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REVIEW

More than just scanning: the importance of cap-independent mRNA translation initiation for cellular stress response and cancer

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Abstract The scanning model for eukaryotic mRNA translation initiation states that the small ribosomal subunit, along with initiation factors, binds at the cap structure at the 5′ end of the mRNA and scans the 5′ untranslated region (5′UTR) until an initiation codon is found. However, under conditions that impair canonical cap-dependent translation, the synthesis of some proteins is kept by alternative mechanisms that are required for cell survival and stress recovery. Alternative modes of translation initiation include cap- and/or scanning-independent mechanisms of ribosomal recruitment. In most cap-independent translation initiation events there is a direct recruitment of the 40S ribosome into a position upstream, or directly at, the initiation codon via a specific internal ribosome entry site (IRES) element in the 5′UTR. Yet, in some cellular mRNAs, a different translation initiation mechanism that is neither cap- nor IRES-dependent seems to occur through a special RNA structure called cap-independent translational enhancer (CITE). Recent evidence uncovered a distinct mechanism through which mRNAs containing N^6-methyladenosine (m^6A) residues in their 5′UTR directly bind eukaryotic initiation factor 3 (eIF3) and the 40S ribosomal subunit in order to initiate translation in the absence of the cap-binding proteins. This review focuses on the important role of cap-independent translation mechanisms in human cells and how these alternative mechanisms can either act individually or cooperate with other *cis*-acting RNA regulons to orchestrate specific translational responses triggered upon several cellular stress states, and diseases such as cancer. Elucidation of these non-canonical mechanisms reveals the complexity of translational control and points out their potential as prospective novel therapeutic targets.

Keywords Eukaryotic translation initiation · Repression of global protein synthesis · Non-canonical translation initiation · *Cis*-acting RNA regulons · Cellular stress · Disease · Cancer

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Introduction

Protein synthesis—or mRNA translation—is the most energy-consuming process in the cell and is essential for gene expression regulation [1]. Translation involves the coordinated interaction of mRNA, auxiliary factors, ribosomes, and tRNAs, and is tightly controlled [1–4]. Misregulation of the translation machinery may lead to several disease states, including carcinogenesis [5–7]. Regulation of mRNA translation occurs at all steps of the process—initiation, elongation, termination and recycling [1–4]. Also, it is generally accepted that translation initiation is the rate-limiting step and the most tightly regulated [1, 4–9]. Translation initiation of most eukaryotic mRNAs occurs through a mechanism that has been named "cap-dependent scanning" [7–12]. This canonical mechanism requires that the small ribosomal subunit, together with several eukaryotic initiation factors (eIF), recognizes the m^7GpppN cap structure at the 5′ end of the transcript,

previously bound to the trimeric complex eIF4F, and scans the 5′ untranslated region (5′UTR) until it reaches the first initiation codon (usually AUG) in a favorable context [11, 13–15].

Although this mechanism is used in most circumstances, under various stress conditions, canonical translation initiation is impaired and overall protein synthesis is drastically reduced [4, 5, 7, 16]. However, repression of global protein synthesis is often accompanied by selective translation of mRNAs that encode proteins that are crucial in cell survival and stress recovery [4, 5, 7]. Synthesis of these stress-responsive proteins can occur through alternative mechanisms of translation initiation that are evolutionarily conserved and greatly impact translation in organisms as diverse as yeast or humans. This translational reprogramming is achieved through mechanisms that can involve specific mRNA features, such as small structural elements that interact with *trans*-acting factors, upstream open reading frames (uORFs), or other *cis*-acting RNA regulons [4, 17]. Moreover, translational reprogramming occurs via a cap- and/or scanning-independent mechanism of ribosomal recruitment [18–22]. However, non-canonical translation initiation can also occur via a cap-dependent but scanning-free mechanism. Examples of this include those taking place on mRNAs with extremely short 5′UTRs or with highly complex 5′UTRs that promote ribosome shunting, as well as those that may occur in transcripts with 5′UTRs that form highly stable structures such as RNA G-quadruplexes or pseudoknots [23–32]. Nevertheless, many oncogenes, growth factors and proteins involved in stress response and in the regulation of programmed cell death are encoded by mRNAs that have alternatives to the cap-dependent mechanisms of translation initiation to sustain protein synthesis under stress conditions or disease [4, 6, 7, 33, 34]. The most widely described cap-independent mechanism of translation initiation consists of 40S ribosomal subunit recruitment into a position that is either on or upstream the initiation codon, via a specific internal ribosome entry site (IRES) element [21, 22]. However, cap-independent translation initiation can also occur in the absence of an IRES. One distinct mechanism, known as “cap-independent translational enhancer (CITE)-mediated translation”, is utilized by some cellular mRNAs under apoptotic conditions and remains dependent on 5′ end scanning [35–37]. Notably, it has also been recently described that mRNAs containing N^6-methyladenosine (m^6A) in their 5′UTR can also be translated in a cap-independent manner [38–40].

As stated above, not only is selective translation by cap-independent initiation mechanisms important for cellular survival under stress, but it also is used by cells in disease states, such as autoimmune diseases, neurodegeneration and cancer [5–7, 34, 41, 42]. Interestingly, many proteins encoded by IRES-containing mRNAs play decisive roles in cell survival (BAG1 [43], XIAP [44], Bcl-xL [45], cIAP1 [46], Bcl-2 [47]), proliferation (FGF2 [48], Myc [49], PDGF2 [50], IGF2 [51]), cell cycle (p27 [52], p53 [53], PITSLRE [54]) and angiogenesis (VEGF-A [55], HIF1α [56])—all these processes are vital in cancer onset and progression.

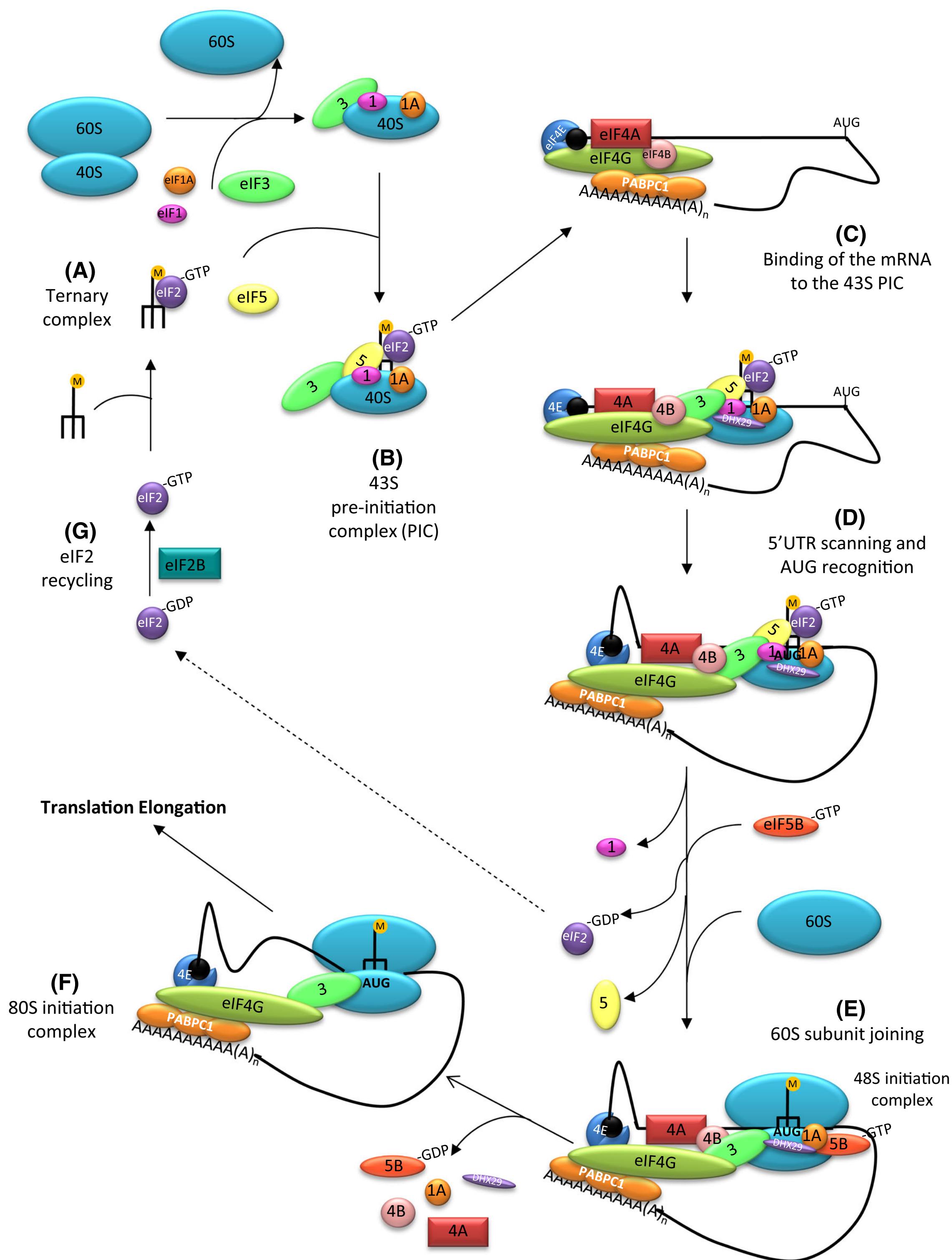
Here, we will review the mechanisms of cap-independent translation initiation, how these alternatives work individually or cooperate with other 5′UTR *cis*-acting RNA regulons, contributing to the selective translation of proteins that are required for stress recovery and cell survival. In addition, we will illustrate how cap-independent translation of selected transcripts plays a major role in cancer.

Overview of the canonical translation initiation mechanism

Translation initiation is a complex process involving ribosome loading, scanning, and start codon selection, before elongation commitment [1–4] (Fig. 1). Translation initiation starts with the formation of the ternary complex, which consists of eukaryotic initiation factor 2 (eIF2) binding to both Met-tRNAi and GTP (Fig. 1a) [57]. Its assembly is controlled by the guanine nucleotide exchange factor eIF2B [58]. GTP is hydrolyzed after AUG start codon recognition; this results in GDP-bound eIF2, whose affinity for Met-tRNAi is tenfold lower [59].

Once the ternary complex is assembled and active, it must bind to the 40S ribosomal subunit. According to current models based on studies in reconstituted eukaryotic systems, this binding is aided by eIF1, eIF1A, eIF3 and eIF5 (Fig. 1b) [59–64]. Although eIF1 and eIF1A promote scanning, eIF1—and possibly the C-terminal domain of eIF1A—must be displaced from the P decoding site to permit base-pairing between Met-tRNAi and the AUG codon, as well as to allow subsequent phosphate release from eIF2–GTP [12, 65]. On the other hand, a large factor, such as eIF3, might distort the conformation of the entire 40S subunit to allow easier access of eIF2 with its attached Met-tRNAi [66]. eIF5 also affects ternary complex recruitment as it is crucial for the assembly of the eukaryotic pre-initiation complex, working as an adaptor between 40S subunit-bound eIF3 and the ternary complex [66]. The binding of the ternary complex to the 40S ribosomal subunit, together with the aforementioned initiation factors, is known as “43S pre-initiation complex” (PIC) (Fig. 1b).

Once assembled, the 43S PIC must bind to the cap structure at the 5′ end of the mRNA molecule (Fig. 1c), so that it is able to scan the UTR until the correct initiation codon in the proper context is recognized. eIF4F (eIF4E/



60S
40S
eIF1A
eIF1
eIF3
(A)
Ternary complex
eIF5
GTP
eIF2
M
(B)
43S
pre-initiation
complex (PIC)
(C)
Binding of the mRNA
to the 43S PIC
eIF4E
eIF4A
eIF4G
eIF4B
PABPC1
AUG
AAAAAAAAAA(A)n
(D)
5'UTR scanning and
AUG recognition
DHX29
(E)
60S subunit joining
48S initiation
complex
eIF5B
GDP
(F)
80S initiation
complex
Translation Elongation
(G)
eIF2
recycling
eIF2B

◀**Fig. 1** Model of canonical translation initiation in eukaryotic cells. **a** Translation initiation starts with the formation of the ternary complex, composed of eIF2 bound to Met-$tRNA_i$ and GTP. **b** Once the ternary complex is assembled and active, it must bind the 40S ribosomal subunit with the aid of eIF1, eIF1A, eIF3 and eIF5, forming the 43S pre-initiation complex. **c** Then, the 43S pre-initiation complex must bind the cap structure at mRNA 5′ end, so it can scan the mRNA to find the initiation codon. **d** The scanning stops when the 43S complex finds the first initiation codon in a favorable context, thus forming a stable complex known as 48S initiation complex. **e**, **f** After the 48S initiation complex is formed, several events take place so that the 60S subunit may join and form the 80S ribosome. This reaction requires eIF5B, which hydrolyzes the eIF2–GTP, thereby releasing the initiation factors, including eIF2–GDP, from the small ribosomal subunit, leaving the initiator $tRNA_i$ bound to the start codon. Following eIF2–GDP dissociation, eIF5B–GTP binds to the 40S subunit and accelerates the rate of 60S subunit joining by its hydrolysis. **g** Once the initiation step is finished and the ribosome has entered the elongation phase, eIF2 is recycled to enable, yet again, ternary complex formation for another round of translation

eIF4G/eIF4A) is essential for 43S to recognize the m^7G cap structure [67]. Apart from being the cap structure-binding protein, eIF4E stimulates eIF4A helicase activity. eIF4A is a DEAD-box RNA helicase, whose ATPase activity is required for duplex unwinding in vitro [68]. It is held in its active conformation by eIF4G, which enables it to unwind the 5′UTR of the mRNA, producing a single-stranded binding site for the 43S PIC near the 5′ cap [69–71]. eIF4G is a high molecular weight protein that acts as a scaffold for binding eIF4E and eIF4A. In addition, eIF4G helps recruit the 43S PIC by directly interacting with eIF3 through eIF3c, -d, and -e subunits [72, 73]. Altogether, at the 5′ end of the mRNA, the binding of the PIC to the mRNA involves the cooperative activities of eIF4F, eIF3 and eIF4B. Recent findings demonstrated an eIF4A-independent role for eIF4B in addition to its function as eIF4A cofactor in promoting PIC attachment or scanning on structured mRNAs [74].

After proper assembly at the 5′ end of the mRNA, the PIC needs to scan the mRNA to find the initiation codon (Fig. 1d) [10, 11]. If the 5′UTR is unstructured, a minimal 43S complex (comprising only a 40S, eIF1, eIF2-Met-tRNAi and eIF3) is capable of scanning without any requirement for ATP hydrolysis or factors associated with it. Nevertheless, if—as in most cases—the 5′UTR is at least mildly structured, this scanning process requires the hydrolysis of ATP, eIF1, eIF1A, and DHX29—a protein that binds directly to the 40S subunit and eIF1A [75, 76]. In addition, hydrolysis of ATP helps eIF4A or DDX3/Ded1p (both DEAD-box family members with helicase activity) to either actively translocate the ribosome in a 5′ to 3′ direction, or simply to unwind the structures in the mRNA, allowing a diffusive movement of the ribosome that is prevented from backsliding due to reforming of the unwound structures behind it [77]. Then, scanning stops when the PIC finds the first initiation codon in a favorable context, i.e., a purine in position −3 and a guanine in position +4 (Fig. 1d) [10].

Once the AUG codon is in the ribosomal P site, it becomes base-paired with all three nucleotides in the anticodon of the tRNAi, stabilizing the conformation of the tRNAi while allowing it to displace the basic loop of eIF1 [65]. This results in a stable complex, known as “48S initiation complex”. Furthermore, eIF1 has a major role in start codon selection, as it is needed for 43S PIC to discriminate between cognate and non-cognate initiation codon [78]. eIF5B is then required to stabilize Met-tRNAi in the P site, operating only after AUG recognition and release of eIF2–GDP from the 48S initiation complex [79]. After the AUG codon is recognized, several events take place so that the 60S subunit may join and form the 80S ribosome (Fig. 1e). This reaction requires eIF5B, which hydrolyzes eIF2–GTP, thereby releasing the initiation factors, including eIF2–GDP, from the small ribosomal subunit, but keeping the initiator tRNAi bound to the start codon [80]. Following eIF2–GDP dissociation, eIF5B–GTP binds to the 40S subunit and accelerates the rate of 60S subunit joining [81–83]. At this stage, 80S ribosomal complex is assembled and ready to start decoding the mRNA sequence and eventually originate a polypeptide (Fig. 1f).

As soon as the ribosome starts the elongation phase, eIF2 released from the ribosome is bound to GDP, which must be replaced by GTP to again allow ternary complex formation, so that another round of translation may take place [83, 84]. As eIF2 has a greater affinity for GDP, eIF2B works in promoting guanine nucleotide exchange (Fig. 1g). The formed eIF2–GTP is not stable unless Met-$tRNA_i$ joins in to form the ternary complex. This is one of the rate-limiting steps of translation initiation [58].

Rate-limiting steps in canonical translation initiation mechanism

Canonical translation initiation may be impaired at different stages under stress conditions. Regulation of protein synthesis is partly influenced by phosphorylation of eIF2 (via the α subunit), which is a part of the eIF2–GTP–Met-tRNAiMet ternary complex [85]. When the α subunit of the heterotrimeric eIF2 is phosphorylated at serine-51, it acts as a competitive inhibitor of eIF2B, by binding and sequestering eIF5B (reviewed in Ref. [2]). Since the cellular level of eIF2B is thought to be lower than that of eIF2α, only partial phosphorylation of eIF2α may be sufficient to inhibit all of the eIF2B, resulting in a failure of eIF2α to exchange GDP for GTP [9]. This occurs if there is amino acid starvation, heme deficiency, a viral infection, or

an unfolded protein response and subsequent endoplasmic reticulum stress [85].

Binding of the cap by eIF4E is often considered the rate-limiting step of cap-dependent initiation, and the concentration of eIF4E is a regulatory nexus of translational control [2]. Naturally, a small percentage of this initiation factor is phosphorylated. Another regulator is 4E-BP, which binds to the initiation factor eIF4E and inhibits its interactions with eIF4G, thus preventing cap-dependent initiation [85]. Hyper-phosphorylation of the 4E-BPs decreases their affinity for eIF4E and therefore enables the latter to bind to eIF4G and recruit an mRNA for translation [85]. 4E-BPs are phosphorylated by mTOR, which, in turn, is regulated through a complex net of signal transduction pathways (reviewed in [86]). Depending on the extent of 4E-BPs phosphorylation, cap-dependent initiation can be severely or only partially inhibited [85]. However, several mRNAs may be translated without the involvement of eIF4E, and thus evade this regulatory mechanism. Such mRNAs are translated via cap-independent translation initiation mechanisms as covered in the following paragraphs.

Cap-independent translation initiation mechanisms

Although the canonical scanning model is widely accepted as the most important mechanism of translation initiation in eukaryotes, it fails to explain how some proteins continue to be translated under conditions that impair m^7G cap structure recognition at mRNA 5′ end. Aiming to answer this question, most studies have shown that cap-independent initiation mechanism involves recognition of an IRES in the mRNA [22, 87]. However, some cellular mRNAs capable of cap-independent translation do not seem to contain any IRES elements. Below, we review the known cap-independent mechanisms that help circumvent difficulties in the normal initiation of eukaryotic translation.

IRES-mediated translation

In 1988, Pelletier and Sonenberg [88] discovered that some viral mRNAs from polioviruses are translated by a mechanism that enables ribosomes to initiate translation effectively on highly structured regions located within the 5′UTRs. Up until then, the only known mechanisms of translation initiation were dependent on the binding of eIF4E to the 5′ cap of mRNAs, but these authors showed that some mRNAs have a mechanism to bypass the need for eIF4E binding. This mechanism was called IRES-mediated translation [88, 89] (Fig. 2a). This mode of translation initiation is generally independent of 5′ cap structure recognition, but it may involve scanning in search of an initiation codon or directly recruit the 40S ribosome to the vicinity of the initiation codon. The 40S subunit recruitment can occur either in the complete absence of any other protein factors (dicistrovirus intergenic IRES) or with the aid of various combinations of canonical initiation factors (such as eIF4G and eIF3) and auxiliary proteins (reviewed in Ref. [90]). Since these discoveries, it has been found that many viruses contain IRES sequences in their mRNA 5′UTRs. Such sequences can direct translation of viral proteins without the aid of all translation initiation factors [90, 91]. These viruses are able to hijack the host eukaryotic translation machinery by cleaving factors necessary for canonical cap-dependent translation initiation but dispensable for IRES-mediated, free 5′ end-independent translation [88]. In this way, viral mRNAs are able to surpass eukaryotic mRNAs for ribosome binding and, in many cases, become the most abundant transcript being translated [88]. Most viral IRESs possess defined secondary and tertiary structures that account for their efficient interaction with the 40S ribosome. This interaction may be direct, or partially indirect, requiring the assistance of some canonical initiation factors and IRES *trans*-acting factors (ITAFs) [92]. ITAFs are known to assist in recruiting the 40S ribosomal subunit onto the mRNA through specific interactions or stabilization of specific active conformations of the IRES [4, 22, 90, 91, 93] (Fig. 2a). Based on (1) their need for factors; (2) the proposed secondary structure of the IRES; (3) the location of the start codon in relation to the IRES, and (4) the IRES' ability to operate in rabbit reticulocyte extract (with or without supplementation), the viral IRESs are divided into four groups [94]. Group 1 comprises the IRES RNAs that bind directly to the ribosome and require neither protein factors nor Met-$tRNA_i$, and are highly structured and compactly folded, operating essentially as an all-RNA-based ribosome recruitment apparatus [95]. Group 2 includes IRES RNAs that also bind directly to the 40S subunit, but can use a subset of canonical eIFs (eIF3, eIF2) as well as Met-$tRNA_i$; such IRESs are mostly extended, but maintain some structured and tightly packed regions [96]. Group 3 contains IRES RNAs that require some canonical eIFs, Met-$tRNA_i$ and ITAFs; they work in rabbit reticulocyte lysate and initiate translation at the 3′ end of the IRES; they are extended and largely flexible IRES RNAs [94, 96]. Finally, group 4 IRES RNAs require some canonical eIFs, Met-$tRNA_i$ and ITAFs; this type of IRES works efficiently in rabbit reticulocyte lysate only when it is supplemented with extracts from other cell types, and initiate at an AUG codon somewhat downstream of the IRES [94]. The more structured and packed the IRES, the lower the requirement for ITAFs and canonical factors [95]. The need for ITAFs on the less-structured viral IRES RNAs raises the question of ITAF

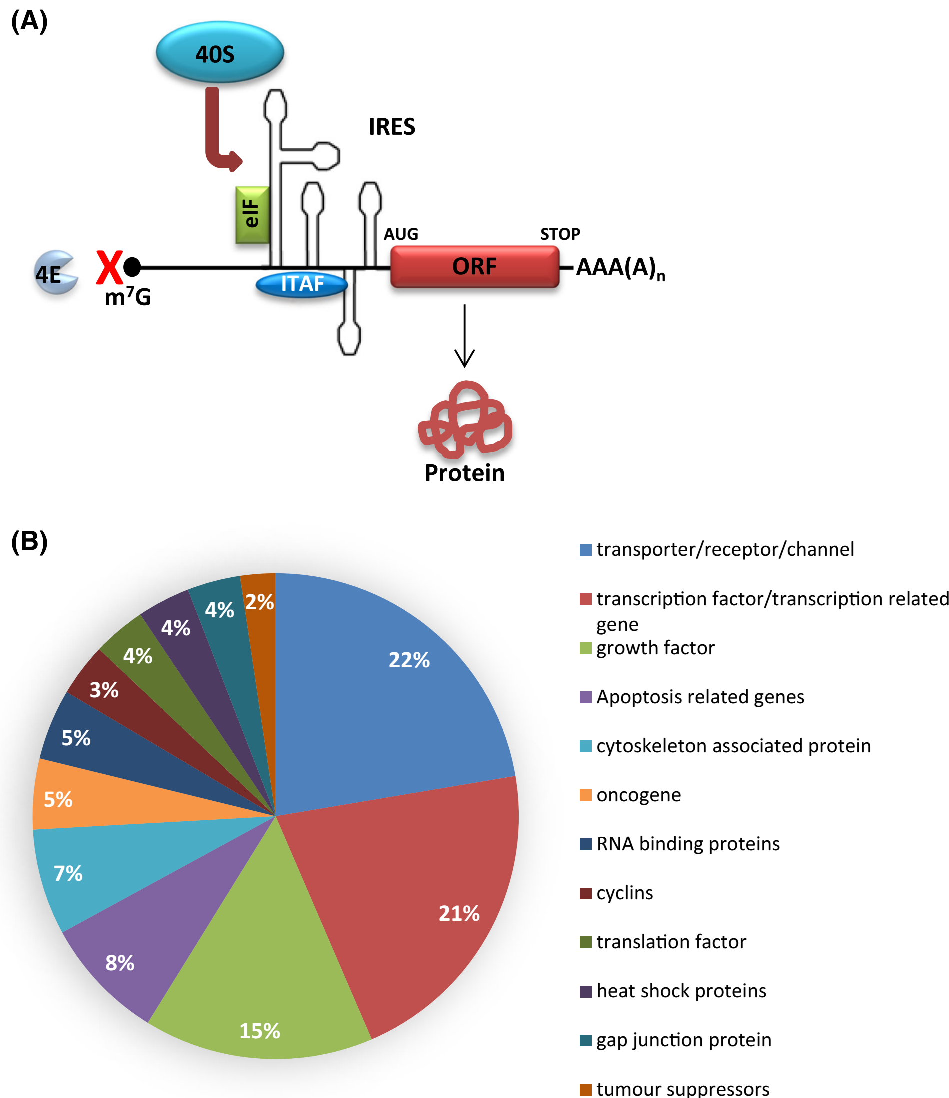


Fig. 2 Model of internal ribosome entry site (IRES)-dependent translation initiation, and distribution of IRES-containing transcripts by functional gene families. **a** IRES-mediated translation. Strong mRNA secondary structures (represented by stem loops) can directly recruit the 40S ribosomal subunit to the initiation codon (AUG) of the open reading frame (ORF) or its vicinity, skipping, or not, the scanning process. This interaction may be direct, or partially indirect, requiring the assistance of some canonical initiation factors (eIFs) and/or IRES *trans*-acting factors (ITAFs). **b** Distribution of the known IRES-containing transcripts according to functional gene families. According to what has been described in the literature, most IRES-containing transcripts encode transcription factors or transcription-related genes, transporters, receptors or channels, and growth factors; nevertheless, several other classes of proteins have been described as synthesized via an IRES-dependent mechanism of translation initiation. The latter include apoptosis-related genes, heat-shock proteins, tumor suppressors, cytoskeleton-associated proteins, gap junction proteins, oncogenes, RNA-binding proteins, cyclins, and translation factors. All these proteins need a fine-tune regulation of their synthesis, inasmuch as they are somehow involved in crucial processes of cell fitness and survival

function. One hypothesis is that they stabilize a specific IRES RNA conformation that enables binding of other factors or the ribosome [95]. This is supported by the fact that the more structured IRESs (which are assigned to group 1 or 2), such as the HCV-like IRESs, do not need ITAFs to bind the ribosome or eIFs [96]. Also, it is known that binding of PTB and ITAF45 to the foot-and-mouth disease (FMDV) IRES induces structural changes in the IRES, and synergistically enhances eIF4G and eIF4A binding [97].

Several eukaryotic cellular mRNAs can also be translated in an IRES-dependent way. The first cellular IRES in eukaryotes was discovered by Macejak and Sarnow [98] in the mRNA encoding the immunoglobulin heavy-chain binding protein (BiP). Since this discovery, many transcripts containing IRES structures within their 5′UTRs

have been described, and it has been estimated that 10–15% of the cellular mRNAs could be translated by an IRES-dependent mechanism [99]. Accordingly, recent data from a systematic screen for IRES-mediated translation activity have shown that about 10% of human 5′UTRs have the potential to be translated by this cap-independent mechanism [100]. Apart from the most recent discoveries, they are included in the IRESite, which presents carefully curated experimental evidence of many viral and cellular IRES elements [101]. Much like viral IRES-containing mRNAs, cellular mRNAs containing IRES elements were found to be preferentially translated under conditions of inhibition of cap-dependent initiation, such as endoplasmic reticulum stress [102], hypoxia [103], nutrient limitation [104], mitosis [105], and cellular differentiation [106]. Also, the reduced need for canonical initiation factors and/or requirement for specific ITAFs (often shared by viral and cellular IRESs), appear to be quite similar in viruses and eukaryotic cells [107, 108]. A striking feature of many ITAFs is that they belong to the group of heterogeneous nuclear ribonucleoproteins (HnRNP A1, C1/C2, I, E1/E2, K and L) known to shuttle between the nucleus and the cytoplasm [107, 109, 110]. Although the exact mechanism(s) underlying ITAF function remain(s) unknown, hypotheses include: (1) remodeling of IRES spatial structures to produce conformations with higher or lower affinity for components of the translation apparatus; (2) building or abolishing of bridges between the mRNA and the ribosome, in addition to those provided by canonical initiation factors; and (3) taking the place of canonical factors in building bridges between the mRNA and the ribosome [107, 109, 110]. Depending on the IRES, one out of three mechanisms of cellular IRES-mediated translation can be selected: (1) most, if not all, canonical initiation factors and many ITAFs are required; (2) a limited number of canonical factors and ITAFs are required; and (3) canonical factors are dispensable, but some ITAFs may be required. Several ITAFs, such as La, PTB, Unr or ITAF45 are common to viral and cellular IRESs [106, 108], as they are responsible for sensing changes in cellular metabolism and influence IRES activity [107, 109, 110]. Also, several canonical factors like eIF2, eIF3, eIF4A, eIF4G, eIF4B, eIF1A can be used as ITAFs by several picornaviral IRESs [111]. However, cellular IRES elements are often less structured than their viral counterparts [107, 108]. In 2006, Baird and collaborators found that length, number of upstream AUGs (uAUGs) and %GC content of 5′UTRs of the human transcriptome have a similar distribution to those of published IRES-containing UTRs [112]. Thus, none of these criteria is specific enough to be used in further identification of putative IRES sequences. IRESs tend to be found in longer 5′UTRs. However, half of all 5′UTRs from human transcripts are at least 150 bases long, which means that a large portion of 5′UTRs are long enough to contain IRES elements [112]. Upstream AUGs are common in 5′UTRs and, although IRES-containing 5′UTRs might have more uAUGs than what is observed in a normal distribution, IRES-containing 5′UTRs with no uAUGs are also common [112]. It is often stated that IRES-containing 5′UTRs tend to have greater levels of secondary structure. Although uORFs and specific inhibitory stable RNA structures may hinder the cap-dependent scanning mechanism for translation, it is not clear whether all IRES-containing 5′UTRs include these elements [112]. The overall %GC content appears no different for IRES-containing 5′UTRs from that found in the normal distribution of %GC content with 5′UTRs of human transcripts [112]. Moreover, a common Y-shaped structure has been predicted for cellular IRESs [113]. This pattern has been adapted for PatSearch—a flexible and fast pattern matcher that can find specific combinations of oligonucleotide consensus sequences, secondary structure elements and position–weight matrices [114]—to annotate putative IRES motifs. Unfortunately, this pattern is as common in known IRES-containing 5′UTRs as in all 5′UTRs. Overall, in silico identification of IRES elements, based on such unspecific characteristics only, is neither easy nor trustworthy.

By using a high-throughput bicistronic assay, a recent systematic analysis of sequences mediating IRES-dependent translation in human and viral genomes revealed that the fraction of sequences that mediate IRES-dependent translation is higher in viruses than in the human genome and that, in general, viral IRESs are more active than human counterparts, as previously predicted [100, 115]. Furthermore, this recent analysis also revealed that viral 5′UTRs with IRES activity have lower GC content and higher minimal free energy compared to their human counterparts. On the other hand, analysis of GC content and minimal free energy for all active and inactive 5′UTRs, from both human and viral origins, unexpectedly revealed that active 5′UTRs have lower GC content and higher minimal free energy [100]. This apparent contradiction remains to be understood. This study also revealed that there are two functional classes of IRESs: (1) those for which expression is reduced only when a specific position is mutated; and (2) IRESs for which mutation in most positions greatly reduces expression [100]. These two classes may represent differences in the underlying IRES activity mechanism. IRESs can either act through a short sequence motif—such as ITAF-binding sites, in which only mutations in a specific motif reduce activity (local sensitivity)—or involve the formation of a secondary structure, in which mutations at various positions can disrupt the overall structure and result in reduced activity (global sensitivity) [100].

The mechanism of IRES-mediated translation has been further investigated using the *XIAP* mRNA as a model [115]. XIAP protein is encoded by two mRNA splice variants that differ only in their 5′UTR regions. The most abundant transcript is shorter and produces the majority of XIAP protein under normal growth conditions via cap- and eIF2α-dependent translation [116]. However, during cellular stress, the longer transcript, containing the IRES element, directs efficient translation despite attenuation of global, cap-dependent translation [113]. Upon serum deprivation, the *XIAP* IRES-dependent translation switches to an alternative, eIF5B-dependent mode to circumvent attenuation due to eIF2α phosphorylation [117]. The cell's ability to evade ternary complex requirements suggests that cells have developed an alternative, eIF2α-independent mechanism of tRNA delivery to support a "rescue mechanism" for translating critical survival proteins under conditions when the "normal mechanism" is unavailable [117].

Interestingly, a limited investigation of other cellular IRES-containing mRNAs (*Bcl-xL*, *cIAP1*, *Apaf-1*, and *p97/DAP5*) suggests that not all cellular IRESs use eIF5B-dependent mode of tRNA delivery during serum deprivation [118]. Nonetheless, in order to better understand how IRESs allow direct association of the mRNA with the ribosome without the requirement of eIF4E, a different study revealed that *BCL2* IRES-dependent translation involves the association of DAP5 protein (an eIF4G homolog) with eIF2β and eIF4AI [105]. Likewise, a previous study revealed that eIF4A elicits potent activity on the lymphoid enhancer factor-1 (*LEF-1*) IRES, and, on the contrary, hippuristanol inhibition of eIF4A stalls *LEF-1* IRES-mediated translation [119].

Recent discoveries revealed that a eukaryotic viral IRES—cricket paralysis virus intergenic region IRES (CrPV IGR IRES)—can initiate translation in living bacteria [120]. Using crystal structure-solving data, these authors revealed that, in spite of differences between bacterial and eukaryotic ribosomes, this IRES binds directly to both and occupies the space normally used by tRNAs [120]. CrPV IGR IRES domain 3 is a precise mimicry of the anticodon loop-codon structure of an initiator tRNA. This suggests that docked CrPV domain 3 can form all the specific intermolecular contacts that occur between the ribosome and an authentic anticodon loop–mRNA complex within the decoding center [121]. This is an interesting observation given that the tRNA affinity for the ribosome reaches its highest value in the P site [122]. In bacteria, the P site performs a diverse set of functions through very specific contacts with the codon–anticodon structure [123]. The authors propose that the structured IRES RNA forms interactions with bacterial ribosomes that are transient and weaker than the highly tuned interactions that occur in eukaryotes, but allow internal ribosome entry into the message. Recruited subunits or ribosomes are repositioned to a downstream start codon where protein synthesis initiates [120]. This primitive mechanism suggests that RNA structure-driven or structure-assisted initiation may potentially be used in all domains of life, driven by various RNAs—perhaps possessing tRNA-like character or decoding groove-binding capability, thus bridging billions of years of evolutionary divergence.

The existence of IRES in capped cellular mRNAs raises the question of their pathophysiological function and of the advantage of a cap-independent translation [115]. In fact, several reports have demonstrated that cellular IRESs work in various physiological processes including spermatogenesis, neuron plasticity, and cell differentiation [20, 124–126]. Yet, reports have shown that some IRESs are also activated during cell cycle mitosis [127, 128] and apoptosis [7, 129], or are aberrantly activated in tumor cells, and are thus involved in dysregulation of gene expression in cancer [6, 20, 34, 41, 130]. Furthermore, cellular IRES activity is stimulated during various cellular stresses when cap-dependent translation is blocked [5, 7, 20, 56, 115, 131, 132]. Hence, perhaps cellular IRESs exist to play a crucial role in decisive moments of cell life, when cap-dependent translation initiation is compromised, in order for the cell to cope with environmental changes that affect its viability. As IRES-containing transcripts occur throughout every functional class of protein-encoding genes, we decided to search in PUBMED and cluster them according to the encoded protein's function, in order to understand which proteins are more prone to be translated via an IRES-dependent mechanism (Fig. 2b). Data showed that most IRESs described so far were found in transcription factor mRNAs (21%), in messages encoding growth factors (15%), and in mRNAs encoding transporters, receptors and channels (22%). Transcription factors like c-MYC and HIF1α, for instance, are key players in gene expression regulation, since they respond to quick environmental changes and adapt transcription levels to the cells' needs in a specific context [133, 134]. As for growth factors (e.g., FGF and VEGF protein families), they are of the utmost importance for the growth of specific tissues and play a major role in promoting cell proliferation and differentiation, and in regulating cell survival [133–137]. Transporters, receptors and channels (CAT-1, voltage-gated potassium channel, estrogen receptor α, among others) are the main vehicle for cell–cell communication, and play a critical role in signal transduction. Consequently, they are fundamental elements in cellular homeostasis, as they respond to extracellular environmental alterations. The greater relative proportion of IRES elements in these protein families can be a consequence of the high number of proteins (over 1000 transcription

factors [138] and receptors [138], and over 100 growth factors [139]) belonging to each of these functional classes in human cells. On the other hand, although there are few proteins in other functional classes, as is the case of cyclins (11 proteins [140] and corresponding subfamily members), 3% of these transcripts contain an IRES element, which account for the accurate gene expression regulation these proteins must suffer. Thus, disturbance in their function and expression is associated with profound alterations in cellular function and significantly contribute to the development and progression of disease [135]. All gene families presented in this graph (Fig. 2b) include proteins with pivotal roles in cellular processes that demand a fine-tuned regulation and whose expression levels need adjustment, in response to external cues that interfere with regular mechanisms of translation initiation and concomitant protein synthesis. Furthermore, alterations of their expression levels may account for many cancer types in human population, as will be further discussed below.

CITE-mediated translation

Some cellular mRNAs previously thought to contain IRESs fail to pass stringent control tests for internal initiation, thus raising the question of how they are translated under stress conditions. Terenin and co-workers [36] showed that the insertion of an eIF4G-binding element from a viral IRES into 5′UTRs of mRNAs that are strongly translated via the canonical mechanism greatly reduces their cap requirement in mammalian cells. This mechanism has been proven to be different from the internal entrance, because these mRNAs fail the bicistronic test, meaning they need a free 5′ end for the pre-initiation complex to bind [36]. Thus, although this is a cap-independent mechanism, it is 5′ end-dependent and involves special elements, the so-called "cap-independent translation enhancers" (CITEs) [35] (Fig. 3). CITEs are located within the untranslated regions of mRNAs and attract key initiation factors, in order to promote the assembly of translation initiation complexes [35, 37].

Most CITEs have been described within the 3′UTRs (3′CITEs) of plant viral mRNAs and are thought to function by establishing long-distance base-pairing interactions between 3′ and 5′UTRs to allow the 43S complex to entry the mRNA and to scan the 5′UTR until the former reaches the AUG (Fig. 3) [141–145]. In the case of 5′CITEs, a CITE is located within the 5′UTR and is capable of additional, presumably rather weak, interactions with initiation factors of the scanning machinery [35]. Although cap recognition by eIF4E still plays a major role in mRNA recruitment, the primary mRNA binding is also possible in the absence of this interaction, solely due to some interplay of key initiation factors (or the 40S ribosomal subunit itself) with 5′CITEs. Some components of the translation apparatus, for example eIF4G and eIF3 are able to be directly or indirectly recruited onto the 5′UTR via RNA–protein interactions with concomitant recruitment of other components of the scanning apparatus [37, 146]. In this way, the 5′UTR of an mRNA creates in its vicinity a high concentration of translational components. This also helps to overcome the competition for factors from other cellular mRNAs.

This mechanism has been described as the one that the human *APAF-1* mRNA uses to initiate translation under conditions of suppression of the cap-binding factor eIF4E [37]. Indeed, it has been shown that *APAF-1* 5′UTR can mediate an m^7G cap-independent but 5′ end-dependent scanning, even under apoptosis [37]. *APAF-1* plays a central role in DNA damage-induced apoptosis, and thus, its depletion contributes to malignant transformation [147]. Indeed, inactivation of *APAF-1* gene is implicated in disease progression and chemoresistance of some

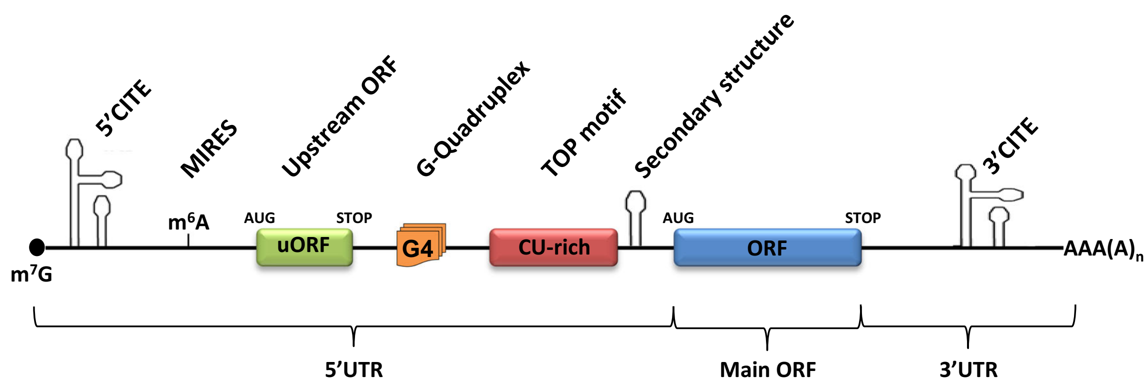


Fig. 3 Schematic mRNA with a 5′ untranslated region (5′UTR), coding region (ORF) 3′UTR, cap structure (m^7G), and poly(A) tail, showing different *cis*-acting elements involved in translational control: cap-independent translation enhancer (CITE), N^6-methyladenosine (m^6A)-induced ribosome engagement site (MIRES), upstream open reading frame (uORF), G-quadruplex (G4), and oligopyrimidine (TOP) motif

malignancies, such as metastatic melanomas [148]. In this regard, CITE-mediated translation under apoptosis may contribute extensively to the maintenance of APAF-1 protein levels, and to its tumor suppressor activity in stress conditions. It has been previously shown that *APAF-1* 5′UTR also has IRES activity that is triggered by UV-induced apoptosis [149]. How these data can be reconciled is yet to be determined.

m^6A-mediated translation

A feature of many eukaryotic mRNAs is N^6-methyladenosine (m^6A), a reversible base modification, seen in the 3′UTR, the coding region, or the 5′UTR [150, 151]. Although the biological function of the m^6A in 3′UTRs has been explored [151–154], the role of m^6A in the 5′UTRs has just recently been unveiled [39]. Data have shown that m^6A in the 5′UTR works as a 5′ cap alternative to stimulate translation initiation at sites called "m^6A-induced ribosome engagement sites" (MIRES) [39] (Fig. 3). In addition, data have shown that the m^6A in the 5′UTRs can bind eIF3, which is sufficient to recruit the 40S ribosomal subunit to initiate translation in the absence of the cap-binding factor eIF4E [39]. It seems, however, that the m^6A-mediated translation initiation involves a 5′ end-dependent 5′UTR scanning mechanism [39], as opposed to internal ribosomal entry [22]. As m^6A-mediated cap-independent translation initiation still requires 5′UTR scanning, it seems to behave similarly to what has been previously described for mRNAs with eIF4G-binding viral IRES domain within their 5′UTRs [36, 37, 39]. How m^6A is recognized by the translation machinery and facilitates cap-independent initiation needs deeper research, though. The importance of 5′UTR m^6A residues has been observed in both ribosome profiling datasets and individual cellular mRNAs. Data using *HSP70* mRNA revealed that a single m^6A modification site in its 5′UTR enables 5′ end N^7-methylguanosine cap-independent translation initiation, providing a mechanism for selective mRNA translation under heat-shock stress [39, 40]. Since it has been previously shown that *HSP70* 5′UTR also possesses IRES activity [155–157], it remains to be examined whether both features/properties cooperate to increase cap-independent translation following heat shock. In addition, it will be of great importance to know whether m^6A-mediated translation is involved in triggering disease states, such as carcinogenesis, and/or in the corresponding response to chemotherapeutics. Putative cellular IRESs often lack the complex structural elements that exist in viral IRESs [22, 87] and many assays that test cellular IRES function have inherent flaws. Most of these assays have been based on plasmid DNA transfections and, consequently, until it is definitively proven that there are no monocistronic FLuc mRNAs generated from the bicistronic DNA construct with a given IRES sequence, we cannot be sure whether the measured luciferase production is the result of an IRES or, for example, a consequence of cryptic promoter(s), or alternative splice sites, that are at the origin of monocistronic mRNAs [22, 158] (for a review on the tests required to truly validate an IRES element, see Ref. [22]). Considering this, the utility of m^6A in the 5′UTRs may be an additional and/or alternative mechanism to explain the occurrence of cap-independent translation. In addition, it is interesting to note that 5′UTR methylation in the form of m^6A is dynamic, and UV-, interferon-γ- and heat shock-inducible [38–40]. It will be important to analyze whether other stress-response pathways also induce m^6A modifications in 5′UTRs, and therefore use such modified sequences to mediate cap-independent translation initiation under stress conditions. Thus, stress-inducible 5′UTR methylation with promotion of cap-independent translation initiation establishes a new pattern of translational control. Of note, recent data have revealed that RNA cytosine hydroxymethylation can favor translation in *Drosophila* cells [159]. Also, YTH domain-containing proteins have been identified as a class of RNA-binding proteins that preferentially bind m^6A-methylated RNA in mammals and yeast [153, 160]. Interestingly, while the presence of the YTH domain of *Arabidopsis* AtCPSF30 (a protein shown to be involved in oxidative stress responses [161] and required for programmed cell death and immunity [162]) is dependent on alternative splicing, the YTH domain is completely absent in yeast and mammalian CPSF30 homologs [163]. These stress-responsive functions are independent of the YTH domain and raise questions about the possible roles of YTH domain-containing AtCPSF30 in regulating m^6A-containing RNAs [164]. It remains to be determined whether this RNA modification also occurs in mammalian cells and mediates cap-independent translation.

Cooperation between IRESs and other *cis*-acting RNA regulons

mRNA regulatory elements, often within 5′ and 3′UTRs, are the focus of many studies aimed at understanding translational regulation. However, the field is only now beginning to appreciate the rich regulatory information that directs selective translation of transcripts [165]. This is of great importance if we consider the variety of *cis*-acting regulatory elements, which include terminal oligopyrimidine tracts, pyrimidine-rich translational elements, cytosine-enriched regulator of translation, RNA secondary structures, G-quadruplexes, translation inhibitory elements (TIEs), uORFs and IRESs, among others (Fig. 3) [165–167]. These sequence motifs or structures can drive regulation through processes as diverse as cap-dependent

recognition and scanning, cap-independent translation, start codon usage, translation elongation rates, localization, and transcript stability [33, 165, 168]. Primarily, these sequence/structure motifs confer transcript-specific translational control. Here, using specific examples, we will discuss how IRES-driven cap-independent translation may be affected by the presence of other *cis*-acting RNA regulons.

G-quadruplexes are special secondary structures formed by G-rich DNA and RNA sequences that fold into a four-stranded conformation, and that are involved in many biological processes, including telomere elongation, transcription regulation, pre-mRNA splicing and translation [169–171]. G-quadruplex formation decreases cap-dependent translation initiation [170] from 35% in proteins like neural cell adhesion molecule 2 and thyroid hormone receptor α, up to 85% for estrogen receptor α [172]. However, there are cases when RNA G-quadruplex formation has been shown to actually promote translation. The human *FGF-2* IRES is 176 nucleotides long, is highly structured and contains two RNA stem-loops and one G-quadruplex motif—each of these structural domains contributes to IRES activity [129, 173]. G-quadruplexes also promote translation in the human *VEGF* mRNA [174]. The *VEGF* 5′UTR is 1038 nucleotides long and GC-rich, and able to initiate translation via IRES. Indeed, this untranslated region harbors two separate IRESs [55, 174, 175]. A 293-nucleotide portion, IRES-A, initiates translation at the canonical AUG and is known to maintain *VEGF* translation under hypoxia [55, 175]. This region also includes a sequence containing more than four G-stretches (nucleotides 774–790), which provides enough redundancy to ensure the formation of RNA G-quadruplex structures [174]. When the sequence is mutated in a way that disrupts the formation of the G-quadruplex, IRES activity is completely abolished [174, 175]. This suggests that a G-quadruplex structure must be formed in order to maintain the IRES function and hence promote translation. A more recent study has shown that the G-quadruplex within the *VEGF* IRES is dispensable for cap-independent function and activation in stress conditions [176]. However, stabilization of the *VEGF* G-quadruplex by increasing the G-stretches length, or by replacing it with the one of NRAS, results in strong inhibition of IRES-mediated translation of *VEGF* [176], i.e., the G-quadruplex ligands stabilize the *VEGF* G-quadruplex and inhibit cap-independent translation in vitro [176]. These findings have implications for the in vivo applications of G-quadruplex-targeting compounds and for anti-angiogenic therapies [176].

Likewise, a uORF within a transcript's 5′UTR can also cooperate with an IRES in the same transcript, in order to control protein synthesis. There are several pieces of evidence showing that many uORF-containing transcripts with an IRES element are involved in cell growth and differentiation, such as platelet-derived growth factor (*PDGF*) [177], *GATA-6* [178], *CAT*-1 [179, 180], *VEGF-A* [55, 175], and *FGF9* [181]. Thus, their expression needs to be fine-tuned through the interaction between IRES and uORFs co-existing within the same 5′UTR, and even small alterations in this interaction might have a role in cancerogenesis. For example, FGF9 protein synthesis is normally controlled by uORF-mediated translational repression, which keeps the protein at a low level [181]. In contrast, it is upregulated in response to hypoxia, through a switch to IRES-dependent translation [181]. Thus, *FGF9* IRES works as a cellular switch to turn FGF9 protein synthesis "on" during hypoxia, a mechanism likely to underlie FGF9 overexpression in cancer cells [181]. In another example, Yaman and colleagues have shown that the *CAT-1* 5′UTR has a uORF that modulates the activity of the co-existing IRES [179]. These results suggest a model for the regulation of the *CAT-1* IRES, which is dependent on uORF translation. In the absence of uORF translation, the mRNA 5′UTR exists in a structure that locks the IRES in a dormant state [179]. However, translation of the uORF disrupts this structure, allowing the sequence to form the IRES structure [179]. Under amino acid starvation, an ITAF binds the inducible IRES, leading to increased translation initiation at the *CAT-1* ORF [179]. This model suggests that uORF translation plays different roles in fed and starved cells. In the former, uORF translation inhibits downstream initiation by preventing the ribosome from reaching the *CAT-1* ORF. In the latter, uORF translation unfolds the 5′UTR, allowing the ITAF that is synthesized in response to eIF2α phosphorylation to bind the IRES and initiate CAT-1 protein synthesis [179]. Increased *CAT-1* translation during amino acid starvation requires both GCN2 phosphorylation of eIF2α, which leads to the increased translation of the transcription factor GCN4, and the uORF translation within the mRNA 5′UTR [182]. Amino acid starvation also induces translation of a monocistronic *CAT-1* 5′UTR-containing mRNA, in an eIF2α phosphorylation-dependent and uORF translation-dependent manner [182]. The uORF is translated within a bicistronic mRNA via an IRES that is located downstream of the uORF initiation codon $A^{-224}UG$ [179]. This model of *CAT-1* translation proposes that the uORF plays the role of a zipper that opens and closes the IRES [179, 180] and suggests that the mechanism of induced CAT-1 protein synthesis is part of the cell's adaptive response to amino acid limitation [182]. Likewise, there may be uORFs translated via an IRES-dependent mechanism. The expression of *GATA-6* and different *VEGF-A* isoforms is regulated by a small uORF located within an IRES and is translated through a cap-independent mechanism [55, 178].

On the other hand, the uORF may be located upstream the IRES as is the case of *FGF9* [181]. Under normal conditions, the uORF is generally translated in order to repress the expression of the main ORF and keep a low level of protein synthesis. Under specific environmental conditions, such as hypoxia, the high levels of FGF9 expression are achieved by activating the *FGF* IRES, which might be due to a switch of the ribosomes from the uORF AUG to the AUG of the main ORF. Thus, these two elements play opposite roles for *FGF9* translational control to fine-tune the level of FGF9 protein expression, either in normoxia or under hypoxia [181].

Recent evidence have shown that the ribosome itself, at the level of ribosomal proteins (RP), can also play a highly regulatory role, as it can stimulate a specialized translation of specific subpools of mRNAs [18]. Indeed, it has been shown that the group of mRNAs encoding homeobox (Hox) proteins has a mechanism to recruit "specialized ribosomes" containing the RPL38 protein [18]. This protein is dispensable for general cap-dependent translation, but is required for the translation of a subset of the 11 HoxA mRNAs (Hoxa4, Hoxa5, Hoxa9, Hoxa11 [18]). It facilitates 80S complex formation on these mRNAs, as a regulatory component of the ribosome, to confer transcript-specific translational control [183]. Such specialized ribosomes are the result of heterogeneity in ribosome composition resulting from differential expression and post-translational modifications of ribosomal proteins, ribosomal RNA (rRNA) diversity, and the activity of ribosome-associated factors [184]. They have a unique composition, or specialized activity, which renders regulatory control in gene expression. Examples of heterogeneity in ribosomes can include: (1) diversity in the composition and post-translational modifications of subsets of ribosomal proteins; (2) variations in rRNA sequences; or (3) their binding to ribosome-associated factors. All these may contribute to the occurrence of specialized ribosomes in different cell types [184]. In addition, even core ribosome components that show little variation may exert more specialized activity by virtue of their interactions with specific *cis*-acting regulatory elements in subsets of mRNAs [184]. In addition, *Hox* mRNAs present *cis*-regulatory TIEs in their 5′UTRs, which inhibit cap-dependent translation, and an IRES is activated relying on the RPL38 protein to promote *Hox* translation [18]. These RNA regulatory elements, together with the specialized ribosomes, provide a new mechanism of protein expression control, with implications in organismal development [18, 168].

As seen above, the interplay between different *cis*-regulatory RNA sequences/structures adds another level to translational regulation mechanisms with crucial roles in human health and disease, as we will discuss in further detail for carcinogenesis.

Cap-independent translation and cancer

Cancer is a disease caused by oncogene activation and tumor suppressor gene inhibition. Deep-sequencing studies identified numerous tumor-specific mutations, not only in protein-coding sequences, but also in non-coding sequences. These coding-independent mutations in regulatory elements, UTRs, splice sites, non-coding RNAs and synonymous mutations are able to affect gene expression from transcription, splicing, mRNA stability to translation (reviewed in Ref. [185]). In addition, the process of tumorigenesis involves back-up mechanisms that allow tumor cells to cope with stress, including those involved in the synthesis of proteins required for stress adaptation [6, 7, 34, 186–189]. Many transcripts with relevance in cancer, but with no specific tumor-associated mutations, are able to initiate translation through a cap-independent mechanism, namely through an IRES element. Accordingly, several oncogenes, growth factors and proteins involved in the regulation of programmed cell death are translated via IRES elements in their 5′UTRs (Fig. 2b). In this review, we will discuss data supporting the hypothesis that selective translation of these factors may contribute to the survival of cancer cells under stress situations induced within the tumor microenvironment—such as lack of nutrients, hypoxia, or therapy-induced DNA damage—and to understand which cancer cells are resistant to conventional therapies.

It is known that 4E-BP activation, in response to hypoxia and mTORC1 inhibition, dictates a switch from cap-dependent to cap-independent translation to support tumor growth and angiogenesis [186, 187]. Indeed, Braunstein et al. [187] demonstrated that most of the highly advanced breast cancers overexpress the translation regulatory protein 4E-BP1 and the initiation factor eIF4G. Overexpression of these two proteins leads to cap-independent mRNA translation, which promotes increased tumor angiogenesis and growth at the level of selective mRNA translation. This switch results in selective translation of IRES-containing mRNAs. These include a number of mRNAs that encode proteins involved in signal transduction pathways, gene expression and development, differentiation, apoptosis, angiogenesis, cell cycle, or stress response [6, 7, 20], as is the case of *VEGF-A* [190], *HIF1α* [191, 192] and *FGF2* [193], among others. For example, hypoxia reduces vascular endothelial growth factor C (VEGF-C) cap-dependent translation via the upregulation of hypophosphorylated 4E-BP [56]. However, IRES-mediated translation initiation of VEGF-C is induced by hypoxia, but independently of HIF1α signaling [56]. Notably, the VEGF-C IRES activity is higher in metastasizing tumor cells in lymph nodes than in primary tumors,

most likely because lymph vessels in these lymph nodes are severely hypoxic [56]. However, some studies assessing IRES activities of *HIF1α* and *VEGF* showed very low translation activity from these elements, suggesting that cryptic promoter activity in constructs used for those studies may interfere [22, 158]. Of note, Young et al. [194] confirmed that *VEGF* transcripts are selectively translated under hypoxia, even without significant IRES-mediated translation, suggesting that selective and alternative IRES-independent translation mechanisms may sustain VEGF synthesis under these conditions. Silvera and Schneider [195] have shown that inflammatory breast cancer cells have adapted to mimic a state of prolonged hypoxia at the translational level. Likely, this is to optimize the production of proteins required for tumor emboli survival and dissemination, a state promoted by high levels of eIF4GI protein coupled with a constitutively active 4E-BP1, leading to higher rates of translation of IRES-containing mRNAs, namely *VEGF* and p120 catenin, which are responsible for maintaining high rates of angiogenesis and membrane associated E-cadherin, respectively [196].

Fibroblast growth factors (*FGF*) play a critical role in the processes of proliferation and differentiation of a wide variety of cells and tissues; thus, their translation has to be tightly regulated, so that the expression levels are maintained within a range that promotes healthy growth and development. Some FGFs, such as *FGF1* and *FGF2*, contain IRES elements within their 5′UTRs that allow cap-independent translation initiation [197, 198]. These factors have been shown to be expressed at increased levels in prostate cancer [199]. Moreover, the role of IRES-mediated regulation of *FGF2* translation in tumorigenesis is considered a critical step, not only in solid tumors, but also in multiple myeloma, in a way that the *FGF2* IRES is the non-cytotoxic primary molecular target of thalidomide and should be considered the target for the development of immunomodulatory drugs in multiple myeloma [109, 200]. *FGF9* is another instance of an FGF family member, whose aberrant expression usually results in human malignancies [201]. Overexpression of *FGF9* has transforming potential in fibroblasts and stimulates the invasion of epithelial and endothelial cells, suggesting it might result in uncontrolled cell proliferation and malignancy [181, 201]. Under normoxia, FGF9 protein levels are maintained low due to the presence of a uORF that represses its expression [181]. In response to hypoxia, FGF9 protein expression is upregulated through a switch to IRES-dependent translational control, which may be the likely mechanism underlying *FGF9* expression in cancer cells, namely colon cancer cells [181].

Another case of a protein whose expression is upregulated during tumorigenesis by activation of IRES-mediated translation is specificity protein-1 (Sp1). It is accumulated during hypoxia in an IRES-dependent manner and is strongly induced at protein, but not mRNA, level in lung tumor tissue, suggesting that translational regulation might contribute to the accumulation of Sp1 during tumorigenesis [202]. Further studies have revealed that IRES-mediated translation of *Sp1* occurs through the recruitment of nucleolin to the 5′UTR of *Sp1* mRNA [203].

CDKN2A/p16INK4a is an essential tumor suppressor gene that controls cell cycle progression and replicative senescence, and is the main melanoma susceptibility gene. Its mRNA is also subjected to IRES-mediated translation. In fact, *p16INK4α* 5′UTR acts as a cellular IRES and Y-box binding protein 1 (YBX1) acts as its ITAF under hypoxic stress, both in cancer-derived cell lines, and p16INK4a wild-type lymphoblastoid cells obtained from a melanoma patient [204]. Interestingly, a germline sequence variant found in the *p16INK4α* 5′UTR (c.-42T>A) of a multiple primary melanoma patient results in local flexibility changes in RNA structure, impairing the binding of YBX1 and its stimulatory effect on IRES-dependent translation efficiency [204]. This sequence variant appears to alter p16 protein expression levels [204]. Impaired p16 translation under hypoxia could provide a mechanistic clue to explain melanomagenesis associated with this germline variant [204].

In a different study, data showed that in multiple myeloma cells under ER stress induced by thapsigargin, tunicamycin or the myeloma therapeutic bortezomib, the *c-MYC* IRES activity is also activated and requires the proteins hnRNP A1 and RPS25 as ITAFs for c-MYC protein levels to be maintained [102]. In addition to hnRNP A1 and RPS25, *c-MYC* IRES has been shown to be able to use P97—an eIF4G-related protein that has been described both as an inhibitor of translation and as a modulator of apoptosis—using a HeLa-based cell-free translation system [205].

Translation of specific transcripts, in response to nutrient deprivation, also occurs through cap-independent mechanisms. Specifically, synthesis of two amino acid transporters, namely cationic amino acid transporter-1 (CAT-1) and sodium-coupled neutral amino acid transporter 2 (SNAT2), which are required to promote recovery of amino acid balance, are controlled by IRESs under amino acid or glucose starvation [206, 207]. As referred above, under amino acid starvation, eIF2α phosphorylation, by GCN2 kinase, induces synthesis of an ITAF that binds the *CAT-1* IRES and initiates translation [179, 208, 209]. In tumor cells under glucose deprivation, translation from the *CAT-1* IRES is also induced, but through phosphorylation of eIF2α by the transmembrane endoplasmic reticulum kinase, PERK [207]. Moreover, phosphorylation of eIF2α by GCN2 in response to amino

acid deprivation also induces *SNAT2* IRES-mediated translation [188].

Conditions of growth factor deprivation also induce IRES-mediated translation of specific transcripts. It is the case of the mRNAs encoding the X-linked inhibitor of apoptosis protein (XIAP) and the sterol regulatory element-binding transcription factor 1 (SREBP-1), which are translated via an IRES, in the absence of growth factors in tumor cells, thus protecting cells from apoptosis [116, 210–212]. IRES-mediated translation of these proteins is involved in cell survival under nutritional stress, and might constitute an advantage for cancer cell survival [213]. In addition, the anti-apoptotic protein XIAP is upregulated under γ-irradiation via IRES-mediated translation, which makes tumor cells resistant to radiotherapy [214, 215]. Accordingly, it has been shown that inhibition of XIAP by RNA interference enhances chemotherapeutic drug sensitivity and decreases myeloma cell survival [215].

In a different study, it was found that paclitaxel (PTX), a drug commonly used in the chemotherapy of ovarian cancer, induces IRES-mediated translation of β-catenin in human ovarian cancer cell lines, and this regulates the expression of downstream factors (c-MYC and cyclin D1), reducing PTX sensitivity [216]. Thus, the regulation of the IRES-dependent translation of β-catenin may be involved in the cancer cell response to PTX treatment [216].

Several other anti-apoptotic proteins are also translationally controlled by IRESs under oxidative and genotoxic stress. These include the c-MYC cancer-associated transcription factor and Bcl-2-associated athanogene 1 (BAG-1) [217], which promotes resistance of tumor cells to DNA damage-inducing drugs [43, 218–220]. In addition, synthesis of cIAP1 and Bcl-2 is enhanced by etoposide and arsenite treatments through IRES-mediated translation [221–224].

The transcriptional master regulator of the oxidative and genotoxic stress response p53 is also IRES translated [53, 104, 225]. Indeed, the p53 transcript has two IRES structures that control the translation of full-length p53 and an N-terminally truncated isoform (Δ40p53) from the same mRNA [53, 225]. IRES-mediated translation of both isoforms is enhanced under different stress conditions that induce DNA damage, ionizing radiation and endoplasmic reticulum stress, oncogene-induced senescence and cancer [104]. Polypyrimidine tract-binding protein (PTB), an ITAF, stimulates IRES-mediated translation of both p53 isoforms in response to doxorubicin, following PTB relocalization from the nucleus to the cytoplasm [226]. This regulation is altered in the presence of melanoma-associated mutations in the *p53* 5′UTR [227]. In addition, human double minute 2 homolog (HDM2) and HDM4 act as other ITAFs which synergistically increase *p53* IRES activity under DNA damage following HDMX phosphorylation by ATM [228]. On the other hand, it was shown that glucose depletion induces *p53* IRES activity of both isoforms through the involvement of the scaffold/matrix attachment region-binding protein 1 (SMAR1)—a predominantly nuclear protein that becomes abundant in the cytoplasm under glucose deprivation—while PTB does not show nuclear–cytoplasmic relocalization, highlighting SMAR1's function as an ITAF under stress [104]. Other ITAFs have been reported to control *p53* IRES activity, such as eIF4G2 (also known as DAP5), Annexin A2 and PTB-associated splicing factor (PSF) [229, 230]. Furthermore, a different mechanism of p53 inactivation that links deregulation of IRES-mediated p53 translation with tumorigenesis was identified in two breast cancer cell lines [231, 232]. Here, the connection between IRES-mediated *p53* translation and p53 tumor suppressive function was established through the identification of two novel *p53* ITAFs, translational control protein 80 (TCP80), and RNA helicase A (RHA), which positively regulate *p53* IRES activity [231]. Recently, Candeias and collaborators [233] have shown that a wild type (Δ160p53) and not mutant p53 protein bears pro-oncogenic traits. Δ160p53-overexpressing cells behave in a similar manner to cells expressing mutant p53: they exhibit "gain-of-function" cancer phenotypes, such as enhanced cell survival, proliferation, invasion and adhesion, altered mammary tissue architecture and invasive cell structures [233]. In contrast, an exogenous or endogenous mutant p53 that fails to express Δ160p53 due to specific mutations or antisense knock-down loses pro-oncogenic potential [233]. These data support a model in which "gain-of-function" phenotypes induced by p53 mutations depend on the shorter p53 isoforms [233].

NRF2 is another master regulator of the response to oxidative stress that is translationally induced through an IRES under oxidative stress [234–236]. While NRF2 synthesis is blocked under basal conditions due to the presence of a highly structured inhibitory hairpin element present in its 5′UTR, its synthesis is enhanced by oxidative stress through stimulation of an IRES element also present within its 5′UTR [236]. IRES-mediated translation of *NRF2* requires the binding of the ITAF La autoantigen [236, 237].

Examples of other transcription factors induced by oxidative and genotoxic stress through IRES-mediated translation are the octamer-binding protein 4 (OCT4), which is synthesized upon H_2O_2 treatment in breast cancer and liver carcinoma cells [238], and runt-related transcription factor 2 (RUNX2), whose translation is stimulated by mitomycin C [239, 240]. All these examples support a model whereby under oxidative and genotoxic stress, IRES-mediated translation of key regulators and pro-survival factors provide tumor cells with mechanisms for attaining resistance to chemotherapy and radiotherapy [34].

On the other hand, the presence of IRES within transcripts coding for tumor suppressor proteins can help the cell maintain the levels of these proteins and prevent the outbreak of cancer. The oncogene-induced senescence (OIS), a critical cellular response that counteracts cellular transformation, is characterized by cell cycle arrest and induction of *p53*, thus restraining the proliferative potential of preneoplastic clones [241]. Bellodi et al. [242] have demonstrated that during OIS, there is a switch from cap-dependent translation to IRES-dependent translation, during which an IRES element positioned in the 5′UTR of *p53* is engaged to promote *p53* translation; hence, specialized translational control of mRNAs, such as *p53*, provides a molecular barrier for cellular transformation. Montanaro et al. [243] demonstrated that increased p53 activity in breast cancer is dependent on dyskerin-mediated increase in IRES-mediated translation but independent of effects on telomerase.

Induction of the aforementioned proteins expression (summarized in Table 1) provides a key factor for cancer cells to survive and proliferate under stress conditions, demonstrating the importance of IRES-mediated translation in the process of tumorigenesis and how the IRES structures may be considered important targets in cancer treatment.

Concluding remarks

Alternatives to the canonical process of mRNA translation initiation in mammalian cells include mechanisms to bypass the m^7G cap requirement, enabling a cap-independent mode of translation initiation. As discussed above, the most widely characterized cap-independent initiation mechanism involves the recognition of an IRES in the mRNA. The recently described mechanism of cap-independent translation initiation associated with RNA modification (adenosine methylation), not only expands the breadth of the non-canonical translation initiation mechanisms and their physiological functions, but also opens new

Table 1 Examples of transcripts that allow IRES-mediated translation during the process of tumorigenesis

Transcript	Cellular conditions	ITAFs	Cancer type	References
VEGF-A	Hypoxia	HnRNP L	Lymphoma, inflammatory breast cancer	[56, 108, 190, 195]
HIF1α	Hypoxia	PTB	Colon cancer	[54, 192]
FGF2	Hypoxia, tumorigenesis	hnRNP A1	Prostate cancer, multiple myeloma, colon cancer	[109, 181, 193, 199, 200]
Sp1	Tumorigenesis, hypoxia	Nucleolin	Lung cancer	[202, 203]
CDKN2A/p16INK4α	Hypoxia	YBX1	Melanoma	[204]
c-MYC	Endoplasmic reticulum stress	hnRNP A1, RPS25, GRSF-1, YB-1, PSF, p54nrb, P97	Multiple myeloma	[102, 205]
CAT-1	Amino acid or glucose starvation	hnRNP L, PTB	Colorectal cancer	[179, 206–209]
XIAP	Absence of growth factors, nutritional stress, γ-irradiation	PTB-1, hnRNP C1/C2, La, hnRNP A1	Myeloma	[101, 116, 210, 213–215]
SREBP-1	Absence of growth factors, nutritional stress	hnRNP A1	Endometrial cancer	[116, 210–213]
β-Catenin	Paclitaxel (PTX) treatment		Ovarian cancer	[216]
BAG-1	Oxidative and genotoxic stress	Members of the poly(rC)-binding protein family	Breast cancer	[43, 217–220]
cIAP1	Oxidative and genotoxic stress	DAP5, IGF2BP1	Rhabdomyosarcoma	[101, 221–223]
Bcl-2	Oxidative and genotoxic stress	DAP5	Lymphoma	[108, 221, 222, 224]
p53	DNA damage, ionizing radiation, endoplasmic reticulum stress, oncogene-induced senescence, glucose deprivation	PTB, HDM2, HDM4, DAP5, Annexin A2, PTB-associated splicing factor (PSF), SMAR1, TCP80, RHA	Melanoma, breast cancer, cervical cancer, leukemia, head and neck squamous cell carcinoma	[56, 104, 225–232]
NRF2	Oxidative and genotoxic stress	La autoantigen	Colorectal cancer	[234–237]
OCT4	Oxidative and genotoxic stress		Breast cancer and liver carcinoma	[238]
RUNX2	Mitomycin C treatment		Osteosarcoma	[239, 240]

research prospects in the area of cap-independent translation initiation mechanisms. These promising studies, as well as a deeper knowledge of how these mechanisms are involved in translational control in response to cellular stresses or pathological conditions, will be of great value in designing new treatment strategies (including those by abrogation/modulation of IRES-mediated translation) for many human conditions, including cancer.

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