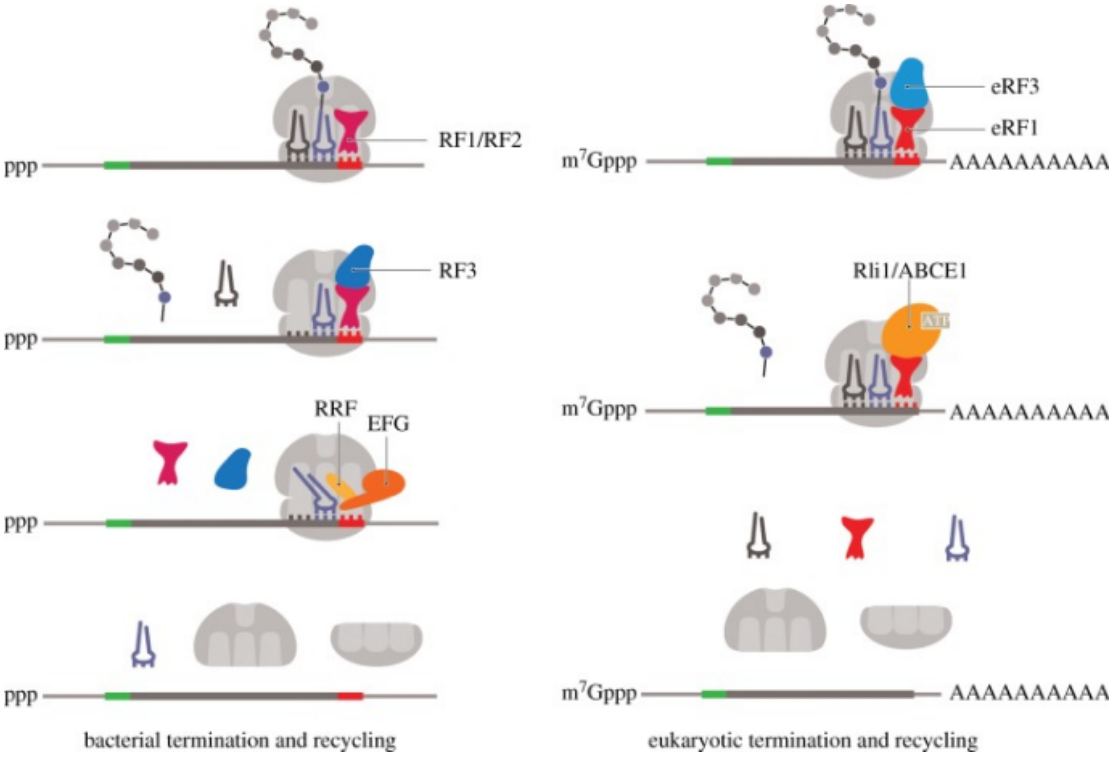

Figure 1.6: Different translation termination and recycling mechanisms [6].

The ribosome is a molecular factory, consisting of smaller and larger subunits, and translate mRNA to the corresponding polypeptide chain. The assembly of the peptide chain is orchestrated by catalytic action of RNA present in the larger subunit which link one amino acid to the next [30]. The primary structure and the core mechanics of ribosomes are alike in all biological life forms, but differences can be recognized in the form of size, increased structural complexity and enhanced functionality [31]. In both the prokaryotes and eukaryotes the stop codons are conserved but translation termination and ribosome recycling mechanics are different [Fig 1.6]. In bacteria, the stop codons are sensed with high precision by RF1(detects UAA & UAG) and RF2(detects UAA & UGA) factors whereas in eukaryotes, eRF1 recognizes every known stop codon. *Freistroffer et al.* [32], point that the release factor RF3 , in bacteria, accelerated the separation of RF1/RF2 factors from the ribosome.

1.2.2 Ribosomal occupancy and Ribosome profiling

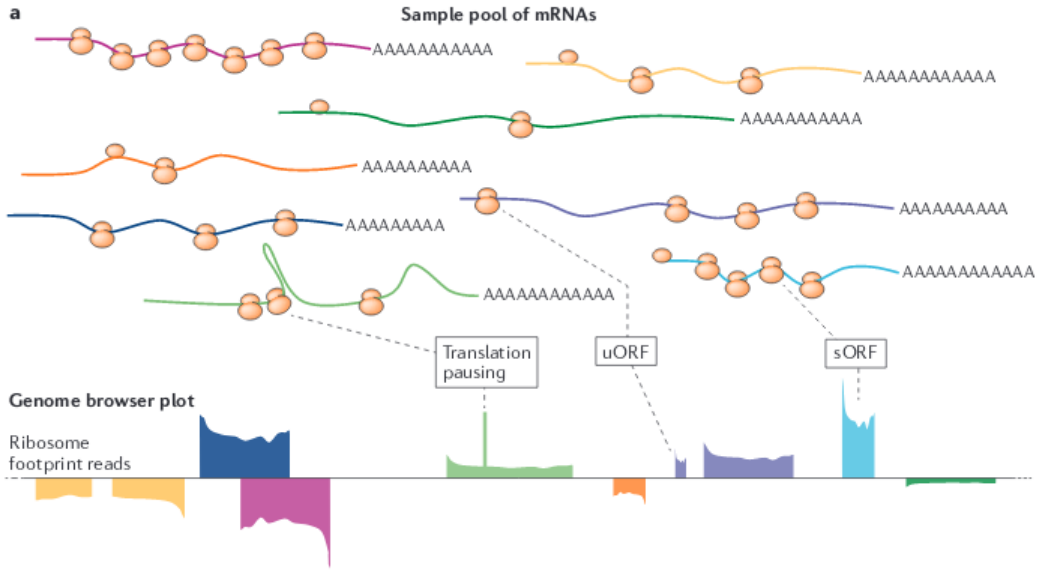

Figure 1.7: Ribosome Profiling NGS technique and different pausing sites of ribosomes [7].

Ribosome occupancy is a quantized measure of the percentage of mRNA being translated by one or more ribosomes. At any given time, a healthy mammalian cell will have more ribosomes than floating mRNA in its cytoplasm [33]. During mRNA translation, the floating mRNAs engage with the available ribosomes at the same time. The mRNA from genes having easy access to ribosomes are expressed more than mRNAs competing for occupancy. The fidelity of the ribosomal translation process is in direct relation to the quality of the transcribed mRNA [34]. Based on the aspect of mRNA structure it will be effectively translated, otherwise it will lead to either inaccurate translation (due to mutations in mRNA) or lead to ribosomal stalling. Ribosome profiling is an NGS technique to assay and gather information *in vivo* about the occupancy, pausing/stalling of ribosomes and also quantize mRNA and protein synthesis levels(based on the caveat that all ribosomes finish translation successfully) [Fig 1.7] [7]. Newer methods have been introduced for disclosing ribosomal pausing in single nucleotide resolution *in vivo* [35].

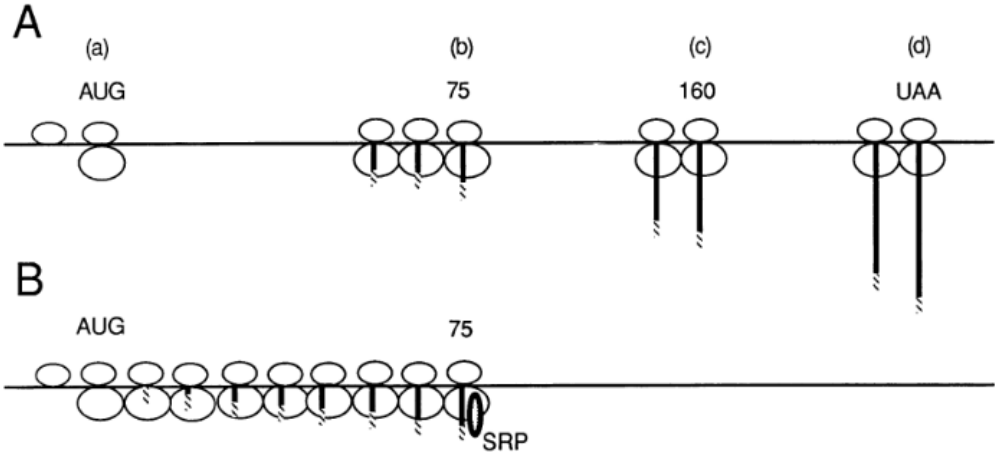

Figure 1.8: Ribosome Pausing(A) & Stalling(B) [8]. (A) (a-d) Common ribosomal pausing positions. (B) Ribosomal stalling.

1.2.3 Ribosomal Pausing and Stalling

Ribosomes do not progress at the same speed while elongating. Codon contexts can influence translation elongation rate, thus based on the arrangement of codons the ribosome can stream through the mRNA at different rates. Evidence suggests that endogenous ribosomal pausing [Fig 1.8] is an act of the cell for maintaining protein homeostasis [36] and other co-translational processes such as protein-folding and protein targeting [37]. Shine-Dalgarno(SD) [AGGAGG] sequence is a series of nucleotides of length 6 located upstream of a start codon in bacterial and archeal mRNA. The contrasting SD sequence (known as anti-SD sequence) is known to cause ribosomal pausing in *E. coli* & *B. subtilis*, but a similar event could not be observed in *S. cerevisiae* [38]. Factors such as gene regulation and mRNA structure should be accounted for so as to not classify pausing as stalling. Detection of stalling [Fig 1.8] can be either due to a longer pause (after which the ribosome may be re-initiated for translation) or error in elongation. Given the complexity involved in the dynamics of ribosomal stalling, it is not an exclusively pathological event. From ribosomal profiling, hints of ribosomes stalling in situ, in locations of perfectly transcribed mRNA in healthy cells shows that this phenomenon is not limited to faulty mRNA mechanisms. These mRNA strands were not abnormal and were not degenerated by the mRNA surveillance pathway (*Quality control mechanisms for mRNA strands*). Peptides can also influence stalling,for example, *proline* is an amino acid (encoded by all codons starting with CC) with a high bias in ribosome profiling data with respect to ribosomal stalling [39]. Alternatively, several self-regulating nascent peptides are known to stall ri-

bosomes quite frequently and with high efficiency [40]. This discovery has been confirmed by analysis of distribution of nascent peptides that cause ribosomal stalling in *S. cerevisiae* [41].

1.2.4 Stalling Associated Quality Control

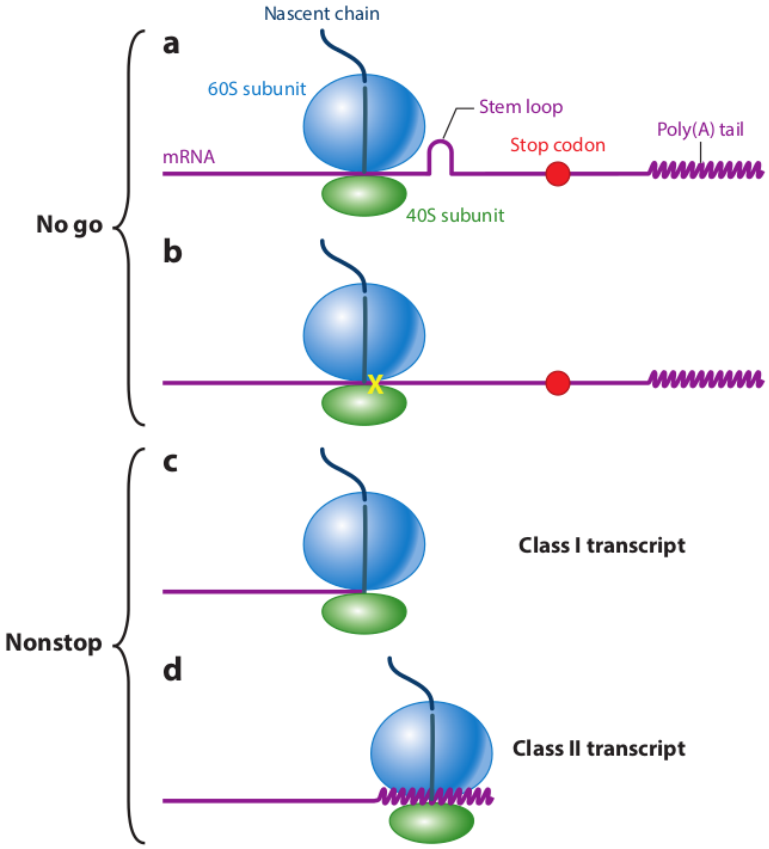

Figure 1.9: Ribosome Quality Control [9].

Ribosomes can stall for a variety of reasons and can be classified under 'nonsense mediated', 'no-go' or 'non-stop mRNA' [Fig 1.9]. After stalling, the nascent polypeptide chain is cleaved, the ribosome unit is recycled, and the mRNA is pushed to degradation [42]. Dom34, a protein which dissociates ribosomal subunits is known to specifically target and dissociate stalled ribosomes in the 3' UTR regions [43]. MRNA degradation is initiated at a time when a ribosome begins it's final translation routine, this phenomenon is known as *"co-translational degradation"* and was identified using 5PSeq in *S. cerevisiae* cells. It uncovers ribosomal pausing in rare codons which would have been masked by the treatment process in

other studies [44]. Adding to these factors, anti-codon loops in tRNAs and mRNA secondary structures (eg, stem loops) also play a major role in translational efficiency and provocative ribosomal stalls. *Ishimura et al.* [45] provides the first example of ribosomal stalling in mice due to mutation in tRNA molecules which leads to neurodegeneration and death. Decreased ribosome density at the stop codon is directly associated with a decrease in the level of eEF3(eukaryotic elongation factor) in yeast [46]. Pathological stalls due to point nonsense mutations or aberrant mRNA usually invoke ribosome associated quality control mechanisms after which the ribosome is disassembled and the mRNA is degraded. ZNF598 (*zinc finger protein*) which is a known translation repressor during embryonic development also has a cellular function of a quality control sensor for collided diribosomes [47]. Astonishingly, ZNF598 can detect if a stall is either physiological or pathological but the actual process behind it is unclear.

1.2.5 Auto-regulative Stalling and Protein Homeostasis

Combining FTRs, ribosomal stalling(or more appropriately queuing)and gene regulatory mechanisms disclose a new form of temporally-auto-regulative stalling mechanism for protein homeostasis. This idea arose from an *in silico* kinetic(motion) simulation experiment of ribosomal elongation in starved *E. coli* cells which produced contradictory results unlike the widely accepted existing kinetic models (*Traffic Jam & SAT models*) [48] based on which *Ferrin et al.* propose a new model where the collision of ribosomes act as a timer for quality control pathways. This proposition raises some concerns because of its purely computational nature and inconclusive evidence, but, the concept turns out to be conclusive in the wake of AMD1 coding mRNA. AMD1 has oncogenous properties and excessive production leads to the growth of aggressive tumors, thus the cell should take high caution and invoke tight regulatory mechanisms to stop it from developing into a putative carcinoma. *Yordanova et al.* found an interesting effect of ribosomal stalling in HEK293T cell line, where the ribosomes parked themselves at the 3' tail ORF and the ribosomal queue length is proportional to the number of AdoMetDC molecules synthesized from the AMD1 mRNA [Fig 1.10]. The mRNA has a read-through context and when the read-through was mitigated by replacement with a sense codon, lead to a complete loss of protein levels. This is an interesting amalgamation of concepts and is termed as "Molecular Memory formation" as the cells keeps the AMD1 levels in check based on the number of parked ribosomes in the uORF [10]. This cyclic dependency leads to the self renewal of AdoMetDC molecules and protects it from endogenous misregulation. What happens to the parked ribosomes and how they escape quality control is not clear yet but detecting this novel mechanism only foreshadows that there is more to be understood about cells and how they function.

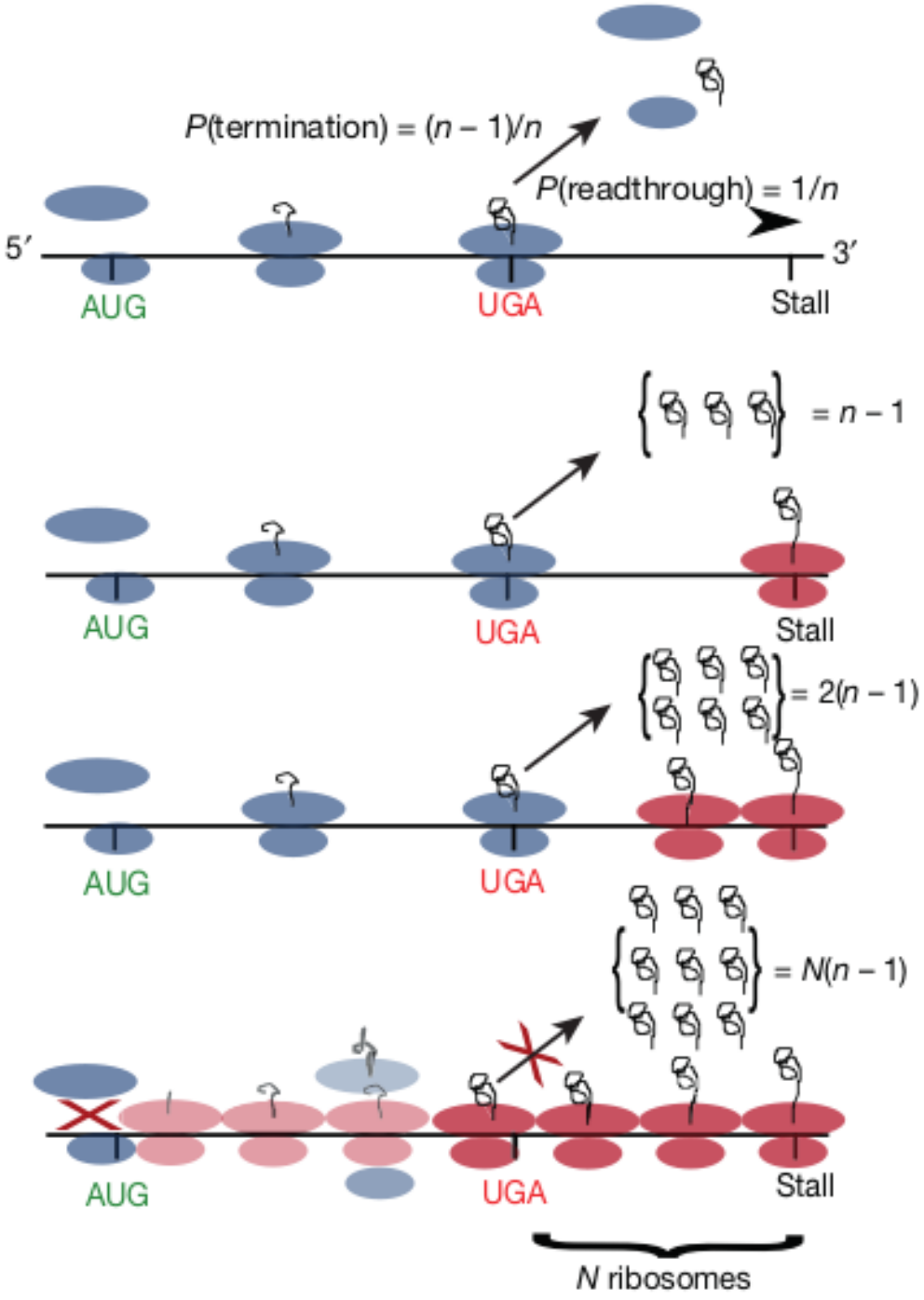

Figure 1.10: Auto-regulative temporal ribosome stalling regulating the number of AdoMetDC molecules synthesized by AMD1 mRNA [10].

1.3 Discussion

The review is a summary of read-through & ribosomal stalling mechanisms and it's consequences on translation elongation step. Identifying read-throughs in both prokaryotes & eukaryotes explain about the level of conservation of this mechanism. On studying the fidelity of ribosome translation and stop codon efficiencies, the multiple factors involved in read-through initiation and execution were captured. Comparing normal and read-through contexts of translation elongation that read-throughs consequences are not limited to non-functional products. This provides a better picture of the evolutionary pressure acting on the stop codons. Finally, when conjugating ribosomal stalling mechanics with read-throughs, a novel & important gene regulatory mechanism of "Molecular Memory Formation" was discovered. This discovery opens new ventures in molecular biology to tweak future studies based on current advances and takes one step further in decrypting the enigma called 'life'.

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