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mRNAs translation and tumorigenesis

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INTRODUCTION

Translational control in cancer

Proteins are the most important molecules for life processes as they catalyze most of the reactions on which life depends and they serve numerous structural, transport and regulatory roles in all organisms.
Accordingly, a large proportion of the cell's resources is devoted to translation, the process by which the mRNAs are "transformed" in proteins. Because of its importance, translation is closely monitored and regulated[(1)].
Particularly, translational control has an important role in pathways involving cell proliferation and growth, cellular responses to stress such as hypoxia and nutrient deprivation, and stimulation by mitogenic signals[(1)]. As a consequence translational control is emerging as an important component of cancer etiology. It has been known for more than 100 years that nucleoli, the sites of rRNA synthesis and ribosome assembly, are considerably enlarged and more numerous in highly transformed cells[(2)]. But, in general the connections between increased rates of protein synthesis and cell proliferation was recognized in the 1970s. For example, engineered reduction of protein synthesis by half is sufficient to drive cells into the quiescent G0 resting phase of the cell cycle, in normal but not in transformed cells causing growth arrest[(3)]. Changes in translation associated with cancer development and progression observed involve alterations of the expression of eukaryotic initiation factors (such as eIF4E, eIF2α, eIF4G and eIF3), of translation regulatory factors (such as 4E-BP that blocks eIF4E function and therefore cap-dependent mRNA translation), as well as ribosomes and alterations of the signalling pathways that activate the mRNA translation (primarily the PI3K-AKT-mTOR and Ras pathways)[(4-11)]. These changes are manifested in a variety of ways, including up-regulation of global protein synthesis, increased translation of individual mRNAs, and selective translation of

antiapoptotic, proangiogenic, proproliferative, and hypoxia-mediated mRNAs. Other transformation-associated changes in translation are directed to uncoupling of signal transduction and translational control pathways that suppress translation during physiological stresses and impair cell growth (cell mass) and cellular proliferation (cell division)[1].

In a multistep process as the protein synthesis is, the control can be driven at different levels: initiation, elongation and termination. Even if there are well documented cases of translation control during the late stages such as the elongation phase, the principal control regards the initiation step. This observation is in line with the biological principle that is more efficient to govern a pathway at its outset than to interrupt it in midstream and have the deal with accumulation of intermediates and recycling. So that initiation is the step that limits the speed of the translational process. That's why its impairment is deeply involved in cancer. Therefore, I will focus on it during my discussion.

Cap-dependent mRNA translation initiation and cancer

In the eukaryotes, translation initiation is the step that leads the assembly of an elongation-competent 80S ribosome from small (40S) and large (60S) ribosomal subunits. The "standard" translation initiation requires separated 40S and 60S, involves at least 12 eukaryotic initiation factors (eIFs) and the binding and the hydrolysis of GTP. Indeed, most important is the recognition of the m^7G[5']ppp[5']N cap (m7G-cap, where N is any nucleotide) at the 5'-terminal of the mRNAs. For that reason the canonical translation initiation is called cap-dependent[1].

Cap-dependent translation initiation: an overview

The cap-initiation process consists of a series of steps that can be summarized as follow[1]:

- Selection of an aminoacylated initiator methionyl-transfer RNA (Met-tRNA$_i$) from a pool of tRNAs by the binding with the eukaryotic initiation factor 2 (eIF2) to form a ternary complex eIF2/GTP/Met-tRNA$_i$. The ternary complex together with other initiation factors (eIF3, eIF1 and eIF1A) binds the 40S to form a new complex called 43S ribosome pre-initiation complex;
- Recruitment of the 43S ribosome complex on the m^{7}G[5']ppp[5']N cap at the 5'-terminal of the mRNAs. The association of the 43S ribosome complex with the cap is mediated by the cap-binding complex eIF4F. eIF4F is composed of three IFs that are eIF4G, eIF4E and eIF4A. Also associated with eIF4F and involved in the initiation are several initiation factors such as eIF3 and other mRNA binding protein like the polyA-tail binding protein (PABP);
- Ribosomal scanning on the mRNA starting from the 5' untranslated region (UTR) directed to the first initiation AUG codon (5'-3' direction) to form the 48S complex;
- Recognition of the initiation codon due to its complementarity with the anticodon of the Met-tRNA$_i$;
- Release of the initiation factors from the 48S surface to permit the binding of the 60S and the formation of the elongation-competent complex 80S. This step involves the presence of some many others initiation factors like eIF5 and eIF5B.

Cap-dependent translation initiation is summarized in Figure 1.

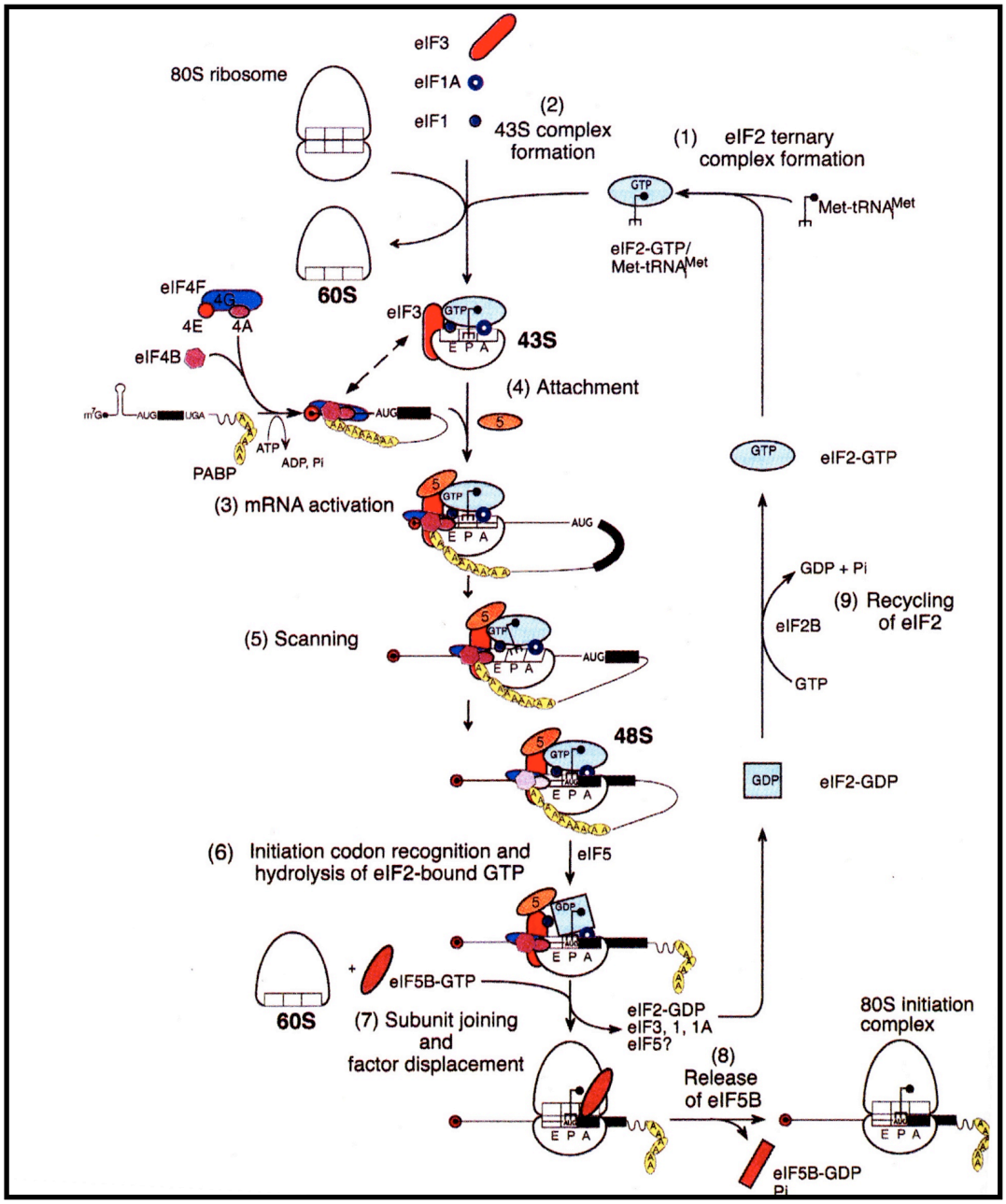


Figure 1. Schematic representation of the cap-dependent translation initation[1]. The canonical pathway of eukaryotic translation initiation can be divived into eight steps. These steps result in the formation of an elongation competent 80S ribosomal complex.

Most important, each step of the cap-dependent translation initiation described is well regulated in normal eukaryotic cells both by regulation of the activity or the level of expression of the initiation factors and by the regulation of the major pathways involved in the activation of protein synthesis. An alteration of such regulatory mechanisms can be implicated in transformation and tumour progression. An overview of the steps of the cap-

translation initiation that can be involved in cancer is presented in Figure 2 and each step will be described in the next paragraph.

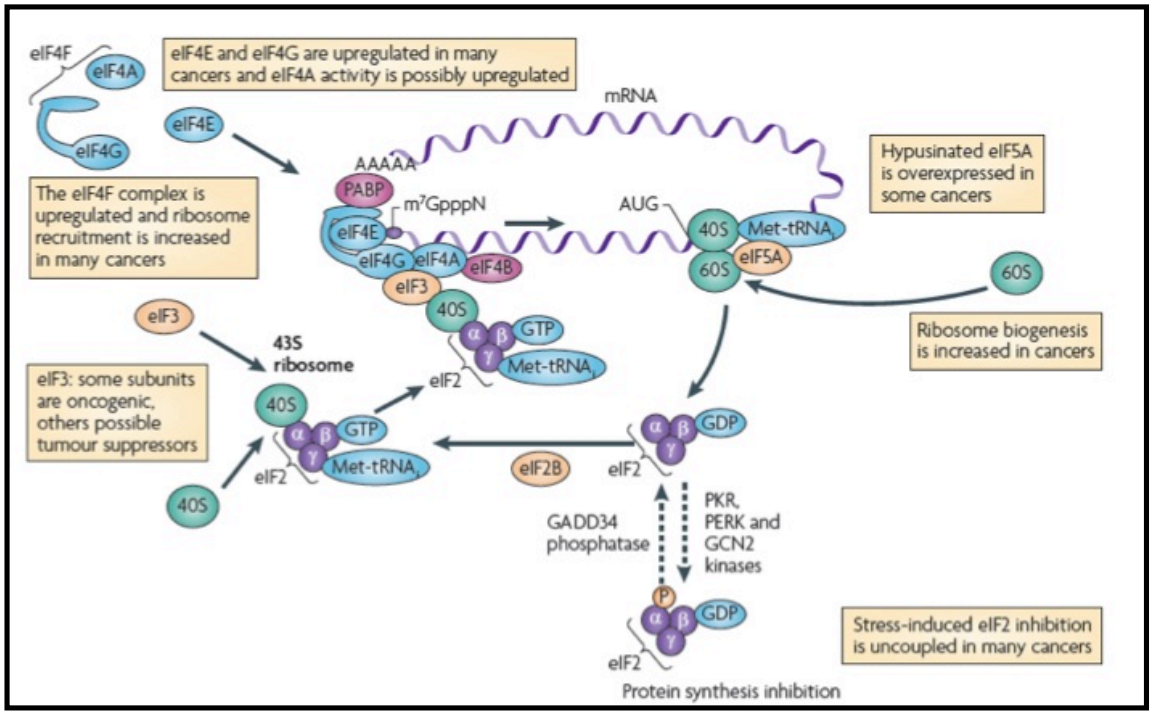


Figure 2. Eukaryotic cap-dependent mRNAs translation steps and cancer[52]. The major regulated steps for the cap-translation initiation are shown. eIF4F formation is rate-limiting for initiation, and increased abundance of its components occurs in many cancers. The different expression of one or more subunits of eIF3 complex can interfere with the reclruitment of the 43S ribosome complex in cancer. The regulation of protein synthesis by eIF2α phosphorylation has a complex role in cancer too.

Alteration of cap-dependent translation initiation in cancer

As described before, the first step of translation initiation is the formation of the 43S ribosome initiation complex. Particularly the activity of this complex is regulated by the phosphorylation state of eIF2[12]. eIF2 is an eterotrimeric complex composed of α, β and γ subunits capable of binding GTP. Only when eIF2 is bound to the GTP (a process mediated by eIF2B), the ternary complex eIF2/GTP/Met-tRNA$_i$ can recruit the 40S to form the 43S ribosome initiation complex. When the eIF2α subunit is phosphorylated, eIF2 cannot recycle GDP in GTP and the protein synthesis is inhibited (Figure 3).

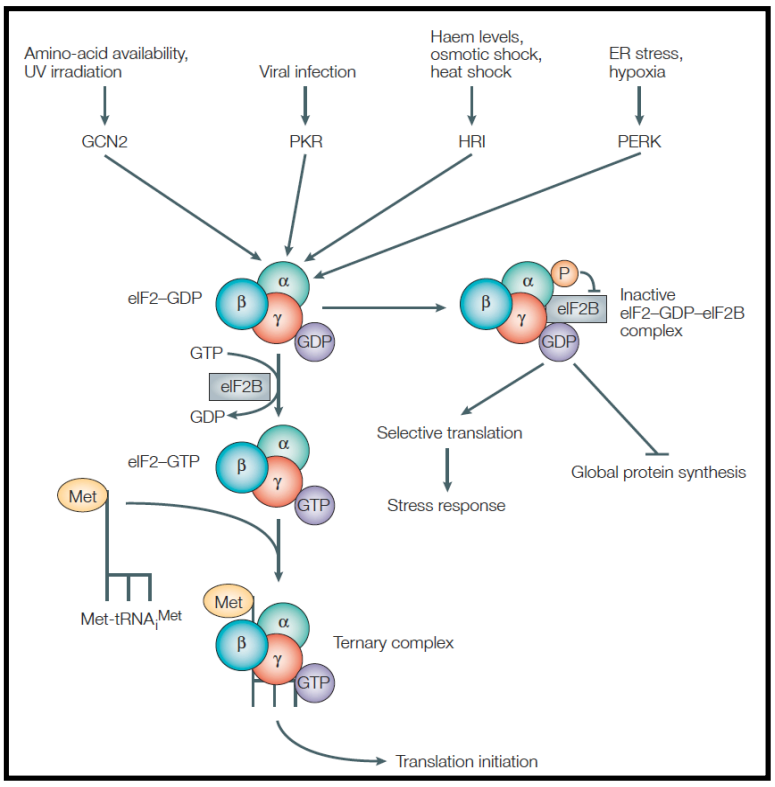


Figure 3. Eukaryotic initiation factor 2 and the phosphorilation of its α subunit[13]. eIF2α is a subunit of eIF2 (together with eIF2β and eIF2γ) that is part of the ternary complex. Phosphorylation of eIF2α inhibits the GDP–GTP exchange by reducing the dissociation rate of eIF2B. Ultimately, this results in the inhibition of global translation.

The phosphorylation of the α subunit is mediated by a related group of protein kinases in response to various physiological stresses (e.g. hypoxia) that block protein synthesis. These kinases are: heme-regulated inhibitor kinase (HRI or EIF2AK1) that is active in erythroid cells under condition of heme deprivation, protein kinase RNA (PKR or EIF2AK2) that is active in response to viral infection, PKR-like endoplasmatic reticulum (PERK or EIF2AK3) that is active in response to the presence of unfolding proteins in the endoplasmatic reticulum and general control non-derepressable-2 (GCN2 or EIF2AK4) that is active in response to nutrient deprivation. Instead the de-phosphorylation is catalized by GADD34 (Figure 2).

It's now well known that a deregulation of eIF2α subunit phosphorylation can be involved both in the stimulation and in the prevention on cancer probably accordingly with this context. In some studies in mice overexpression of a mutant form of eIF2α that cannot be phosphorylated or of a dominant interfering form of PKR, promotes tumorigenesis[14,15]. Accordingly, increased levels of eIF2α subunits (demonstrated by immunohystochemical experiment) are found in colon carcinoma and adenoma, malignant melanoma, bronchioloalveolar carcinomas and more aggressive brain cancers[16,17,18] .
In contrast to these results, elevated level of active PKR (which oppose eIF2 function and was suggested to have tumour suppressor activity) are found in melanoma lymph node metastases and colon carcinomas[19]. In breast cancer low levels of PKR are reported in preneoplastic breast lesions, whereas higher levels are reported in the more aggressive invasive ductal carcinomas[20,21]. One explanation to this could be that the phosphorylation of eIF2α can occur in the early stages of the disease as a response to severe stress (e.g. hypoxia) to reduce protein synthesis and permit cancer cell surviving [22]. Indeed, even if phosphorylation of eIF2α leads to decrease the global translation rates, translation of some mRNAs like growth factors (such as Vascular Endothelial Growth Factor) or anti-apoptotic factors (like X-chromosome-linked Inhibitor of Apoptosis) continues or is even enhanced since their translation is controlled by alternative kinds of translation called cap-independent. This kind of translation could be mediated by special secondary structures on the 5'UTR of some mRNAs called Internal Ribosome Entry site (IRES). I'll discuss about this kind of translation later in this thesis [23]. It's clear that more research needs to be done to understand the role of eIF2 phosphorylation in human's tumours.

The second step of translation initiation is the recruitment of the 43S ribosome pre-initiation complex on the mRNA that is mediated first by the presence on the mRNA of the eIF4F cap-binding initiation complex and

second by the involvement of another important initiation factor complex eIF3. eIF4F is a multiprotein complex formed from 25-, 46- and 220-kDa subunits, called eIF4E, eIF4A, and eIF4G, respectively. eIF4E is a cap-binding protein, eIF4A is an ATP-dependent helicase (the activity of which is stimulated by the RNA binding protein eIF4B) and eIF4G is a large scaffolding protein[1] (Figure 4).

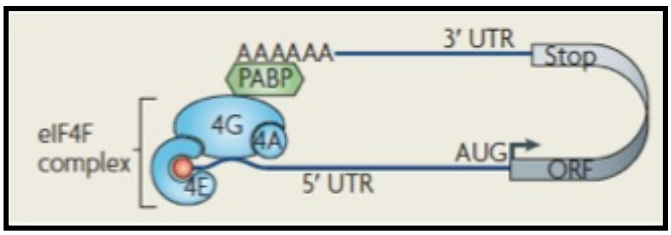


Figure 4. eIF4F complex[24]. The figure shows the components of eIF4F complex and the its binding on the m^7G[5']ppp[5']N cap at the 5'-terminal untransalted region of the mRNAs.

eIF4E is responsible for binding the m7Gcap found on all eukaryotic mRNAs. In 1990 was published the first report implicating eIF4E in oncogenesis[25]. That report clearly indicated that the overexpression of eIF4E promotes transformation of immortalized murine NIH-3T3 cells in vitro and confer them solid-tumor growth properties if subcutaneously transplanted in nude mouse models. The transformation of immortalized cells could be attributed to the direct overexpression of eIF4E as antisense inhibition of it profoundly represses tumor growth and malignancy. Since these reports in the early 1990s, numerous articles have now substantially enhanced our understanding of the role for eIF4E in malignancy. Particularly, overexpression of eIF4E has been demonstrated in colorectal, lung, head and neck, thyroid, bladder, skin and breast cancers as well as in non-Hodgking's lymphomas[26,27]. Disputing the correlation between overexpression and prognosis or survival, eIF4E expression is strongly associated with worse clinical outcome and decreased survival. The role of eIF4E in transformation and cancer progression seem not to be due by the global increase of translation but probably depends on the selectively increased translation of a subset of mRNAs containing structured 5'UTR[28,29]. These mRNAs encode

proteins with notable functions in all aspects of malignancy, including angiogenesis (vascular endothelial growth factor A (VEGFA) and fibroblast growth factor 2 (FGF2)), cell cycle progression (c-MYC) and survival (survivin). Interestingly, most mRNAs that are characterized by short, unstructured 5'UTRs (e.g., h-actin) are largely unaffected by changes in eIF4E expression[28,29]. Recently most importance is given not only on eIF4E abundance but also to its availability and/or activity. We know, in fact, that eIF4E availability and/or activity are regulated by its phosphorylation at Ser 209 and by the eIF4E inhibitory binding proteins (4E-BPs). Both of them seem to be important in the oncogenesis.

eIF4E phosphorylation is mediated by its binding to eIF4G which brings it into proximity with the MNK1 and MNK2 kinases. The phosphorylation of eIF4E stimulates translation initiation in vitro. Mitogen-enhanced eIF4E phosphorylation usually correlates with increased protein synthesis[30] and phosphorylation increases the binding of eIF4E to capped mRNA and to eIF4G[31]. The location of Ser 209 adjacent to the cap-binding pocket is consistent with an effect of phosphorylation on mRNA binding (Figure 5).

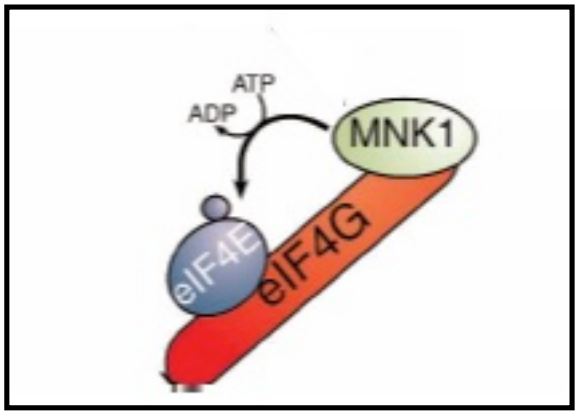


Figure 5. eIF4E phosphorylation[32]. Both MNK1 and eIF4E interact with eIF4G, bringing the two proteins in close proximity, resulting in more efficient eIF4E phosphorylation.

Overexpression of phospo-eIF4E is found in 63% of human cancer of different origin (lung, colorectal, stomach, liver, kidney, breast, ovary, head and neck and brain) compared with 30% of adiacent non-cancer tissues[33]. Indeed p-eIF4E expression seems to be significantly higher in the early

stages of disease than in the advanced stages in certain types of cancers (e.g., colorectal and gastric cancers). Thus, it seems that p-eIF4E may play an important role in the earlier stages of malignant transformation than in the late stage of these types of cancers[33].

As mentioned eIF4E availability is due to the presence of 4E-Binding proteins, the most important of which is 4E-BP1. When 4E-BP1 is hypophosporilated is able to bind the initiation factor eIF4E blocking his link with the other components of the initiation complex. Instead when, thanks to the activation of the mTOR pathway, 4E-BP1 is hyperphosphorilated eIF4E is reliase and available to form the complex[32] (Figure 6). Less is known about 4E-BP1 status on cancer. For example it's known that in advanced prostate cancer[34] and higher-grade breast cancer[35] there are high levels of phospho-4E-BP1 which in turns correlates with high amount of available eIF4E. On the other hand in some advanced but clinically non-metastatic cancers, like the oesophageal one or the locally advanced breast cancer (LABC) there is an overexpression of active 4E-BP1 and a consequent sequestering of eIF4E[36,37]. In this case it's probably that alternative mechanisms of initiation can be favoured.

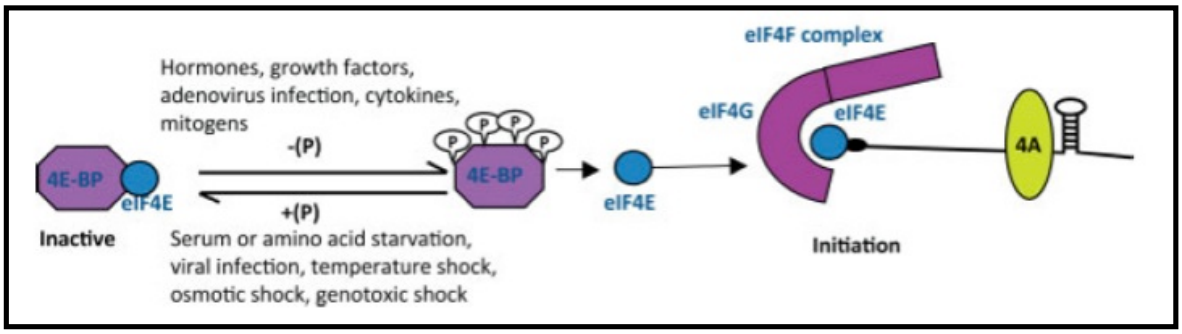


Figure 6. The binding of the 4E-BPs to eIF4E is regulated by phosphorylation[93]. Various stimuli increase the phosphorylation of the 4E-BPs. Hyperphosphorylated 4E-BPs have a relatively low affinity for eIF4E. Conversely, a decrease in 4E-BP phosphorylation increases the affinity of the 4E-BPs for eIF4E.

Little attention is paid, right now, to the other eIF4F components.

eIF4G, the scaffolding protein of eIF4F, when overexpressed in NIH-3T3 cells drives theirs transformation in the absence of eIF4E overexpression[38]. In inflammatory breast cancer (IBC) is shown an overexpression of eIF4G

itself without changes in eIF4E or 4E-BP1[39]. That's why much more than in cap-dependent translation seems to have an important role in the so-called cap-independent translation. This is not the first time that I've mentioned the alternative way for translation to initiate and its possible implications in cancer and that's why I will focus on them later.

Once eIF4F is bound to the mRNA another complex is essential to permit the interaction with the 43S pre-initiation complex. This complex is eIF3, a complex of 10-13 proteins. The roles of eIF3 in cancer can be clarified by examining the individual contribution of each subunit[40]. The major subunits involved in cancer are eIF3a, eIF3b, eIF3c, eIF3h, eIF3f and eIF3e[41]. Particularly, overexpression of eIF3a is associated with breast, cervical, lung, oesophageal and stomach cancer[42-46]. Overexpression of eIF3c is associated with testicular seminomas and meningiomas[47,48]. Overexpression of eIF3h is associated with high grade prostate cancer[49]. In all these cases the overexpression seems to have a double role: promote protein synthesis by iperactivating the translation initiation and inhibit the translation of subset of mRNA involved in tumour suppressor such as p27 (for eIF3a) or merlin (for eIF3c). On the other hand loss of expression of eIF3f is associated with pancreas, breast, ovary, small intestine tumours and melanomas[50] and loss of expression of eIF3e is associated with breast and lung carcinomas[51]. In the first case is known that eIF3f has tumour suppressor properties, instead remain unclear the translational mechanisms by which eIF3e can drive tumour progression.
A list of the initiations factors with a description of the cancers in which their different expression/regulation is involved is summarize in Table1.

Factor	Function	Observed modification	Cancer association
PKR	eIF2α kinase	Decreased expression	Progression from benign to malignabt HNSCC: indolent papillary thyroid carcinomas
		Increased expression	Colon adenocarcinomas and carcinomas aggressive IDC and hight grade viral HCC
eIF2α	Forms an eIF2–GTP–Met-tRNAi ternary complex that binds to the 40S subunit, thus mediating ribosomal recruitment of Met-tRNA	Increased expression	Benign and malignant melanomas and colon cancers: associated with more aggressive brain cancers
eIF3a	Binds 40S subunits, eIF1, eIF4G and eIF5; stimulates binding of eIF2–GTP–Met-tRNAi to 40S subunits; promotes attachment of 43S complexes to mRNA and subsequent scanning; and possesses ribosome dissociation and anti-association activities, preventing joining of 40S and 60S subunits	Increased expression	Breast,cervical, lung, oesophageal and stomach cancers
eIF3c		Increased expression	Testicular seminomas; meningiomas
eIF3h		Increased expression	High-grade prostate cancers and gene amplification in NSCLC24
eIF3f		Decreased expression	Pancreas, breast, ovary and small intestine tumours; gene loss in melanomas
eIF3e		Decreased expression	Breast and lung carcinomas
eIF4E	Binds to the m7GpppG 5′ terminal 'cap' structure of mRNA	Increased expression	Correlates with worse clinical outcome and decreased survival in breast, head and neck, colorectal, lung, prostate, bladder, skin and cervical cancers, and lymphomas; correlates with increased malignancy in meningiomas, glioblastomas and astrocytomas; associated with decreased survival in advanced prostate cancers; LAEC
Phospho-eIF4E		Increased phosphorylation	In prostate cancer compared with normal tissue; correlates with anti-apoptotic gene expression in DLBCL and Burkitt's lymphoma;associated with serous histological type and better survival in ovarian tumours; observed in most human cancers; associated with lower stage colorectal, lung and gastric cancers; no correlation with malignancy in meningiomas, glioblastomas and astrocytomas despite eIF4E correlation
eIF4G	Binds eIF4E, eIF4A, eIF3, PABP, SLIP1 and mRNA and enhances the helicase activity of eIF4A	Increased expression	Associated with decreased metastatic progression in LABC; associated with IBC and formation of metastatic cancer cell emboli; associated with increased CCND1 translation in squamous lung carcinoma
4E-BPs	Regulates eIF4E availability	Decreased expression	Reduced survival in advanced prostate cancers
		Increased expression	Associated with reduced tumour grade in breast cancers reduced metastatic progression in LABC, and with LAEC
Phospho-4E-BP1		Increased phosphorilation	Decreased survival in advanced prostate cancers; higher grade and reduced survival in breast cancers; poor differentiation and higher mitotic rates in ovarian tumours; LAEC

Table 1. Translation factors and translation regulatory factor alteration in human cancer. eIFs functions and their alterations in cancer are shown.CCND1= cyclin D1; DLBCL= diffuse large B cell lymphoma; HCC=hepatocellular carcinoma; HNSCC=head and neck squamous cell carcinoma; IBC, =inflammatory breast cancer; IDC,=nvasive ductal carcinoma; LABC=locally advanced breast cancer; LAEC= locally advanced oesophageal cancer; NSCLC,=non-small-cell lung cancer.

Cap-independent mRNA translation initiation and cancer

For long time the "cap-dependent" or "scanning" mode of initiation was considered the only way through which translation of eukaryotic mRNAs could be initiated. During the years, it started to be clear that a number of cellular physiological and pathological stress responses (such as hypoxia or nutrient deprivation) involve inhibition of one or more general translation initiation factors, yet the adaptive responses to stress require new protein synthesis. Logically, cells need mechanisms that could allow mRNAs encoding key regulatory proteins to escape the general inhibition of translation. The observation that some cellular mRNAs continue to be translated in poliovirus-infected cells after the inhibition of cap-dependent initiation (through cleavage of eIF4G by a virally encoded protease) is consistent with this hypothesis[53]. Indeed mRNAs with very long 5'UTRs or containing numerous predicted stem-loop structures or upstream AUG codons within their 5'UTRs could be translated with reasonable efficiency, despite evidence that such features can significantly reduce translation of model mRNAs[54-56]. All these observations led the researchers to investigate the presence of alternative mechanisms of translation initiation that, in the complex, are called as cap-independent translation. Most of the knowledge on this matter came from the study made on virus translation initiation.

Ribosome shunting is an alternate mechanism of translation initiation in which ribosomes bind to the mRNA in a normal cap-dependent mode, then jump upstream (5'→3') of the initiator AUG codon. It appears less dependent on eIF4F than is scanning-mediated initiation, so it may also support translation of specific mRNAs under conditions in which eIF4F activity is reduced, such as viral infections and heat stress[57] (Figure 7).

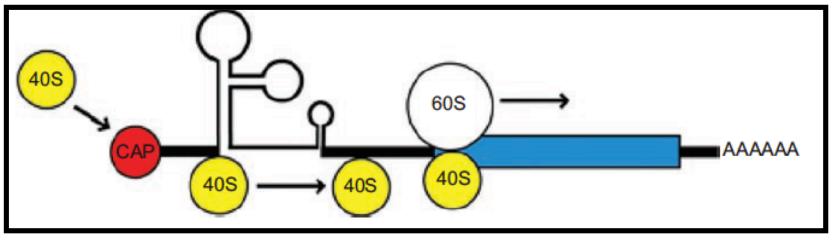


Figura 7. Ribosome shunting[58]**.** The ribosome shunts over a large segment with extensive secondary structure, possibly containing start codons.

For some mRNAs, shunting has been shown to require various mRNA elements, some of which are thought to base pair to 18S rRNA. The data obtained provide direct evidence that ribosomal shunting can be mediated by mRNA-rRNA base pairing, a finding that may have general implications for mechanisms of ribosome movement[59].

Studies of viral gene expression in the late 1980s led to the discovery of an alternative mode of translation initiation in eukaryotic cells that allows the 40S ribosome to be directly recruited to the vicinity of the initiation codon bypassing the binding of the cap[60]. The mRNA regions required for this direct recruitment of the 40S ribosomal subunit are termed Internal Ribosome Entry Sites (IRESs) to emphasize that the process is independent of the 5'-end recognition. It has been shown that specific eukaryotic mRNAs can have IRESs too[61] and that viral and cellular IRES-driven translation initiation is typically utilized when cap-dependent initiation is compromised (Figure 8).

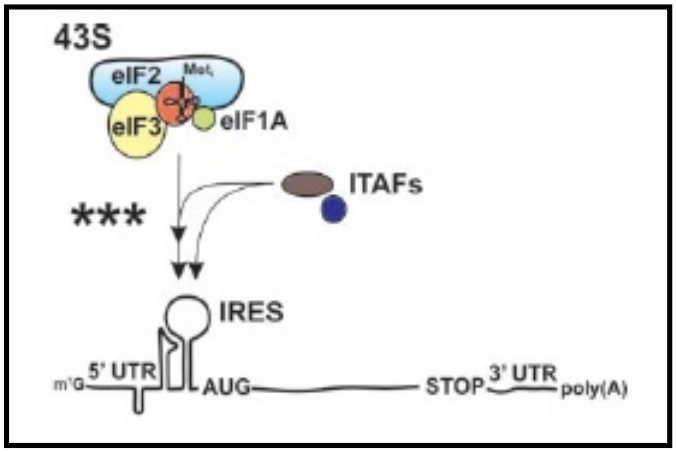


Figura 8. IRES-mediated translation initiation[61]. This translation initiation mechanism is generally independent of the recognition of the 5'cap-mRNA end and involves direct recruitment of the 40 S ribosome to the vicinity of the initiation codon (directed by an IRES element). The 40 S recruitment is assumed to be accompanied by the simultaneous recognition of the initiation codon.

As might be thus expected, IRES-driven translation has a generally reduced requirement for canonical translation initiation factors, particularly members of the eIF4F complex (initiation factors eIF4E and eIF4G). Moreover, in certain "extreme" cases, initiation can proceed without involvement of any of the canonical initiation factors[62]. In addition, a number of proteins have been identified that are capable of modulating (typically enhancing) internal initiation called IRES trans-acting factors (ITAFs)[63]. Because this is the most well studied alternative (cap-independent) mechanism for translation initiation I'll focus on that deeply later.

Finally, in the discussion the alternative way of translation initiation some new updates might be mentioned. It seems to be reasonable that the cap-independent translation does not need to be necessary directed by an IRES but it can be 5′ end- and scanning-dependent[64]. Some studies, using transfection of cells with capped and uncapped reporter mRNAs, found that the contribution of the cap is not similar for various 5′ UTRs of cellular mRNAs, though none of the tested cellular 5′ UTRs had an IRES[65]. Some of them were stimulated by the 5′ cap much stronger than the other. Notably, there was no correlation between the magnitude of the stimulation effect and the length or the overall stability of the 5′ UTR secondary structure. This means that even in the absence of the eIF4E-cap interaction, the translational machinery is able to recognize the 5′ end, bind there and then scan to the 3′ end in a search for the initiation codon[65-68]. All these works clearly demonstrated that although the stimulation effect was cap-independent, it still required 5′ end-dependent ribosome binding. Therefore, there must be some elements within eukaryotic mRNAs that can promote a cap-independent translation initiation but are not truly IRESs. Some indications about the mechanisms come again from virus studies.

The mRNAs of a large portion of all plant viruses lack the m7GpppN-cap

structure and they employ a strategy alternative to the internal ribosome entry. They use special elements termed Cap Independent Translation Enhancers (CITE)[69-71]. The majority of CITEs is located in 3′ UTRs (so called 3′ CITEs). CITE is thought to recruit components of translational apparatus (such as eIF4E or eIF4G) and then to deliver it to the 5′ end of mRNA through long distance base pairing between 5′ and 3′ UTRs (Figure 9).

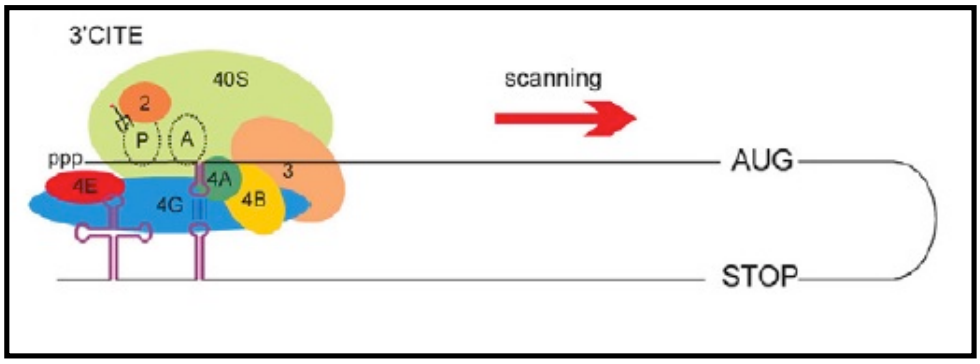


Figure 9. 3′ CITE-assisted initiation[64]**.** 3′ CITEs are shown in violet color. In this particular case, eIF4E and eIF4G bind by means of CITEs to the 3′ UTR of an mRNA. A long distance interaction of stem-loop structures results in circularization of the mRNA and positioning of the scanning machinery near its 5′ end.

It should be noted that the initiation on the 5′ UTR of these mRNAs directed by the 3′ CITE is cap-independent, but 5′ end dependent and requires scanning to locate the authentic initiator codon. Moreover, when placed in 5' UTRs, CITEs can direct cap-independent translation too[72]. Finally is demonstrated that the CITEs don't work as IRES when placed between two cistrons[70]. Thus, the CITE: a) can recruit eIF4F, b) can stimulate a cap-independent, but 5′ end-dependent translation when placed at the 5′ end of mRNA, c) cannot direct an internal initiation. This allows hypothesizing that some kind of CITE-like elements may exist within 5′ UTRs of mammalian mRNAs (5′ CITEs) as illustrated in Figure 10.

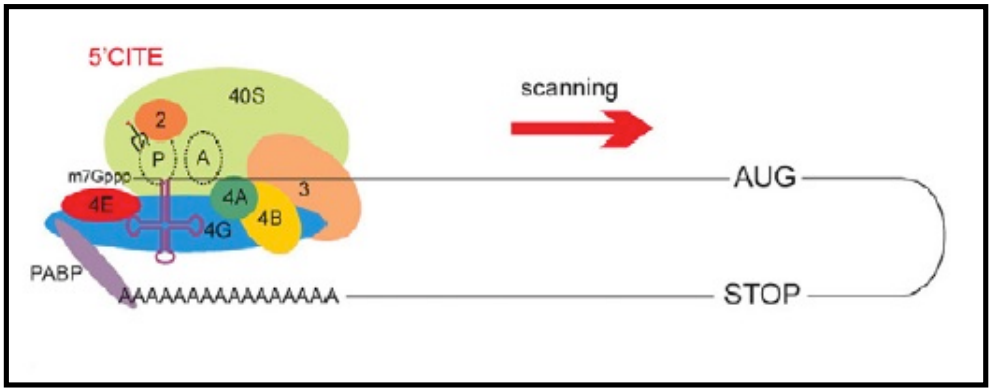


Figure 10. 5′ CITE assisted initiation initiation[64]**.** In this case, a CITE is located within the 5′ UTR and is capable of additional, presumably rather weak interactions with initiation factors of the scanning machinery. Although the recognition of the cap by eIF4E still plays a major role in the mRNA recruitment, the primary binding of the mRNA is still possible in the absence of this interaction, solely due to some interactions of key initiation factors (or the 40S ribosomal subunit itself) with 5′ CITEs.

They allow these mRNAs to be preferentially translated under conditions when translation of "canonical" cap-dependent mRNAs are strongly inhibited. It could be also possible that such translational enhancer elements can recruit dedicated ribosome binding proteins (RBPs)[73]. Although it is not yet clear how many RBPs might similarly bridge interactions between cellular 5-UTRs and the translation machinery, even the relatively small yeast genome is predicted to encode 300 RBPs (exclusive of ribosomal proteins), each with a specific set of RNA targets[73].

IRES-mediated translation initiation

Under relevant conditions which downregulate cap-dependent mRNA translation (for example, amino acid starvation, mitosis, hypoxia and apoptosis), the expression of key genes that are involved in cell proliferation, differentiation and apoptosis is needed. As mentioned above, translation of these mRNAs occurs by "alternative" translation initiation mechanisms the most common and studied of which is known as IRES mediated-

translation[74]. Little is known about the exact mechanisms by which initiation by the IRES occurs. Translation of viral and cellular mRNAs IRESs has received increased attention during recent years due to its emerging significance for many physiological and pathological stress conditions. The existence of an IRES within the 5'UTR of a viral or cellular mRNA is verified by the use of the bicistronic assay[1]. In the bicistonic assay, the bicistronic mRNA is engineered to contain two cistrons (or open reading frames ORFs) with the putative IRES element inserted between them. The first cistron is translated by the cap-dependent scanning mechanism, whereas translation of the second cistron does not happen unless internal initiation at the IRES element occurs. The commonly used vector for the bicistronic assay is the pRF vector which contain the *Renilla* and the *Firefly* luciferases coding regions as the first and the second cistrons as shown in Figure 11. Of course other reporter such as the chloramphenicol acetyltransferase (CAT) can be used.

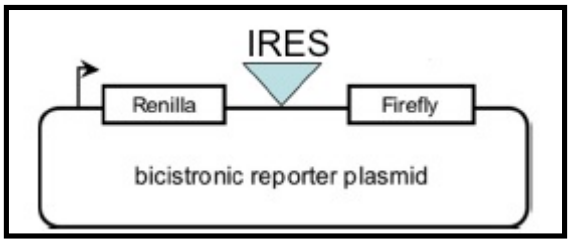


Figure 11. Typical bicistronic reporter plasmid used for IRES assays[73]. A strong promoter such as SV40 drives expression of a bicistronic mRNA. Renilla luciferase activity reports the level of cap-dependent initiation in the experiment. Firefly luciferase activity is very low unless the intercistronic region contains an IRES.

Viral-IRESs

Viruses use the components of the host cell for gene expression and replication. Soon after infection, the host cell often tends to limit viral production and replication by shutting-off global translation. This regulatory mechanism generally targets events from the initiation phase because it is the rate-limiting step that determines overall protein production for most mRNAs. Many viral genomes have evolved to bypass this general inhibition

of translation by developing mechanisms of initiation independent of the classical recognition of an m7G cap structure at the 5′ end of the mRNA. These mechanisms imply the utilization IRESs which can promote 5′ end independent initiation[60]. The existence of internal ribosome entry in viruses was proven in two laboratories simultaneously, by Sonenberg (MacGill, Canada) and Wimmer (Stony Brook, USA)[64]. In spite of a large variety of structures of viral IRESs, there is one property of well-studied viral IRES elements which is common to all of them, regardless of their organization and mechanism of functioning. As clear from the data described above, they all have at least one specific site within their structure with a high affinity to a key translational component. These highly specific and strong binding sites for key ribosome recruiting components or ribosome itself are real hallmarks of viral IRESs. Their existence helps to understand how and why 40S ribosomes are directed to a defined internal region of the 5′ UTR of an mRNA rather than to the 5′ end or any other sequence within this mRNA. The existence of specific and strong binding sites for initiation factors or ribosomes within 5′ UTRs is a mandatory but not the only specific property of true viral IRES-elements. Another property is a highly specific secondary and tertiary structure of these IRES-elements that are probably needed for the sequences in vicinity of the initiation codon to be accommodated in the ribosome mRNA-binding channel. The mechanism of this accommodation is still poorly understood. Probably, it involves a change of conformation of the small ribosomal subunit[64]. Viruses IRESs are divided into four major structural groups, epitomized by poliovirus (PV; Type 1), encephalomyocarditis virus (EMCV; Type 2), hepatitis C virus (HCV; Type 3) and cricket paralysis virus (CrPV; Type 4)[75]. The Type 1 and 2 IRESs belong to the picornaviridae mRNAs, the Type 3 IRESs belong to the Flaviviridae mRNAs and the Type 4 IRESs belong to the Dicistroviridae mRNAs.

All picornaviruses share a long and structured 5′ UTR (600 to 1200 nt

depending of the virus) containing multiple upstream AUG triplets that are never used to start translation. As mentioned before, based on biochemical, structural and phylogenetic studies the picornaviral IRESes are divided into two major groups: type I IRESes are found in the mRNAs of enteroviruses (i.e. PV) and rhinoviruses and type II are found in the mRNAs of aphthoviruses (i.e. FMDV), and cardioviruses (i.e. EMCV)[60].

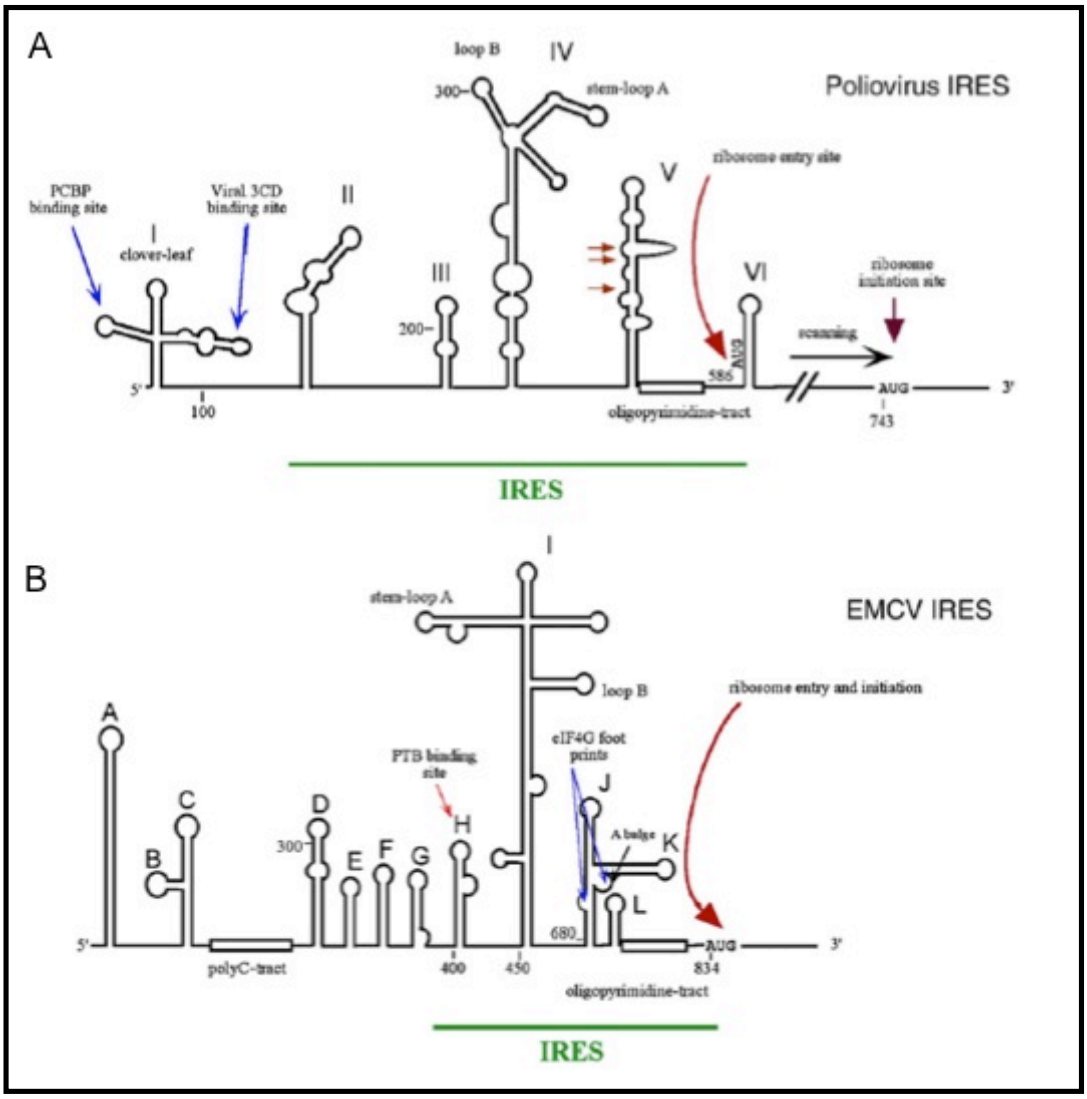


Figure 12. Schematic representation of the two picornaviral IRESs[60]. (A) Structures of the 5′UTR of PV (type I IRES). The body of the IRES within the 5′UTR is underlined in green. The binding sites for the viral proteins PCBP and 3CD on the clover leaf structure are indicated. The ribosome enters the RNA at nucleotide 586 just after the oligopyrimidine tract and then scan down until it reaches the authentic initiation site located at position 743. (B) Structures of the 5′UTR of EMCV (type II IRES). The core of the IRES within the 5′UTR is underlined in green. The binding site for PTB and eI4G are indicated. The ribosome lands and initiates at the AUG located at the end of the polypyrimidine tract.

All picornaviral IRESes share some characteristics in common which is the presence of a GNRA motif in the central domain which adopts a tetraloop conformation and a small polypyrimidine rich tract that lies about 20 to 25 nucleotides upstream of an AUG triplet (Figure 12). In type I IRESes this AUG codon is not utilized to start translation and the ribosome has to scan some 100 to 150 nucleotide downstream to initiate protein synthesis at the next AUG (Figure 12). Studies on EMCV type II IRESes have shown that the distance between the oligopyrimidine tract and the initiator AUG is critical and that the 40S ribosome seems to be loaded directly, or, at the immediate vicinity of this AUG without any scanning process[(60)]. Experiments using a reconstituted translation assay in which all the components required for translation initiation (initiation factors, amino acids, ribosomes, mRNA and tRNAs) are added sequentially to form a preinitiation complex, demonstrate that virtually all of the canonical translation factors were needed to recruit the 40S subunit, on the EMCV IRES, with the exception of eIF4E and the aminoterminal of eIF4G. The initiation factor eIF4G also appears to be a key player in the recruitment of the 43S complex onto the IRES sequence. It has been shown that eIF4G has the ability to directly contact the IRES sequence of EMCV at the J–K domain and the stem–loop V of PV in association with eIF4B (see Figure 12). If some picornaviral IRESes such as EMCV are able to efficiently drive translation in the rabbit reticulocytes lysate (RRL) with the sole set of canonical initiation factors, this is clearly not the case for members of the polio-/rhino-virus genera. Translation of these type I IRESes in the RRL results in a very low yield of protein production. Interestingly, such profile can be corrected by supplementation of the reticulocyte lysate with HeLa cell extracts[(60)] suggesting that some cellular factors that are present in HeLa but absent from the RRL are needed for ribosomal entry. These factors were called IRES trans-acting factors (ITAFs). A schematic representation of the Type I and Type II IRES-mediated translation is shown in Figure 13.

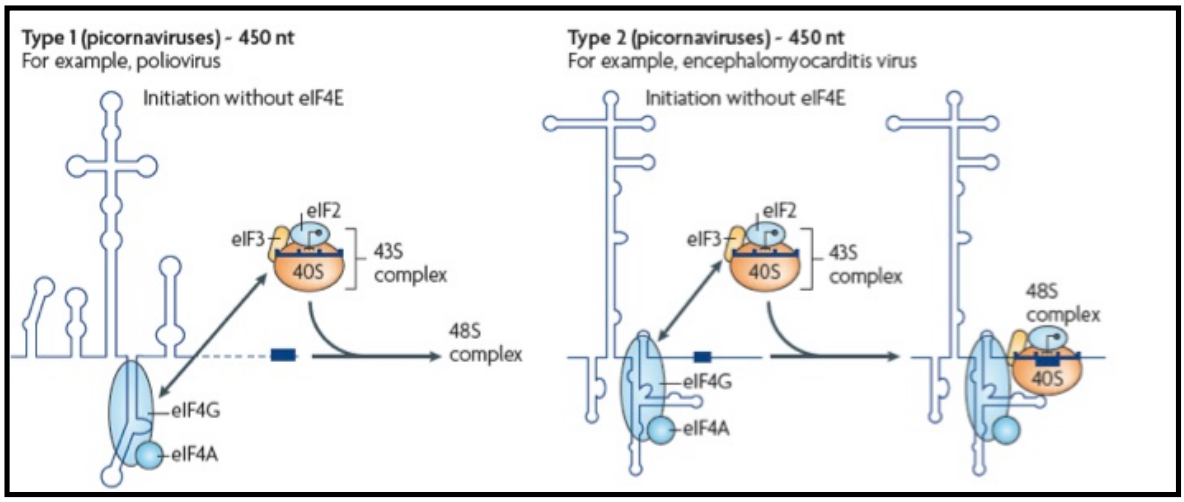


Figure 13. Type I and II IRES-mediated translation[76]**.** Initiation on type 1 and type 2 IRESs involves their specific binding to eIF4G, which is enhanced by eIF4A. The eIF4G–eIF4A complex recruits 43S complexes to type 1 and type 2 IRESs without the involvement of eIF4E. Initiation on type I IRESs also requires IRES trans-acting factors (ITAFs)-RNA-binding proteins that are thought to stabilize the optimal three-dimensional IRES conformation.

The Hepatitis C Virus (HCV), the most representative member of the Flaviviridae, was shown to contain an IRES in its 5′ UTR. HCV IRES is composed of a 5′ UTR of 341 nt long (up to the AUG codon) and folds into four major structural domains numbered I to IV[60]. An interesting feature of the HCV IRES is the fact that the sequences required for IRES activity are located between domains II and III and IV and extend beyond to the AUG codon on the first 30 nucleotides of the ORF. Domain I appears to be dispensable for translation activity. Secondary structures of the HCV IRES are summarized in Figure 14. Domain II is a 75 nt long hairpin structure with 3 internal and a terminal loop that enhances significantly IRES activity although it is not absolutely necessary. Domain III is the largest RNA structure and comprises several helices and hairpins named IIIa to IIIf. Domains IIIa– IIIc and IIIe–IIIf form a three-way helical junction with the IIIf stem– loop being part of the pseudoknot that is essential for ribosomal recruitment (Figure 14).

The mechanism of ribosomal recruitment onto the HCV IRES is quite different from that described for the picornaviruses.

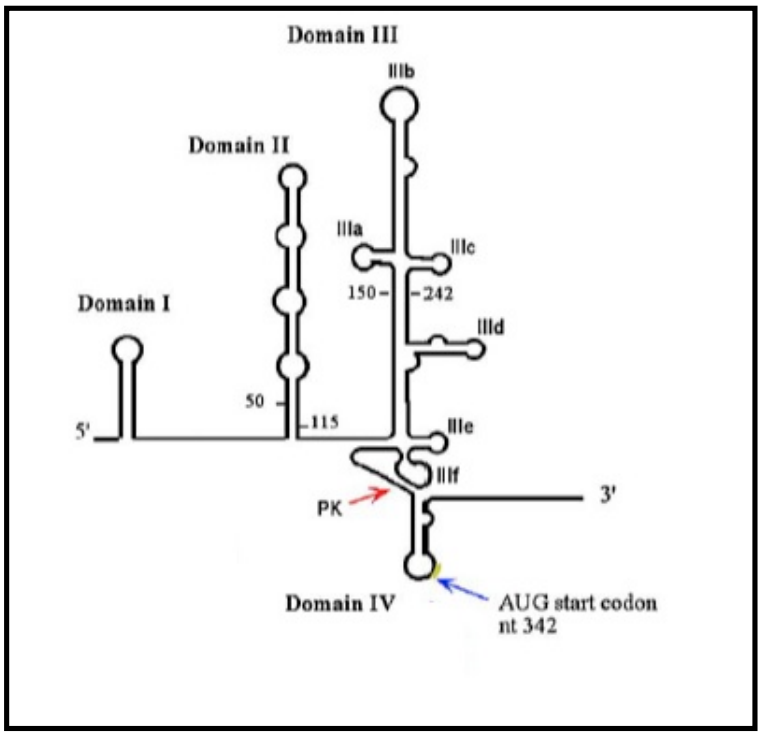


Figure 14. Schematic representation of the secondary structure of the 5'UTR of HCV[60]. The IRES is composed of domains II–III–IV whereas domain I is dispensable for IRES activity. The AUG initiation codon is part of the loop of domain IV extending.

The assembly of the 48S initiation complex is independent of eIF4A, eIF4B, and eIF4F. In fact, a crude 40S ribosomal subunit can directly bind to stem–loops IIId or IIIe of the HCV IRES, and intermolecular contact zones have been reported on most of the IRES sequence[60]. These contacts are due to interactions between RNA motifs from the IRES and ribosomal proteins that constitute the 40S subunit. As a result of this interaction, the positioning of the HCV IRES on the surface of the 40S ribosomal subunit induces a conformational change that clamps the mRNA and positions the AUG codon in the P site of the ribosome with no need for ribosomal scanning. Furthermore the IRES can also directly bind to the initiation factor eIF3 via loops IIIa and IIIb and the junction domain IIIabc. This interaction is essential for the recruitment of the eIF2–tRNAi Met ternary complex and the initiation of translation (Figure 15). Thus, direct interaction between the

IRES and the 43S ribosome results in the correct positioning of the initiator AUG in its P site, in a "prokaryotic mode"; after recruitment of the ternary complex stabilized by eIF3, the hydrolysis of eIF2-bound GTP (catalyzed by eIF5) allows the joining of the 60S subunits. Interestingly, it has been shown that the HCV IRES could support the reduced availability of eIF2–GTP–$tRNA_iMet$ ternary complex that is found in stress conditions, by switching to an eIF2 independent mechanism when this factor is inactivated by phosphorylation[60]. Thus, this low requirement in initiation factors certainly represents a selective advantage for efficient replication of HCV even under unfavourable physiological conditions.

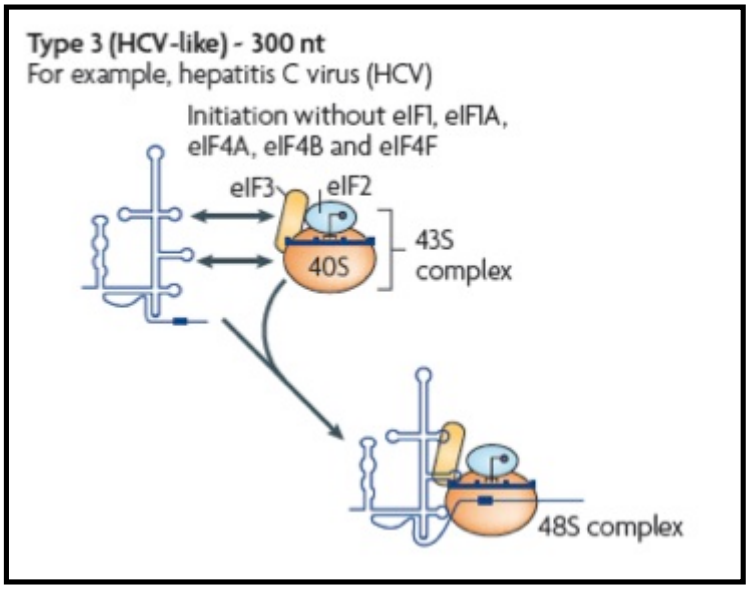


Figure 15. Type III IRES-mediated translation[76]. initiation on type 3 IRESs involves their interaction with the eIF3 and 40S subunit components of 43S complexes. Type 3 IRESs directly attach 43S complexes to the initiation codon independently of eIF4F, eIF4B, eIF1 and eIF1A.

The dicistroviridae family is composed of 11 members amongst whitch the Cricket paralysis virus (CrPV) is used like a model. The genome is organized in two large open reading frames, ORF1 and ORF2, that are translated as long polyproteins which are later processed by a virally encoded protease. These two ORFs are separated by an intergenic region (IGR) of approximately 200 nucleotides in length (Figure 16A) and are expressed by

two distinct IRESes. The first one lies within the 5′ UTR and was shown to be functional in a wide range of translational systems whereas the second IRES sequence is located in the intergenic region and has the unique ability to recruit a ribosome in the absence of initiation factors and without the tRNAiMet (see below). Although the length of the intergenic region varies among dicistroviruses, the overall secondary and tertiary structures of the IGR IRESes appear to be conserved. The structure can be split in three different domains, which all contain a pseudoknot domain (PK); PKII and PKIII overlap to form a stable folded domain whilst PKI is attached to them by a linker and forms a more independent domain 3 which is able to dock into the ribosome decoding groove (see Figure 16B).

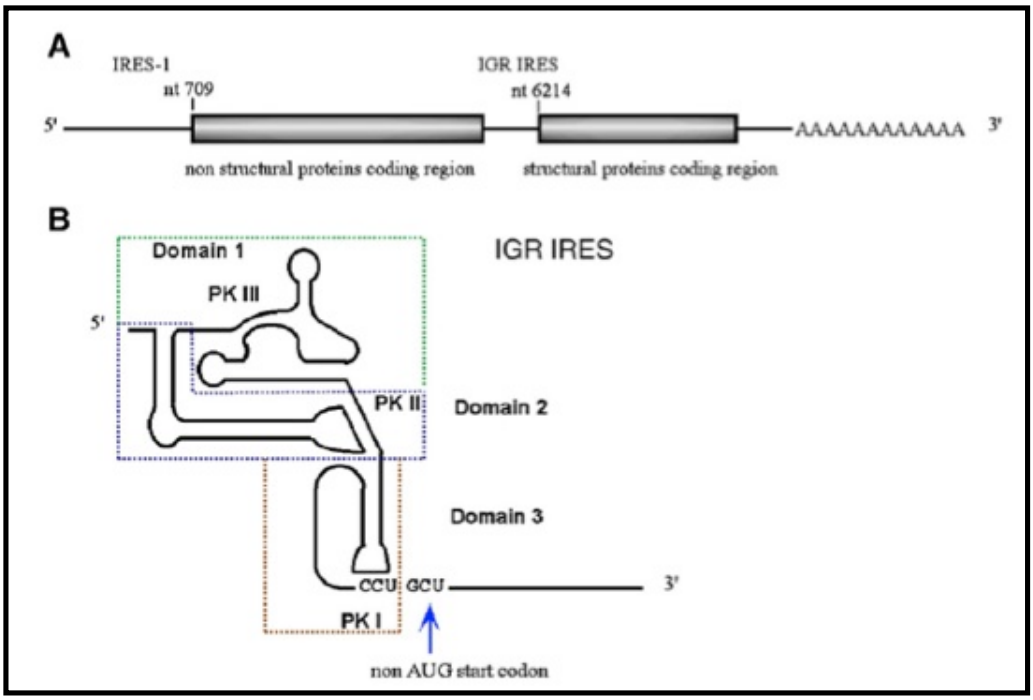


Figure 16[60]. (A) Genome organization of CrPV. The first IRES is located on the 5′UTR part of the molecule. The first ORF ends at nucleotide 6022 when begins the intergenic region and the IGR IRES. (B) CrPV IGR IRES secondary structure. Domains 1, 2 and 3 are limited by coloured dashed lines. The CCU triplet at position 6214–6216 fits in the P site of the ribosome and determines the open reading frame used, the following GCU is the first codon to be translated**.**

The CrPV IGR IRES occupies the region involved in tRNA binding in the P and E sites. This is mainly due to domain 3 which seems to be positioned in the P site and able to adopt a tRNA–mRNA conformation which mimics the

conformation of a tRNAiMet bound to its initiator codon. In the 80S ribosome the IGR IRES gets positioned in the precise hybrid state that can be found during the transition between a P/E hybrid tRNA and a P site tRNA. This P/E hybrid state could explain how the initiation can start with no requirement for a peptide bond formation with the amino acid loaded in the A site. The IGR IRESes use a unique and remarkable mechanism for translation as they are able to directly bind to a 40S subunit and to assemble the 80S ribosome without any requirement for any initiation factor, tRNAiMet or hydrolysis of GTP. The total absence of requirement for initiation factors confers to this IRES the ability to drive viral protein production in extreme cellular physiological conditions that can be found after induction of the unfolded protein response with a high level of eIF2 phosphorylation or following amino acid starvation. Thus, these IRESes are well adapted to the physiological status of the cell that is usually found during the infection by dicistroviruses (Figure 17).

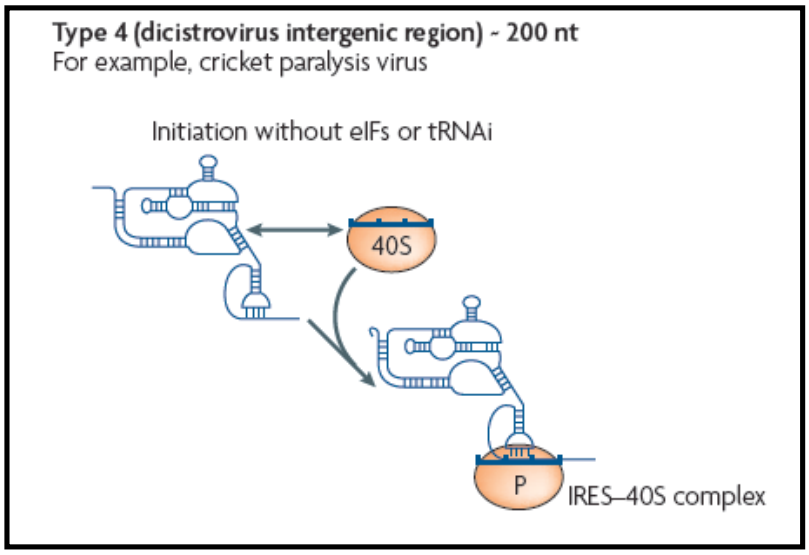


Figure 17. Type IV IRES-mediated translation initiation[75]. Initiation on type 4 IRESs involves their binding to 40S subunits. Type 4 IRESs initiate without eIFs or tRNAiMet. In fact the P-site of the 40S subunit is occupied by an IRES domain that mimics codon–anticodon base pairing.

Cellular-IRESs

While first discovered in the RNA genomes of picornaviruses, IRES elements have also been identified in a subset of cellular mRNAs (Table 2).

Gene	Cellular conditions for translation	Known ITAFs
Apaf-1	Apoptosis	PTB, unr, DAP5
XIAP	Apoptosis	La, hnRNP C1/C2, DAP5
c-myc	Apoptosis, development, genotoxic stress, cell cycle	PCBP2, PCBP1, hnRNP C1/C2, hnRNP K, DAP5, IRP, unr, YB-1, GRSF-1, PSF, P54nrb
DAP5	Apoptosis	DAP5
Reaper	Apoptosis, heat shock	
Hsp70	Apoptosis, heat shock	
Bcl-2	Apoptosis	
HIAP2/c-IAP1	Apoptosis, ER stress	DAP5
Antennapedia	Development	
Ultrabithorax	Development	
ODC	Cell cycle	
PITSLRE	Cell cycle	unr
hnRNP A/B	Cell cycle	
Hairless	Cell cycle	
Notch2	Cell cycle	
IGF-II	Cell Cycle	
VEGF	Hypoxia	PTB
HIF-1α	Hypoxia	
Cat-1	ER stress, hypoxia	
Bip	Heat shock	La, NSAP1
BAG-1	Heat shock	
FGF-2	Tissue/cell specific	hnRNP A1
FGF-1	Tissue/cell specific	
Kv1.4	Tissue/cell specific	
LEF-1	Oncogenesis	

Table 2[85]. Some cellular IRES elements. Some IRES elements that have been identified in cellular mRNAs are shown, including known cellular ITAFs that function in translation for each particular IRES. Note that only non-canonical ITAFs are listed; this list does not include any canonical eukaryotic translation initiation factors (eIFs). Apaf-1, apoptotic protease activating factor 1; XIAP, X-linked inhibitor of apoptosis protein; DAP5, death-associated protein 5; Bcl-2, B-cell CLL/lymphoma 2; HIAP2/c-IAP1, inhibitor of apoptosis protein; ODC, ornithine decarboxylase; IGF-II, insulin-like growth factor 2; VEGF, vascular endothelial growth factor; HIF-1α, hypoxiainducible factor 1; Cat-1, cationic amino acid transporter 1; BiP, immunoglobulin heavy chain-binding protein; BAG-1, BCL2-associated athanogene 1; FGF, fibroblast growth factor; LEF-1, lymphoid enhancer-binding factor 1.

The IRESite database presents evidence of many eukaryotic cellular internal ribosome entry site elements and the list is growing[77]. An increasing body of evidence indicate that these cellular IRESs have two major physiological functions: first they support low levels of translation initiation for cellular IRES-containing mRNAs with highly structured 5'-UTRs (incompatible with efficient scanning) under normal physiological conditions when cap-dependent translation is fully active and second they support robust translation of cellular mRNAs under a variety of physiological conditions such as mitosis, when cap-dependent translation is compromised[63]. All

cellular mRNAs are presumed to be capped and should be capable of binding the eIF4F complex. However, it is generally believed that conventional scanning from the m7Gcap is not efficient for most IRES-containing cellular mRNAs because their 5'UTRs are typically long, GC-rich, highly structured and may contain several upstream translation initiation codons. It is now apparent that under conditions of decreased cap-dependent initiation, cellular IRES-mediated initiation takes over[78] like occurs for viral ones.. It has been demonstrated that many physiological, pathophysiological and stress conditions that lead to inhibition of cap-dependent translation cause a substantial increase in cellular IRES-mediated translation[13]. Such conditions include, but are not limited to, endoplasmic reticulum (ER) stress, hypoxia, nutrient limitation, mitosis and cell differentiation. Since cap-dependent translation is suppressed under these conditions, it is believed that IRES-containing mRNAs become more competitive for the available pool of ribosomes and translation initiation factors, including both canonical initiation factors and ITAFs (Figure 18). It is striking that many of the cellular mRNAs that contain IRES elements[77] encode proteins that are involved in protection of cells from stress or, alternatively, induction of programmed cell death (apoptosis). It should be noted, however, that in contrast to viral IRES elements whose mechanism of action is becoming better understood, very little is currently known about the mechanism underlying cellular IRES function. No common sequence and/or structural motifs have been identified to allow prediction of cellular IRES elements from an mRNA sequence. The vast majority of cellular IRES elements are located within the 5'-UTRs immediately upstream of the initiation codon.

Nevertheless, there are cases in which the IRES is downstream of the initiation codon or located in the coding regions of the mRNA, thereby triggering synthesis of a truncated protein. Cellular IRESs, as found for viral IRES elements, are characterized by complex structures that often include stem loops and pseudoknots. These motifs can be 150-300 nucleotide long

even if there are cases in which 22 nucleotides long fragments are indicated[61].

Similar to viral IRESs, cellular IRES elements likely participate in multiple interactions with components of the translational machinery (canonical initiation factors, ITAFs and 40S ribosomal subunits). However there have not been extensive systematic studies of the ability of cellular IRES elements to bind the 40S ribosomal subunit or of the requirements for canonical translation initiation factors in cellular internal initiation. It remains possible that certain cellular IRESs may utilize the mechanism typical for picornavirus IRES elements. IRESs found in c-*myc*, L-*myc* and N-*myc* mRNAs were suggested to utilize this mechanism of internal initiation[79]. In addition, it has been postulated that some cellular IRESs, such as a short nine nt IRES from the mRNA of the human homeodomain protein Gtx and a 90 nucleotide IRES found in the human proto-oncogene IGF1R mRNA may operate through a Shine-Dalgarno-like interaction between the IRES and the 18S rRNA[80,81]. Clarification of these issues is an important goal since: a large number of cellular IRESs have been experimentally verified[77]; under stress conditions, 3–5% of the mRNAs are translated efficiently as determined by their association with polyribosomes[82]; 10–15% of cellular mRNAs were suggested to rely on cap-independent mechanisms of translation initiation, independently of stress [13] and several recent reports highlight the in vivo significance of IRES-mediated translation of specific mRNAs.

Given the prevalence of IRES-mediated initiation under stress conditions, it is notable that in most cases of cellular internal initiation, the cap-binding protein eIF4E and the scaffolding protein eIF4G (which undergoes caspase-mediated cleavage during stress) seem not to be required. For example, some studies revealed that the c-*myc* and N-*myc* IRESs do not require eIF4E or intact eIF4G for their activity, but do require eIF4A and eIF3[79]. In this regard, these IRESs are similar to many viral IRES elements, such as the EMCV. Recently, the first case of a cellular IRES element that seem to be capable of direct binding to 40S ribosomal subunits was reported for the c-

Src kinase mRNA[83]. This feature of the c-Src kinase IRES element makes it similar to Type III and IV viral IRESs. However, it is currently unclear how many other cellular elements utilize the same pathway.

The role of eIF2 has also been investigated for cellular internal initiation. Many cellular IRES-containing mRNAs (such as cat-1, N-*myc*, s-Src, etc.) were shown to be insensitive, or much less sensitive than mRNAs without IRESs, to the inhibition of protein synthesis caused by eIF2 phosphorylation[13]. Some viral IRESs (HCV), as well as some cellular IRESs are insensitive to this mode of translation inhibition. These observations suggest that different mRNAs and, in particular, different IRES-containing mRNAs might differ in their requirements for the active ternary complex and/or might utilize different pathway(s) to deliver Met-tRNAiMet to the ribosome. The latter pathway(s) might involve initiation factor eIF5B and/or Ligatin or perhaps some other proteins[63]. It cannot be excluded that some other/additional proteins can promote Met-tRNAiMet binding to ribosomes in eukaryotes. To summarize, the complex nature of regulation of cellular mRNA translation under different pathophysiological conditions suggests that there may be multiple diverse pathways leading to cellular IRES-mediated initiation. An overview of them is shown in Figure 18.

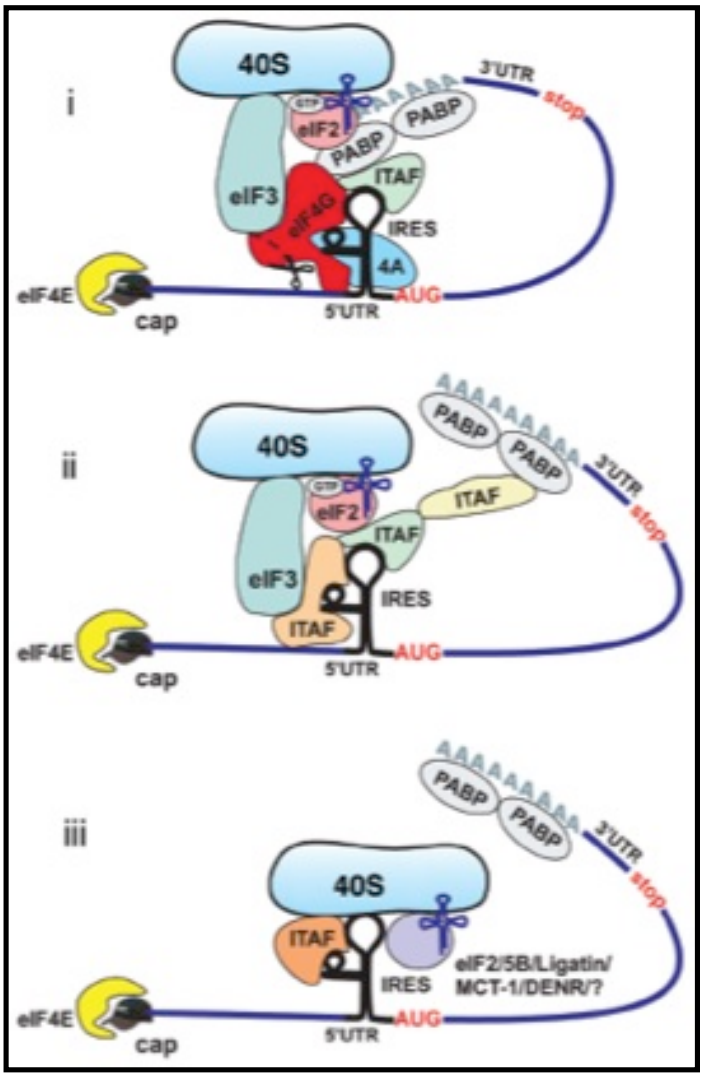


Figure 18. IRES-mediated mechanisms of translation initiation in eukaryotic cells[63]. Cellular IRES-mediated translation generally does not require the cap-binding protein eIF4E and/or intact eIF4G. The requirement for canonical initiation factors and ITAFs can vary between different IRES-containing mRNAs. Potential mechanisms of cellular IRES-mediated translation: (i) most, if not all, canonical initiation factors and many ITAFs are required; (ii) a limited number of canonical factors and ITAFs are required; and (iii) canonical factors are dispensable, but some ITAFs may be required.

The role of ITAFs in cellular IRES-mediated translation starts to be studied. 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. In addition to their participation in a variety of cellular activities (like RNA splicing and/or export), ITAFs are generally believed to be able to increase or, in certain cases, decrease, the affinity of binding between IRESs and

components of the translational apparatus (canonical initiation factors and ribosomes). Although the exact mechanism(s) underlying ITAF function is unknown it can be hypothesized that they remodel IRES spatial structures to produce conformations with higher or lower affinity for components of the translation apparatus or that they build or abolish bridges between the mRNA and the ribosome in addition to those provided by canonical initiation factors and that they take the place of canonical factors in building bridges between the mRNA and the ribosome. Overexpression and/or depletion of specific ITAFs in normal cells can affect the activity of the cellular IRESs that normally utilize those ITAFs without altering cap-dependent translation[84]. Thus, it is clear that the intracellular concentration of ITAFs plays an important role in modulating the activity of IRESs, but the mechanism(s) responsible for regulating ITAF concentration have not been fully defined. Two alternative mechanisms have been proposed to explain the effects of ITAF compartmentalization. In one model, nuclear localized ITAFs were suggested to associate with their target IRES-containing mRNAs and sequester them in the nucleus away from the translational machinery. Alternatively, ITAFs in the nucleus were suggested to be primarily in an mRNA-unbound form, separated from their target IRES-containing messages residing in the cytoplasm. Following the appropriate signals (caused by stress or other physiological conditions), either the ITAF-bound mRNAs (in the first model) or the unbound ITAFs themselves (in the second model) were proposed to translocate from the nucleus to the cytoplasm, allowing translation of the mRNAs to proceed. Additional studies need to be done to better understand both the cellular IRES-mediated translation initiation steps and the role of ITAFs in this process.

IRES-mediated translation initiation in cancer

As the data suggest that IRES-mediated translation occurs under conditions of cell stress (like exposure of cells to chemotoxic agents, apoptosis and hypoxia) in which cap-dependent initiation is usually inhibited and as the tumour cells very often live under such conditions, IRES-mediated translation initation is probably involved in the tumorigenesis. This hypothesis id enhanced even more if considered that, as shown in Table 2, many growth promoting and cell survival proteins such as c-Myc, VEGF-A, XIAP and Apaf-1 are encoded by mRNAs that contain IRESs. For this an increasing attention is taken to these processes.

Mitogenic signals such as growth factors, hormones and cytokines activate the protein kinase Akt (Figure 19), which in turn phosphorylates and activates mTOR, the kinase component of mTOR complex 1 (mTORC1). Activated mTOR phosphorylates and inactivates the eukaryotic 4E-BPs, freeing eIF4E to bind eIF4G and promoting cap-dependent translation. Downregulation of mTOR activity by physiological stresses such as hypoxia, nutrient deprivation, leads to hypophosphorylated (activated) 4E-BP proteins that compete with eIF4G for binding to eIF4E, preventing cap-dependent translation. The eIF4E/4E-BP complex might remain bound to the m7Gcap (as shown), possibly aiding the inhibition of cap-dependent mRNA translation owing to blockade of the mRNA 5′ end. Increased levels of hypophosphorylated 4E-BPs, in conjunction with elevated levels of eIF4G, can then function as a switch, impairing the initiation of translation on purely cap-dependent mRNAs but enhancing translation of dual mechanism mRNAs that also contain an internal ribosome entry site (IRES) to which eIF4G may bind directly. These mRNAs include vascular endothelial growth factor A (VEGFA), fibroblast growth factor 2 (FGF2), B-Cell Lynfoma-2(BCL2) and hypoxia-inducible factor 1α (HIF1A). This switch is activated by hypoxia and other stresses to preserve tumour cell viability and promote tumour angiogenesis[52]. The presence of this switch is indirectly justified by the

identification of many mRNAs whose is induced under conditions of mTOR inhibition following treatment with its inhibitor rapamycin in glioblastoma cell lines[89]. Many of these transcripts remained on actively translated polysomes or shifted from monosomal to polysomal translational states following the global inhibition of cap-dependent translation. That's why those cells continue to proliferate.

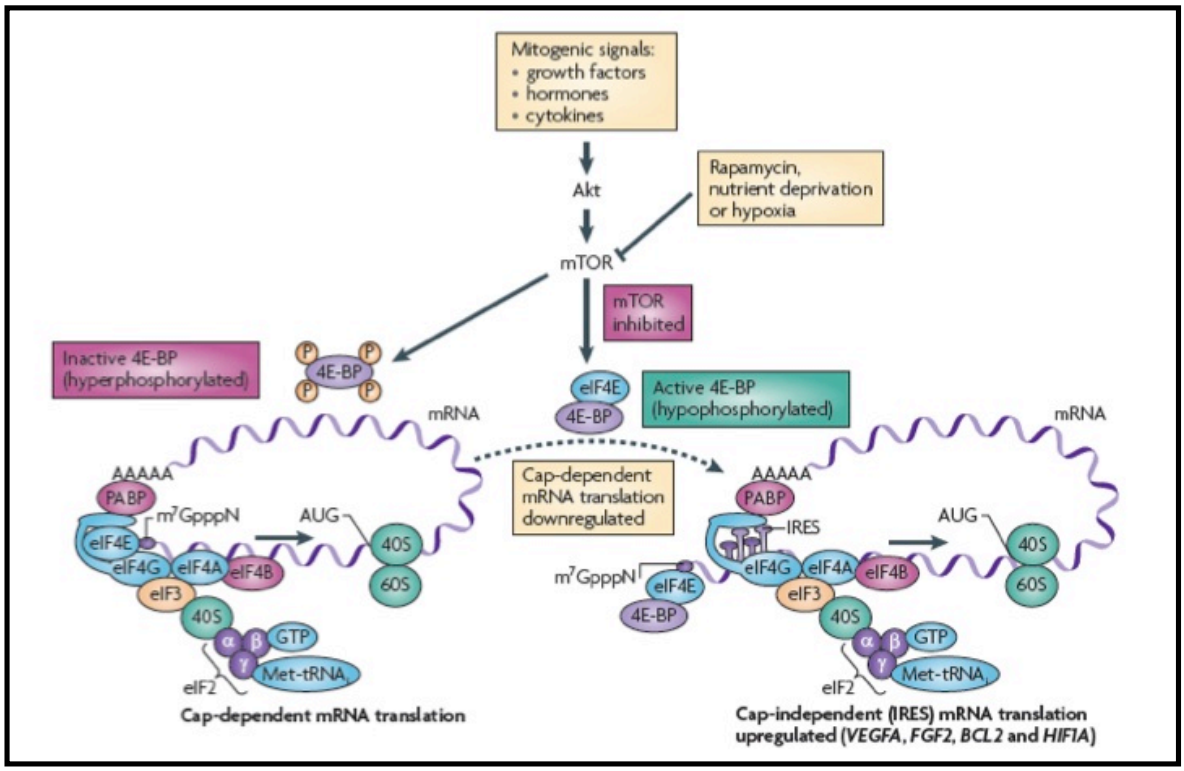


Figure 19[52]. A cap-dependent to cap-independent translation switch mediated by the akt/mTOR/4E-BP pathway.

In local advanced breast cancer (LABC), the overexpression of eIF4G1 and 4E-BP1 was shown to function as a hypoxia-activated switch in animal models, reducing eIF4F-dependent mRNA translation and stimulating IRES-dependent translation. This promotes increased tumour size, angiogenesis and survival under hypoxic conditions through increased IRES-dependent translation of mRNAs encoding HIF1α, VEGFA, BCL-2 and other dual mechanism mRNAs that contribute to tumour growth and survival[74]. It is

not known how overexpression of 4E-BP1 and eIF4G1 are related to decreased metastasis in LABC. The overexpression of eIF4G1 that is observed in most inflammatory breast cancers (IBCs), in the absence of increased levels of eIF4E and 4E-BP1, was shown to maintain high levels of IRES-dependent mRNA translation in animal models. Two mRNAs that are required for IBC survival and metastasis were identified: VEGF-A and p120 catenin (P120CTN; also known as CTNND1)[39] which promote the formation of highly metastatic cancer cell clusters known as emboli. Several IRES-containing mRNAs clearly have important and direct roles in cancer development through cap-independent translation. One of the most widely investigated examples is that of MYC. Increased MYC expression can be acquired by gene amplification, increased transcription, or deregulated translation of its mRNA, which occurs by both cap-dependent and IRES-dependent mechanisms. As observed for VEGF-A, HIF1α, P120CTN and several other IRES-containing mRNAs, the MYC IRES also uses eIF4G for initiation, along with several ancillary factors. It is interesting that 42% of patients with multiple myeloma have a C to T mutation in the MYC IRES sequence that results in increased cap-independent translation[86] through more efficient binding of the heterogeneous nuclear ribonucleoprotein K (HNRNPK), which is an ancillary factor for MYC IRES activity. The increased IRES-mediated translation of MYC mRNA in multiple myeloma cells is also reportedly due to interleukin-6-induced binding of another HNRNP (HNRNPA1) to its IRES element.

A crucial event in tumour progression is the gain of invasive properties of carcinomas cells by epithelial to mesenchymal transition (EMT). This process is characterized by the loss of epithelial cell polarity and acquisition of a fibroblastoid phenotype enabling tumor cells to leave epithelial cell organization. EMT is induced by the IRES-mediated translation of a number of mRNAs, including SNAI1[88]. It has been shown that in hepatocellular carcinoma cells (HCC) the IRES-mediated translation of some proteins such as Laminin B1 (LamB1) is enhanced and drives the EM transition[87].

Importantly in this case the IRES-mediated translation seems to be favored by the overexpression of some ITAFs. In fact, as just mentioned above, cellular IRESs generally require ITAFs for function. So, it would be expected that over-expression of ITAFs would enhance IRES-mediated translation, and it has been shown that increased expression of PTB (that is one of most "popular" ITAF) is associated with metastasis, and that up-regulation of YB1 both induces epithelial–mesenchymal transition (EMT) and enhances metastasis[88].

In some case, cells respond to physiological and pathophysiological stress by phosphorylation of the α subunit of eIF2 (as just descrived above) which results in an attenuation of global protein synthesis. Phosphorylation of eIF2α leads to reduced availability of ternary complex, and a concomitant decrease in global translation rates. However, translation of some mRNAs (such as ATF4 and GCN2) continues (or is even enhanced) under conditions that cause eIF2α phosphorylation since their translation is controlled by upstream open reading frames, a setting in which low levels of ternary complex promote translation of the downstream open reading frame[23]. Continuous translation of several IRES-containing mRNAs during conditions of phosphorylated eIF2α and reduced ternary complex availability has also been reported. For example, translation of VEGF-A was substantially increased during tumor hypoxia, while the IRES-mediated translation of X-chromosome linked Inhibitor of Apoptosis (XIAP) sustained during ER stress or was increased in response to serum starvation, low-dose gamma irradiation or glucose deficiency. The up-regulation of XIAP IRES-mediated translation enhances the survival of non-small cell lung carcinomas cells in an animal model[90]. It is not clear, however, how IRES-mediated translation of cellular mRNAs proceeds under these conditions, and what is the precise molecular mechanism that allows cellular IRES to function in an eIF2a -independent manner. A reasonable hypothesis is that the eIF2α –dependent pathway is utilized for IRES-dependent translation initiation during normal growth conditions, whereas cellular stresses that inactivate eIF2αby phosphorylation

cause IRES-dependent translation to switch to an eIF5B-dependent mode[23].
Alterations in ribosome modification have also been shown to affect IRES-mediated translation. In patients with X-linked dyskeratosis congenita (X-DC), the mutated DKC1 gene encodes dyskerin, a pseudouridine synthase that modifies ribosomal RNA. This mutation leads to a specific defect in IRES-mediated translation of the tumour suppressor p27 (Kip1), Bcl-XL and XIAP. Patients with this disorder have an increased susceptibility to cancer, and it is suggested that the reduced expression of p27 is partially responsible for this aspect of the phenotype of DKC[96].

The role of hypoxia in cancer

Hypoxia is a reduction in the normal level of tissue oxygen tension and occurs during acute and chronic vascular disease, pulmonary disease and cancer[91]. It has been estimated that 50–60% of solid tumors contain areas of hypoxic and/or anoxic tissues that develop as a result of an imbalance between oxygen supply and consumption in proliferating tumors. Low oxygen concentrations may result on one side from increased metabolic activity and oxygen consumption and, on the other side, from increased tumor cell distance from local capillaries and blood supply[92]. Although hypoxia is toxic to both cancer cells and normal cells, cancer cells undergo genetic and adaptive changes that allow them to survive and even proliferate in a hypoxic environment. These processes contribute to the malignant phenotype and to aggressive tumour behaviour.
Particularly, cells undergo a variety of biological responses in response to hypoxic conditions. The earliest recognized pathway was that hypoxic cells undergo a shift from aerobic to anaerobic metabolism. Hypoxia also induces erythropoietin (EPO) production in renal cells (to increase haemoglobin production) and tyrosine hydroxylase synthesis in neural cells (involved in catecholamine production)[91]. One of the most well studied hypoxia responses is the production of growth factors that induce angiogenesis (new blood vessel formation). Many of the cellular responses to hypoxia are

mediated through changes in gene expression. The transcription factors primarily responsible for these changes are the hypoxia-inducible factors (HIFs). HIFs are members of the bHLH-PAS family of proteins and bind to canonical DNA sequences (called hypoxia regulated elements, or HREs) in the promoters or enhancers of target genes. They consist of an α (HIF-α) and a β (HIF-β, or ARNT) subunit and activate the expression of at least 150 genes encoding proteins that regulate cell metabolism, survival, motility, basement membrane integrity, angiogenesis, hematopoiesis, and other functions (Table 3 and Figure 20). To date, two HIFs (HIF1 and HIF2) have been identified that regulate transcriptional programs in response to low oxygen levels.

Oxygen transport and iron metabolism • Ceruloplasmin | erythropoietin | ferritin light chain | heme oxygenase-1 | transferrin | transferrin receptor
Angiogenesis • Adrenomedullin | angiopoietin-2 | cyclooxygenase-2 | endothelin-1 and -2 | fibroblast growth factor-3 | hepatocyte growth factor | histone deacetylase | monocyte chemotactic protein-1 | nitric oxide synthase | osteopontin | placental growth factor | Tie-2 (an angiopoietin receptor) | transforming growth factor (TGF)-**α,** TGF-**β**1,TGF-**β**3 | vascular endothelial growth factor (VEGF)-A | VEGF receptor-1
Glycolysis and glucose uptake • Aldolase-A | enolase-1 |glucose transporter1, 3 (GLUT1,GLUT3) | glyceraldehyde-3-phosphate dehydrogenase| lactate dehydrogenase-A | phosfructokinase-C | phosfructokinase-L | phosphoglycerate kinase-1 | pyruvate kinase-M
Transcription factors • Annexin V | BCL-interacting killer (BIK) | cyclin G2 | differentiated embryo-chondrocyte expressed gene1 (DEC1) | FOS | heat-shock factor | hypoxia-inducible factor (HIF)-1**α**;HIF-2**α**| insulin-like growth factor (IGF) binding protein1,2, 3 | JUN | KIP1| nuclear factor-**κ**B (NF-**κ**B) | NIP3 | NIX | transgelin | transglutaminase-2 | WAF1
Metabolism/pH/neurotransmitters • Acetoacetyl CoA thiolase | adenylate kinase-3 | aminopeptidase-A | carbonic anhydrase-9, -12 | phosphoribosyl pyrophosphate synthetase | spermidine N1-acetyltransferase
Growth factors/cytokines • IGF-2 | interleukin-6 | interleukin-8 | intestinal trefoil factor | macrophage inhibitory factor | platelet-derived growth factor-B | staniocalcin
Stress-response pathways • 150-kDa ORP (oxygen-regulated protein) | growth arrest- and DNA damage-induced gene **(**GADD153**) |** human apurinic apyrimidinic site endonuclease **(**HAP-1**) |** thioredoxin
Cell adhesion, extracellular matrix, cytoskeleton and proteases/coagulation • CD99 | collagen-5**α**1 | Ku70 | Ku80 | low-density lipoprotein receptor-related protein | metalloproteinases | matrix metalloproteinase-13 | neuronal cell-adhesion molecule L1 (L1CAM) | plasminogen activator inhibitor-1 | tissue factor (TF) | | vimentin | **α**-integrin

Table 3. Gene induced by hypoxia[91].

A recent survey of malignant and normal tissues found that the expression of both HIF-1α and HIF-2α are commonly increased in a variety of human tumors, including bladder, breast, colon, glial, hepatocellular, ovarian, pancreatic, prostate, and renal tumors[92]. In clinical specimens, elevated HIF-1 expression correlates with poor patient outcome in head and neck cancer, nasopharyngeal carcinoma, colorectal, pancreatic, breast, cervical, osteosarcoma, endometrial, ovarian, bladder, glioblastoma, and gastric carcinomas, while elevated HIF-2 expression correlates with poor patient outcome in hepatocellular, colorectal carcinoma, melanoma, ovarian, and non-small cell lung cancers[92].

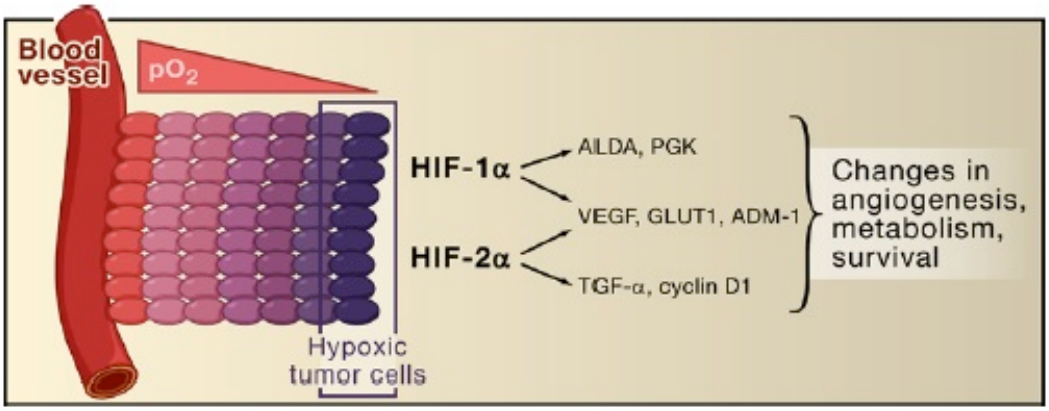


Figure 20. HIFs in Tumor Progression[93]. Stabilization of HIF-α proteins in these cells stimulates the expression of numerous target genes encoding factors that mediate adaptation to hypoxic stress. Some target genes are regulated specifically by HIF-1α, such as those encoding the glycolytic enzymes ALDA and PGK, whereas others are specific targets of HIF-2α, such as those encoding TGF-α and cyclin D1. Most HIF target genes are regulated by both HIF-1α and HIF-2α, including those encoding the angiogenic cytokine VEGF and the glucose transporter GLUT1.

Hypoxia mediate inhibition of general protein synthesis by the modification of eukaryotic translation initiation factors (eIFs) at two steps that are: regeneration of the "ternary complex" (eIF2-GTP and met-tRNA) and regulation of the m7GTP cap binding complex eIF4F (eIF4E/eIF4A/eIF4G). Many non-transformed cells undergo hypoxia inhibition of protein synthesis, whereas highly transformed cells are largely resistant[37,94,95]. Although general translation is downregulated during hypoxia, mRNAs important for

adaptation to hypoxia such as HIF1α, VEGF, and platelet-derived growth factor (PDGF), continue to be translated. Translation in these cases is often cap independent due to the presence of IRES.

Local oxygen concentrations can directly influence stem cell self-renewal and differentiation. One attractive hypothesis is that stem cells, particularly in long-lived animals, might benefit from residing in hypoxic niches where oxidative DNA damage may be reduced. A number of experiments over the past decade support the idea that cancers can grow from a discrete subpopulation of malignant cells with stem cell properties. These transformed cells are formally similar to normal stem cells in that they self renew and produce more committed progenitor or "transit-amplifying" cells whose progeny differentiate, albeit aberrantly, to produce the bulk of the tumor. To date, cells with these and other stem cell properties have been identified in human hematopoietic, brain, and breast cancers, and are likely to be found in other tumors[93]. Consequently it's reasonable to hypothesize that it's hypoxia itself that can promote the generation of cancer stem cells through HIF activity. HIF activity in a rare subset of hypoxic tumor cells may enhance the expression or activity of other gene products including Notch, Oct4, c-Myc, ABC transporters (ABC-T), and telomerase to promote a stem cell-like state. Increased expression of KLF4, Sox2, and other factors could promote further dedifferentiation and confer stem cell-like properties, such as self-renewal, on what was originally a transformed cell with limited replicative potential. Inhibition of HIF activity in the resultant cancer stem cells might block, or reverse, this effect (Figure 21).

The role of the translation control in cancer stem cells generation during hypoxia need to be studied.

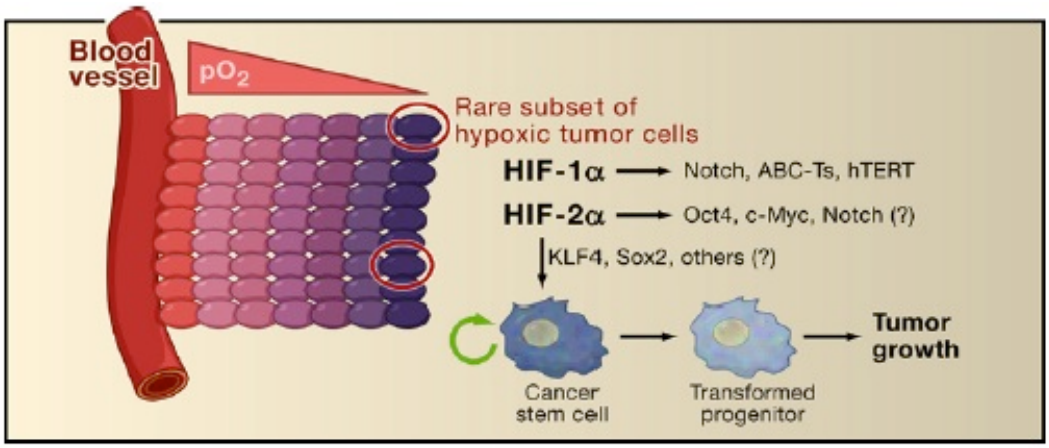


Figure 21. Could Promote Generation of Cancer Stem Cells[93]. HIF activity in a rare subset of hypoxic tumor cells may enhance the expression or specific gene (such as Notch, Oct4, c-Myc, ABC, KLF4, Sox2) to promote a stem cell-like state.

Ribosome biogenesis and its association with cancer

Ribosome biogenesis is a multistep highly coordinated process that led to the production of mature ribosomes. Briefly, all ribosomal RNAs, with the exception of the 5S, are transcribed as a polycistronic transcript known as pre- ribosomal RNA (pre-rRNA or 45S) in the nucleolus. Concomitant with rRNA transcription, the rRNA sequences are extensively modified. Specifically, a large family of small nucleolar RNAs (snoRNAs) guides the site-specific conversion of uridine to pseudouridine (Ψ) in rRNA. In addition, other snoRNAs also guide the formation of 2′-0-methylated nucleosides (CH3) in rRNA. The pre-rRNA precursor is then cleaved at specific sites by RNases to produce a series of characteristic intermediates and finally mature rRNAs 18S, 5.8S and 28S. During rRNA processing, the rRNA species must associate with more than 70 ribosomal proteins, as well as the 5S rRNA in the nucleolus, to form the 40S and the 60S ribosomal subunits, which are assembled and transported to the cytoplasm to initiate protein synthesis (Figure 22). Quantitative and qualitative changes in ribosome biogenesis may be responsible for neoplastic transformation[98](Figure 22). Regarding the quantitative changes in ribosome biogenesis, there is evidence that an up-regulation of ribosome biogenesis induces translational and post-translational

alterations. Particularly the transcription of the 45S rRNA gene is negatively regulated by tumour suppressors such as p53 and retinoblastoma (RB) and augmented on mitogenic stimuli by several kinases that phosphorylate components of the transcription complex that are responsible for 45S synthesis. The accurate regulation of rRNA synthesis can be lost in cancer cells through inactivating mutations in tumour suppressors or upregulation of these kinases[97]. Genome instability and reduced p53 activity might well explain the increased risk of cancer onset in tissues or organs in which hyperplastic and dysplastic lesions or chronic inflammatory processes are associated with ribosome biogenesis upregulation. Indeed, as ribosome assembly involves the association of rRNA with more than 70 ribosomal proteins (made in the cytoplasm), an increase in ribosomal protein production and activity has been observed in many cancer types[98]. Mutations in ribosomal proteins such as S19 have also been associated with a human syndrome that is characterized by increased tumour susceptibility[97].

Concerning the role of qualitative changes in ribosome biogenesis, the qualitative alteration of the proper functioning of the processes involved in ribosome biogenesis may also be responsible for a series of human diseases. These mainly consist of a group of rare inherited disorders in which the genes encoding for factors necessary for ribosome production, such as ribosomal protein or other factors involved in rRNA transcription and processing, are mutated. These disorders include X-linked dyskeratosis congenita (X-DC), Shwachman–Diamond Syndrome (SDS), cartilage hair hypoplasia (CHH), Diamond–Blackfan anemia (DBA) and Treacher Collins syndrome, and are jointly defined as ribosomopathies[98]. Interestingly, X-DC, SDS, CHH and DBA are characterized by a very high incidence of cancer, supporting the hypothesis that specific qualitative defects in the process of ribosome biogenesis may also induce cancer. The mechanisms underlying increased cancer susceptibility in ribosomopathies are still being studied. However, it has been suggested that alterations in the complex structure of ribosomes, which are the effectors of protein synthesis, may be responsible for

alterations in the translation of mRNAs of significance for neoplastic transformation. Such a hypothesis is gradually finding confirmatory evidence in X-DC, which appears to be the ribosomal disorder in which the molecular connections between the qualitative alterations of ribosome biogenesis and cancer have been better characterized and they will be discussed later in this thesis.

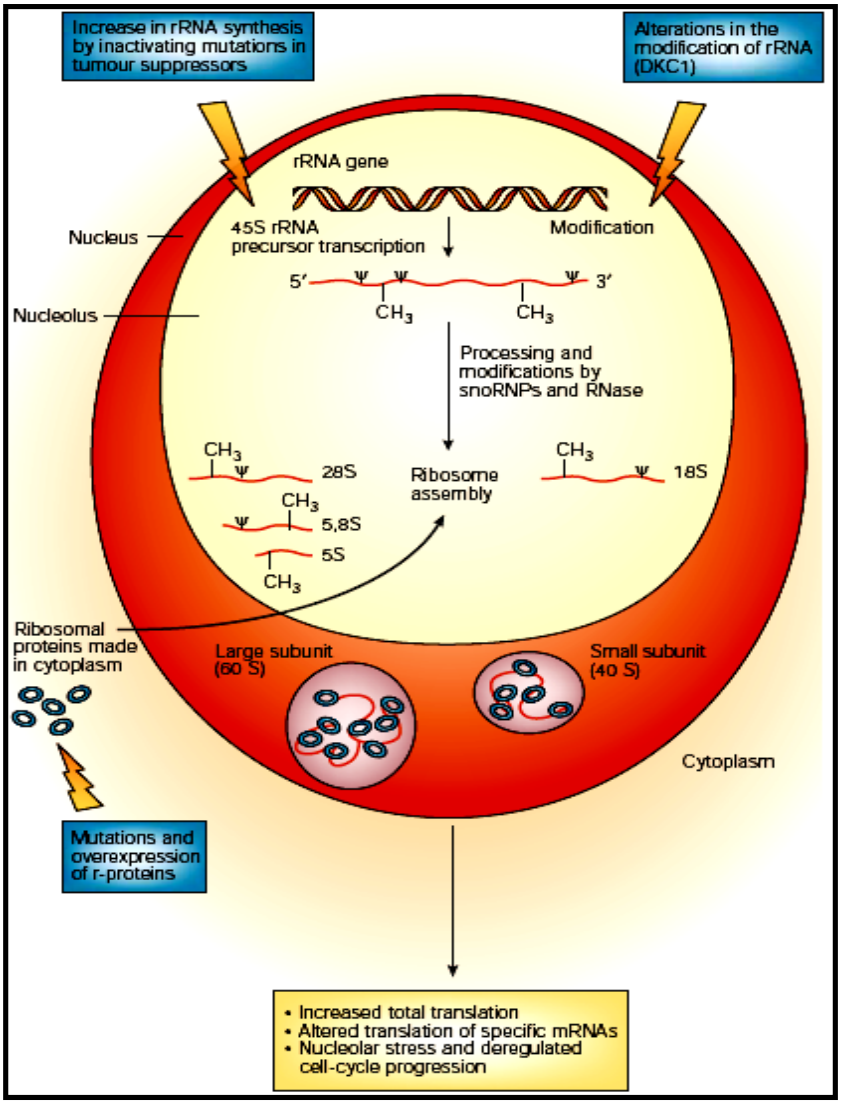


Figure 22. Ribosome biogenesis and the steps involved in cancer if deregulated[97]. The scheme illustrates the main processes involved in ribosome biogenesis. The 45S precursor rRNA is site specifically modified (Ψ=pseudouridylation, CH3=2-O′-ribose methylation and base methylation), processed, and the mature 18S, 5.8S and 28S rRNA are assembled with 5S rRNA and the ribosomal proteins to form the 40Ss and 60Ss. Mature ribosomal subunits are exported to the cytoplasm to constitute the ribosomes. Each of the three blue boxes indicate the steps that could contribute to tumour initiation or cancer progression.

Dyskerin role in translation and cancer susceptibility

X-DC is a disease characterized by a progressive failure of proliferating tissues (including bone marrow and skin) associated with an increased risk (11-fold over the control population) of developing malignancies. X-DC is caused by mutations in the DKC1 gene which product, dyskerin, is a nucleolar protein that performs at least two different major functions. First, dyskerin is one of the core proteins involved in small nucleolar ribonucleoproteic particles (snoRNPs) involved in rRNA processing. In particular, dyskerin is necessary for the site-specific conversion of uridine to pseudouridine present in the rRNA molecules. This function is necessary for the proper pre-rRNA maturation and involves the function of a class of small nucleolar RNA (snoRNA) containing a Hinge-ACA box (and therefore termed H/ACA snoRNA), which guides dyskerin and the other components of the pseudouridylation complex to specific uridine residues to be modified. Second, through the stabilization of the telomerase RNA component, which also contains an H/ACA box, dyskerin makes the proper telomerase enzymatic complex activity possible[99] (Figure 23).

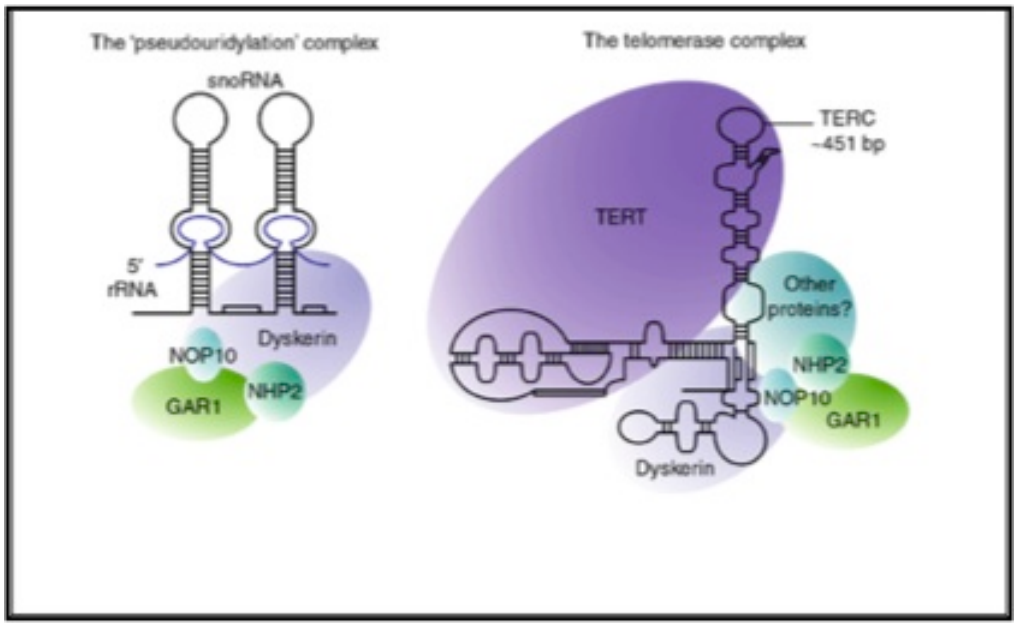


Figure 23. Dyskerin in action[99]. The picture shows the two major function of dyskeryn: pseudouridilation of the rRNA(left) and telomerase stabilitiation (right).

Studies in the DKC1 hypomorphic mouse model indicate that the defect in ribosome biogenesis plays a direct causal role in the cancer susceptibility observed in X-DC patients: indeed, an increase in cancer incidence is already observed in that model from the early generations, when telomeres are still very long. The observation that rRNA uridine modification is defective in the DKC1 hypomorphic mutant model suggested the unconventional hypothesis that the alteration of ribosome function might be involved in determining the cancer susceptibility observed in X-DC. Indeed, the uridine modification sites are placed within specific domains of the ribosome, which are important for tRNA and mRNA binding. The reduction in modified uridine residues in the ribosome might result in an impaired translation of specific mRNAs encoding for products of importance for neoplastic transformation. In line with this hypothesis, it has been reported that, in cells from both X-DC patients and DKC1 hypomorphic mice, the translation of a small sub-set of cellular mRNAs containing the IRES elements is inefficient. In X-DC, the defect in IRES-mediated translation results in the diminished synthesis of protein encoded from IRES containing mRNA. This is particularly evident when a reduction of 5′ cap-dependent translation initiation occurs, as in the case of exposure to different types of stress. Initially, as translational targets of defective dyskerin functions, both the mRNAs encoding the anti-apoptotic factors Bcl-xL and XIAP and the tumor suppressor p27 were identified[100]. In addition, the IRES containing mRNAs encoding for the tumor suppressor p53 have also been recently added to the list[101,102]. These findings indicate that the mechanism responsible for the increased tumor susceptibility in X-DC may be the defective translation of mRNAs encoding for tumor suppressors such as p53 and/or p27 (Figure 24). On the other hand, the defect in p27 mRNA translation characterizing the DKC1 hypomorphic mouse leads to an increase in the susceptibility to pituitary adenomas in vivo in association with the p27 +/− knockout background, rendering its phenotype at this level similar to that of the p27 −/− mouse[103]. There is also evidence indicating that the qualitative defects in ribosome biogenesis may contribute

to cancer development not only in this rare inherited disorder but also in sporadic tumors affecting the general population. In fact the defect in the synthesis of p53 and p27 already described in X-DC and X-DC models has also been described in a sub-set of human breast carcinomas characterized by a strong reduction in DKC1 expression and function, and in a pituitary adenoma bearing a DKC1 mutation, respectively.

Because dyskerin clearly has a role in controlling the IRES-mediated translation initiation and because its implications in the development, it should be a nice models to study the importance of alteration of the cap-independent translation in the tumorigenesis.

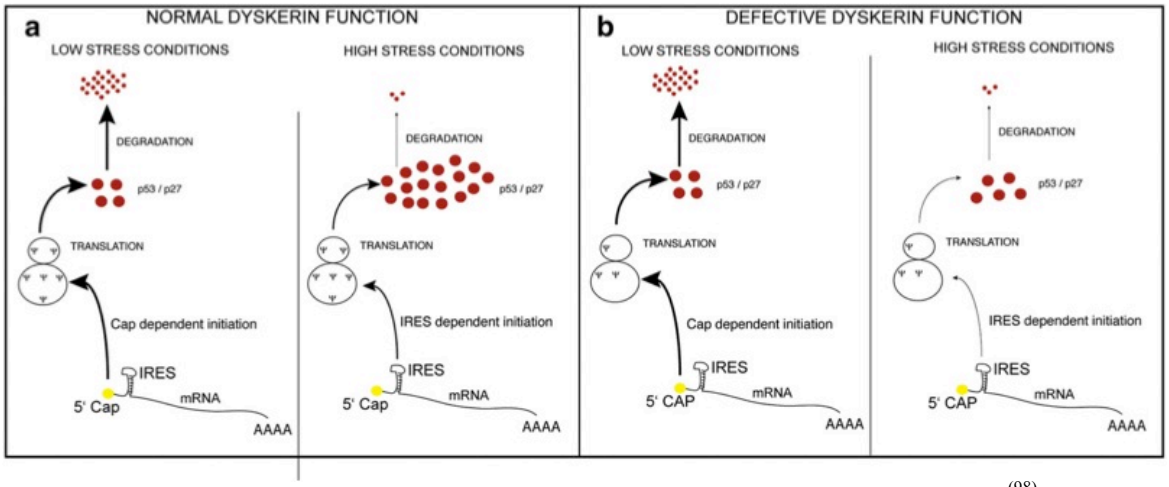


Figure 24. How qualitative defects of ribosome biogenesis may contribute to cancer[98]. Simplified scheme summarizing the consequences of defects in dyskerin function on the translation of IRES containing mRNA encoding for the tumor suppressors p53 and p27. (a)When dyskerin function is maintained, the correctly pseudouridylated ribosomes (**Ψ**=pseudouridine) are able to efficiently translate these mRNAs both in conditions when cap-dependent translation initiation is active, and under stress conditions, including, e.g., genotoxic damage, hypoxia, nutrient deprivation, oncogenic stress, etc., when IRES-mediated translation initiation predominates. (b)Impairment of correct ribosome pseudouridylation, as in the case of dyskerin mutations or dyskerin-reduced expression, negatively affects cap-independent translation initiation under stress, resulting in reduced synthesis and accumulation of tumor suppressors and decreased cellular response.

AIM OF THE STUDY

Translational control has a direct impact on cancer development and progression[1,52]. It is now well defined that quantitative and qualitative changes of cap-dependent translation contribute to neoplastic transformation and progression. These changes may promote a global increase in protein synthesis to match tumour cell proliferation, and a selective translation of specific mRNAs that promote tumour cell survival, angiogenesis, transformation, invasion and metastasis[104,52]. Indeed, alterations of eukaryotic initiation factors (such as eIF4E, eIF2α, eIF4G and eIF3) or of translation regulatory factors (such as 4E-BP1), as well as quantitative and qualitative alterations of ribosomes, and alterations of the signalling pathways that activate the mRNA translation (primarily the PI3K-AKT-mTOR and Ras pathways) [4-11] are associated with cancer development and progression. However, the idea that "alternative" mechanisms of translation initiation, such as IRES-dependent translation, can be involved in the tumorigenesis is emerging. An example of this is X-linked dyskeratosis congenita (X-DC), a ribosomopathy characterized by a very high incidence of cancer. It has been reported that, in cells from X-DC patients, the translation of a small sub-set of cellular mRNAs containing the IRES elements is inefficient. These findings indicate that the mechanism responsible for the increased tumor susceptibility in X-DC may be the defective translation of IRES-containing mRNAs.

Although cap-dependent translation is important during cancer progression, cancer cells can encounter environmental conditions that down-regulate mRNA cap-dependent translation. An example of such conditions is hypoxia. Hypoxia can be defined as a condition of sub-optimal oxygen availability to which cells must adapt if they want to survive. When a tumour cell is insufficiently supplied with oxygen, it undergoes profound modifications involving metabolic changes and the re-programming of gene expression at transcriptional and translational levels [92]. In order to conserve energy, cells

strongly down-regulate the global translation. Nevertheless, in this condition, the ability of cancer cells to survive and proliferate is dependent to the synthesis of specific proteins required for tumor vascularization (VEGF-A), survival (Bcl2, survivin) and the hypoxia response (HIF1a) among other activities. Translation of these mRNAs occurs by IRES mediated-translation. [37]. Little is known about the exact mechanisms by which initiation by the IRES occurs, or the influence of such mechanisms in angiogenesis, transformation, invasion and metastasis.

Because the relevance of this kind of translation initiation in cancer progression is not so well clarified, the purpose of my work was to study the impact of IRES-dependent mRNA translation on tumourigenesis and cancer progression with particular regard for breast cancer. I investigated the consequences of the up-regulation or down-regulation of selective IRESs in the 5'UTR of specific mRNAs (tumour suppressor p53, growth factor VEGF-A and response to stress hsp70) on the capability of the cells to proliferate or die and on the capability of cells to adhere to specific substrates. I also investigated the importance of changes in IRES-dependent translation in the acquisition of a more aggressive phenotype in immortalized but not transformed human epithelial cells.

MATERIALS AND METHODS

Cells and reagents

All the cells used were obtained from ATCC. Human breast cancer derived cell line MCF7 and low transformed MCF10A and HMEC/TERT breast derived cells lines were cultured in monolayer at 37°C in humidified atmosphere containing 5% CO2. MCF7 were grown in RPMI 1640 (Euroclone) supplemented with 10% of fetal bovine serum (FBS, Euroclone), 2mM L-glutamine (Euroclone), 100 U/ mL penicillin and 100 mg/ mL streptomycin (Euroclone). MCF10 A and HMEC/TERT were grown in a 1:1 mixture of Ham's F12 and DMEM (Lonza) supplemented with 5% horse serum, 20 ng/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin, 0,01 mg/ml human recombinant insulin, 72,5 ng/ml hydrocortisone and 4 µg/ml gentamycin.
Subconfluent MCF7 cells were treated with doxorubicin 1 and 4 µM and deferoxamine 100 µM (Sigma-Aldrich) and collected after 72h and 96 h of treatment. For survival assays, cell were either trypsinized and counted after trypan blue staining or formalin fixed and stained with 0.05% crystal violet in 20% methanol for 10 min.
MCF10 A and HMEC/TERT were grown both in normal oxygen concentration and inside a hypoxia chamber at 0.5% oxygen and fed every two days with fresh medium. After 24h or 144h of hypoxia they were trypsinized and processed as described in next paragraphs.

RNA interference

DKC1 double stranded siRNAs and an appropriate control were obtained from Invitrogen: DKC1 RNAi was performed with 3 pooled siRNA oligonucleotides (Invitrogen catalog number HSS102781, HSS102782, HSS102783). siRNA were transfected in MCF7 adherent cells using

Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM medium (Invitrogen) accordingly to manufacturer's procedures. The concentrations of siRNAs used resulted to be capable to reduce the mRNA levels to at least the 80% of control for a duration of 96 h.

RNA extraction and Real-time PCR

Total RNA was extracted from MCF7 cells 96h after siRNA transfection using TRI reagent (Ambion) according to manufacturer instructions. RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems) following the manufacturer instructions. Real-time PCR analysis was performed in a Gene Amp 7000 Sequence Detection System (Applied Biosystems) using the TaqMan approach. For each sample three replicates were analyzed. Sets of primers and fluorogenic probes specific for DKC1 (catalog number Hs00154737_m1), p53 (Hs00153349_m1), VEGF (Hs00900054_m1) and b-actin (Hs99999903_m1) mRNA were purchased from Applied Biosystems. The relative amounts of the studied target genes were calculated using the expression of human β-glucuronidase (Applied Biosystems - 4326320E) and 18S RNA (for polysomal analysis - Hs99999901_s1) as an endogenous control. The final results were determined as follows: N target = $2^{-(\Delta Ct \text{ sample}-\Delta Ct \text{ calibrator})}$, where ΔCt values of the sample and calibrator were determined by subtracting the Ct value of the endogenous control gene from the Ct value of each target gene.

mRNA transfection

Capped mRNA was transcribed from linearized pR-EMCV-IRES-F (gift from Prof. A.C. Palmenberg), pR-HCV-IRES-F (gift from Prof R.E. Lloyd,) pR-CrPV-IRES-F (gift of Dr. Ruggero), pR-p53-IRES-F plasmids (gift from Dr. Mazumder), pR-VEGF-A -IRES-F (gift of Prof. G.J. Goodall) and pR-

Hsp70-IRES-F (gift of Prof. I.N. Shatsky), pR-LUC (Promega) and pRL-OCT4A-5'UTR-A using the mMessage mMachine T7 or SP6 kits (Ambion). Cells were transfected with 0,4μg RNA/sample using Lipofectamine 2000 (Invitrogen) following the manufacturer instructions. After 8 hr transfection, cells were harvested and analyzed with dual-luciferase assay kit (Promega) following the manufacturer instructions.

Western Blot

Whole cell protein extracts and subsequent SDS-PAGE and immunoblot analysis were carried out according to standard procedures. Briefly, total cellular proteins were extracted in the lysis buffer (0.1 M KH2PO4 pH 7.5, 1% Igepal (NP-40) added with 0.1 mM β-glycerophosphate and complete protease inhibitor cocktail (Roche Diagnostics) 1X). Protein concentrations were evaluated using Bradford assay by Biorad Protein Assay (Biorad). Denatured protein samples were separated in 10% SDS polyacrylamide gels and transferred to cellulose nitrate membranes (Hybond C Extra, Amersham). Filters were then saturated with 5% non fat dry milk powered dissolved in TBS-T solution (20 mM Tris-HCl, 137 mM NaCl pH 7.6 added with 0.1% Tween 20, all Sigma-Aldrich) and incubated overnight at 4°C with primary antibodies. The next day membranes were incubated in the presence of horseradish peroxidase–labeled secondary antibody (Santa Cruz Biotechnology, dilution 1:10.000). The horseradish peroxidase activity was detected using an enhanced chemiluminescence kit ECL (GE healthcare). The following antibodies were used: anti-dyskerin (Santa Cruz Biotechnonogy,), anti-p53 (clone BP-53-12, Novocastra), anti-β-actin (Sigma-Aldrich), anti-eIF4G (Cell Signaling), anti-eIF4A (Cell Signaling), anti-eIF4E (BD-Bioscience), anti-phospho-4E-BP1 (Cell Signaling), anti-HIF1α (BD bioscience), anti-Oct4 (Cell Signaling), anti-Sox2 (Cell Signalling), anti-c-Myc (Cell signalling), anti-Snail (Cell Signalling), anti-

Slug (Cell Signalling), anti E-cadherin (Cell Signaling) and anti-fibronectin (Ab Cam).

VEGF-A quantitative evaluation

The quantitative evaluation of VEGF-A was made by using an enzyme-linked immuno-assorbent assay (ELISA, Quantikine Kit, R&D) following the manufacturer instruction. The day before performing the assay, MCF7 medium was changed and a less volume of the same medium was put on the cells in order to concentrate the protein. The day of performing the assay the supernatant was collected and the particulates removed by centrifugation. The results coming from spectrophotometer were elaborated as following described. Average of the duplicate readings for each standard, control, and sample were made and the average zero standard optical density subtracted.
A standard curve was created by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data were linearized by plotting the log of the VEGF concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. Samples concentration was determined on the standard curve. If samples were diluted, the concentration read from the standard curve were multiplied by the dilution factor. Results are presented after relative normalization in respect to the control.

Analysis of protein synthesis in cells

Protein synthesis was measured as the rate of incorporation of labelled leucine during a 30 minutes incubation of the subconfluent MCF7 cell monolayers in complete medium containing 50 mg/l leucine and trace amounts of [^{3}H]leucine. Briefly after the incubation, proteins were precipitated with fresh 10% trichloroacetic acid. Finally the protein were

recovered adding 0,2 N KOH. Part of this solution was used to quantify the proteins and part was used to count the radioactivity. The final level of protein synthesis is obtained after normalize the radioactivity to the total amount of proteins rescue.

Isolation of polyribosomal mRNA

Subconfluent cells were harvest and pelleted. The cellular pellet was lysed in 2 volumes of 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ 0.5% NP-40 for 10 minutes at 4°C. The lysates was then centrifuged at 14000 x g for 10 min at 4 °C and the supernatant was used for the isolation of polyribosomes. Lysates were stratified onto a 15-50% sucrose gradient in 30 mM Hepes/KOH (pH 7.5), 80 mM KCl, 1.8 mM Mg-Acetate, and centrifuged at 4°C for 15 h at 40000 x g. From gradients, 1 ml fractions were collected, reading their 260 nm absorbance. Polyribosomal fractions were pooled and centrifuged at 10000g for 15 h at 4°C. RNA was extracted from pellets using the TRI reagent.

Adhesion Assay

Wells of a 96 well plate were coated with 0.2% gelatin or 0.01% collagen or 20 µg/ml fibronectin (all Sigma–Aldrich) for one hour at 37°C. As negative control, some wells were left uncoated. After washing the wells few times with 0.1% BSA in RPMI medium (washing buffer), wells were blocked with 0.5% BSA in RPMI medium (blocking buffer) at 37°C for 45-60 min. In the mid time, MCF7 were counted and resuspended at the concentration of 400000/ml. After washing the wells, 50 µl of cells suspension was added to each well and a triplicate for each samples were done. The plate was incubated at 37°C for 30 minutes and then rocked at 2000 rpm for 10-15 sec. After washing the cells were formalin fixed and stained with 0.05% crystal

violet in 20% methanol for 10 min. Finally the plate was read in a multiwell-plate spectrophotometer at 450µm. The results are calculated in respect to the value belonged to the plastic samples.

Clonogenic assay

MCF10A and HMEC/TERT cells were seeded in duplicate in a 6 wells plate at the concentration of 250 cells/well in normal medium. The next day the samples were put inside the hypoxia chamber or were left inside the incubator at normal oxygen concentration. The cells were maintained in such condition for 7 days, fed with fresh medium every two day. Then they were fixed in methanol and stained with 0,5% crystal violet in 25% methanol for 20 min. Finally, the colonies were counted. Results are presented as average of the number of colonies counted in each well for each condition.

Colony forming assay (soft agar assay)

This assay was performed in a 6 well plate in duplicate. The bottom layer (base agar) consisted of 2ml/well MCF10A and HMEC/TERT growing medium and 0.7% agarose. The base layer was left 2 hours at room temperature to solidify. 250000 cells were added to 1ml of the same culture medium contaning 0.35% agarose and plated on top of the bottom layer (top agar). Cells were incubated o/n at 37ºC. Next day 1 ml of culture medium was placed on the top of each well. Samples were cultivated in hypoxia or normoxia for 20 days. Bottom medium was replaced every two days with fresh one. Finally the soft agar samples were washed with PBS and stained with 0,02% crystal violet in water for 1 hour at room temperature. The excess of crystal violet was washed with PBS and pictures were made. The results are presented as the percentage of the average of the number or colonies

counted in each slide dividing the colonies in accord with their size (smaller or bigger than 30 µm).

Three-dimensional growth of mammary epithelial acini

45 µl of growth factor reduced Matrigel (BD Bioscience) was added to each well of an 8 well chamber slide (BD Bioscience) on ice and spread evenly. The slides were placed on the cell incubator to harden fro 30-40 min. MCF10A and HMEC/TERT were typsinazed and counted and resuspended in Assay Media (same as MCF10A and HMEC/TERT growth media but with 2% horse serum, 5ng/ml EGF and 2% Matrigel) at a concentration of 5000 cells/well. 400 µl of cell suspension/well was plated on top of hardened matrigel. Cells were grown in normoxia or hypoxia 13 days and media was replaced every 4 days with Assay Media.

Immunofluorence of mammary epithelial acini

During day one, cells were fixed with 2% paraformaldehyde (PFA)(Fisher Scientific) in PBS pH 7.4 for 20 min at RT. The cells were permeabilized with 0.5% Triton X-100 in PBS for no longer than 10 min at 4ºC and then where rinse 3 times with 100 mM glycine in PBS fro 10 min each. The cells were incubated with 200 µl/well of 0.2%Triton X-100, 0.1% BSA, 0.05%Tween 20 and 10% goat serum in PBS (primary block solution) for 1 hour at room temperature. The primary block was aspirated and the cells were incubated with the primary antibody in primary block solution at 4ºC O/N.

During day two, chamber slides were washed three times with 0.2%Triton X-100, 0.1% BSA, 0.05%Tween 20 in PBS (IF wash) for 20 min each. The cells were incubated with the secondary antibody (1:100) in IF plus 10% of

goat serum for 50-60 min at room temperature. The slides were kept in the dark until the end. The cells were rinse two times with PBS for 10 min and incubated with TOPRO-3 (Invitrogen) in PBS (1:200). The cells were rinse one with PBS and the plastic chamber where separated from the glass slide The slide were mounted with the Vectashield Hard-set mounting media+DAPI (Invitrogen) and allowed to dry at room temperature O/N in the dark. Slide were visualized using a confocal microscopy at 63X magnification.

The following primary antibodies were used: anti-LamininV (mouse, Millipore), anti-α6-integrin (rabbit, Santa Cruz Biotechnologies) and anti-GM130 (rabbit, Ab cam). The following secondary antibodies were used: goat FICT anti-mouse (Invitrogen) and goat TRITC anti-rabbit (Ab cam).

Study of the secondary structure of the 5'UTR

To study the structure of Oct4 and Sox2 5'UTRs, the Vienna RNAfold server was used. This is a free server accessible via the Vienna RNA webserver at http://rna.tbi.univie.ac.at/. Input consists of a single sequence that has to be typed or pasted into a text field of the input form. The server predicts the minimum free energy (mfe) structure of a single sequence and the structure graph among other informations. The first 58 nucleotides of isoforms 2 of Oct4 mRNA sequence (NM_002701.4) and the first 438 nucleotides of Sox2 mRNA sequence (NM_003106.3) were used.

Cloning

The pRL-OCT4A-5'UTR-A plasmid was derived from the insertion of first 55 mucleotides presented in the 5'UTR of Oct4 mRNA (NM_002701.4) in to the pRMLA vector[108] after digestion with BamHI and NcoI enzymes. The construct were verify by DNA sequencing.

Statistical analysis.

The Chi-square or Mann-Whitney U tests, when appropriate, were used for the comparisons among groups. All statistics were obtained using the SPSS statistical software package (SPSS Inc., Chicago, IL). Values for p less than 0.05 were regarded as statistically significant.

RESULTS

Modulation of IRES-mediated translation trough DKC1 knock-down and its consequences on the behaviour of cancer cell

To study the impact of IRES-dependent mRNA translation on cancer cancer cells behavior, I first investigated the consequences of the modulation of selective IRESs present in specific mRNAs on cell proliferation, survival and adhesion to specific substrates..

To modulate or control the IRES-dependent translation I used the RNA interfering technique to transiently reduce DKC1 mRNA levels in a breast cancer cell line (MCF7). As described in the introduction of this thesis, DKC1 mRNA knock-down (KD) as well as DKC1 mutations, influenced the translation of the IRES containing XIAP, p27 and Bcl-XL mRNAs. After checking the effective reduction of dyskerin expression by Real time RT-PCR and/or Western blot analyis, I evaluated the IRES-mediated activity of viral (EMCV, HCV and CrPV) and cellular (tumour suppressor p53, growth factor VEGF-A and response protein hsp70) mRNAs by using a bicistronic assay. Particularly the cells are transfected with a bicistronic mRNA in vitro transcribed in which the IRES sequence of my interest is inserted between the two reporter cistrons. Finally the activity of the two reporter luciferases *(Renilla* and *Firefly*) is evaluated. Then I estimated the capability of the DKC1 siRNA transfected cells to proliferate and the cell death rate. Finally I investigated the ability of these cells to adhere to different substrates may mediate metastatisazion. The results of this first part of my thesis are shown below.

DKC1 KD impairs viral IRES-mediated translation in MCF7 breast cancer cell line

To evaluate the translation mediated by each of the four IRES types in which the viral IRESs have been classified, I decided to use the EMCV IRES for Type1 and 2, HCV IRES for Type 3 and CrPV IRES for type 4. The results show that strong reduction in DKC1 mRNA and dyskerin protein by transient RNAi in breast cancer cells MCF-7 (Figure 26,A) led to a significant reduction of the translation mediated by the CrPV IRES (Figure 26,B). In contrast, DKC1 KD doesn't impair the translation mediated by the HCV (Figure 26,C) and EMCV IRESs (Figure 26,D). These results can be interpreted considering the different regulation on which each viral IRES classes can be subjected.

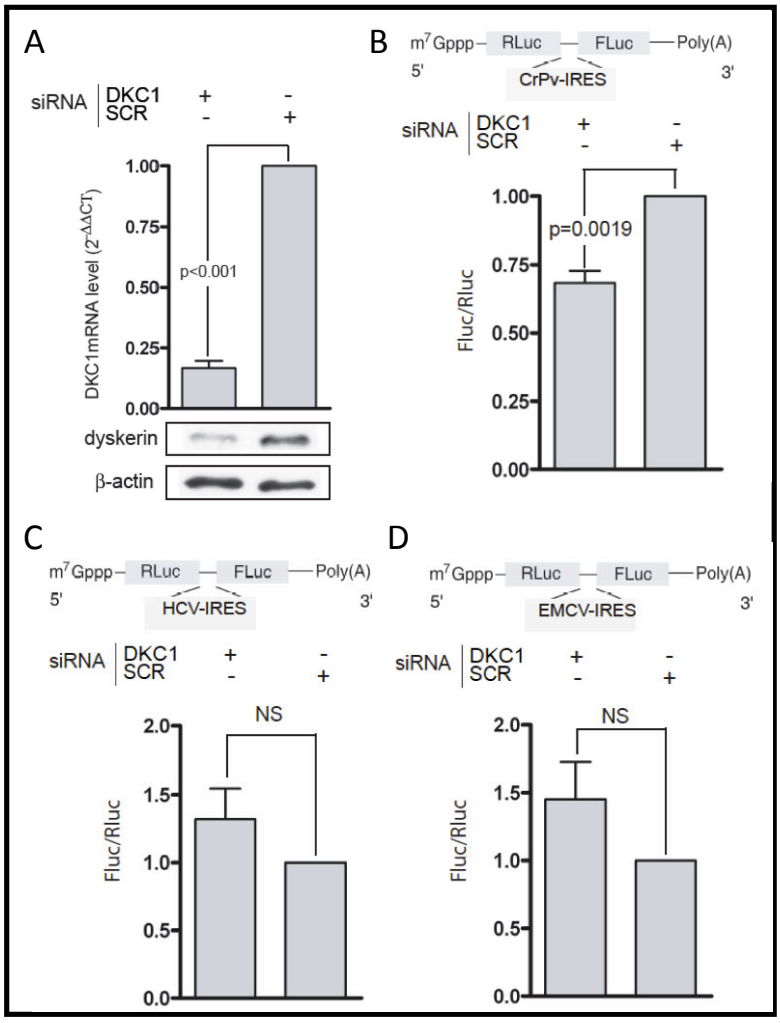


Figure 26. Dyskerin knock down impairs viral IRES translation inititation. A, dyskerin expression by Real-time RT-PCR and Western blot analysis in MCF-7 cells after transfection with DKC1-specific or control (SCR) siRNAs. B,C,D. IRES-mediated translation assessed by measuring the FLuc and RLuc activity in MCF-7 cells after DKC1 and control (SCR) siRNA transfection and 8 hours after transfection with a bicistronic mRNA transcribed from pRL CrPV-IRES (B), pRL HCV-IRES(C) and pLR EMCV-IRES(D). siRNA transfection was performed 96 hours before cell harvesting. Histograms represent means and SDs from three independent experiments. P value <0,05 are considered significative. NS= not significant.

DKC1 KD impairs cellular IRES-mediated translation in MCF7 breast cancer cell line

As the rate of the viral IRESs translation initiation seems to vary accordingly with the viral type, I wondered if cellular IRES translation initiation can have a similar heterogeneous behaviour in cell within reduced DCK1 expression and if it is true, if this led the breast cancer cells to be committed to a more aggressive phenotype. To answer the questions I decided to analyze the IRES-mediated translation of proteins that can have important role in cancer. Through a research in the IRESsite database, I decided to consider the translation mediated by the IRESs of a tumour suppressor (p53), of a growth factor profoundly involved in the tumour angiogenesis (VEGF-A) and of a stress response protein clearly associated with loss of differentiation in breast cancer (Hsp70).

DKC1 KD strongly reduced the p53-IRES-mediated translation (Figure 27,A) but increased the VEGF(Figure 27,B) and Hsp70-IRES(Figure 27C) mediated translation. The results show for the first time that translational alteration due to reduced dyskerin levels can up or down regulate IRES-translation initiation of specific mRNAs. As a consequence it can be speculated that IRES-mediated translation of mRNAs is important for cell proliferation, angiogenesis, etc., can drive cell to a more “aggressive” behavior.

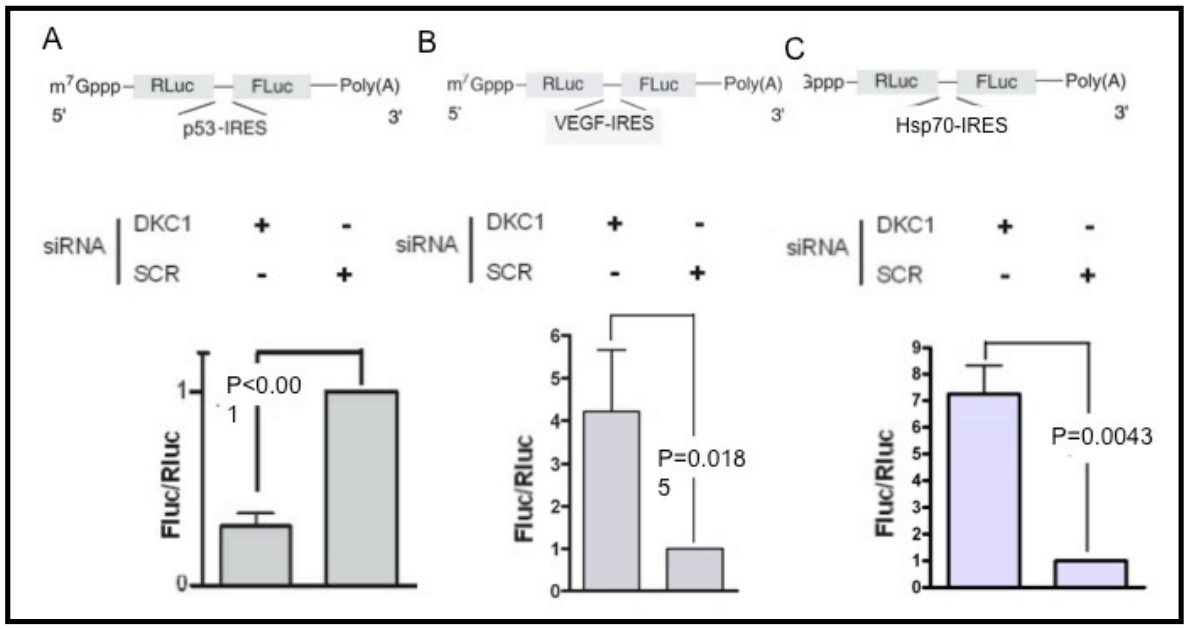


Figure 27. Dyskerin knock down impairs cellular IRES translation inititation. A,B,C, IRES-mediated translation assessed by measuring the FLuc and RLuc activity in MCF-7 cells after DKC1 and control (SCR) siRNA transfection and 8 hours after transfection with a bicistronic mRNA transcribed from pRL-p53-IRES (A), pRL- VEGF-A-IRES(B) and pRL Hsp70-IRES(C). siRNA transfection was performed 96 hours before cell harvesting. Histograms represent means and SDs from three independent experiments. P value <0,05 are considered significative. NS= not significant.

Impairment of cellular IRES-dependent translation is combined with altered protein expression in humanMCF7 breast cancer cell

I then investigated if the observed translational defect of p53 and VEGF-A mRNAs could affect p53 and VEGF-A protein levels. Measuring the levels of p53 by western blot, I found that DKC1 KD strongly down-regulated the its levels (Figure 28,A). As VEGF-A is a soluble protein that, once produced, is secreted, using an ELISA array I measured the level of the VEGF-A protein in the supernatant of the MCF7 (Figure 28,B). DKC1 KD leads to an elevated increase of secretion of the protein.

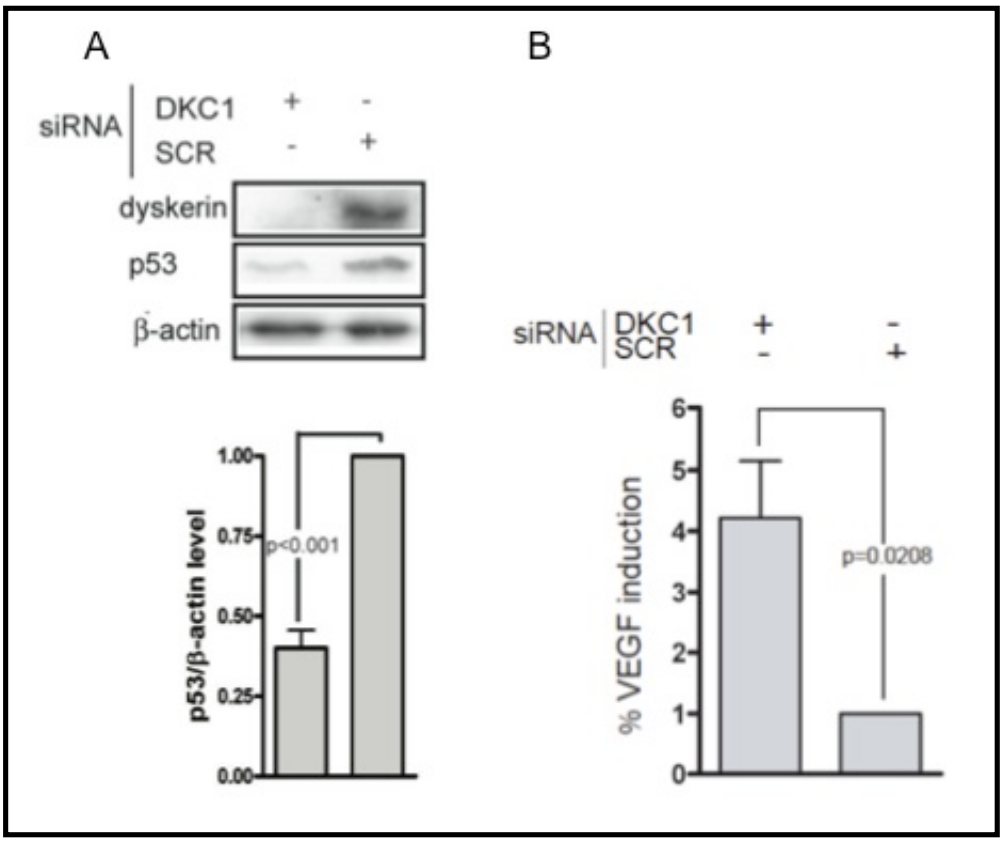


Figure 28. Impairment of cellular IRES-dependent translation is combined with altered protein expression. A, representative immunoblots showing steady state p53 levels in DKC1 KD and control MCF-7 cells; B, histogram showing the level of VEGF-A protein detected using an ELISA array. Experiments were performed in MCF-7 cells. siRNA transfection was performed 96 hours before cell harvesting. Histograms represent means and SDs from three independent experiments. P value <0,05 are considered significative.

Protein expression is not combined with mRNAs levels

Because the reduction of dyskerin levels did not cause impairment in protein synthesis as well as in mRNA translation (Figure 29), to clearly address the effect of translation on p53 and VEGF-A protein expression showed in Figure 28 I decided to investigate the levels of p53 and VEGF-A mRNAs in MCF7 with DKC1 KD and in the control both in the total mRNAs extract and in the polysomal fractions. Polysomes are clusters of ribosomes

associated with mRNAs, indicating that the mRNA is translationally active. It is possible to isolate the polysomal component as far as the 40S, 60S and 80S components on the basis of their molecular weight by ultracentrifugation on a sucrose gradient and to extract the mRNA bounded.

While DKC1 KD had no significant effects on global p53 mRNA levels, it strongly decreased the global VEGF-A mRNA levels (Figure 30,A). Interestingly, DKC1 KD strongly reduced the recruitment of the p53 mRNAs to polysomal fractions whereas the VEGF-A mRNAs levels seems to be recovered (Figure 30,B). As mentioned above, previous studies indicated that as for DKC1 mutations, DKC1 KD influence the translation of a limited list of cellular IRES containing mRNAs including those encoding for XIAP, Bcl-Xl, p27[(100)]. I selected XIAP to confirm that results in my system. From Figure 30 it's clear that DKC1 KD also influenced the translation of the IRES containing XIAP mRNA, while it did not affect the translation of the beta-actin house-keeping mRNA. These results clearly demonstrate that the effect on the expression of two proteins important for cancer development and progression as p53 and VEGF-A is due to the translational control and might be explained by the alterations in the IRES-mediated translation.

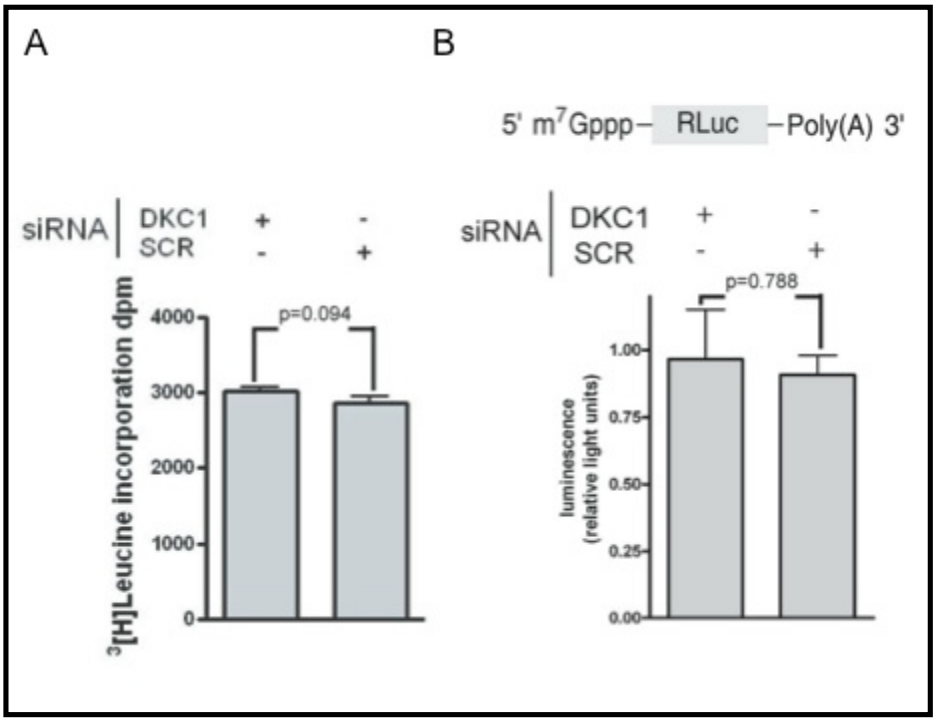


Figure 29. Global protein synthesis and mRNA translation in MCF7 DKC1 KD cells. [^{3}H]-leucine incorporation (A) and Renilla luciferase (Rluc) activity (B) after transfection of an in vitro transcribed capped mRNA in DKC1 KD and control MCF-7 cells. siRNA transfection was performed 96 hours before cell harvesting. Histograms represent means and SDs from three independent experiments. p values are > 0,05 so the differences are not significative.

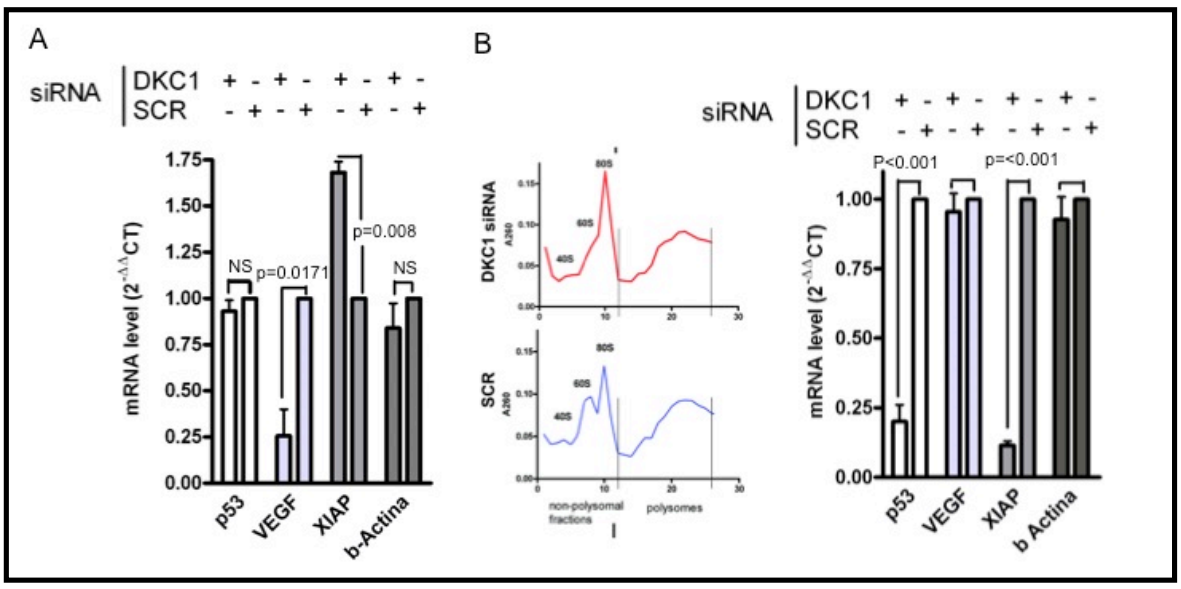


Figure 30. mRNA expression in MCF7 DKC1 KD cells. Total (A) and polysome-associated (B) p53, VEGF-A, XIAP and β-actin mRNA levels assessed by real time PCR after DKC1 KD. Representative polysomal profiles are shown. P value <0,05 are considered significative.

Contribution of the IRES-mediated translation on cancer cells behavior

To evaluate the functional consequences of the translational impairment observed in the IRES-translation on the neoplastic progression, I evaluated the capability of DKC1 siRNA transfected MCF-7 cells to growth or to die in respect of their controls. Indeed I exposed the cells to different kind of stresses. Particularly I treated the cells with the genotoxic agent doxorubicin and the hypoxic mimetic agent desferoxamine (DFX) and then analyzed the capability of the cells to respond to these stresses in term of proliferation and death rate. In basal conditions, DKC1 siRNA transfected MCF-7 cells have a growth advantage and death resistance. Significantly, after DKC1 KD, cells on one side, became resistant to doxorubicin-induced death and to DFX-mediated cell death and on the other side they acquire a growth advantage under doxorubicin treatment.(Figure 31,A,B,C)

An important phase of the tumorigenesis is the capability of cancer cells to invade tissues, a property that drives the metastatization. The first step in this process is the cellular adhesion to the extracellular matrix. Consequently I evaluated the ability of MCF7 with DKC1 KD to adhere to substrates that are present in the extra cellular matrix. Particularly I selected laminin, fibronectin and collagene. The DKC1 KD MCF7 cells showed the capability to better adhere to fibronectin and collagene substrates if compared to the control cells (Figure31,D).

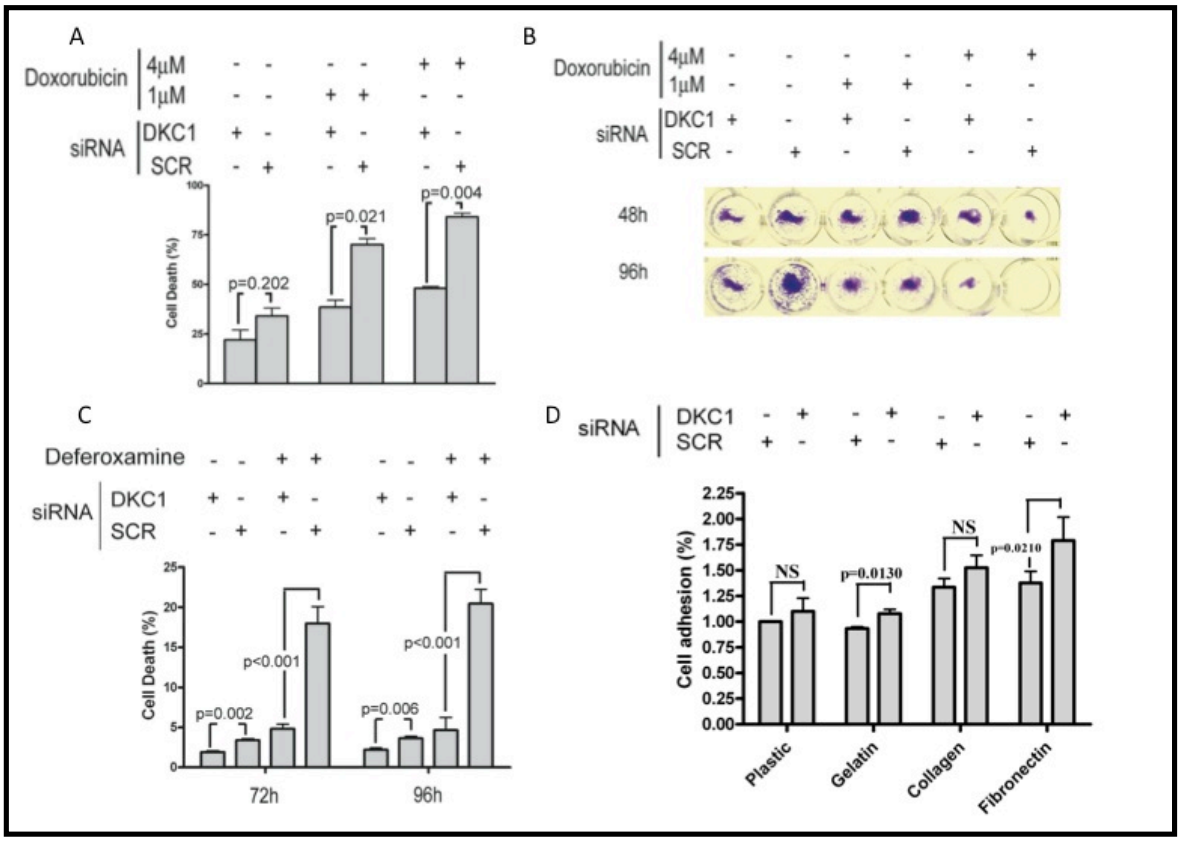


Figure 31. Contribution of IRES-mediated translation to malignancy. A, cell death by Trypan blue exclusion test under doxorubicin. B, growth after crystal violet staining analyses under doxorubicin treatment. C, cell death analysis under DFX treatment is also shown. D, adhesion assay performed with gelatin, collagene and fibronectine. Experiments were performed in MCF-7 cells. siRNA transfection was performed 96 hours before cell harvesting. Histograms represent means and SDs from three independent experiments. P<0,05 are considered significant.

In summary the data obtained indicated that (I) DKC1 KD can up or down regulate the IRES-mediated translation of viral or cellular mRNA; (II) the impairment in the IRES-mediated translation on one side led to reduce the expression of proteins (such as p53 as here demonstrated or p27 as previously demonstrated[100]) that usually block cell proliferation and on the other side led to increase the expression of proteins (such as VEGF-A or Hsp70) that drive the cell proliferation; (III) the effect on the protein expression can be ascribed to an alteration in translational control; (IV) IRES-translation deregulation led the breast cancer cell to have a more aggressive phenotype especially when subjected to stresses.

Hypoxia impacts on the expression of the cancer stem cells markers through translational control

As hypoxia is a condition in which mRNA cap-dependent translation is down-regulated and as it seems to be important in the maintenance of the cancer stem cells (CSC) population of a tumor and in the epithelial/mesenchymal transition (EMT), I focused my attention on how hypoxia impacts on breast tumour stem cells through translational control. For these studies I used a hypoxia cell growth chamber in which I could control oxygen levels very accurately. I used MCF10A cells, a poorly transformed (immortalized) early stage breast ductal carcinoma line and normal human mammary epithelial cells immortalized with telomerase (HMEC/TERT). I performed all experiments at 0.5% O_2 to reproduce the low levels of oxygen in the tumour mass. Cells were subjected to 24 hours and 144 hours of hypoxia. I first verified the status of the cap-dependent translation by immublotting and then evaluated the expression of stemness (Oct4, Sox2 and c-Myc) and EMT markers (Slug and Snail). Indeed I investigated if the cells under hypoxia conditions can acquire the capability to self renew and to better growth in respect of cells grown in normal oxygen conditions. Finally, the involvement of the IRES-mediated translation is evaluated.

Hypoxia inhibits cap-dependent translation and drives the expression of stemness markers in low transformed cell lines

I verified the expression of the major proteins involved in the translation initiation such as eIF4A, eIF4G, eIF4E and the expression of the eIF4E regulator, 4E-BP1 (Figure 32,A). The data show no differences in the expression of the initiation factors but demonstrate a reduction in the

phosphorylation state of 4E-BP1. This is in line with the principle that under hypoxic conditions, there is a strong downregulation of the mTOR pathway that results in the hypophosphorylation of the negative regulator of cap-dependent protein synthesis, the protein 4E-BP1.

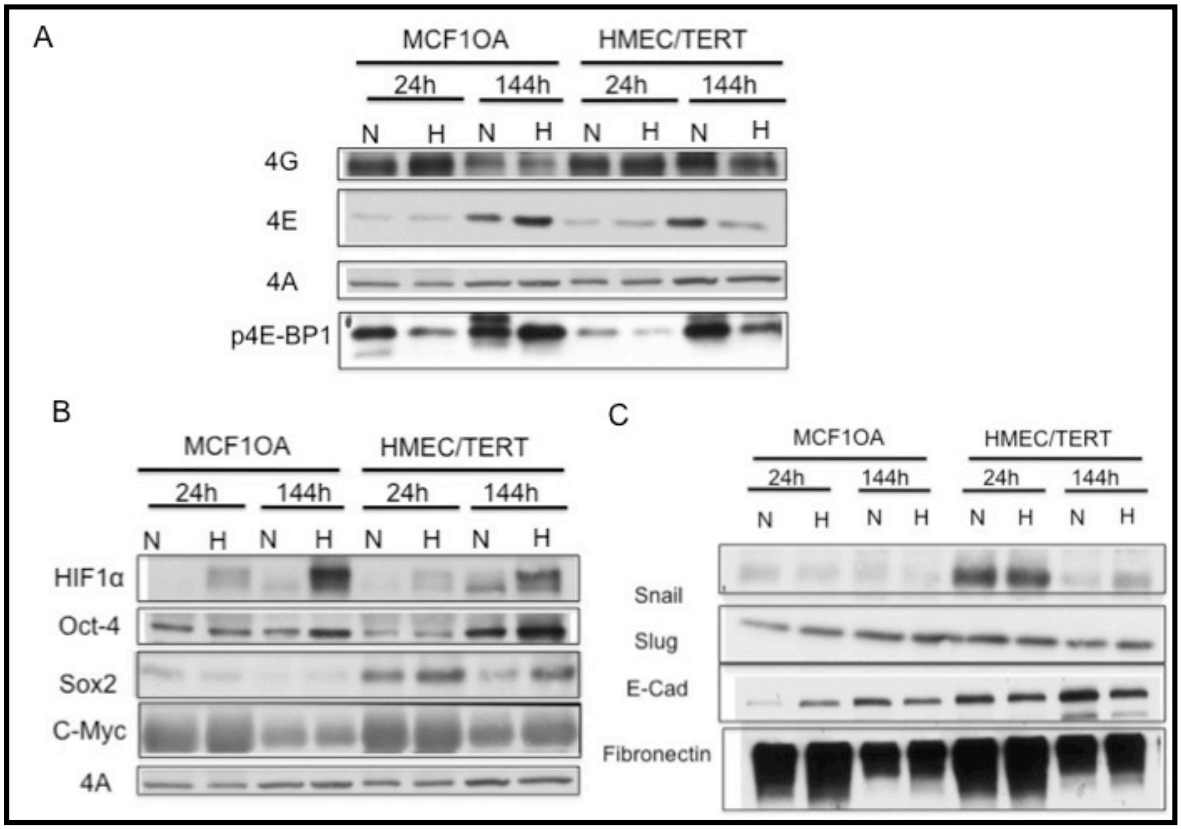


Figure 32. Hypoxia inhibits cap-dependent translation and drives the expression of stemness markers in MCF10A and HMEC/TERT. A, rappresentative immunoblotting for the expression of the major proteins involved in translation initiation. B, rappresentative immunoblotting fort he expression of the three stemnness master factors. C, rappresentative immunoclotting of some EMT markers. Experiments were performed three times in MCF10A and HMEC/TERT under normoxia (N) or hypoxia (H, 0,5% O_2) for 24 or 144h. The right loading of the gel was evaluated in respect of eIF4A expression.

After I verified that the cells were really under hypoxic conditions by the Western Blot analysis of the induced expression/stabilization of HIF1α protein, I examined the expression of factors that are well established to be involved in the promotion of cell "stemness", that is, stem like characteristics. The three essential master factors of cell stemness established in literature are Oct4, Sox2 and c-Myc. Under hypoxia in both MCF10A and HMEC/TERT cells, there is a significant increase in the

expression of Oct4, Sox2 and c-Myc, especially after 144 hours of hypoxia (Figure 32,B). Instead in highly transformed MDA MB-231 the expressions of the three masters factors is undetectable (data not shown). Since stemness is very often associated with EMT I examined the expression of factors that drive directly this mechanism (Figure 32,C). The results obtained demonstrated that in weakly transformed cells under hypoxia, the EMT is actually blocked.

Contribution of hypoxia to cancer cells behaviour

To investigate the consequences of the up regulation of the stemness markers into the selection of a more aggressive subgroup of cells I first performed a clonogenic assay. Clonogenic assay is based on the ability of a single cell to grow into a colony. The assay essentially tests every cell in the population for its ability to undergo "unlimited" division. Only a fraction of seeded cells retains the capability to produce colonies. Results showed no significant differences in the number of colonies formed under hypoxia by the MCF10A cells but an increased in the number of colony formed by HMEC/TERT (Figure 33). This means that the HMEC/TERT has higher capability of cell renewal compared with MCF10A. The fact the MCF10A are much more transformed than HMEC/TERT could explain the non-significance observed in the MCF10A cells.

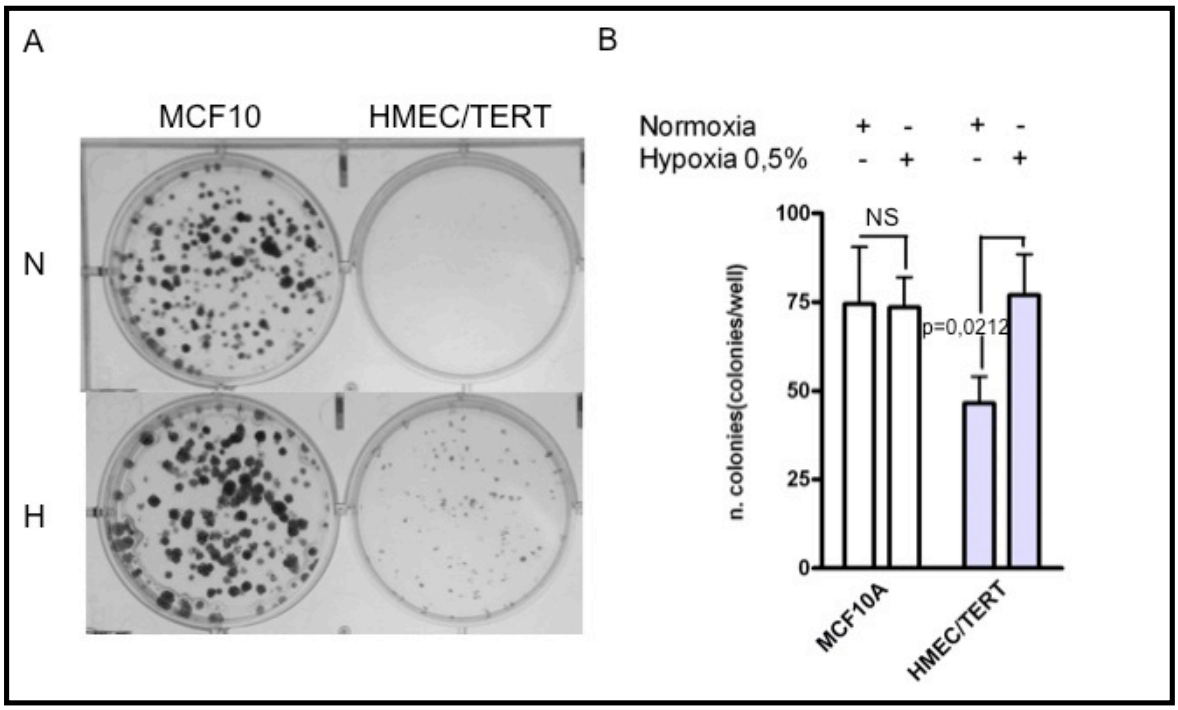


Figure 33. MCF10A and HMEC/TERT ability to self–renew is increased under hypoxia. Clonogenic assay of MCF10A and HMEC/TERT. A, Pictures represent the colonies formed after cultivating MCF10A and HMEC/TERT under normoxia (N) or hypoxia (H). B, histograms representing the number of colonies counted at the end of the treatment. Histograms represent means and SDs from three independent experiments. P<0,05 are considered significant.

Compared to non-transformed cells, neoplastic cells are much less contact-inhibited, exhibit anchorage-independent growth, and can proliferate in the absence of exogenous growth factors. As shown in Figure 34,A MCF10A in hypoxic conditions form bigger colonies. Instead for HMEC/TERT (Figure 34,B) no differences are shown. Importantly even if there were no differences in the number of the colonies, the colonies growing in hypoxic conditions, were really bigger for both the cells lines.

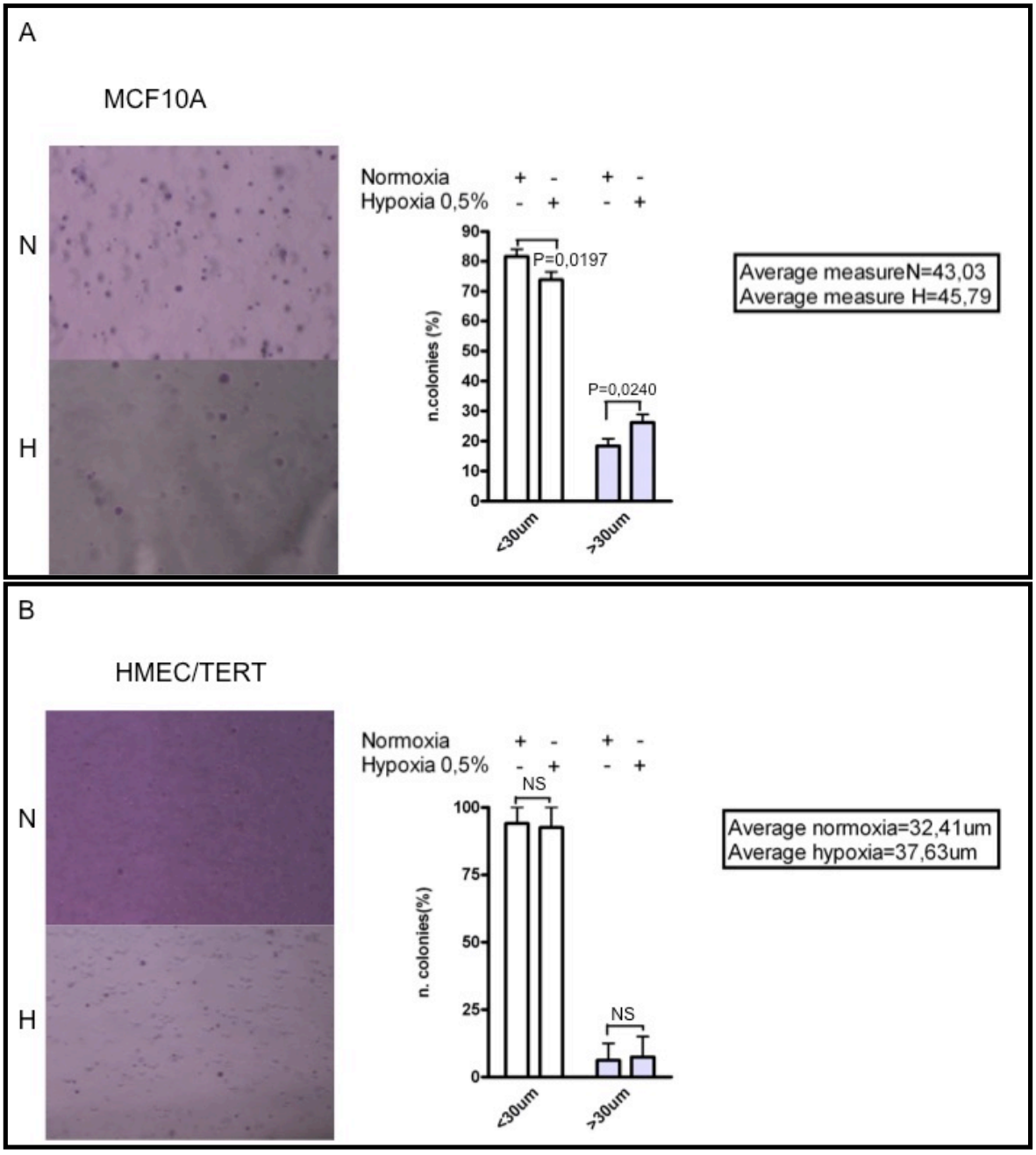


Figure 34. The growing rate of MCF10a and HMEC/TERT is increasing under hypoxia. Soft agar assay of MCF10A and HMEC/TERT. On the left side of panel A and B representative pictures of MCF10A (A) and HMEC/TERT (B) cells colonies grown in a semisolid medium in normoxia (N) or hyoxia (H) are reported. The colonies were divided in two groups accordingly to their size (< 30μm and >30μm). Histograms (right side of each panels) represent the number of colonies counted in each groups at the end of the treatment. Histograms represent means and SDs from three independent experiments. P<0,05 are considered significant. In the bold square of each panel. the average size of the colonies is reported.

Finally, because cells grown as monolayers don't recapitulate the glandular structure of epithelium in vivo they don't provide a complete system to fully understand how proliferation, cell death and differentiation can influence the form and function of the glandular epithelium during the early stages of the tumor formation. That's why I decided to use a three-dimensional (3D) culture system to investigate if hypoxia can influence these characteristics. The 3D culture system permits to recapitulate some features of the breast epithelium, including the formation of acini-like spheroids with an inner lumen, apicobasal polarization and basal deposition of the basement membrane components. Importantly transformed cells lose polarization, acquiring the ability to move somewhere else especially if transformed. Results reported in Figure 35 showed that MCF10A acinis grown in hypoxia are not perfect spheroids. Indeed it's possible to see the partial lost of cells polarization as GM130, a Golgi marker apically located, and α6-integrin, a membrane marker basolaterally located, are distributed differently. The same results can be observed in the HMEC/TERT (Figure 36).

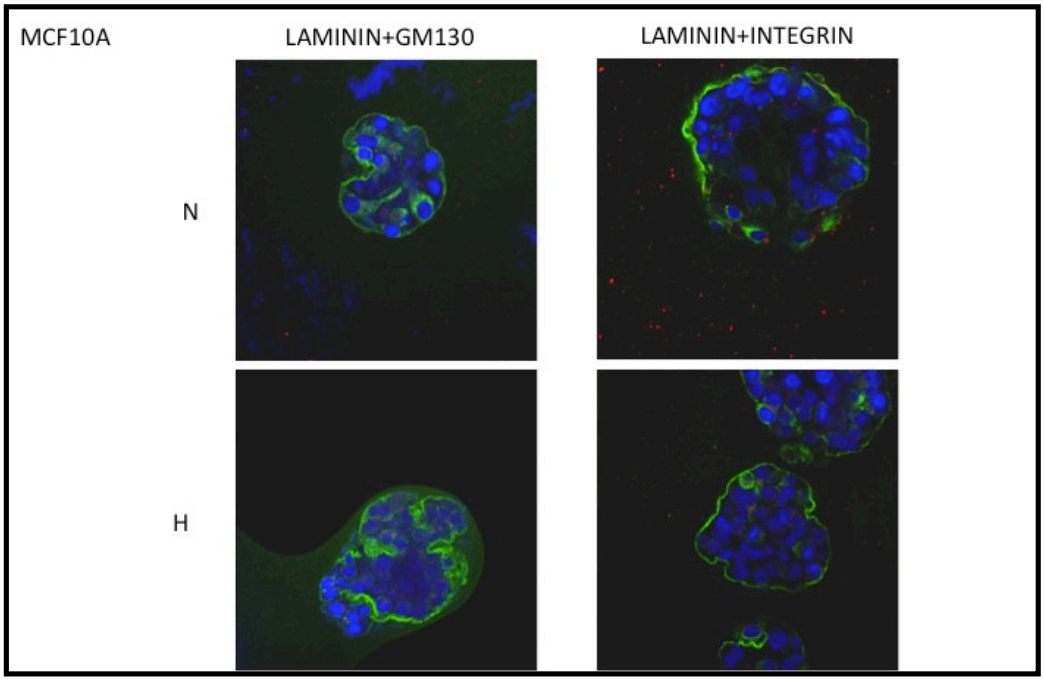


Figure 35. Under hypoxia MCF10A lost the normal acini conformation. Three dimensional culture of MCF10A. Representative pictures of three dimensional culture of three indipendent experiments of MCF10A cells grown in normoxia (N) or hypoxia (H). Green=laminin staining, Red=GM130 or α6-integrin staining as indicated, Blue=nuclei staining.

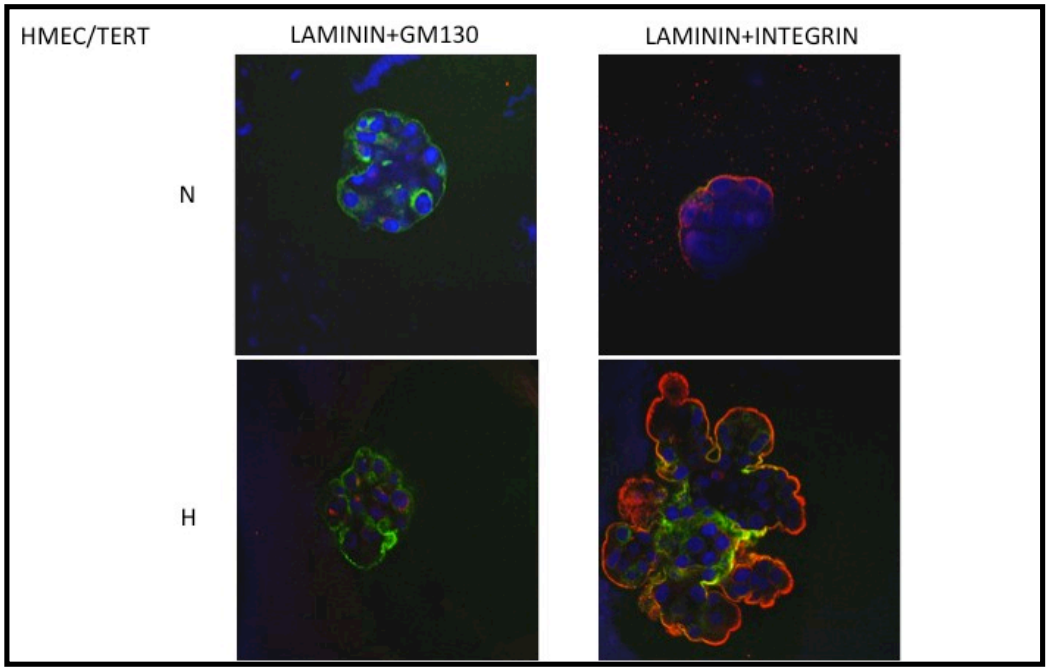
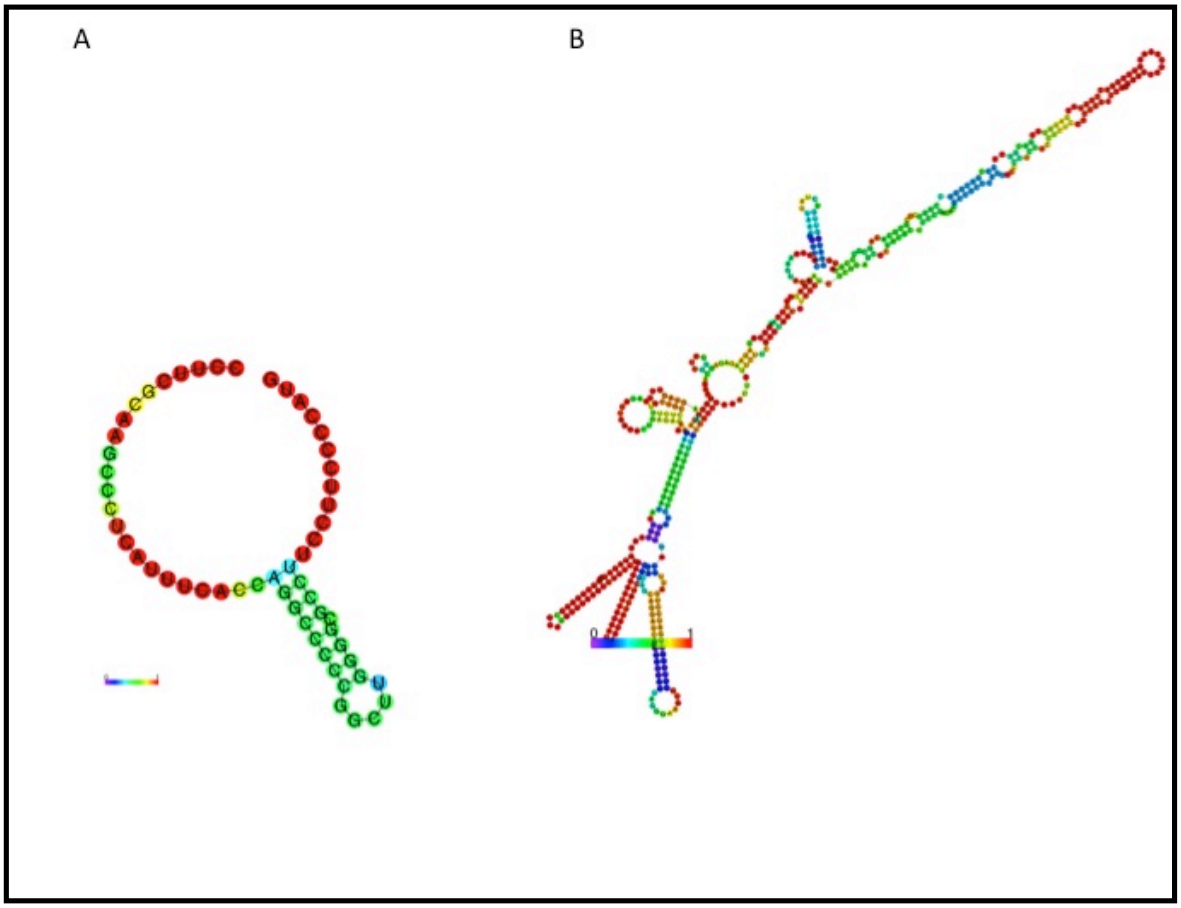


Figure 36. Under hypoxia HMEC/TERT lost the normal acini conformation. Three dimensional culture of HMEC/TERT. Representative pictures of three dimensional culture of three indipendent experiments of HMEC/TERT cells grown in normoxia (N) or hypoxia (H). Green=laminin staining, Red=GM130 or α6-integrin staining as indicated, Blue=nuclei staining.

How translational control drives the phenotype in hypoxia

Another important issue is to understand whether the induction of stemness factors in "non transformed" cell lines is driven by alternative mechanisms of translation initiation. To achieve this aim, I initiated analyzing the sequence of the 5'UTRs in the mRNAs of Oct4 and Sox2 using a computational server (Vienna RNAfold) (Figure 37). I discovered that the 5'UTR of the A isoform of Oct4 (Oct4A), the isoform clearly implicated in stemness, is a 55 bases long sequence with a stem region that is very rich in C-G basepairs and sufficiently stable (free energy is -14,59 kcal/mol)(Figure 37,A). Sox2 5'UTR is a 438 bases long RNA with different stable secondary structures (free energy is -173,18 kcal/mol)(Figure 37,B).



Because the western blot analysis showed that the expression of Oct4 was higher then the expression of Sox2 during hypoxia, I decided to start cloning the Oct4 5'UTR sequence into a bicistronic mRNA translation vector to verify if in this system the expression of the second cistron can be driven under hypoxia by the 5'UTR of Oct4A. I started with MCF10A (Figure 38). Unfortunately there are no differences in the expression of the second cistrons in normoxia and hypoxia. This doesn't exclude the fact that alternative mechanisms of translation initiation can be implicated. Unfortunately, I could not investigate the activity of the 5'UTR of Oct4A in HMEC/TERT and the activity of the 5'UTR of Sox2.

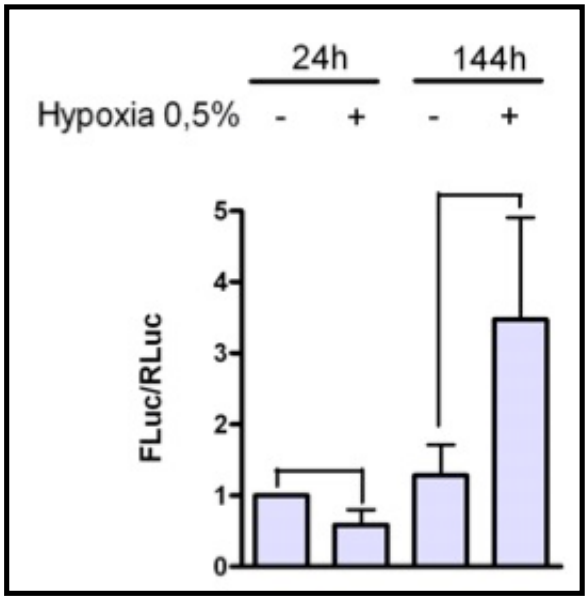


Figure 38. Oct4A 5' UTR has no IRES-activity. Oct4A 5'UTR translation assessed by measuring the FLuc and RLuc activity in MCF10A cells under normoxia or hypoxia for 24 and 144h. Histograms represent means and SDs from three independent experiments. The differences are not significant.

Taken together, the results showed that low levels of oxygen permit the expression of stemness factors in cells that are only weakly transformed. Further studies are necessary to better understand how this can be linked to translational control as the 5'UTR of the Oct4 mRNA seems not to function as an IRES and the possible IRES activity of Sox2 5'UTR mRNA has not been tested yet, but this is the first demonstration of selection of cells with stem cell characteristics (cancer stem cells?) from a pool of less transformed cells under extreme hypoxia condition in which the cap-dependent translation is inhibited.

DISCUSSION

There is increasing evidence that changes in the IRES-dependent mRNA translation may contribute to tumourigenesis and cancer progression. However, the mechanisms involved are far from being clearly defined. For this reason the purpose of my work has been to study how changes in IRES-dependent mRNA translation may led to cancer and facilitate tumour progression. To reach the aim, I modulated the IRES-dependent translation. This was achieved with the use of two different methodological approaches. The first methodological approach regarded the use of the technique of the RNA interfering to transiently reduce in a breast cancer cell line (MCF7), DKC1 mRNA levels, encoding dyskerin. Defect in dyskeryn function, results in a reduction of IRES-dependent translation activity.[100] Thus dyskerin clearly has a role in controlling the IRES-mediated translation initiation. In MCF7 cancer cells line, the control of IRES-mediated translation trough loss of dyskerin led to the decrease in the activity of the tumor suppressor p53 that in turn facilitate cell proliferation. In the same system I observed an increase in VEGF-A IRES-mediated translation associated with an increased in protein secretion. As known, VEGF-A is an important mediator in the angiogenesis, the process that drives the formation of new vessels. It is worth noting that the expression of VEGF is very often associated with a worse prognosis in different kind of cancer, particularly in breast cancer[106]. Furthermore I found that the IRES-mediated translation of Hsp70 is increased in the same system. Importantly, in breast carcinomas the expression of Hsp70 is associated with the lose of differentiation, increased proliferation, metastasis and worse prognosis[105]. The differences shown in the regulation of IRES-mediated translation can be due to the ribosomes qualitative changes consequent to DKC1 KD. Even if an intrinsic functional

ribosomal defect has never been clearly demonstrated, the analysis of the mRNA associated with the polysomal fractions indicated that all the changes in proteins expression can be ascribed to the translational activity.

It is possible to postulate that the defect in the pseudourydilation can led to ribosome structural modification, that in turn encourages a cap-independent translation initiation. Moreover, the present results did not exclude the possibility that the different modulation of IRES-mediated translation shown ca be ascribed to the different expression of the ITAFs, the "alternative" initiation factors mentioned in the previous sections.

The second methodological approach used to study the involvement of the cap-independent translation in cancer was to modulate IRES-mediated translation by exposing two immortalized cell lines (MCF10A cells and HMEC/TERT) to hypoxia. Immortalized but not transformed cells under hypoxia undergo inhibition of protein synthesis, whereas highly transformed cells are largely resistant[37,94,95]. There is evidence that in breast cancer cells, hypoxia activates IRES-dependent translation of mRNAs encoding HIF1α, VEGF-A, p120 catenin and BCL-2 [74] which are responsible for angiogenesis and survival. This was due to the fact that hypoxia induced the overexpression of eIF4G1 and 4E-BP1 whereas reduces eIF4F dependent translation initiation[39]. In addition to the mechanism reported above by which hypoxia can control IRES-dependent translation, local oxygen concentrations can favor the tumorigenesis, influencing stem cell self-renewal and differentiation. In this context, data reported that stem cells, might benefit from residing in hypoxic niches where the oxidative DNA damage is reduced[93]. Consequently it is reasonable to hypothesize that hypoxia can promote the generation of cancer stem cells trough a mechanism that involved IRES-dependent translation changes. For this reason I carried out experiments maintaining two immortalized cell lines (MCF10A and HMEC/TERT) at 0.5% of oxygen, a condition that is very similar to the oxygen concentration within the tumor mass. Hypoxia usually induced a great reduction of the cap-dependent translation, affecting particularly the

initiation phase of this process. Consequently, I verified the expression of the major proteins involved in the translation initiation like eIF4A, eIF4G, eIF4E and the expression of eIF4E's regulator 4E-BP1. The data did not show differences in the expression of the initiation factors but demonstrated a decrease in the phosporylation state of 4E-BP1 demonstrating a reduction of the translation initiation in the cells. In this condition I further investigated the expression of the three stemness master factors, Oct4, Sox2 and c-Myc. In both cell lines, hypoxia induced a greater expression of all stemness markers. Because stemness if very often connected with the EMT, I verified the expression of the factors that drive the EMT, Slug and Snail and the expression of some epithelial markers that had to be lost during the epithelial-mesenchymal transition. However I did not find any changes in these factors in MCF10A and HMEC/TERT in hypoxic conditions. These results may appear to be in contrast with those reported in some papers showing an increased in the EMT markers expression. However those results were obtained analyzing mRNA and not protein expression.

Another interesting finding was that the overexpression of the stemness markers in hypoxic conditions improves the capacity of breast cancer cells to self renewing. Using MCF10A and HMECT/TERT the number of colony units under hypoxia condition was higher that in the control cells. Furthermore, in a contest in which cap-dependent translation is reduced and IRES-dependent translation increased, MCFC10A and HMEC/TERT cells increased the ability to growth in a semisolid environment, that can be considered the first phase of transformation[109,110]. In these experimental conditions, cells seeded in three dimensions and exposed to hypoxia, lose the polarity and seem to "escape" from the normal acinal shape.

As reported above, hypoxia reduced the cap-dependent protein synthesis in MCF10A and HMEC/TERT. This is not the case for cap-independent translation. I consequently hypothesed that the higher expression of Oct4 and Sox 2 might be due to the IRES-translation activity. Unexpectedly I found that the expression of Oct4 was not due to the IRES-mediated translation.

Two possible explanations can be suggested. The first one is the presence of drawbacks into the methodologies used to study the Oct4 5'UTR structure. The second one is that the expression of Oct4 may be due to cap-independent mechanisms not linked to IRES-mediated translation.

CONCLUSIONS

The data obtained in this study clarify the function of cap-independent translation in cancer. Particularly the results suggested that the deregulation of IRES-dependent translation, such as characterized Dyskeratosis congenital, can be considered a sort of pro-oncogenic stimulus characterized by the inhibition of the expression of some proteins that block cell growth and proliferation and by the over expression of other proteins that contributed to cell survival. Under stress condition, such as a hypoxic status, changes in IRES dependent translation in an increased ability to survive and even proliferate and therefore in the acquisition of a more aggressive phenotype. In addition in immortalized epithelial cell lines, changes in cap-independent translation are associated with an induction of expression of stem cell markers, and with the selection of a sub group of cells that have an increased ability to self-renewing.

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Notes

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