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How are circRNAs translated by non-canonical initiation mechanisms?

by

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ABSTRACT

Circular RNAs (circRNAs) are covalently closed RNA loops produced by a very large number of expressed eukaryotic genes. Initially considered as splicing background and/or splicing side products, recent studies have shown that they are evolutionary conserved and abundant in cells. Yet, their functions remain largely unknown. Because of their circular shape, they were initially categorized as non-coding RNAs. However, recent studies based on mass spectrometry analysis indicate that some cytoplasmic circRNAs are effectively translated into detectable peptides. This raises the interesting question of which mechanisms regulate the translation initiation of those circular transcripts, i.e. unable to recruit the small ribosome subunit through the 5' cap. A possible mechanism for alternative translation initiation is the presence of an IRES (Internal Ribosome Entry Site) that allows direct recruitment of initiation factors and ribosomes on the RNA independently from the cap. This is the case for several circRNAs that exhibit IRESs upstream from the start codon. Yet, another process seems to be involved in initiating the translation of circRNAs: the presence of N⁶-methyladenosine (m⁶A) residues. These m⁶A can promote cap-independent translation and have been shown to be enriched in circRNAs. Interestingly, these two alternative translation initiation processes are generally activated under cellular stress to allow expression of specific stress response genes. These discoveries therefore link circRNA translation to cellular response to stress conditions, raising new enquiries about the regulation of circRNA expression under stress conditions and their functions. This review provides a state of the art on this emerging area.

Keywords: circRNA, translation, IRES, N⁶-methyladenosine

1. Introduction

Circular RNAs (circRNAs) are endogenous to eukaryotic cells. Discovered more than 25 years ago, circRNAs have for long been considered as irrelevant artefacts of RNA processing [1,2]. However, recent studies have uncovered their peculiar properties and large scale involvement in physiological and pathological gene expression [3–6]. Indeed, data arising from RNA-seq projects demonstrated the significant amount of circRNAs in eukaryotic cell and the evolutionary conservation of this new category of RNAs, clearly stating that they are not merely splicing background. The first article describing a massive presence of circular RNAs in eukaryotic cells was from Salzman et al. [7]. In that study, the authors aimed to identify cancer-specific exon scrambling events, corresponding to non-colinear exon junctions. For this, they selected and analyzed non-colinear sequencing reads, evidence of “scrambled” exons and potential circRNAs in RNA-seq data. In this article they identified a total of 2,748 scrambled isoforms, or circRNAs, in HeLa and H9 embryonic cells. At the same time, a study conducted by Memczak et al. extended the exploration to different species and led to the identification of approximately 2,000 circRNA in humans, 1,900 in mice and 700 in nematodes and showed that circRNAs are often expressed with specific expression patterns depending on tissues or developmental stages [8]. Subsequently, Jeck et al. modified the approach by adding a 3' exoribonuclease (RNase R) treatment prior to sequencing, thus enriching the population of circular RNAs in the samples. This enhancement led to the identification of a significantly higher number of circular RNAs, and the results suggested that 14.4% of actively transcribed genes produced circular RNAs in human fibroblasts [9]. It is also the development of specific bioinformatics tools, based on split mapping of RNA-seq reads, that allowed the efficient prediction of circular RNAs from RNA-seq data [8,10–13].

A relatively large number of actively transcribed genes generate circular RNAs in mammalian cells. They mostly originate from coding genes by a spliceosome-mediated alternative

splicing of the pre-messenger RNA called backsplicing [3,7–9,14]. Using the spliceosome to link a splice acceptor (3' splice site) to a downstream splice donor (5' splice site) on the same exon or further, circRNA biogenesis can compete with the maturation of its linear counterpart [7–9,15,16]. This non-colinear splicing forms a backspliced junction specific to each circular RNA species and absent from the canonical linear RNAs. This process is promoted by the presence of complementary inverted repeats and RNA binding proteins (RBPs) binding motifs in introns flanking the two splice sites (Figure 1). The pairing of complementary sequences or formation of RBP dimers brings the circularized exons close together [9,14,17–19]. In primates, Alu repeats are the most abundant form of repeated sequences and have thus been associated with circRNA biogenesis in human cells. Formation of these double stranded RNA structures can also imply *trans*-acting RBPs that will modulate backsplicing either positively, by stabilizing the structure as is the case with NF90/NF110, or negatively, by destabilising the base-pairing like the editing enzyme adenosine deaminase that acts on RNA (ADAR1) [20,21].

The covalently closed circular structure of circRNAs renders them resistant to 3' or 5' exonucleases. From this characteristic derives their high stability with a half-life reaching up to 10 times that of linear RNAs (median half-life 2.5 times longer on 60 analyzed circRNAs) [22]. In eukaryotes, the length of circRNAs is variable but tends to average between 200-800 nucleotides (nt) with a range of 100 to 4000 nt in length [23]. One estimate that in humans, circular RNA may account for 1% as many molecules as poly (A) RNA [7].

On the functional level, circRNAs are tuned to retain tissue and developmental stage specificity and can be sensitive to cellular stresses [8,24]. They could also be involved in pathological processes such as cancer [25,26]. The circular transcripts have well-conserved sequences among eukaryotes. Based on the type of sequences they contain, they are divided into exonic (EcRNA), intronic (CiRNA) which have a different biogenesis mechanism and

exon-intron (EIcRNA) circRNAs. Like in other RNAs, these differences in structure and sequence define their sub-cellular localisation and function [27,28] (Figure 1). Some intronic and exon-intron circRNAs are reported to be sequestered in the nucleus [27,29], while exonic circRNAs are mostly exported to the cytosol via a length-dependent mechanism involving *Drosophila*'s Hel25E dead-box helicase and its homologues in human cells [30]. Circular RNAs are predominantly exonic and thus predominantly cytosolic [7,9].

In the nucleus, circRNAs participate in gene expression regulation at different levels. First, circRNA biogenesis is largely post-transcriptional and use the canonical splicing apparatus, thus competing with the maturation of its linear counterparts [15,16,31,32]. Second, exemplified by circPAIP2 in human cells, intronic and exon-intron circRNAs have been described as transcriptional regulators of their parental genes by interacting with RNA polymerase II, snRNPs and hnRNPs [27,29,33] (Figure 1-1).

In the cytoplasm there is an accumulation of EcRNAs with two types of functional outcomes described so far. Firstly, because they share parts of the mature linear RNA sequence, exonic circRNAs act as competing endogenous RNAs, sequestering microRNAs (miRs) and RBPs away from their cytosolic targets (Figure 1-2). For example, one of the first and most studied circRNAs, circCDR1as/ciRS-7 in brain tissue harbours 63 conserved binding sites for miR7, acting as an effective miR7 sponge. Over-expression of the human circCDR1as/ciRS-7 revealed a negative impact on brain development in zebrafish [8,28]. CircRNA interaction with RBPs is illustrated by the cases of circMbl and circFoxo3. The *Drosophila* muscleblind gene (*Mbl*) produces a circular RNA (circMbl) when the Mbl protein, involved in circRNA biogenesis, accumulates. CircMbl then binds and retains the excessive Mbl protein [34]. In mammalian cells the tumour suppressor gene *Foxo3* generates a circRNA (circFoxo3) that regulates cell cycle progression by binding to cell division protein kinase 2 (CDK2) and cyclin-dependent kinase inhibitor 1 (p21) [35]. Hence, circRNAs participate in miR and RBP

transportation, storage and availability creating yet new ways of post-transcriptional regulation.

In addition, interesting recent studies have shown that a subset of exonic circRNAs is found associated with polysomes and most of them comprise the native start codon of their mRNA counterparts and exhibit favourable open reading frames [25,26,36–43]. This revealed an unexpected translation potential for circRNAs. Originally mislabelled as "noncoding", circRNAs can actually be translated into regulatory peptides (Figure 1-3). These observations nevertheless raise the question of translation initiation for this particular class of circular transcripts.

2. circRNA translation

The question of circRNAs translation in cells is highly relevant since they are mainly cytoplasmic and comprised of exonic sequences (EcRNAs), often carrying an initiator codon and sometimes the canonical AUG of the associated mRNA with potential open reading frames. In addition, Chen and Sarnow have proven, in early studies in 1995, that synthetic circular RNAs could recruit the 40S ribosomal subunit and initiate translation of detectable peptides in human cells through an internal entry site [44]. However, that study does not investigate the case of endogenous circRNA molecules. Therefore, several teams investigated whether endogenous circular RNAs were translated. The early results provided rather negative responses to that problematic. In a first study Jeck et al. selected three EcRNAs that had a translation start site (common with the corresponding mRNA) and found out that they were absent from the polysome fraction [9]. This indicated that these three circular RNAs were not bound to the ribosomes and therefore not translated. Another study, by Guo et al. systematically searched for evidence of circRNA translation by examining data from

ribosome footprinting of non-poly(A) selected RNA-Seq [45]. They concluded that no evidence supported the possibility of circRNAs undergoing translation in cells. The only demonstration of EcRNAs translation by ribosome footprinting is the detection of reads spanning the backsplicing junctions. In this case, it is presumably this constraint combined with the lesser abundance of circRNAs, compared with corresponding linear RNAs, that could explain these results (i.e., no evidence of circRNA translation). In a later study, Schneider et al. investigated the association of circRNAs with proteins by sucrose gradient fractionation [46]. Like the study by Jeck et al. they focused on a limited number of abundant circRNAs and failed to find any association with polysomes. Despite these negative responses, the investigations continued and, from 2017 to this date, seven distinct circular RNAs have been deemed translated in human cells and, for some, the function of the deriving peptide have been identified [25,26,36,40,47–49]. In addition to that, a recent large scale study by Ragan et al., identified circRNAs with a great potential of translation [50]. By performing TriP-seq (Transcript Isoform in Polysomes sequencing, [51]) they found approximately 15% of all circRNA read counts in the ribosomal fraction, representing 177 potentially translated circRNA candidates in HEK293 cells [50]. Most of these candidates for translation are associated with the monosomal to tetrasomal fractions, whereas linear mRNAs are mostly associated with hepta and octasomal fractions. These results, considering the seven studies mentioned above, support the coding potential of many more endogenous circular RNAs and raises the question of the mechanisms by which their translation is initiated.

2.1 cap-independent translation initiation mechanisms

The circular structure of circRNAs necessarily implies a cap-independent and internal mechanism to initiate translation. Indeed, eukaryotic mRNAs have their 5' end modified by

the presence of a m⁷G (7-methyl guanosine) cap which is recognized by the eIF4E translation initiation factor [52]. The eIF4E factor combines with eIF4G (a scaffold protein) and eIF4A (an helicase protein) to form the eIF4F complex [53,54]. The interaction between eIF4E and the cap is modulated by the mTOR (mammalian Target Of Rapamycin) kinase pathway. When the cells are submitted to a stress like hypoxia, nutrient starvation, oxidative stress, etc... the mTOR kinase is inactivated, leading to an overall inhibition of the cap-dependent translation due to hypophosphorylation of 4E-BPs (eIF4E-binding proteins). Hypophosphorylated 4E-BPs compete with eIF4G for eIF4E binding and thus prevent eIF4F assembly and the 43S ribosome recruitment at the 5' end of the transcripts [55]. This is one of the main pathways for translation initiation control in eukaryotes [56]. The other major mean to modulate translation initiation is through the eIF2 α factor phosphorylation. A number of serine kinases phosphorylate eIF2 α . Those kinases are activated by stresses such as amino acid deprivation (GCN2), ER stress (PERK), the presence of dsRNA (PKR), heme deficiency (HRI) or interferon [57]. When eIF2 α initiation factor is phosphorylated, there is a blockade of GDP to GTP exchange in the eIF2 complex, which in turn prevents the assembly of the ternary complex eIF2-GTP-tRNA_i^{Met}. Thus, during stress, the cap-dependent translation is largely inhibited [58]. Nevertheless, a subset of messenger RNAs maintain their translation during stress by using cap-independent translation initiation mechanisms [59,60].

One possibility in the cells, for cap-independent translation initiation, is the presence of an IRES (Internal Ribosome Entry site) structure in the 5'UTR of mRNAs. IRESs are sequences that form secondary structures on RNA and allow translation initiation in the total or partial absence of canonical translation initiation factors, replaced by ITAFs (IRES Tran-Acting Factors) (for review, see [61]). Although eIF2 α is, in principle, required for the initiation of cap-dependent and cap-independent translation, several reports have shown that IRES-dependent translation can occur despite eIF2 α phosphorylation [62]. The first IRES elements

have been discovered in viruses [63] but are also present in the 5'UTR of many cellular mRNAs like the immunoglobulin heavy-chain binding protein (BiP) mRNA, the fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) mRNAs [61][64]. On cellular mRNAs, IRESs are particularly functional under stressful conditions. They allow the maintenance or activation of translation for a pool of RNAs equipped with IRESs, providing the cell with important proteins for its survival. These genes include master regulators like transcription factors, growth factors, etc....

Another mechanism described recently is the cap-independent translation initiation under stress through methylated adenosine residues in the form of N⁶-methyladenosines (m⁶A) in the 5' untranslated region (5'UTR) of some transcripts [65]. 5'UTR m⁶A residues can directly recruit the eukaryotic initiation factor 3 (eIF3), which is sufficient to recruit the 43S complex and initiate translation in the absence of the cap-binding factor eIF4E. 5'UTR m⁶A residues can also modulate translation initiation during integrated stress response by redirecting ribosomes on different ORFs. This is illustrated by the case of ATF4 mRNA, which is regulated by both the eIF2 α signaling pathway and the presence of m⁶A residues, upon amino acid starvation [66]. Indeed, ATF4 mRNA is m⁶A methylated in the uORF2 located in the 5'UTR. This methylation is decreased upon amino acid starvation in a ALKBH5 and FTO demethylase-dependent manner. In fact, ribosome retention increases on the uORF2 m⁶A residues, leading to a slower ribosome scanning, which in turn promotes translation initiation at non-canonical upstream initiation sites. Ultimately, there is a decrease of translation initiation on ATF4 main ORF. This concept has been enlarged to other transcripts by global profiling of m⁶A and translation initiation sites occupancy [66]. Thus, both m⁶A and IRES dependent translation initiation are potential mechanisms for cytoplasmic circular RNAs translation.

To date, only seven translated circRNAs have been uncovered by Legnini, Pamudurti, Zhang, Yang, Zheng and Liang et al. in separate studies published during the last two years [25,26,36,37,40,48,49]. In the following segments we will present each of these studies, focusing on the crucial question of which cap-independent translation initiation mechanisms are triggered by circular RNAs. Consequently, we exclude the study by Zheng et al. on circPPP1R12A for which the translation initiation mechanisms were not addressed [48].

2.2 IRES driven circRNA translation

IRES-driven translation of circZNF609

In the study by Legnini et al. the authors showed that circZNF609 is the backsplicing product of the ZNF609 gene exon 2, encoding a poorly characterized transcription factor belonging to the zinc-finger protein family [37]. Interestingly, the Open Reading Frame (ORF) of circZNF609 starts at the same AUG codon as its linear counterparts but encounters a stop codon 3 nucleotides after the backspliced junction where a frameshift occurs. Supporting its *in vivo* translation, circZNF609 has been found in the heavy polysomes fraction after RNase R treatment. The authors then tested whether the sequence upstream from the initiation codon (i.e. 80 nt from the 3' end of exon 2 and across the backspliced junction fused to the UTR) displayed an IRES activity. This sequence cloned into a bicistronic vector, containing the Renilla luciferase under cap-dependent translation and the Firefly luciferase under the putative IRES-dependent translation, did not display any significant IRES activity. However, when they added small fragments of the endogenous intronic sequences in the construct, in order to mimic the backsplicing occurring to generate circZNF609, they obtained a stronger IRES activity than that of EMCV. Thus circZNF609 exhibits a splicing dependent IRES activity. This observation is particularly interesting because it suggests that factors necessary

for IRES activity may be recruited through splicing. It is entirely conceivable, for example, that proteins of the hnRNP family involved in splicing could be recruited onto circRNA in the nucleus, afterwards acting as IRES Trans-Acting Factors (ITAFs) in the cytosol. This process has already been described for several hnRNPs acting as ITAFs for mRNAs [61]. Additionally, protein production from circZNF609 is increased after a heat-shock stress, which is known to activate IRES activity [58] and modulate alternative splicing [67]. These two processes may be a functional link between stress and circRNA translation initiation.

IRES-driven translation of circMbl

In the same issue of *Molecular Cell*, Pamudurti et al. presented their study in which they show that a subset of circRNAs are associated with translating ribosomes in vivo in *Drosophila* by ribosome footprinting [36]. They focused on a circRNA, circMbl, generated from the muscleblind locus. Like circZNF609, circMbl shares the same start codon as the linear muscleblind mRNA and the ORF stops a few nucleotides after passing the backspliced junction. Similarly, they have found an IRES activity arising from the UTR of the circRNA (cUTR) cloned into a bicistronic vector. In their experiments, they also measured Firefly and Renilla activities in the presence or absence of 4E-BP, mimicking the blockade or not of cap-dependent translation. Overexpression of 4E-BP resulted in an increase by more than 4 fold of the Firefly luciferase/Renilla luciferase luminescence ratio, sustaining cap-independent translation initiation through the circMbl cUTR in a context mimicking cellular stress. Importantly, the circMbl-encoded protein was also detected by mass spectrometry.

IRES-driven translation of circSHPRH

In another study, Zhang et al. identified a new circRNA produced by the gene coding the E3-ligase SNF2 histone linker PHD RING helicase (SHPRH), called circSHPRH [40]. In this case, the backsplicing of exons 26 to 29 forms circSHPRH and organises a large ORF of 146

amino acids. The particularity of circSHPRH compared to the two previous examples is that the ORF occupies the totality of the circRNA by using a start codon overlapping with two tandem repeat stop codons: UGAUGA. The authors have identified an IRES able to drive translation initiation of circSHPRH. However, this IRES is located downstream from the start codon in contrast to circZNF609 and circMbl. It is also interesting to note that the functional IRES is formed by the joint sequences of exons 26 and 29 and thus spans across the backspliced junction. Indeed, if a portion of the backspliced junction arising from either exon 26 or exon 29 is deleted, the IRES activity is dramatically reduced. This illustrates yet another example of splicing dependence for IRES mediated translation initiation of a circRNA, and points again at the possibility of ITAF recruitment through splicing. Significantly, circSHPRH produces a novel 146 amino acids protein that was detected by mass spectrometry and displayed a tumour suppressor activity by acting as a decoy, protecting the related full-length SHPRH protein from degradation.

IRES-driven translation of circFBXW7

In a similar way, Yang et al. have characterized a circRNA produced by the tumour suppressor E3 ligase FBXW7 [25]. The circular form of FBXW7, circFBXW7, derives from the backsplicing of exon 3 to 4 and forms a 620 nt circular RNA. In the case of circFBXW7, the authors have found a 120 nt long IRES in the cUTR that is located upstream from the AUG and downstream from the backspliced junction. The partial deletion of this sequence induced a decrease in the IRES activity. Unfortunately, it seems that the authors did not test the IRES activity of a longer sequence stretching upstream to cover the backsplicing junction, which would have been interesting to compare with the previous occurrences of circRNA IRESs. Similarly to circSHPRH, the 185 amino acids protein encoded by the circFBXW7 (FBXW7-185aa) was detected by mass spectrometry and displayed a tumour suppressor activity in a glioblastoma model. The analysis of circFBXW7 expression in glioblastoma

clinical samples confirmed its effective translation in vivo and the potential tumour suppressor activity of FBXW7-185aa, as the protein levels were reduced in the samples compared with their paired tumour-adjacent tissues.

IRES-driven translation of circLINC-PINT

A second study by Zhang et al. reported last year the translation of a circRNA originating from the long non-coding RNA and p53-induced transcript LINC-PINT [26]. The circLINC-PINT is generated by the circularisation of exon 2. The authors identified circLINC-PINT as a potential coding circular RNA from the analysis of ribosome nascent chain-sequencing data. This 1084 nt circular RNA contains several small open reading frames. The study has proven one of them to produce a 87 amino acid peptide, called PINT87aa, that participates in the control of cell proliferation and tumorigenesis in cancer cells. The authors cloned the sequence of 478 nt between the backspliced junction and the start codon and found an IRES activity able to drive cap-independent translation initiation in a bicistronic vector. In this case too, the cloned region tested for IRES activity did not span the backspliced junction. Interestingly, the most potent region that retained IRES activity, when they performed deletion experiments, was the region closest to the backspliced junction (i.e. the first 231 nt display a comparable IRES activity to the full length 478 nt).

IRES-driven translation of circ β -catenin

This year, Liang et al. have published the latest article to date reporting the translation of a circular RNA into a novel β -catenin isoform [49]. Circ β -catenin is formed by the backsplicing of exons 2 to 7 of the β -catenin gene, resulting in a 1129 nt long circular molecule. The authors identified an ORF spanning from the same start codon as the linear β -catenin mRNA (nucleotide 49 in exon 2) across the backspliced junction to a new stop codon (nucleotide 30 in exon 2). The study demonstrated that a 370 amino acids β -catenin isoform was produced

by this ORF and detected by mass spectrometry, on a liver cancer cell line. Further experiments revealed that this protein promotes liver cancer cells growth, both in vitro and in vivo, through the activation of the Wnt pathway. By acting as a decoy for the canonic β -catenin, the circRNA derived isoform protects it from degradation and thus promotes its action. The authors identified a putative IRES sequence of 107 nt, upstream of the start codon, displaying a significant activity when cloned into a dual luciferase reporter system, strongly suggesting the presence of an IRES in this region.

Thus, like for circZNF609 and circSHPRH, the location of the stop codon a few nucleotides upstream the start codon, with respect to the size of the IRESs that are described, implies that the sequence containing the IRES overlaps the translated region. In addition, the possibility of a similar layout for the other four circRNAs is not ruled out, if not comforted in the case of circLINC-PINT. The reoccurrence of this configuration in such a small population of translated circRNAs is interesting to note as it could be a specific feature of circular RNA IRESs. Indeed, for cellular IRESs, only one example of an IRES in a translated region has been reported in the literature. It is the case of the CAT-1 mRNA, which possesses an IRES overlapping a small ORF upstream of the main start codon. In this case, the translation of the small reading frame induces a conformational change that activates the IRES [68]. Could this apply to circRNAs ? Though, in the case of the CAT-1 mRNA, the activation of the IRES depends on the cap-dependent translation of an upstream ORF, whereas for circular RNAs, the IRES would need to be active enough to initiate a pioneering translation round and then be further activated. It will be interesting to carry out an in-depth mechanistic study of these IRESs overlapping translated regions in circular RNAs to answer these questions in the future.

2.3 m⁶A driven circRNA translation

Nevertheless, it seems that mRNAs without IRES activity can still be translated independently of the eIF4F complex. Several recent articles report that methylation in the form of N⁶-methyladenosine (m⁶A) in the 5'UTR of messenger RNAs facilitates the translation of some RNAs, in a context where translation initiation via eIF4F is blocked but in an IRES-independent pathway [69]. Therefore, this mechanism seems to constitute a third way to initiate translation in eukaryotic cells and an alternative to the use of IRESs for cap-independent translation initiation. The mechanism for m⁶A-initiated translation is not well known but involves the methyltransferases METTL3, METTL14 and Wilm's tumor associated protein [70,71]. This system is reversible since translation by m⁶As can be inhibited by the demethylases FTO (fat mass and obesity-associated protein) and ALKBH5 (alkylated DNA repair protein alkB homolog 5) [72,73]. Interestingly, the initiation of m⁶A-dependent translation seems to be finely regulated under stress conditions and could constitute an additional mechanism to modulate the translation of a pool of specific transcripts under stress conditions in parallel with the pool of IRES-controlled mRNAs [66]. Here again, this mechanism, which allows the initiation of translation in an eIF4F-independent way, can be applied to translation initiation for circular RNAs, as reported by some recent studies [38]. In the article by Yang et al., the authors show that m⁶A residues are enriched on all circRNA sequences and that a single m⁶A is sufficient to initiate translation. This circRNA m⁶A-dependent translation relies on the eIF4G2 factor, and the YTHDF3 m⁶A reader. It is activated under stress conditions such as heat-shock or by the overexpression of METTL3/14 and is inhibited by the FTO demethylase. The authors performed an RNA-Seq analysis of m⁶A-targeted immunoprecipitated RNAs, treated with RNase R. They estimate that at least 13% of circular RNAs carry the m⁶A modification, but this is probably an underestimated value since the technical approach focuses only on the analysis of m⁶A motifs adjacent to

backspliced junctions. This study identified 250 circRNAs associated with polysomes and some may be actively translated since this association is sensitive to puromycin treatment, although this fact alone does not constitute absolute proof of translation.

3. Translation of synthetic circular RNAs

Very recently, studies have been initiated to produce synthetic circular RNAs in order to investigate the biological characteristics of circular RNAs and their biogenesis. For this purpose, authors have designed a plasmid expression system where backsplicing is facilitated by the hybridization of complementary sequences in the synthesized “pre-circRNA” [74]. With an IRES upstream of the reading frame, the circRNAs derived from these expression vectors can produce a given protein. Yang et al. adopted this strategy to design a vector where a GFP reading frame is assembled by backsplicing [75]. The construct can therefore be used to study the efficiency of backsplicing or IRES dependent translation initiation. Considering the stable properties of circRNAs, this type of vector is also a promising tool in biotechnology for a prolonged production of recombinant proteins [75]. The IRES-dependent GFP split construct has already been transposed into an adenoviral genome to produce recombinant proteins in vivo [76]. Finally, very recently, a team proposed an original variant where the translation is initiated by an "eukaryotic IRES-like site" i. e. an m⁶A motif. In this new vector, the stop codon of the reading frame has been removed and the presence of a T2A sequence allows the ribosome to continuously re-initiate translation. Therefore, the entire sequence is translated. The use of an m⁶A motif drastically reduces the size of the sequence allowing translation initiation [77]. This system enables the stable and continuous production of a protein in a given cell. The combination of the stability of circular RNAs and their high potential for continuous translation could make them major tools for future use in

biotherapies. Nevertheless, much work remains to be done to optimize these vectors, especially by a thorough understanding of the translation initiation mechanisms on circular RNAs.

4. Outlook

New perspectives are opening for the field of research on RNAs and particularly on circular RNAs. There is no doubt that new protein coding circRNAs will be discovered in the near future. It will be essential to uncover and understand the various cellular functions of these novel proteins. These mechanisms are of utmost relevance in physiopathological contexts since circRNA derived proteins are very likely involved in stress responses, due to their cap-independent and stress responsive translation initiation, whether IRES or m⁶A-dependent. Their implications in cell differentiation and tumor cell development are proof enough of the potential of circRNA derived proteins in deciphering complex systems and finding new therapeutics. There is no doubt that a growing number of publications will enlighten us on the importance of circular RNAs in many cellular processes, including cell metabolism, which has been poorly studied so far.

Conflict of interest

The authors declare no conflict of interest

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Figure captions

Figure 1: Circular RNAs biogenesis and functions.

Circular RNA (circRNA) biogenesis uses an alternative splicing mechanism called backsplicing, which is facilitated by inverted Alu repeats (thick arrows) in flanking introns. Once generated, circRNAs engage in different paths depending on their structure. CircRNAs are either strictly exonic (EcRNA: Exonic circRNA) or intronic (CiRNA: Circular intronic RNA) or comprised of both intron and exon (ElciRNA: Exon-Intron circRNAs) (1.) CiRNA and ElciRNA are sequestered in the nucleus. The latter can participate in the transcriptional regulation of their parental gene expression by interacting with the RNA polymerase II (RNA pol II) and other snRNP partners like snRNPU1. Exonic circRNAs are exported from the nucleus via length-dependent mechanisms involving evolutionarily conserved helicases. In the cytosol exonic circRNAs have two known functions. (2.) They can act as endogenous competing transcripts by sponging RNA Binding proteins (RBPs) or microRNAs (miRNA), participating in their transport and luring them away from their targets. (3.) Finally, circRNAs can be translated. Translation initiation can occur via two alternative cap-independent mechanisms: the presence of an internal ribosome entry site (IRES) or a N⁶-methyladenosine (m⁶A) motif. These structures allow the internal recruitment of the 40S ribosomal subunit. The resulting proteins then participate in multiple cellular functions.

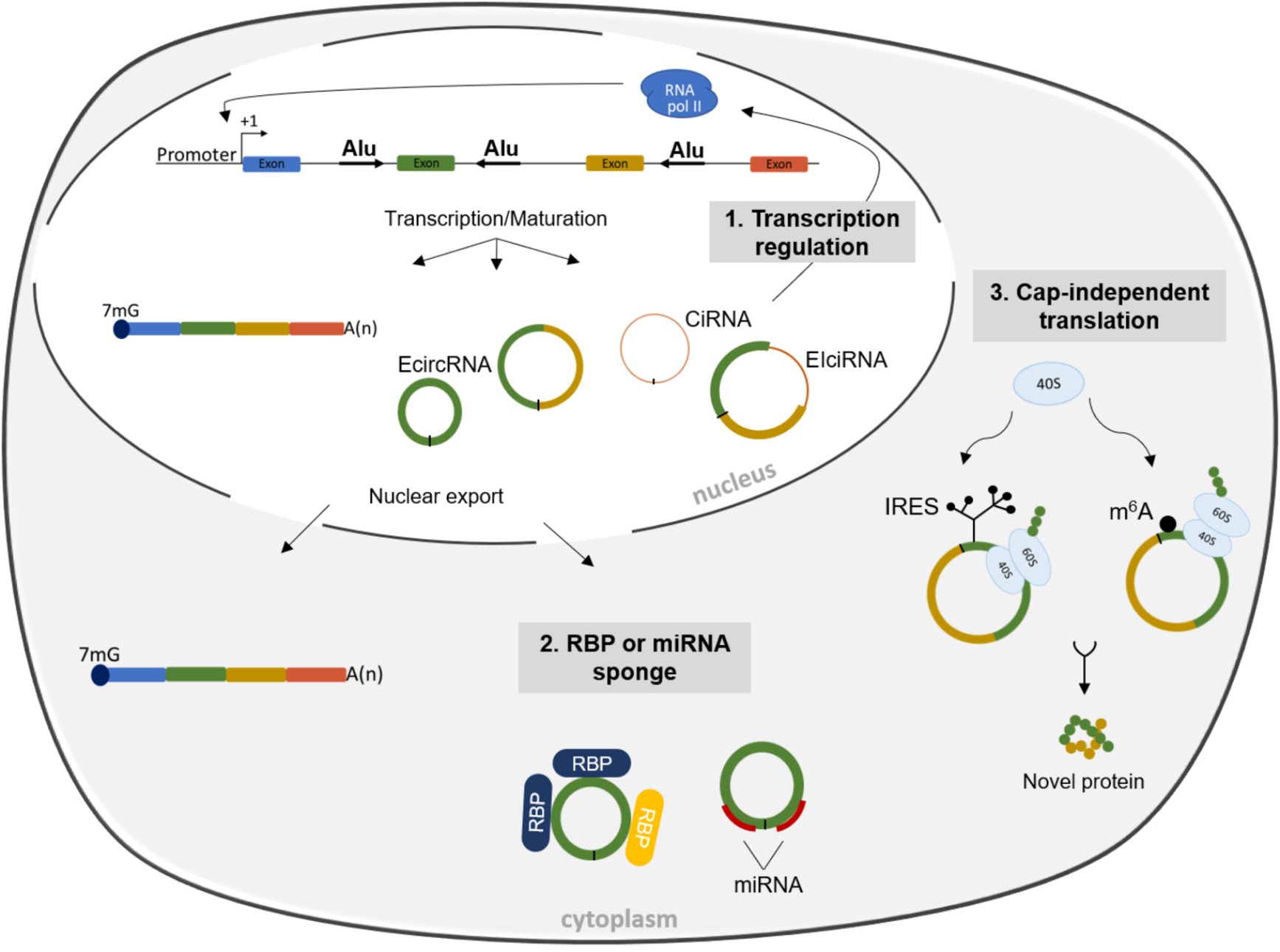
Figure 2: Circular RNA translation

Without any stress, cytoplasmic circRNA may not be translated because cap-dependent translation is predominant. For IRES-dependent translation initiation, ITAFs may be inactive or absent; for m⁶A dependent translation initiation, m⁶A may be erased or m⁶A reader absent

or inactive. Under stress conditions cap-dependent translation is repressed and circRNA translation is favoured through IRES activation due to the presence of ITAFs, or ITAF activation, or through methylation of adenosines (m^6A) or the presence of m^6A readers. circRNA are translated and produce detectable proteins.

Highlights

- A subset of circular RNAs segregate with heavy polysomes
- CircRNA derived proteins are detectable in vivo by mass spectrometry
- Circular RNA translation initiation is cap-independent and mediated by IRESs and/or m^6A motifs
- CircRNA derived proteins regulate cell differentiation, proliferation and malignancy

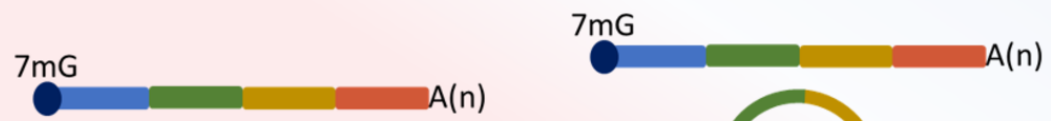


STRESS

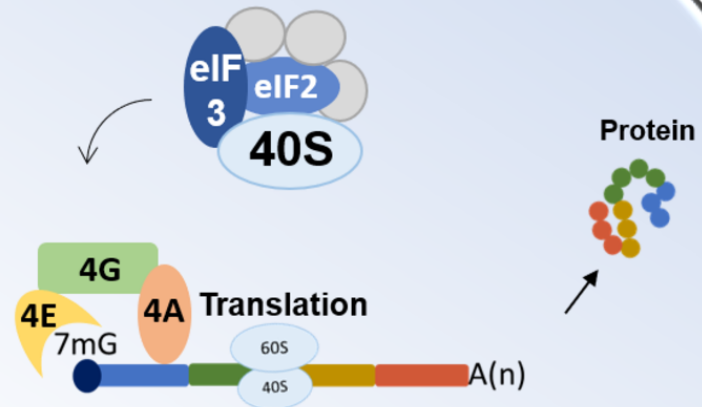
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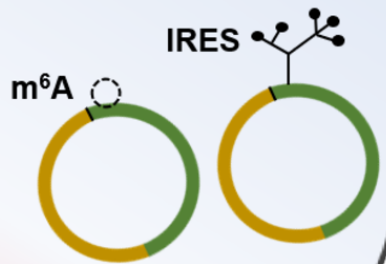
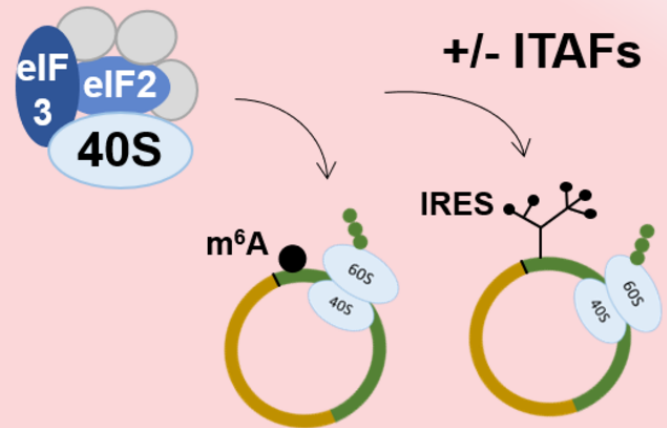
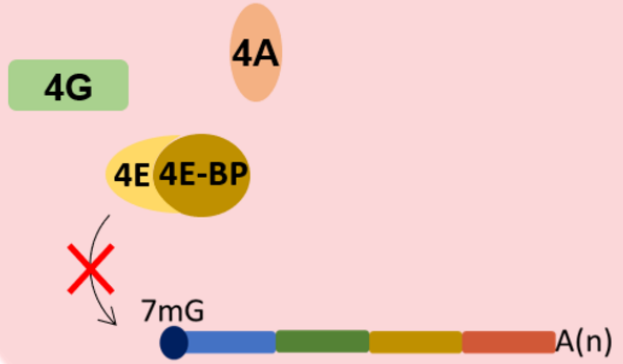
Transcription/Maturation



Cap-dependent translation



Cap-independent translation



cytoplasm

nucleus

