Circular RNA, the key for translation

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Abstract: It was thought until the 1990s that the eukaryotic translation machinery was unable to translate a circular RNA. However internal ribosome entry sites (IRESs) and m^{6}A-induced ribosome engagement sites (MIRESs) were discovered, promoting 5'end-independent translation initiation. Today a new family of non-coding RNAs, circular RNAs (circRNAs), has emerged, revealing the pivotal role of 5'end-independent translation. CircRNAs have a strong impact on translational control via their sponge function, and form a new mRNA family as they are translated into proteins with pathophysiological roles. While there is no more doubt about translation of covalently closed circRNA, the linearity of canonical mRNA is only theoretical: it has been shown for more than thirty years that polysomes exhibit a circular form and mRNA functional circularization has been demonstrated in the 1990s by the interaction of initiation factor eIF4G with poly(A) binding protein. More recently, additional mechanisms of 3'-5' interaction have been reported, including m^{6}A modification. Functional circularization enhances translation via ribosome recycling and acceleration of the translation initiation rate. This update of covalently and non-covalently circular mRNA translation landscape shows that RNA circular shape is the rule for translation with an important impact on disease development.

Keywords: Translation, ribosome, circRNA, RNA circularization, IRES, m^{6}A, MIRES, 3'UTR

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1. Introduction

The potential of circular RNA to be translated has been studied since the 1970s. In 1979, an experiment was designed to determine the ability of circular mRNA to attach ribosomes (1). To this end, a synthetic RNA was circularized with T4 RNA ligase and the binding of bacterial 70S ribosomes versus wheat or rabbit 80S ribosomes was assessed, showing that only the prokaryotic ribosomes were able to attach to RNA circles while the eukaryotic ribosomes were not. This demonstration supported the hypothesis of a ribosome scanning mechanism, depending on the RNA 5' end, to explain initiation of translation in eukaryotes. According to this model, the 40S ribosome small subunit was expected to be recruited only at the mRNA capped 5' end (2). Consequently it was thought for years that eukaryotic ribosomes were unable to initiate translation by internal entry, rendering impossible the translation of circular RNA.
The essence of science is to overcome dogmas: ten years later, the discovery of internal translation initiation mediated internal ribosome entry sites (IRESs) broked the rule (3-5). Furthermore, the presumed inability of eukaryotic ribosome to bind circular RNA was contradicted in 1995: artificial circular RNA containing an IRES was generated (6). The authors observed a significant translation of circular RNAs containing the IRES of encephalomyocarditis virus (EMCV). This work definitely demonstrated two main points: in contrast with earlier suggestions (i) the 40S ribosomal subunit is not necessarily recruited at the mRNA 5' end but can be recruited internally onto an IRES, and (ii) a circular RNA can be translated.
Despite these demonstrations, IRES function in cellular mRNAs remained questioned for a long time, while obvious in the case of picornaviruses whose genomic mRNAs are uncapped (7, 8). From here on,

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many studies have demonstrated the role of IRESs to permit translation of specific classes of capped mRNAs, mostly coding proteins with regulatory functions, in conditions when the cap-dependent initiation mechanism is blocked, which occurs during stress (9, 10). The IRES-dependent mechanism has now revealed its crucial role in the translational response to stress, and is regulated by specific proteins called IRES trans-acting factors (ITAF) (3). IRESs are also responsible for an increased translation of these mRNAs in cancer cells, a process related to abnormal rRNA modifications (11).

Covalently closed RNA circles resulting from splicing were identified at the beginning of the 1990s (12, 13). They were first considered as aberrant splicing products. More than 20 years later, it appears that hundreds of human and animal genes express circular RNA isoforms, called circRNAs. They are post-transcriptional regulators and in several cases they are translated, mostly via IRESs (14-16). Translation of cellular circRNAs thus provides full physiological relevance to IRES-dependent translation.

CircRNAs may also be translated by another cap-independent mechanism, mediated by the methylation of the nitrogen at position 6 in the adenosine base within mRNA (16, 17): N^6-methyladenosine (m^6A), a reversible epitranscriptomic modification that is present in many eukaryotic mRNAs (10). When present in the 5′ untranslated region (5′UTR), a single m^6A promotes cap-independent translation at sites called "m^6A-induced ribosome engagement sites" (MIRESs) (18). As IRESs, MIRESs stimulate selective mRNA translation in stress conditions by a mechanism involving direct binding of the initiation factor eIF3 (18).

Translation of circRNAs definitively put an end to the debate about 5′ end requirement and IRES existence in cellular mRNAs (8, 19). CircRNAs form a new class of mRNAs whose stability is far more important than that of their linear counterpart.

In parallel to these studies on covalently closed circular (CCC) RNA, a series of reports have shown that translation involves the functional circularization of mRNA. Already in the 1980s, circular polysomes were observed by electron microscopy: 80% of the polysomes bound to the endoplasmic reticulum are circular, with commonly six or seven ribosomes for each circle (20). It was demonstrated a few years later that the mRNA 3′ untranslated region (UTR) is functionally interacting with the 5′UTR, leading to the closed-loop model that the mRNA 5′cap and 3′poly(A) tail are close to each other via an interaction between the poly(A)-binding protein (PABP) and the initiation factor eIF4G (21, 22). This mechanism of mRNA circularization involves both cap-dependent and -independent translation, as eIF4G can bind to the mRNA via IRESs, independently of the cap-binding factor eIF4E (23). The closed-loop phenomenon promotes ribosome recycling and thus enhances translation. Functional circularization of mRNAs occurs through several mechanisms in addition to interaction of PABP with eIF4G. It appears as a pivotal parameter of ribosome recycling and translation efficiency enhancement.

Can we still consider an mRNA as linear? That is the question. This review article aims to propose an update of data about covalently as well as non-covalently circular mRNA translation, as the landscape in the field has strongly evolved in the last years.

2. Circular RNAs, from the artefact to a new gene family

The first RNA circles were observed in 1976 by electron microscopy in viroïds (plant pathogens), then in 1979 in human HeLa cell cytoplasm (24, 25). More than 10 years later in the 1990s, the existence of such circles was confirmed and attributed to a scrambled splicing process, using the acceptor site of an exon located upstream of the donor splice sites (12, 13). The authors described the first cases of circular RNA generated from pre-mRNA processing, but the biological significance of such RNA molecules remained questioned. Today, we know that these studies described what is presently called backsplicing (16).

Shortly after, a circular transcript was identified, after RNase H digestion of RNAs extracted from adult mouse testis, as the most abundant transcript expressed from the *Sry* sex determination gene (26). This transcript, specific to adult testis, shows a cytoplasmic localization and a strong stability despite the absence of cap and poly(A). The stability of RNA circles was not a surprise, as they do not give access to exoribonucleases; moreover such a stability had been observed previously for the circular RNA

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genome of hepatitis delta virus, as well as for plant viroids and virusoids (27, 28). A long open reading frame (ORF) was detected in the *Sry* circular RNA and the authors made the assumption that it could have either a positive role by being translated by internal ribosome entry, to give a SRY protein isoform with an unknown function, or a negative role by preventing efficient translation, the *Sry* circular RNA being a result of an alternative splicing generating a non-translated transcript (26). When suggesting a link with translation, the authors were in the right direction even though there is no evidence that SRY circular RNA itself is translated: twenty years later, *Sry* circRNA has been shown to function as a sponge for the microRNA miR-138, with 16 putative sites for that miRNA (29). *Sry* circRNA thus indirectly acts on translation by preventing miR-138 binding and preventing the translational inhibition of its targets, involved in activation of tumor cell growth and invasion (30).

In the last decade, the emergence of RNA deep sequencing technologies and of sharp bioinformatics analyses generated a major leap forward in the field of circRNAs. The abundance of the circular transcript observed for *Sry* in 1993 turned out to be a general feature for thousands of genes in human and mouse, and in various cell types (14, 15). RNA-seq analyses revealed that many scrambled splicing isoforms are expressed at levels comparable to that of their linear counterparts. The circular status of these scrambled isoforms was demonstrated using RNase R, a 3'-5' exoribonuclease that degrades all linear RNA molecules. Most circRNAs are located in the cytoplasm. The expanded landscapes of circRNAs have been determined by RNA-Seq in 44 tissues of human, macaque and mouse, revealing 104,388, 96,675 and 82,321 circRNAs from the three species, respectively (31). Initially considered as splicing background noise, circRNAs constitute according to the current studies 20% of the top 1,000 most abundant transcripts in human and macaque, while only 8% in mouse. In human, 61% of the coding genes express at least one circular transcript (31). All these reports demonstrate that expression of circRNAs is far from being an epiphenomenon.

CircRNAs exhibit different modes of action, depending on their composition which itself affects their localisation. CircRNAs that contain intronic (called ciRNAs) or intronic plus exonic sequences (EIciRNAs) are nuclear and mainly regulate the expression of their parental gene. EIciRNAs have been shown to interact with RNA polymerase II subunits, with U1 snRNP and with the parental gene promoter where they behave as transcriptional enhancers (32). Another study has shown that circRNA expression can influence the splicing of the parental gene by competing with canonical splicing (33). The third class of circRNAs, composed of exonic sequences exclusively (ecRNAs), are cytoplasmic and act via two types of mechanisms: on the one hand they act by sponging miRNAs or RNA binding proteins (RBPs), on the other hand they can be translated (16, 34).

3. circRNAs as translational activators or repressors through sponge and protein carrier functions.

In parallel to the discovery of thousands of circRNAs expressed in mammalian cells, it has been established in 2013 that that natural RNA circles exhibit the function of miRNA sponges (14, 29). As mentioned above, Hansen et al reported that *Sry* circRNA is a sponge for miR-138. At the same time, two reports characterized the sponge function of circular transcript ciRS-7 (circular sponge for miR-7), also known as CDR1as, that is highly expressed in human and mouse brain (14, 29). CiRS-7 has more than 70 binding sites for miR-7 and is associated in a miR-7 dependent manner with Argonaute 2 protein (AGO2), a pivotal component of the RNA-induced silencing complex (RISC). Therefore, ciRS-7 is able to block the miR-7 silencing activity on its mRNA targets while being completely resistant to miRNA-mediated target destabilization (29). This observation revealed that circRNAs are more stable than linear RNAs and that their sponge role is more efficient than that of other long non-coding RNAs. Today this miRNA sponge role has been generalized to several dozens of circRNAs, providing them with a strong impact on epigenetic/epitranscriptomic regulation of gene expression with important consequences in the control of cell proliferation and development of pathologies (35). The primordial function of miRNAs is the inhibition of cap-dependent mRNA translation: the miRNA machinery RNA induced silencing complex (RISC), after binding to mRNA 3'UTR, interacts with the cap-binding complex and blocks the cap-recognition process (36, 37). Several IRESs are also sensitive to miR-dependent inhibition

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of translation, as shown for one of the two vascular endothelial growth factor A (VEGFA) IRESs that is regulated by miR-16 (38). By preventing miR-controlled mechanisms, circRNAs behave as translational activators.

In addition to being miRNA sponges, circRNAs are also sponges for RBPs. It has been shown for Foxo3 circular RNA that forms a ternary complex with cell division protein kinase 2 (CDK2) and its inhibitor p21, which blocks the CDK2 function and arrests cell cycle progression (39). RBP sponges also impact translation when the bound RBP is involved in translational control. This is the case for circPABPN1 which interacts with the protein HuR, a translational activator of the poly(A)-binding protein nuclear 1 (PABPN1) mRNA (40). CircPABPN1 thus lowers PABPN1 mRNA translation. This phenomenon may be generalized to other circRNAs, as HuR associates with many of them and is involved in the control of translation and stability of many mRNAs (40). Recently, interaction between HuR and circPABPN1 has been shown to decrease the translation of the autophagy-related gene 16L1 (ATG16L1) mRNA, whose translation is activated by HuR binding to its 3'UTR in the intestinal epithelium (41). Also, the circRNA BACH1, a circRNA highly expressed in hepatocellular carcinoma (HCC), inhibits the translation of $p27^{kip}$ mRNA by facilitating HuR translocation to the cytoplasm. As HuR is a negative ITAF able to inactivate the $p27^{kip}$ mRNA IRES, its presence in the cytoplasm would promote inhibition of $p27^{kip}$ mRNA translation and prevent cell cycle inhibition by $p27^{kip}$ (42, 43). In this case the circRNA plays a role of carrier rather than sponge.

With these different mechanisms of sponge or protein carrier, circRNAs have a strong impact on translational control as regulatory non-coding RNAs.

4. CircRNAs, a novel class of mRNAs mainly translated by the IRES-dependent mechanism.

We have seen above that circRNAs have a strong impact on translation as as non-coding RNAs. However, they can no longer be considered as non-coding RNAs, due to the growing evidence that many of them are translated.

In spite of the continuous controversy about the ability of cellular mRNAs to be translated independently of their 5' end, the first demonstration of translation of a natural circular RNA was provided in 2014 for the covalently closed circle (CCC) 220 nt-length RNA of the virusoid associated with rice yellow mottle virus (RYMV) (44). The CCC RNA translated in a wheat germ extract system produced a polypeptide of 16 kDa, that was also identified by LC-MS/MS analysis in total proteins from RYMV-infected rice. Larger proteins were also identified, resulting from continuous synthesis around the circular RNA (Fig. 1).

The absolute proof of translation of cellular circRNAs was then provided in 2017 by Pamudurti et al (8, 19). These authors identified, from ribosome footprinting (RFP) datasets, sequencing reads spanning the backsplice junction (i.e. non colinear splicing junctions) which is the signature of the circRNAs. They found 151 circRNAs associated to polysomes in Drosophila, while 34 and 158 circ-RNAs presented RFP reads in rat and mouse, respectively. These so-called ribo-circRNAs show a strong bias towards 5'UTR and 40% of them were predicted to share the start codon with the parental gene. The authors focused to the fly *muscleblind* (mbl) locus that produces several highly expressed circRNAs and identified, by mass spectrometry (MS), a 37kDa peptide produced by the circRNA CircMbl3. Furthermore they identified IRESs in several circRNAs, circMbl, circCdi, circPde8 and circTai by using the well-known bicistronic vector strategy, and by measuring their resistance to 4E-BP overexpression (which inhibits cap-dependent translation) (3, 5, 19). This study provided multiple lines of evidence supporting circRNA translation by the IRES-dependent mechanism, thus confirming the initial data of Chen & Sarnow in 1995 with an artificial circRNA (6)(Fig. 1A, left panel). Interestingly, Padumurti et al. suggested that circRNA translation may be particularly important for the control of synaptic function in brain, as MBL proteins (and their human orthologs MBLN) are involved in neuromuscular pathologies (45).

In the same issue, Legnini et al identified the translation of another circRNA, circ-ZNF609, expressed in murine and human myoblasts (46). A global change of circRNA expression was observed during

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myoblast differentiation, and myoblasts from Duchenne muscular dystrophy (DMD) patients exhibited a unique signature in terms of circRNA expression levels. The circular/linear ratio tends to increase with myoblast differentiation, a feature probably linked to the high stability of circRNAs. An RNAi-based circRNA functional screening allowed these authors to target 25 circRNAs, revealing the important role of circ-ZNF609 in myoblast differentiation (46). Circ-ZNF609 is downregulated during myogenesis, but remains strongly expressed in DMD cells. This circRNA contains a 753 nt long ORF whose translation initiation is controlled by an IRES and sediments with heavy polysomes, both in human and mouse. By tagging the endogenous circRNA with the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 technology, Legnini et al were able to detect the protein produced by circ-ZNF609 by mass spectrometry. They identified an IRES in the circ-ZNF609 UTR, that was significantly more efficient than that of EMCV IRES used as a positive control in these muscular cells, but whose activity requires the presence of the original splice junction of circ-ZNF609.

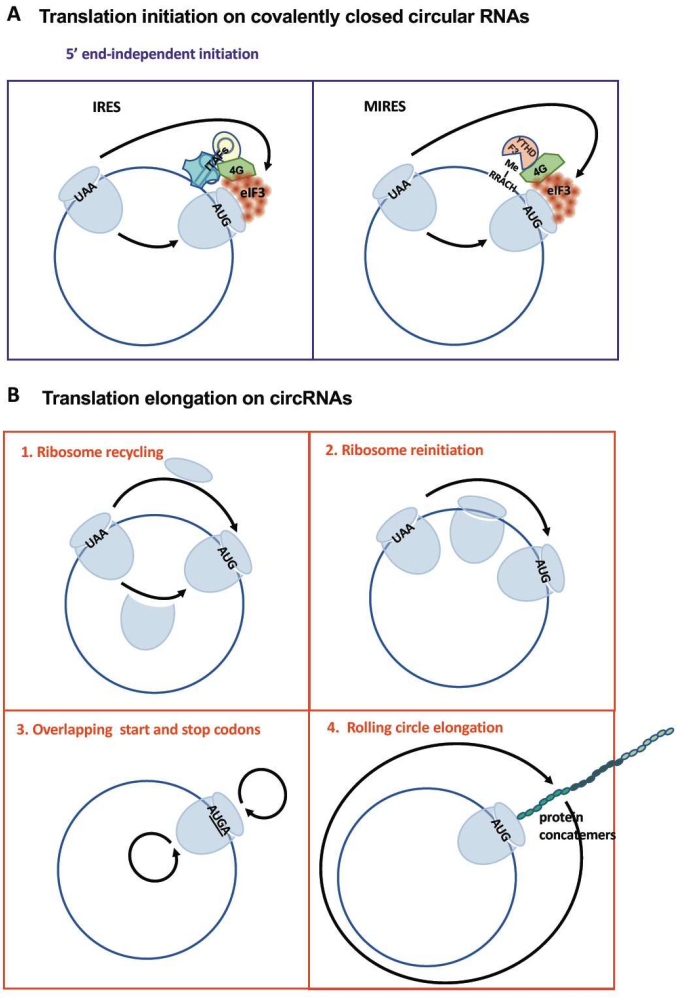

Figure 1. Translation of circRNAs

A. Translation initiation on circRNAs by IRES (left) and MIRES (right) is schematized with the main proteins involved in the complexes. Details are provided in the text (sections 4 and 5).

B. Mechanisms of translation elongation on circRNAs: even though 5' end-independent initiation may be less efficient cap-dependent initiation, translation efficiency is enhanced through ribosome recycling (1), reinitiation (2), overlapping start and stop codons (3) or rolling circle elongation (4). Details are provided in the text (section 10).

Following these two pioneer articles, several additional studies have identified, since that date, proteins expressed from circRNA translation whose translation initiation depends indeed on an IRES as demonstrated by the systematically use of bicistronic vectors. Zhang et al revealed the existence of the circRNA circ-SHPRH, whose expression is repressed in glioblastoma (47). Circ-SHPRH expresses an isoform of histone-linker, PHD and RING finger domain-containing helicase (SHPRH), the 17 kDa protein SHPRH-144aa, which has a role of tumor suppressor. Interestingly, translation of this circRNA uses overlapping initiation and termination codons (Fig. 1B, panel 3). The same laboratory also identified the circRNAs circFPXW7 and circPINTexon2 (circular form of the long intergenic non-protein-coding RNA p53-induced transcript LINC-PINT). Translation of these circRNAs produces peptides of 10 kDa and 26 kDa, respectively, which both suppress glioblastoma cell proliferation (48, 49).

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A fourth coding circRNA, circβ-catenin, has been characterized in human cells and tissues (50). It is overexpressed in cancer cell lines and its knockdown drastically attenuates liver cancer cell growth and metastatasis. Here again, the mechanism of translation initiation is IRES-dependent. This has been demonstrated by the use of a bicistronic vector. Circβ-catenin produces a new β–catenin isoform of 50 kDa, β-catenin-370aa, identified by mass spectrometry, whose role is to protect β–catenin from ubiquitination and degradation mediated by the kinase GS3Kβ. The β-catenin-370aa potentiates the Wnt/β-catenin signaling pathway.
These different examples clearly show the importance of IRES-dependent translation to initiate protein synthesis from circRNAs.

5. MIRES, an alternative mechanism for ribosome recruitment on circRNAs.

All circRNAs characterized above for translation contain an IRES. However, an alternative to IRESs is provided by MIRES, m^{6}A-based ribosome entry sites (18). Supporting that hypothesis, MIRES-dependent circRNA translation has been obtained using an artificial green fluorescent protein (GFP)-circRNA (17). The authors observed that circGFP is translated using different known IRESs, but also with the negative controls. Interestingly, all their negative controls contained the RRACH consensus motif of m^{6}A modification close to the start codon (R=purine, H=pyrimidine or A), suggesting MIRES-dependent initiation (Fig. 1A, rigt panel). It was also observed that m^{6}A predicted motifs are significantly enriched in circRNAs, compared to linear mRNAs, consistent with a higher density of m^{6}A sites identified in the m^{6}A methylome by m^{6}A RNA immunoprecipitation (m^{6}A-RIP) (17, 51). Yang et al finally showed that the presence one or two m^{6}A sites in the GFP circRNA is sufficient to promote reporter gene expression and that this translation is sensitive to the overexpression of the methylases METTL3/14 or of the fat mass and obesity-associated protein (FTO) demethylase. This translation initiation process involves the initiation factors eIF4G2, eIF3A as well as the m^{6}A reader YT521-B homology domain family (YTHDF) 3. The direct interaction of eIF4G2 with YTHDF3 suggested a possible role of the m^{6}A reader in recruiting eIF4G2 to the m^{6}A site, promoting by this way the internal entry of the translation machinery as eIF4G2 may directly recruit eIF3 (17). In the same study, 19 endogenous peptides were identified by MS/MS as translated from/through the circular mRNA junction. The high degree of circRNA m^{6}A methylation was also demonstrated by Zhou et al, who identified 1404 m^{6}A circRNAs by m^{6}A-RIP in human embryonic stem cells, whose methylation is dependent on METTL3 (52).
Very recently, by generating a library of random 10-nt sequences was inserted before the start codon of circRNA-coded GFP, Fan et al. identified 97 IRES-like hexamer sequences enriched in thousands of endogenous circRNAs, whose pervasive translation is supported by mass spectrometry evidences (53). Several of these hexamers contain the RRACH signature for the m6A modification, thus might correspond to MIRESs. 58 RBPs binding these elements were also identified as recruited by IRES-like hexamers. Among them, PABPC1 and heterogeneous nuclear ribonucleoprotein (hnRNP) U are able to activate this cap-independent translation.
MIRESs appear as an alternative mechanism for translation initiation on circRNAs. However, in spite of all the above arguments, MIRES-dependent translation of endogenous circRNAs remains to be demonstrated.

6. CircRNA high stability, an asset for protein production in biological systems.

CircRNAs clearly constitute a new class of mRNAs, with the interesting feature of being much more stable than linear mRNAs. This gives them a high potential for applications in biological systems. Wesselhoeft et al. have engineered a technology of circRNA production for potent and stable translation in eukaryotic cells, based on self-splicing by using a group I autocatalytic intron (54). They found that the most efficient intron is that of Anabaena pre-tRNA, while the optimal IRES is the Coxsackievirus B3 (CVB3) IRES, although the efficiency of the IRES is cell type-dependent. Such circRNAs containing the

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luciferase reporter ORF were produced by in vitro transcription and purified using high-performance liquid chromatography (HPLC). They were then used for transfection of human cell lines, revealing that the circRNA produces 811% more protein than the corresponding capped and polyadenylated linear RNA. CircRNA exhibited a protein production half-life of 80-116 hours, compared to 43-49 hours for the linear counterpart. These authors also reported that circRNAs evade detection by the receptors of innate immunity retinoic-acid-inducible protein 1 (RIG-1) and toll-like receptors (TLR), while linear RNAs induce an immune response via these sensors (55). In this study, synthetic circRNAs containing m^{6}A modifications instead of the IRES failed to produce any translation product, suggesting that m^{6}A-mediated translation would require the binding of nuclear RBPs.
Due to their high stability, circRNAs provide a promising biotechnological tool to produce proteins of interest.

7. The m^{6}A machinery allows circRNAs to be marked as the self.

Other authors also engineered a GFP circRNA based on self-splicing, via permuted *td* autocatalytic intron from T4 bacteriophage, and demonstrated that this self-spliced circRNA is highly immunogenic in mammalian cells (56). It was thus termed circFOREIGN. RNAseq data of cells transfected by circFOREIGN showed that expression of 127 genes involved in innate immunity is increased. This immune response is mediated by RIG-1, as RIG-1 knock-out abrogates the circFOREIGN induced immune response. This article shows that RIG-recognition depends on the intron: circRNA spliced by endogenous spliceosomes are marked as the self, while circRNA generated by autocatalytic splicing is considered as foreign.
In a recent article the same authors have identified the m^{6}A modification as a marker for self (57). They transfected DNA plasmids coding circRNAs generated by protein-assisted (circSELF) or autocatalytic splicing (circFOREIGN) into HeLa cells and found that circSELF, but not circFOREIGN, is associated with the m^{6}A machinery, and that the m^{6}A YTHDF2 reader is required for suppression of immune stimulation by circFOREIGN. The METTL3 writer is also needed for self/non self-recognition. m^{6}A UV-crosslinking and m^{6}A immunoprecipitation revealed that circSELF gained an m^{6}A modification within 50-100 nt downstream from the circularization junction. When circFOREIGN was mutated for all m^{6}A RRACH motifs, its RIG-1 mediated immunogenicity was increased by 10,000 fold (57).
The concept of RNA marking as self or non-self constitutes an important parameter to take into account if using circRNAs as biotechnological vectors for protein expression.

8. mRNA functional circularization enhances translation efficiency via ribosome recycling.

While there is no more doubt about translation of covalently closed circular RNA, the linearity of canonical mRNA can be questioned. It has been shown for more than thirty years that polysomes exhibit a circular or spiral form (20). This was established by electron microscopy in rat somatotropes and mammotropes expressing growth hormone and prolactin, respectively, and concerned about 80% of the polysomes seen in the rough endoplasmic reticulum of these cells. In the two cell types the number of ribosomes in circular polysomes showed a peak at six to seven ribosomes. This study suggested a proximity of the 5′ end with the 3′ termination codon, that would allow ribosome recycling (20). The functional interaction between mRNA 3′ and 5′ UTRs was first demonstrated in yeast, by the demonstration that the poly(A) tail behaves as a translational enhancer, and that the polyA-binding protein Pab1p stimulates 40S ribosome joining (21)(Fig. 2a). One year later, the same authors demonstrated that the Pab1p-poly(A) tail complex interacts with eIF4G (22). The model of mechanism proposed from these data was that, for stimulation of cap-dependent translation, Pab1p binds to the eIF4G/4E complex leading to mRNA circularization and placement of the 40S ribosome subunit, following translation termination, near the mRNA 5′. For cap-independent translation the binding of eIF4G to an IRES would allow 40S internal recruitment (23) (Fig. 2e). The complex between the cap binding factor eIF4E, eIF4G and Pab1p was reconstituted and the authors proved that this complex

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results in mRNA circularization by atomic force microscopy (AFM). Circular RNA molecules were clearly visualized with this technique (58).

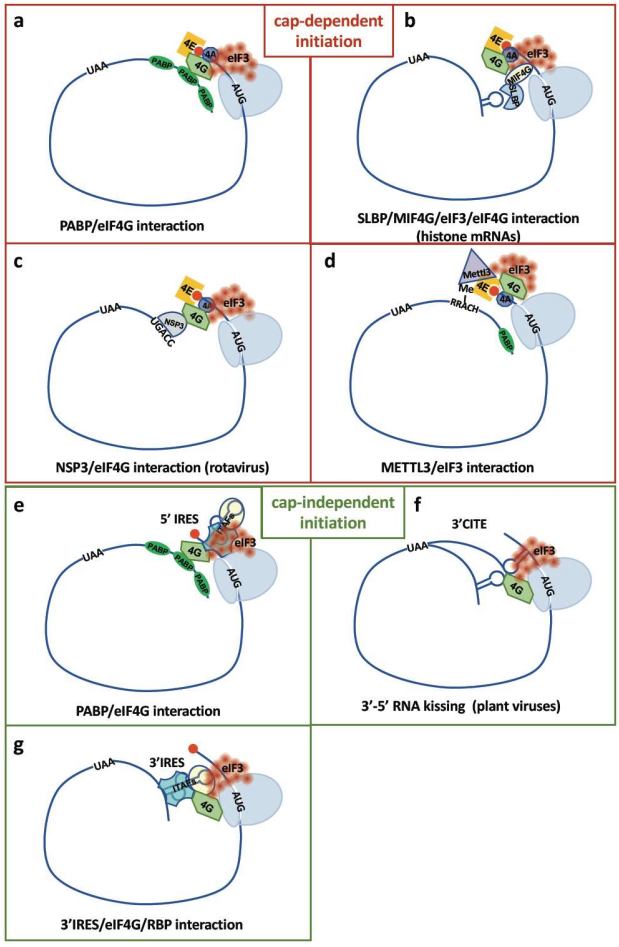

Figure 2. mRNA functional circularization is mediated by different mechanism.
Several mechanisms of mRNA circularization are represented, with the known proteins and RNA elements involved in the 3'-5' interaction, allowing cap-dependent (a-d) and -independent (e-g) translation. Each mechanism is detailed in the text (sections 8 and 9). This list is not exhaustive and the mechanism d (METTL3/eIF3 interaction) may also serve cap-independent translation.

RNA functional circularization is not limited to the interaction between eIF4G and PABP. It also takes place on poly(A) devoid histone mRNAs via a 3' terminal stem-loop that binds the SLBP protein. The 3'-5' bridge is formed by the complex SLBP/MIF4G(SLIP1)/eIF3/eIF4F (59)(Fig. 2b). Several viruses whose mRNAs carry no poly(A) tail have developed specific cyclization systems. Rotaviruses are double-stranded RNA viruses that produce a functional homolog of PABP, NSP3, which binds to the UGACC consensus at the viral mRNA 3' end and circularize the mRNA by interacting with eIF4G (Fig. 2c). In addition, NSP3 shuts down host cell protein by displacing PABP to titrate all the available eIF4G pool for translation of the viral mRNA (60, 61). 5'-3' interaction of the IRES-containing HCV RNA genome is supported by the binding of 40S ribosome subunit and eIF3 to both 5' and 3' regions, involving RNA-RNA long-range interactions as well as binding of several ITAFs (62). These different studies show that mRNA functional circularization is the rule, not the exception.

9. 3'UTR elements and m^{6}A modifications enhance translation by a closed-loop mechanism.

3' translational enhancers (3'TE) have been identified in several plant viruses, such as a pseudoknot in the tobacco mosaic virus RNA 3'UTR that stimulates translation initiation in conjunction with the 5'cap (63). However, many plant positive-strand RNA viruses do not contain a 5' cap, and in that case the 3' TE mimics a 5' cap and has been called a 3' cap-independent translational enhancers (3'CITEs) (Fig. 2f). 3'CITEs bind a component of eIF4F and engage RNA circularization by RNA-RNA kissing interactions (64, 65). In particular, such an element has been described in the barley yellow dwarf virus (BYDV) (66). Activation of translation initiation by elements in the 3'UTR have also been found in animal cell mRNAs, such as the FGF2 mRNA, where a 3'TE modulates the choice of alternative initiation codons (67).
The model of RNA looping was demonstrated in 2014 by Peak et al. who tethered eIF4G fused to MS2 coat protein to the 3'UTR of a reporter mRNA through MS2-binding sites (68). This fusion protein is able to recruit 43S ribosomes at the mRNA 3' and enhanced 5' end-independent translation of the reporter gene located upstream. Cap-independent translation was also stimulated when inserting the

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EMCV IRES downstream from the reporter gene. The authors proposed a model of RNA looping allowing communication between the ribosome recruited in 3′, and the initiation coding located upstream (Fig. 2g). This model was supported by a study aimed to the systematic discovery of cap-independent sequences in human and viral genomes, based on a high-throughput bicistronic assay (69). The authors found a high enrichment of sequences able to generate cap-independent translation in human mRNA 3′UTRs, suggesting that numerous transcripts have the ability to recruit ribosomes in the 3′UTR and then initiate translation by the mRNA looping mechanism. Recently, a large-scale tether function assay (TFA) has been designed to identify RBPs regulating mRNA stability and translation, again based on the MS2 system (70). A library of 690 RBP open reading frames were fused to MS2 coat protein and tethered to the 3′UTR of a reporter mRNA using MS2 binding sites as above. Several proteins were identified as translational enhancers when bound to the mRNA 3′UTR, suggesting that many RPBs are involved in the closed-loop mechanism of translation initiation (70, 71).
Recently, an additional mechanism of mRNA closed-loop model has been described that involves the m^{6}A modification (72). Me-RIP-seq experiments have shown an enrichment of m^{6}A modifications in mRNA 3′UTRs, in particular in the vicinity of termination codon (73). Such modifications have an enhancing effect on translation efficiency mediated by a physical and functional interaction between the m^{6}A writer METTL3 and the eIF3 subunit h (72)(Fig. 2d). Electron microscopy revealed circular polysomes, supporting the hypothesis that METTL3 behaves as a translational activator by the mRNA closed-loop mechanism.
The above evidences indicate that IRESs and m^{6}A, in addition to driving internal initiation of translation, participate in mechanism of mRNA circularization when located in the 3′UTR.

10. Enhancement of translation initiation rate by ribosome recycling and rolling cycle translation

The closed-loop model suggests that translation enhancement would result from engagement into a round of translation of a ribosome recruited to the 3′ UTR, and/or of a re-engagement of terminating ribosomes, via mRNA circularization. This had not been proved experimentally up to now. However a recent article has studied closed-loop assisted reinitiation (CLAR)(74). By monitoring the rate of protein synthesis in the course of translation of capped and polyadenylated mRNA in a Krebs-2 cell-free translation system, this study established that translation has a biphasic kinetics: the first phase exhibits a low initial synthesis rate, and is followed after 18 minutes by a second phase with an acceleration of translation corresponding to a shift of polysome average size from two to five. This acceleration is not caused by the involvement of new mRNAs in translation but reflects an increase of the initiation rate (74). This process requires mRNA 5′-3′ interaction, suggesting that it results from ribosome recycling.
The concept of translation acceleration by ribosome recycling leads us back to translation of covalently closed circRNA, which may be subjected to the ribosome recycling process and are good candidates for the CLAR mechanism and the acceleration rate of translation initiation (Fig. 1B, panels 1-2). While translation efficiency of circRNAs may also be enhanced by processes of reinitation or start/stop codon overlap, the model based on ribosome recycling connects with the observation of continuous translation described for the virusoid CCC RNA in 2014 (44)(Fig. 1B, panels 3-4). Rolling circle translation of covalently closed circRNA has also been described in human cells, using flagged circRNAs synthesized in vitro, transfected into Hela cells (75). The authors showed that in the absence of any IRES, once initiation has occurred on a circular RNA with an infinite ORF, even if it is ineffective, the elongation can revolve around the circle many times and produce high molecular weight proteins. Thus, the rolling-circle amplification mechanism, based on a single although poorly efficient initiation event, may be an additional mechanism a circRNA translation (76). Interestingly, in the recent study cited above by Fan et al, 14% of the endogenous circRNAs containing IRES-like hexamers can produce protein concatemers through rolling-circle translation. This report also confirms that rolling-circle translation directs synthesis of huge amounts of protein concatemers, confirming that initiation of circRNA translation is the rate-limiting step (53).

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11. Impact of covalently or non-covalently closed circular RNA translation in pathologies

The present update has clearly shown the crucial role of RNA circularization for translation efficiency, with important pathophysiological consequences.

Translation of circular RNA, covalently closed or not, has also demonstrated its relevance in many diseases (76, 77). This evidence is growing for covalently closed circRNAs. The first described circRNA translation product, circ-ZNF609, is able to activate myoblast proliferation and is involved in Duchenne myodystrophy (46). Several circRNA products are involved in cancer, by example we can note the tumor suppressor role of circ-FBXW7-185aa and circSHPRH-146aa in glioblastoma (47, 48). Overexpression of circFBXW7 also inhibits proliferation and metastasis of triple negative breast cancer cells (78). These circRNA-derived proteins may act as decoys, as is the case for circSHPRH-146aa, that protects the full-length SHPRH from degradation by the ubiquitin proteasome. The same mechanisms occur with circAKT3 that produces an AKT3 isoform, AKT3-174aa, a dominant-negative variant of AKT with tumor suppressor activity (79). FBXW7-185aa, that competitively interacts with the deubiquitinating enzyme USP28, antagonizes USP28-induced c-Myc stabilization (48). The β-catenin-370aa protein produced by circβ-catenin is highly expressed in liver cancer cells and promotes tumor growth by activating Wnt/β-catenin pathway. It also acts as a decoy protein by binding to the GSK3β ubiquitine ligase, which protects the full-length β-catenin from proteasome-dependent degradation (50). PINT87aa produced by circLINC-PINT, is a tumor suppressor but acts through a different mechanism: it interacts with the polymerase-associated factor 1 which recruits RNA polymerase II and suppresses the transcription of multiple proto-oncogenes (49). Most circRNA translation products described have an effect on cancer progression or suppression (77). However the deregulated expression of circRNAs acting as sponges or being translated is also involved in neurodegenerative diseases, while they appear as key players in aging (80, 81). Interestingly, a global dysregulation of circRNAs was observed in brains of Alzheimer disease patients (82). Globally, a general decrease of expression was observed in diseased brains, contrasting with the general increase observed during aging.

Efficient translation of non-covalently circularized mRNAs also plays a role in disease development. Translation of many viral mRNAs is promoted by a 3'-5' interaction, resulting in a more efficient viral replication, as is the case of rotaviruses with interaction of NSP3 protein with eIF4G. These pathogens are an important cause of gastroenteritis in young animals and children (61). Also, ribosome recycling and acceleration of translation initiation by the closed-loop formation promotes oncogenesis. In particular, mRNA looping generated by METTL3-eIF3h interaction promotes translation promotes translation of a large subset of oncogenic mRNAs, paving the way for the development of new cancer therapeutic strategies (72).

To conclude this review, our last word will be that the concept of RNA circle and of circular RNA translation can no longer be considered as an exception, but as the rule of the translation control in eukaryotes.

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Abbreviations

AFM: atomic force microscopy
AGO: argonaute protein
BYDV: barley yellow dwarf virus
CCC: covalently closed circle
CITE: cap-independent translational enhancer
CRISPR: clustered regularly interspaced short palindromic repeat
DMD: Duchenne muscular dystrophy
eIF: eukaryotic initiation factor
EMCV: encephalomyocarditis virus
FTO: fat mass and obesity-associated protein
GFP: green fluorescent protein
hnRNP: heterogeneous nuclear ribonucleoprotein
HPLC: high-performance liquid chromatography
IRES: internal ribosome entry site
LINC-PINT: long intergenic non-protein-coding RNA p53-induced transcript
MBL: muscleblind
METTL: m^6 A methyltransferase-like
MIRES: m6A-induced ribosome engagement site
MS: mass spectrometry
ORF: open reading frame
PABP: poly(A)-binding protein
RBP: RNA binding protein
RFP: ribosome footprinting
RIP: RNA immunoprecipitation
RISC: RNA-induced silencing complex
SHPRH: histone-linker, PHD and RING finger domain-containing helicase
TE: translational enhancer
TFA: tether function assay
UTR: untranslated region
YTHDF: YT521-B homology domain family

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