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overview

# The growing relevance of cap-independent translation initiation in cancer-related genes

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## Abstract

*Two main mechanisms for eukaryotic initiation of protein synthesis have been described – the canonical cap-dependent and the alternative cap-independent. They mainly differ in their requirement for 7-methylguanosine cap at 5' end of mRNA molecules to initiate translation. In cap-independent translation initiation, an element within 5' untranslated region (5' UTR) of mRNA, defined internal ribosome entry site (IRES), recruits 40S ribosomal subunit directly or close to the start codon without the need for the 5' cap.*

*Some cellular mRNAs – including those encoding for a number of growth factors, oncogenes, receptors, survival proteins, transcription and translation factors – contain IRES elements within their 5' UTR what may allow them to be translated under different physiological or stress conditions (e.g., amino acid starvation, apoptosis, growth arrest, heat shock, mitosis, radiation) when global cap-dependent protein synthesis is suppressed. IRES-dependent translation may escape the control of checkpoints present in cap-dependent regulation causing improper protein synthesis that can lead to cell apoptosis or disease. A growing number of cancer-related genes have been reported whose translation initiation depends on the presence of IRES element in their mRNA. These findings make the quest for discovering and testing new putative cellular IRESes even more meaningful. A deeper understanding of the role of IRES-dependent translation initiation in cancer etiology could ultimately give us a novel targets for cancer therapy.*

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## REGULATION OF EUKARYOTIC PROTEIN SYNTHESIS

Protein synthesis is the final step in the flow of genetic information. In the process of translation the nucleotide sequence encoded in a messenger RNA (mRNA) molecule is converted through genetic code into a protein product. This process is divided into three consecutive stages – initiation, elongation, and termination. It is considered that initiation is the rate-limiting step and is highly regulated in eukaryotes *(1)*. Translational control is one aspect of the post-transcriptional regulation of gene expression and has a significant impact on the cellular proteome composition *(2)*. Selective translation allows rapid cellular adaptation to changes in physiological conditions without involving new mRNA synthesis and transport *(3)*. On the other hand, improper protein synthesis can lead to cell apoptosis or disease *(3, 4)*. In cancer cells, hyperactivated signaling pathways influence translation rates supporting the uncontrolled growth and survival. Consistently, several components of translation initiation system have been found to be mutated, or differentially expressed, and some could act as

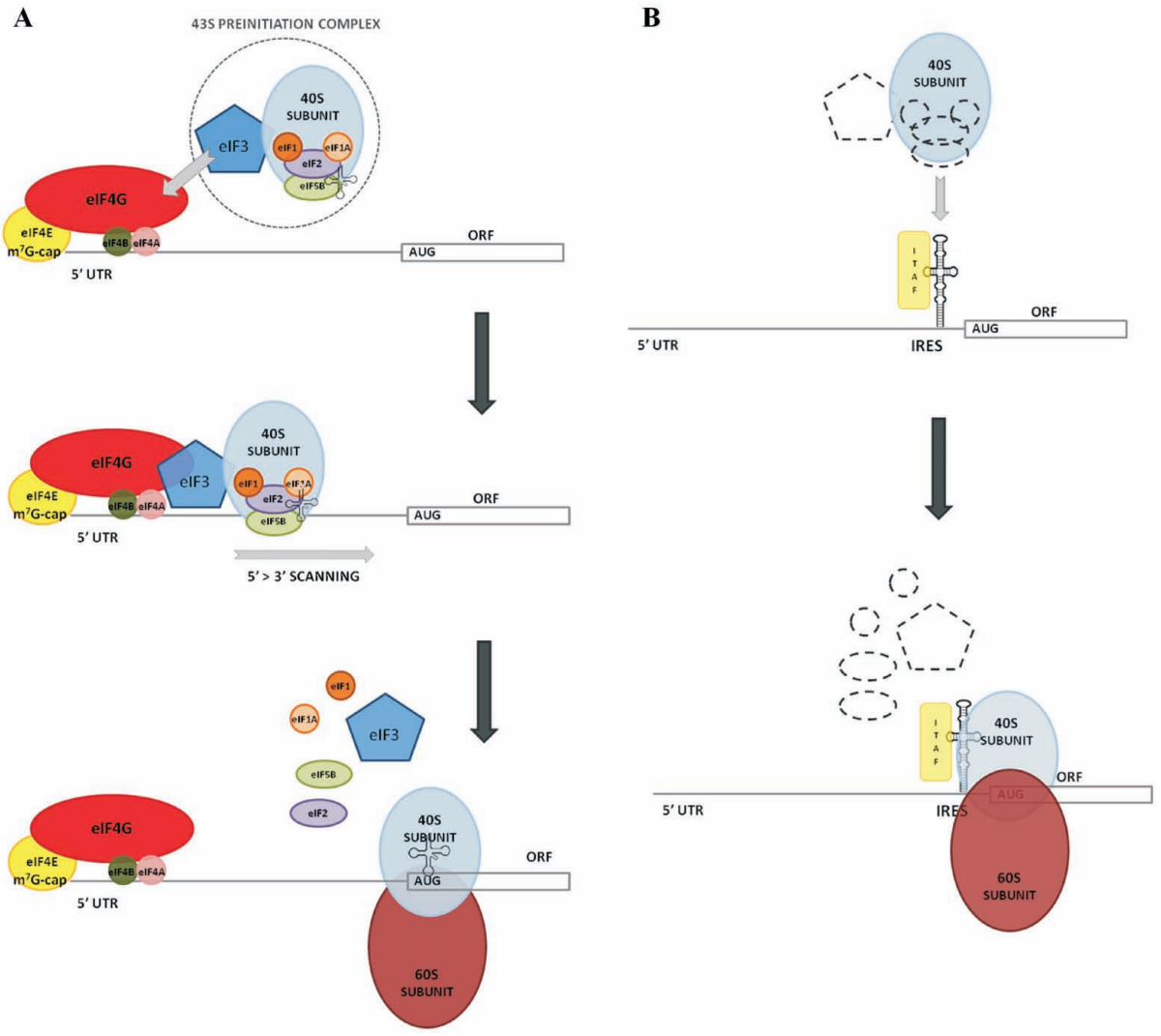


**Figure 1.** *Similarities and differences between canonical cap-dependent and cap-independent internal initiation of translation in eukaryotes. (A) Cap-dependent initiation mechanism requires an mRNA molecule with $m^7$G-cap on its 5' end. A large number of proteins termed eukaryotic translation factors (eIFs) participate in the processes of recognition of the $m^7$G-cap operated by cap-binding complex eIF4F (composed of eIF4E, eIF4A and eIF4G), but also in the recruitment of the 43S pre-initiation complex (composed of 40S ribosomal subunit, eIF2, eIF3 and initiator methionyl-transfer RNA), 5' to 3' directional scanning of the 43S complex along mRNA 5' untranslated region (5' UTR), recognition of start codon (AUG) in appropriate context, and joining of the 60S ribosomal subunit with 40S subunit leading to the 80S ribosome ready to translate an open reading frame (ORF). (B) In cap-independent initiation, the main difference is that an element within mRNA molecule named internal ribosome entry site (IRES) recruits 40S ribosomal subunit directly or close to the translation start codon without the need for $m^7$G-cap and eIF4E. Molecular mechanisms behind cap-independent initiation depend on the presence of an IRES element and may or may not require canonical eIFs and/or auxiliary proteins named IRES* trans*-acting factors (ITAFs).*

oncogenes *(5)*. In cancer, translational alterations can increase the overall rate of protein synthesis as well as activate the translation of specific mRNAs for the proteins that promote cancer progression and metastasis *(5)*.

Generally there are two main mechanisms of eukaryotic protein synthesis initiation – the canonical cap-dependent and the alternative internal cap-independent initiation of translation. Each mechanism relies on a complex interplay involving recognition of mRNA by specific subset of eukaryotic initiation factors (eIFs) followed by recruitment of the ribosome subunits, recognition of the translation initiation codon and initiation of protein synthesis *(6)*.

## CANONINCAL CAP-DEPENDENT TRANSLATION INITIATION

The vast majority of eukaryotic mRNAs initiate translation by a mechanism that require the presence of the 5'-7-methylguanosine structure (termed 'm$^7$G-cap' or merely 'cap') located at the 5'-end of most mRNAs (Figure 1A). A large number of helping proteins termed eukaryotic translation factors (eIFs) mediate these processes. The 5' cap is recognized by cap-binding complex eIF4F that is composed of eIF4E (cap-binding protein), eIF4A (an RNA helicase) and eIF4G (a scaffolding protein). The eIF4G further interacts with eIF3 and the poly(A)-binding protein (PABP) that is bound to the poly(A)-tail of the mRNA, leading to a

**TABLE 1**

Eukaryotic translation initiation factors (eIFs) dysregulated in various human cancers.

| Factor name | Function in translation initiation | Association with cancer | Expression status in cancer | References |
|---|---|---|---|---|
| eIF2α | forms eIF2–GTP–Met-tRNAi ternary complex that binds to ribosomal 40S subunit | melanoma, gastrointestinal carcinomas, non-Hodgkin's and Hodgkin's lymphomas | upregulated | *(12, 13)* |
| eIF3 | binds ribosomal 40S subunit, eIF1, eIF4G and eIF5; stimulates binding of eIF2–GTP–Met-tRNAi to 40S subunit; promotes attachment of 43S complex to mRNA and subsequent scanning; possesses ribosome dissociation and anti-association activities that prevent joining of 40S and 60S subunits | | | |
| eIF3a | | breast, cervix, esophagus, lung | upregulated | *(14, 15)* |
| eIF3b | | breast | upregulated | *(14)* |
| eIF3c | | testis | upregulated | *(16)* |
| eIF3e | | breast, non-small cell lung cancer | downregulated | *(17)* |
| eIF3f | | pancreas, breast, ovary | downregulated | *(18)* |
| eIF3h | | breast, prostate | upregulated | *(19)* |
| eIF4A | DEAD-box ATPase and ATP-dependent RNA helicase | hepatocellular carcinoma, melanoma | upregulated | *(20, 21)* |
| eIF4E | binds to $m^7$G-cap structure of mRNA | bladder, breast, colon, head and neck, liver, non-Hodgkin's lymphoma | upregulated | *(13, 20, 22-29)* |
| eIF4GI | binds eIF4E, eIF4A, eIF3, PABP, SLIP1 and mRNA; enhances helicase activity of elF4A | squamous cell lung carcinoma | upregulated | *(30)* |
| eIF5A | GTPase-activating protein, specific for GTP-bound eIF2; induces hydrolysis of eIF2-bound GTP on recognition of initiation codon | ovary, pancreas, colorectal carcinoma | upregulated | *(31, 32)* |

functional circularization and activation of the mRNA molecule. Binding of eIF4F leads to the recruitment of 43S preinitiation complex that is composed of the 40S ribosomal subunit, eIF2, eIF3 and the initiator methionyl-transfer RNA (Met-tRNA$_i$). The 43S complex with assistance of eIF1 and eIF1A scans the mRNA in a 5' to 3' direction until the first start codon (*AUG*) within an appropriate sequence context (GCC[A/G]CC*AUG*G, termed Kozak consensus sequence) is found, which leads to the formation of the 48S initiation complex. At this step eIF1 is released, eIF5 mediates the hydrolysis of eIF2-bound GTP and eIF5B mediates joining of the 60S ribosomal subunit with the 40S subunit thus assembling a translationally capable 80S ribosome. This cap-dependent translation initiation mechanism is predominantly used by cellular mRNAs to recruit and position the ribosome *(7)*.

## CAP-DEPENDENT TRANSLATION IN CANCER

Overexpression of several components of translation initiation machinery was shown to cause or to strongly correlate with malignant transformation. The cap-binding protein eIF4E, the least abundant eIF and hence considered to be a rate limiting factor for cap-dependent translation, is found upregulated in the majority of human cancers and its high expression levels correlate with poor prognosis *(8)*. Overexpression of eIF4E can also increase the translation of mRNAs with structured 5' untranslated region (5' UTR) that are normally translated with less efficiency *(9)*. Intriguingly, many transcription factors, growth factors, receptors and tyrosine kinases have suchlike mRNAs *(10)*. Many characteristics of eIF4E make it a promising target for cancer therapy *(11)*.

The other eukaryotic translation initiation factors known to be dysregulated in human cancers are listed in Table 1.

## CAP-INDEPENDENT (INTERNAL) TRANSLATION INITIATION

Lack of oxygen (hypoxia), starvation and response to DNA damage-inducing therapy represses cap-dependent translation and leads to reduced levels of overall protein synthesis *(33)*. In parallel, inhibition of cap-dependent protein synthesis allows the subset of mRNAs to be translated in a cap-independent manner using mRNA elements termed internal ribosomal entry sites (IRESes) which are predominantly located in the 5' UTR *(34)*.

As suggested by the definition, cap-independent initiation does not require the $m^7$G-cap to recruit a ribosome to the mRNA (Figure 1B). According to this mechanism, mRNA sequence itself has ability to bind and activate the translational machinery. There are many different mechanisms by

which IRESes can initiate translation – some of them require canonical eIFs while the others can engage auxiliary proteins not normally associated with translation. These RNA-binding proteins named IRES *trans*-acting factors (ITAFs) are thought to help in proper folding of the IRES region facilitating the the recruitment of the translation machinery on the mRNA *(35)*.

The cap-independent mechanism was first discovered in the 5' UTR of two picornaviruses – encephalomyocarditis virus and poliovirus *(36, 37)*. Some picornaviruses can inhibit cap-dependent protein synthesis in infected host cells by virus-encoded proteases that are able to cleave eIF4G and PABP, the key components of cap-dependent translation initiation *(38)*. By this mechanism the virus can block host anti-viral response. The first cellular IRES was found in mRNA encoding for the immunoglobulin heavy chain--binding protein (BiP). It was discovered owing to its persisting activity in poliovirus-infected cells although translation of majority of host mRNAs was stopped *(39)*.

## THEORETICAL AND EXPERIMENTAL CONSIDERATIONS FOR SEEKING IRESes IN CELLULAR mRNAs

The ability of an RNA molecule to function as IRES is entirely encoded in its primary sequence but it is also apparent that higher order secondary and three-dimensional structures are determinants of IRES activity *(40)*. The mRNAs with long 5' UTR (200 to 500 nucleotides), predicted as heavily structured and with high GC content were traditionally considered strong candidates for having IRES elements and at the same time incapable of efficient translation by canonical cap-dependent ribosome scanning mechanism *(41)*. Le and Maizel introduced a prediction rule whereby a Y-shaped double-hairpin structure followed by a small hairpin would constitute an IRES RNA motif that can in fact be found upstream of the start-site codon in a variety of cellular mRNA *(42)*. Still there is no direct experimental evidence for IRES functionality of this motif. Other structural elements that may suggest functional IRESes could be the presence of polypyrimidine tracts, pseudoknots near the start codon, or hairpin-loops mimicking those present in the IRESes of picornavirus, hepatitis C virus (HCV), or the intergenic region (IGR) of dicistrovirus RNA genomes *(43)*. Presence of one or more of these elements may provide hints to select potential cellular IRES for further experimental validation.

The most widely used experimental approach for testing the presence of a cellular IRES in a 5' UTR is based on bicistronic reporter assays *(40, 44, 45)*. The putative IRES sequence is cloned into the inter-cistronic region between two different reporter genes (e.g., *Firefly/Renilla* luciferase). Transcription driven from an upstream promoter should give one bicistronic mRNA transcript. Expression of the upstream reporter should occur through cap-dependent mechanism, whereas the downstream reporter should be translated only if the inserted sequence exhibits IRES activity thus allowing ribosomes to be recruited to the mRNA internally (~1kb downstream from $m^7$G-cap). The same dual reporter vector without inserted fragment ('empty' vector) is often used as a negative control, while a proven IRES sequence is used as a positive control. If activity of the downstream reporter is greater for the putative IRES element than non-IRES control, the candidate RNA is considered as a potential IRES-containing sequence. In addition, to establish that differences in reporter activity are due to translational and not to transcriptional processes, (e.g., that the cloned sequence can act as a cryptic promoter), the bicistronic nature of the reporters' mRNA should be examined by PCR in the control vectors as well as those containing the inserted IRES candidate. Reporter assays based on dual-cistronic DNA constructs can indeed give false positive results owing to the fact that inserted mRNA acts as a cryptic promoter or can lead to an alternative splicing event resulting in $m^7$G--capped monocistronic mRNA containing just the downstream reporter. To avoid these potential ambiguities it is better to use *in vitro* transcribed bicistronic mRNA that is purified and transfected in a cell line for the reporter assay *(40, 45)*. Testing potential IRESes with monocistronic reporter assays requires using vector constructs with $m^7$G-cap analogs or hairpin-loop structures positioned upstream of the reporter gene open reading frame that should facilitate utilization of IRES-dependent internal translation initiation *(40)*.

Since there is no single 'gold-standard' procedure that can be used to definitely confirm the presence of IRES within an mRNA molecule, a series of carefully planned and controlled experiments should be designed to eliminate all possible alternative explanations.

**TABLE 2**

Cancer-related genes whose mRNAs are reported to contain cellular IRES elements.

| Protein function | Gene symbol | References |
|---|---|---|
| Apoptotic proteins | APAF1 | *(77)* |
| | BCL2 | *(86)* |
| | EIF4G2 (DAP5) | *(84)* |
| | BIRC2 (c-IAP1) | *(85)* |
| | XIAP | *(52)* |
| Oncogenes | JUN (c-Jun) | *(57)* |
| | MYC (c-Myc) | *(53)* |
| | MYCN (N-Myc) | *(56)* |
| | PIM1 | *(87)* |
| Tumor suppressor | APC | *(89)* |
| | TP53 | *(63)* |
| Differentiation | LEF1 | *(90)* |
| | PDGFB (c-Sis) | *(70)* |
| Hypoxia | FGF2 | *(64)* |
| | HIF1A | *(68)* |
| | VEGFA | *(51)* |
| DNA damage response | SHMT1 | *(91)* |
| Mitosis | CDK11 ($p58^{PITSLRE}$) | *(59)* |
| | CDK1 | *(86)* |
| | ODC1 | *(88)* |

## IRES ELEMENTS IN CANCER-RELATED GENES

Although capped, some cellular mRNAs thay play important role in many cellular pathways contain IRES elements within their 5' UTR that may allow them to be translated under different physiological or stressful conditions (e.g., amino acid starvation, apoptosis, growth arrest, heat shock, mitosis, radiation) when global cap-dependent protein synthesis is suppressed *(46)*. In contrast, IRES-dependent translation is completely or partially resistant to this inhibition. At the moment there are around 70 experimentally verified cellular IRES elements *(47)* and a large number of those are found in cancer-related genes *(48)* (Table 2).

Since IRES-dependent translation relies on different mechanism and requires several unique protein factors compared with cap-dependent translation it was suggested that IRES-dependent regulation may escape the control of check points present in cap-dependent regulation *(49)*. Most cancer-related cellular IRESes are differentially regulated, e.g., IRES element of the oncogene c-Myc is upregulated following genotoxic stress *(50)*, VEGF IRES shows regulation by hypoxia *(51)* and the XIAP IRES element is upregulated after γ-irradiation or anoxia *(52)*. Those condition requires rapid responses that could be achieved by regulation of RNA metabolism (e.g., splicing, turnover, translation) more efficiently than by *de novo* mRNA synthesis.

### Oncogenes and Tumor Suppressors

c-Myc is a member of the mammalian transcription factors of the bHLH-zip family and a potent oncogene. Deregulated expression of myc genes is frequent in cancers and ectopic expression is sufficient to induce cell cycle progression. Translational control of c-Myc was suggested by the discovery of an mRNA isoform that lacks the 5' UTR and is more translationally efficient. It was subsequently discovered that c-Myc 5' UTR contains a functional IRES element directing c-Myc translation *(53, 54)*. In 42% of patients with multiple myeloma there was an acquired single nucleotide mutation in the c-Myc IRES element resulting in a dramatic increase in c-Myc translation *(55)*.

Also N-Myc *(56)*, c-Jun *(57)*, and ornithine decarboxylase 1 (ODC1) can be translated by IRES-dependent mechanisms *(58)*. Translation of both c-Myc and ODC1 was shown to be upregulated during cell cycle progression.

Another cell cycle regulated gene translated through IRES-dependent manner is protein kinase $p58^{PITSLRE}$, a member of cyclin-dependent protein kinases (CDK) *(59)*. $p58^{PITSLRE}$ is often deleted in neuroblastomas, malignant melanoma, non-Hodgkin lymphomas, childhood endodermal sinus tumors and its levels correlate with aggressive tumor growth *(60)*. The inhibitor of CDK CDKN1B ($p27^{Kip1}$) is also translated through IRES during cell cycle *(61)*. CDKN1B plays a central role in the regulation of cell proliferation and differentiation. The levels of $p27^{Kip1}$ are frequently downregulated in cancer cells and often correlate with poor clinical outcome *(62)*.

The tumor suppressor protein p53 has a key role in maintaining genomic integrity by controlling cell-cycle progression and cell survival. Two IRESes have been described that can mediate the translation of both full-length and ΔN-p53 isoforms. The IRES directing the translation of full-length p53 is in the 5' UTR, whereas the IRES mediating the translation of ΔN-p53 extends into the protein-coding region. The two IRESes show distinct activities depending on the cell-cycle phase – the IRES for full-length p53 is active at G2–M transition and that for ΔN-p53 shows highest activity at the G1–S transition *(63)*.

### Growth Factors

Fibroblast growth factor-2 (FGF2) promotes epithelial proliferation and angiogenesis. FGF2 mRNA contains a long and structured 5' UTR and its translation is initiated either at primary AUG codon or at one of three upstream CUG codons. Translation of all four isoforms is IRES--driven with the same efficiency *(64)*. Interestingly, many breast carcinomas that show elevated levels of eIF4E also exhibit translational deregulation of FGF2 *(28)*.

Although vascular endothelial growth factor A (VEGFA) is not itself considered an oncogene, it is upregulated in tumorigenesis and it is important for pathological angiogenesis in solid tumors *(65)*. Blood-vessel formation is critical in tumorigenesis and prevention of angiogenesis may lead to tumor regression. Two hypoxia-responsive IRESes are involved in VEGFA translational upregulation *(51, 66)*.

Translation of hypoxia-inducible factor-1α (HIF1A), a major transcriptional activator of hypoxia-inducible genes, is also mediated by an IRES element *(67)*.

The proto-oncogene c-Sis encodes for the B chain of platelet-derived growth factor (PDGFB) and its mRNA possesses an extremely long 5' UTR that inhibits translation by cap-dependent mechanism *(68)*. On the contrary, an IRES element that was identified within this 5' UTR very effectively initiates translation of PDGFB *(69)*. Activity of IRES is enhanced during cellular differentiation which suggests that IRES-dependent regulation could restrict expression of PDGFB to the required cell types. PDGFB regulates mesenchymal cell migration and proliferation, and its aberrant expression was implicated in both cancer development and progression *(70)*.

Human insulin-like growth factor 2 (IGF2) regulates fetal development and growth, and is connected with various human cancers, e.g., lung, colon, prostate, and hepatocellular carcinoma *(71)*. Transcription from IGF2 locus generates four diverse transcripts that are differentially expresses depending on the developmental state. Transcripts have different 5' UTR but share identical coding region, and one of them (leader 2) can be translated in a cap-independent manner *(72)*.

### Proteins Involved in the Regulation of Apoptosis

Almost all cancer therapies trigger apoptosis, programmed cell death in target cells. Protein synthesis is mostly halted during apoptosis as many initiation factors, e.g., eIF4G, eIF4B, eIF2α and 4EBPs are targeted and cleaved by caspases thus disabling the protein synthesis machinery *(73)*. Several genes that are involved in the regulation of apoptosis

are translated by IRES-dependent mechanism, suggesting that IRES-directed translation of cellular mRNAs represents a crucial regulatory point for the survival and proliferation of cells under transient apoptotic stress *(49)*.

X-linked inhibitor of apoptosis protein (XIAP) is the most potent intrinsic inhibitor of caspases *(74)*. Like other inhibitor of apoptosis proteins (IAPs), XIAP inhibits both the initiator (caspase-9) and effector (caspase-3, caspase-7) caspases. XIAP mRNA contains an extremely long 5' UTR containing an IRES element that is translationally active under cellular stress conditions such as serum starvation *(75)*, low dose γ-irradiation-induced apoptosis *(76)* or anoxia *(77)*. IRES-mediated translation of XIAP is upregulated in response to irradiation and enhances the survival of some cancer cell lines, suggesting that IRES translation may be critically involved in cancer progression *(52)*.

BCL2-associated athanogene (BAG1/RAP46) encodes for a multifunctional protein BAG-1 produced as several different isoforms with distinct cellular functions including regulation of cell survival *(78)*, protein refolding *(79)* and signal transduction *(80)*. The translation of the most highly expressed isoform, p36/BAG-1S, is mediated by both cap--dependent and IRES-dependent mechanisms *(81)* of which the latter is preferentially utilized in cells exposed to heat shock *(82)*.

Apoptotic protease-activating factor (Apaf-1) is an essential component of the apoptosome and interacts with the inactive procaspase-9 facilitating its proteolytic cleavage and activation *(83)*. The 5' UTR of APAF1 contains an IRES that is enhanced following the induction of apoptosis by etoposide suggesting that the IRES-directed translation is used for ensuring sufficient levels of Apaf-1 protein to propagate apoptotic signal *(77)*. Caspase-cleaved fragments of initiation factors eIF4GI and p97/DAP5/NAT1 selectively activate IRES elements in apoptotic cells *(77, 84)* which suggests the existence of a regulatory loop that disables cap-dependent translation and at the same time enables IRES-mediated translation of some mRNAs.

Although the number of reports on cellular IRESes is increasing, their existence still raises skepticism and is often subject of scientific debates, mainly due to concerns about the lack of unambiguous experimental verifications *(45, 92)*. The fact that there are a growing number of cancer-related genes whose translation regulation can be subjected to cap-independent initiation makes the quest for discovering and testing new putative cellular IRESes even more meaningful. Deciphering their sequence, structure, molecular mechanisms of action and requirements for additional *trans*-acting factors will tell us more about how cancer cells can maintain their growth and sustain their progression in conditions when general protein synthesis is considerably reduced. Actual presence of an IRES element within 5' UTR of certain cancer-related genes could also reveal more about their role in cancer etiology. A deeper understanding of the mechanisms underlying cap-independent translation regulation in malignant cells could ultimately lead to novel therapeutic strategies, that would not affect protein synthesis of normal cells.

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