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Viral Replication Strategies: Manipulation of ER Stress Response Pathways and Promotion of IRES-Dependent Translation

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1. Introduction

Translation initiation is a rate-limiting step of protein synthesis. Therefore, it is highly regulated by different mechanisms, which depend upon the structural characteristics of a given mRNA. Most cellular mRNAs are translated by a cap-dependent mechanism that requires the binding of the trimeric complex of eukaryotic initiation factors (eIF)4F, comprised of eIF4G, eIF4E and eIF4A, to the 7-methyl GpppN cap structure at the 5′ end of the mRNA. However, many viral and some cellular mRNAs have evolved a cap-independent mechanism of translation initiation that uses a highly structured internal ribosome-entry site (IRES) sequence located in the 5′ untranslated region (5′UTR) of their mRNA (Holcik & Sonenberg, 2005). The IRES was first discovered in poliovirus (a typical member of picornaviruses) and later in other viruses such as hepatitis C virus (HCV), HIV, Herpesviruses, etc., and also in many cellular mRNAs (Jang, et al., 1988, Labadie, et al., 2004, Locker, et al., 2011, Pelletier & Sonenberg, 1988). Cellular physiological conditions dictate when a given mRNA uses cap-dependent or IRES-dependent translation initiation. Under normal conditions, cellular mRNAs translation is initiated by a cap-dependent mechanism; however, under stress conditions, such as starvation, irradiation, heat shock, hypoxia, toxin and viral infection, the translation initiation is switched from cap-dependent to an IRES-driven mechanism, which may be on the same mRNA (Komar & Hatzoglou, 2005, Spriggs, et al., 2005).

Several viral infections trigger endoplasmic reticulum (ER) stress responses in a variety of ways inside the host cell. One of the most significant effects is the shutting off of global, cap-dependent translation, which results in activation of IRES-dependent translational mechanisms. This is quite apparent in picornaviruses because their viral mRNA does not

contain a cap structure at the 5'end. Also, its IRES located in the 5'UTR recruits ribosomes and other factors, which then scan to reach the initiation codon without the requirement of the eIF4E (Jang, et al., 2009, Jang, 2006). IRES containing viruses are able to benefit from the ER stress response, enhancing their own protein synthesis while also enhancing their self-defense capability. There are several mechanisms by which virus infections and other stress signals achieve inhibition of cap-dependent translation of cellular mRNAs, including: i) site specific cleavage of cellular translational initiation factors, such as the eukaryotic translation initiation factor 4GI (eIF4GI) by picornaviral and HIV proteases (Chau, et al., 2007, Etchison, et al., 1982, Lamphear, et al., 1993, Ohlmann, et al., 2002) or by cellular caspases (Marissen & Lloyd, 1998). ii) phosphorylation of eIF2α and other co-factors of translation. The site specific cleavage or modification of translation factors does not affect IRES-driven translation, but instead promotes IRES-containing mRNA to utilize the cleaved translation initiation factor or specific IRES transacting factors (ITAFs) for their translation (Morley, et al., 2005, Raught, 2007). (iii) overproduction of homologous proteins of cap-binding protein eIF4E (e.g. 4E-BP), which compete with eIF4G limiting its binding (Marcotrigiano, et al., 1999) to eIF4E iv) suppression of eIF4E expression by certain microRNAs (Ho, et al., 2011, Mathonnet, et al., 2007).

The rapid inhibition of cellular cap-dependent protein synthesis has been demonstrated as a critical precursor to cell fate. In this context, it is noteworthy that the IRES-containing cellular mRNAs are found to be preferentially involved in the control of cell fate by functioning to promote cell growth and survival or apoptosis (Jackson, et al., 2010, Sonenberg & Hinnebusch, 2009, Spriggs, et al., 2005). Notable genes include the B-cell lymphoma-2 (Bcl-2) family proteins, apoptotic protease activating factor 1 (Apaf-1), checkpoint homolog kinase 1(chk-1), eIF4GII, p53 and 78kDa Glucose-regulated protein 78 or Binding immunoglobulin protein (GRP78/BiP) (Komar, et al., 2005, Spriggs, et al., 2005). It was therefore suggested that IRES-mediated translation plays a critical role in regulation of cell fate (Spriggs, et al., 2005). Cellular genes containing IRESs in their mRNA are continually being discovered, some amid controversy as being true IRESs (Shatsky, et al., 2010). Previous studies have indicated that the cell fate decision is made based on the severity and duration of the stress signal. Under a transient stress or during the early phase of infection, the IRES will mediate translation initiation of genes promoting cell survival/growth, which enhance cellular capability to combat viral infection. However, under a severe or prolonged stress such as persistent infection of picornaviruses, translation initiation will selectively express the genes responsible for inducing cell apoptosis (Henis-Korenblit, et al., 2002, Lewis, et al., 2008), effectively destroying the host cells and potentially limiting the viral infection of surrounding cells. In any circumstance, the host cell will employ an alternate way to defend itself. In this chapter, we will discuss the recent advances in the understanding of IRES-mediated translational control of genes under stress conditions, with a particular focus on ER stress caused by picornaviral and other viral infections.

2. Viral Manipulation of ER stress pathways and components

The ER stress response or unfolded protein response (UPR) is a major component of disease (Tabas & Ron, 2011). Many viral infections induce ER stress and have adapted mechanisms

to modulate the stress response and its effectors. On the cellular level, ER stress may be triggered by many factors, including serum starvation, hypoxia, changes in calcium homeostasis, viral infections, as well as other perturbations (Chakrabarti, et al., 2011). In general, ER stress is triggered by the accumulation of misfolded or unfolded proteins in the ER lumen. In response to this stress, a coordinated adaptive program termed the unfolded protein response (UPR) is activated and serves to minimize the accumulation and aggregation of misfolded proteins (Chakrabarti, et al., 2011). The molecules and signaling pathways of the UPR may vary slightly dependent upon cell type. The stress response or UPR is regulated by master regulatory protein, BiP or GRP78. The initial, transient phase of the ER stress response functions to increase the removal or degradation and folding of misfolded or unfolded proteins. In its non-stressed state, BiP is bound to the ER luminal domain of the transmembrane proteins including PKR-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) (Chakrabarti, et al., 2011). These are the three major arms of the UPR. Viral infection causes the rapid accumulation of viral and other cellular proteins trafficked to the ER. When excess proteins accumulate in the ER lumen, BiP dissociates from its three transmembrane sensors, resulting in the functional activation of the 3 major arms of the UPR. PERK and IRE1 are activated and undergo homodimerization and auto-phosphorylation (Bollo, et al., 2010, Liu, et al., 2000, Oikawa & Kimata, 2010), triggering their downstream genes. The activation of the IRE1 pathway leads to the splicing of X box binding protein 1 (XBP-1) (Lee, et al., 2002). This spliced form of XBP-1 mRNA encodes an active transcription factor that binds to the promoter of unfold protein response element (UPRE) to induce expression of a subset of genes encoding protein degradation enzymes, resulting in ER-associated misfolded protein degradation (Lee, et al., 2003). The activation of PERK results in the phosphorylation of eIF2 on its α subunit (Raven & Koromilas, 2008). eIF2α phosphorylation effectively shuts down global, cap-dependent protein synthesis and causes a shift in translation to that of cellular mRNA containing IRESs reducing the burden of accumulating proteins in the ER (Harding, et al., 2002). This constitutes a translational switch to IRES-mediated translation initiation. UPR activation also involves the trafficking of ATF6 by BiP, resulting in its migration to the Golgi apparatus, where it is cleaved by S1P and S2P proteases, releasing a soluble fragment that enters the nucleus and bind to promoters containing the ER stress response elements (ERSE) and ATF/cAMP response elements (CREs) to activate ER chaperone genes, such as BiP, GRP94, and calreticulin (Yoshida, et al., 2001). These newly synthesized chaperones refold misfolded proteins in the ER in an effort to relieve ER stress. ATF6 also promotes XBP1 splicing (Lee, et al., 2002), indicating the interconnectedness of the three branches of the UPR. The shift from cap-dependent to cap-independent translation mediated by ER stress is critical to both cell fate and viral infection productivity. Many viruses, particularly RNA viruses, such as members of the *Picornaviridae* family, have evolved to replicate through cap-independent mechanisms, thus the shut-off of global protein synthesis induced by ER stress is of major strategic importance.

When ER stress is chronic or prolonged, it leads to the induction of ER mediated apoptosis (Tabas, et al., 2011). As is the case in viral infection, viral proteases also inhibit select cellular translational components, which may be initiated by ER stress. Our group has demonstrated

that CVB3 protease 2A and 3C can cleave eIFGI and induce cell apoptosis (Chau, et al., 2007). Viral proteins, such as picornaviral protein 2B, have been shown to contribute to the depletion of calcium stores within the ER (Wang, et al., 2010), furthering the viral life cycle by contributing to viral release. Prolonged and sustained severe ER stress eventually drives the cell to apoptosis (Mekahli, et al., 2011). Although significant progress in our understanding of apoptosis initiated by ER stress has been made in recent years, the molecular mechanisms of ER induced apoptosis are yet to be fully elucidated. During prolonged/severe ER stress, the functions of the three branches of the UPR (IRE1, ATF-6 and PERK) act in concert during prolonged/severe ER stress to induce apoptosis. Under those conditions, the endonuclease activity of IRE1 becomes less specific. As a result IRE1 contributes to the degradation of membrane associated mRNA, termed regulated IRE1 dependent degradation (RIDD). RIDD activation and XBP1 splicing highlight the two distinct functions for IRE1 during ER stress, the former being apoptotic and the latter generally regarded as protective (Hollien, et al., 2009). Previous studies indicate a correlation between enhanced ER stress induced apoptosis and the induction of RIDD activity. RIDD activation requires the nuclease domain of IRE1 to be activated, whereas IRE1 induced XBP1 splicing is modulated by IRE1 kinase domain activation (Hollien, et al., 2009). IRE1 has also been shown to bind Bcl-2 homologous antagonist/killer (Bak) and Bcl-2 associated x protein (Bax) (Hetz, et al., 2006), two pro-apoptotic proteins from the Bcl-2 family previously described in mitochondria derived apoptosis. Recently, however, it was shown that Bax translocates not only to the mitochondria, but also to the ER membrane during prolonged ER stress (Gotoh, et al., 2004, Hetz, et al., 2006, McCullough, et al., 2001, Wang, et al., 2010). Once translocated to the ER membrane, Bax permeabilizes the membrane, causing ER luminal proteins to be translocated to the cytosol (Wang, et al., 2010). Normally anti-apoptotic in function, BiP, once in the cytoplasm translocates to the plasma membrane where it becomes an apoptotic inducing receptor for prostate apoptosis response-4 (Par-4) (Wang, et al., 2010). Par-4 has been shown to co-localize with BiP in the ER. The binding of Par-4 to membrane bound BiP activates the extrinsic apoptotic cascade through FADD, caspase8 and caspase3 (Burikhanov, et al., 2009). Interestingly, the secretion of Par-4 is activated by TRAIL (Hart & El-Deiry, 2009). Several viruses including avian H5N1 and HIV have been shown promote cell death through TRAIL activated apoptosis in macrophages by enhancing TRAIL induced caspase10 activation (Ekchariyawat, et al., 2011, Zhu, et al., 2011).

Additionally, during prolonged and severe ER stress, PERK also enhances the translation of specific downstream genes, including ATF-4 (activating transcription factor-4) (Fels & Koumenis, 2006). ATF-4 is able to activate pro-apoptotic C/EBP homologous protein (CHOP) during conditions of prolonged, severe ER stress (Ma, et al., 2002). CHOP acts to induce apoptosis by promoting constitutively expressed Bax translocation to the mithochondria through inhibition of anti-apoptotic Bcl-2 transcription, as Bcl-2 functions to inhibit Bax in pro-survival conditions (Gotoh, et al., 2004, McCullough, et al., 2001). Here we see a connection between apoptosis mediated by IRE1 (by binding to Bax/Bak) and by PERK-mediated CHOP activation through ATF4, stressing the importance of cross talk between the three arms of the UPR. Interestingly, CHOP acts as a negative regulator of eIF2α phosphorylation as well

(Novoa, et al., 2001). The importance of the pathways described above in both global translation attenuation and apoptosis has made them the target of manipulation of many viruses. For example, Hepatitis E virus (HEV) open reading frame 2 protein (ORF-2) is able to modulate ER stress induced apoptosis by increasing eIF2α phosphorylation and activation of CHOP, simultaneously (John, et al., 2011). Our lab also obtained a similar result in studying coxsackievirus B3 (CVB3)-induced apoptosis through phosphorylation of eIF2α and activation of CHOP; however, this activation is not through ATF4 but through ATF6 (Zhang, et al., 2010). For HEV, during infection, CHOP, which normally induces apoptosis and translocation of Bax to the mitochondria, is unable to perform this pro-apoptotic function. This is due to the simultaneous activation and interaction of heat-shock proteins Hsp-70B, Hsp-72 and Hsp-40 by HEV protein ORF-2 (John, et al., 2011). Several members of the heat shock protein family, including Hsp-70, have been demonstrated to contain an IRES element in its long 5′UTR region of mRNA (Ahmed & Duncan, 2004, Hernandez, et al., 2004). This strategic modulation of pro-apoptosis and pro-survival proteins occurs presumably to delay apoptosis, while allowing the viral replication cycle to continue to completion. This demonstrates the careful strategic interplay between the virus and host translational factors as well as host cell components of the UPR. In doing so, a given virus is able to modulate the delicate balance between apoptosis and survival.

3. Structures of IRES

3.1. Classification of viral IRESs

IRES dependent translation initiation was first described in 1988 in the 5′UTR of the RNA genome of poliovirus (PV) (Pelletier, et al., 1988). Since this original discovery, IRES elements have been identified in the long, highly structured 5′UTR of almost all picornaviruses, including encephalomyocarditis virus (EMCV) (Lindeberg & Ebendal, 1999), Foot-and-mouth disease virus (FMDV) (Ohlmann&Jackson, 1999), Coxsackievirus B3 (Yang, et al., 1997) human rhinoviruses (HRV) (Rojas-Eisenring, et al., 1995), and other viruses, such as, Hepatitis A(Ali, et al., 2001), HIV (Weill, et al., 2009) and DNA viruses such as Kaposi's sarcoma-associated herpesvirus (KSHV) (Bieleski,, et al., 2004). Inherit to viral strategy, viruses must hijack cellular translational machinery, facilitating their own translation and replication. Translation initiation is the rate-limiting step of translation, which is the reason that it has evolved as a key strategic process, vital to viral strategy. Picornaviral mRNA, like many RNA viruses, is uncapped or lacks the 5′ terminal m⁷GpppN cap structure found in cellular mRNAs (Belsham, 2009). Instead, picornaviruses and other IRES translating viruses contain a small, virus-encoded peptide or VPg (Jang, et al., 1990). The discovery of IRES elements across a variety of viruses also identified distinct structural and functional differences amongst them, leading to the implementation of an IRES classification scheme. Viral IRESs are subdivided into four categories based on their structure, function and mechanism of initiation of translation. All four IRES types commonly share the necessity of (on some level) involving non-canonical translational factors that interact with IRES and replace the function of some canonical translation initiation factors. The canonical translation factors involved also vary dependent

upon the IRES structure, degree of interaction, and form the basis for IRES designation and classification.

3.2. Type I IRESs

Type I IRESs (fig.1) comprise enteroviruses and rhinoviruses. These IRESs contain a tetra-loop, cloverleaf structure in stem loop position I that resembles the 4-way junction of tRNA. This structure interacts with host cellular protein poly(rC)-binding protein 2 (PCBP2) and viral protein 3CD to form a bridge between the 5' and 3' ends to facilitate multiple rounds of viral replication (Fernandez-Miragall, et al., 2009). Downstream of the cloverleaf stem loop at position I are three distinctive C-rich motifs that precede the stem loop at position II. Two more C-rich regions are present in domain IV. There is also a pyrimidine tract motif located downstream of domain V, with a silent AUG region found 10-15 bases further downstream.
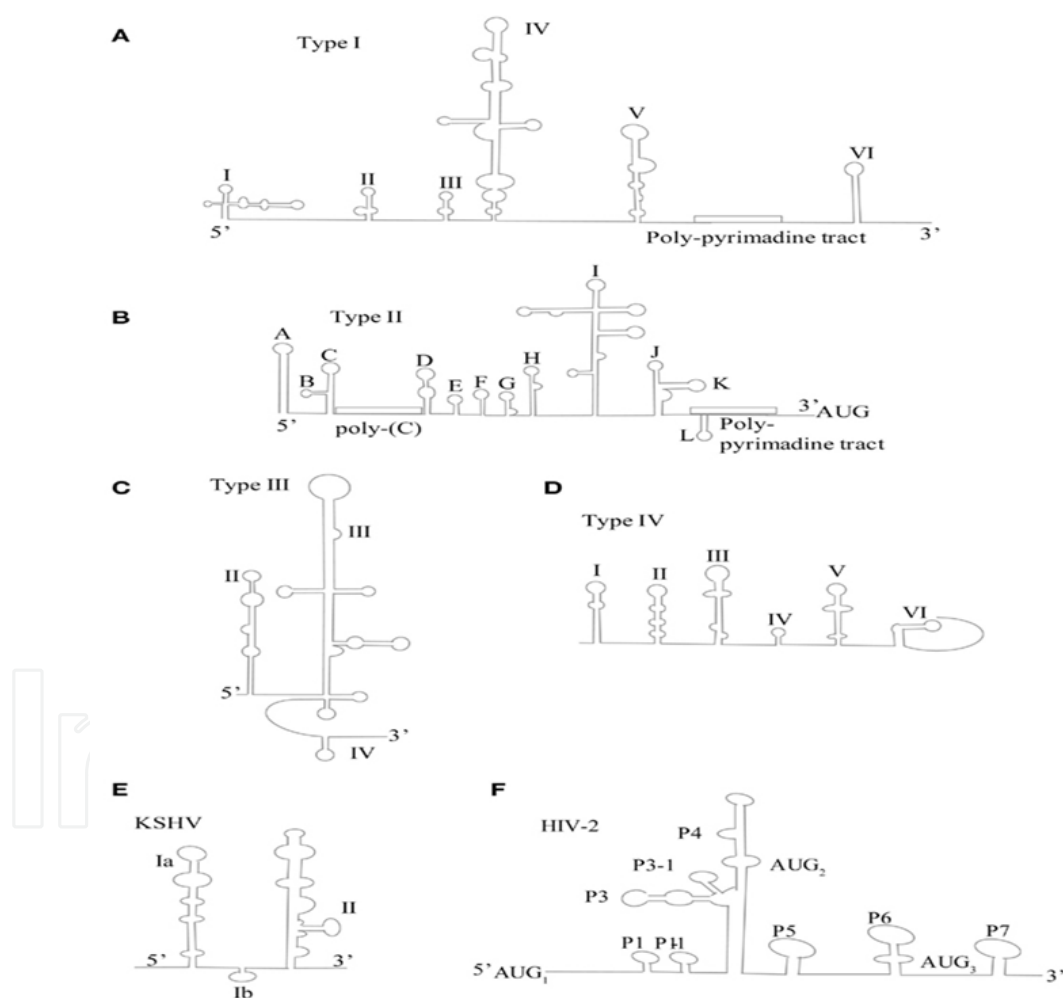

Figure 1. Schematic of proposed secondary structure of viral IRESs. A) Type I IRES represented by PV-1 (adapted from Jang, 2006) B) Type II IRES represented by EMCV (adapted from Jang,, 2006) C) Type III IRES represented by HCV (adapted from Beales, 2003 D) Type IV IRES represented by Plautia stali intestine virus (PSIV) (adapted from Kanamori, and Nakashima, 2001) E) DNA virus IRES represented by Kaposi's sarcoma-associated herpesvirus (KSHV) (adapted from Beales, 2003) F) HIV IRES, represent by HIV-2 (adapted from Locker, 2010)

The functional AUG initiation codon is traditionally further downstream from the silent AUG in type I IRESs, so the ribosome must scan downstream to the next AUG to begin translation initiation. Type I IRESs contain an eIF4G binding site that is absent the N-terminal region. This is due to viral protease cleavage of eIF4G to produce a truncated, yet functional form. This truncation eliminates its N-terminal region that contains a cap-binding domain. It is this feature that allows the ribosome to be recruited independent of the cap-structure, which is the hallmark of IRES-dependent translation. N-terminal deficient eIF4G is the integral translation initiation factor in the recruitment of the 43S ribosomal subunit, a process that is further enhanced by eIF4A. In fact, mutations made to the eIF4G-binding domain of the poliovirus IRES are the basis for the mutation of the PV strain given as the vaccine, further stressing the importance of translation initiation as a rate-limiting step (Malnou, et al., 2004). All together, type I IRESs contain six stem loops termed stem loops I-VI. The authentic IRES structure is located in the stem loop II-VI region, which facilitates initiation and translation of the viral genome (Pelletier, et al., 1988). Many of the canonical translation initiation factors, with the exception of eIF4E and the N-terminal region of eIF4G, are necessary for type I and II IRES translation. For this reason, viral modulation of these cap-dependent translation initiation factors has been identified as a vital component to viral strategy. Type I and II IRESs also utilize non-canonical translation initiation factors, termed IRES-specific cellular transacting factors (ITAFs). Examples of ITAFs include La autoantigen, PTB (pyrimidine tract binding protein) and UNR (upstream of N-Ras) (Costa-Mattioli, et al., 2004, Verma, et al., 2010, Cornelis, et al., 2005). ITAFs allow for the bypass of canonical translation initiation factors that are likely functionally inhibited and the target of viral strategy, either through direct proteolytic cleavage or modulation of pathways (such as UPR modulation).

3.3. Type II IRESs

Type II IRESs (fig.1) comprise the cardio- and apthoviruses of the *Picornavirdiae* family. There are several features of the IRES structure which differentiates the type II from that of the type I IRES. The 5′UTR are significantly longer than their type I counterparts. In place of the cloverleaf structure at stem loop position I, there is a hairpin or S structure. Just downstream of the S structure is an ~200bp C-tract that separates the S structure from the coding region. In between the C rich tract and the coding region there are three structural distinct regions. The first are 2 to 4 pseudoknots, next is the cis-acting replication element (cre) and lastly the IRES element, which spans stem loops II-V, also termed H-L. Just downstream are two AUG triplets that actively initiate protein synthesis. Interestingly, each produces a unique version of the leader protein. Type II IRESs require many of the canonical translation initiation factors. eIF4G, eIF4A and eIF4B have been demonstrated to interact with the SL J/K/L regions of the type II IRESs, with mutations to these domains causing reductions in IRES activity (Jang, 2006). As mentioned above, viral IRESs often utilize ITAFs, which further enhance translation in the absence of the canonical translation factors. The variability of ITAFs and canonical translation factors seen amongst the four types of IRESs is indicative of differences amongst IRES structural components, which are able mimic the function of both.

3.4. Type III IRES

Type III IRES (fig.1) structures demonstrate a new level of IRES-mediated translation initiation in which they are able to induce conformational changes directly to the ribosome that influence its entry, position and stability (Hellen, 2009). Flaviviruses, such as hepatitis C virus (HCV), contain IRESs considered to be prototypical of type III IRESs. The HCV IRES contains 3 distinctive domains, II, III and IV. Domain II is an irregular shaped, long stem loop structure. Domain III is a pseudoknot that also contains several hairpin-structured sub-domains, IIIa-IIIf, whereas domain IV is a short hairpin structure containing the initiation codon. The HCV IRES, like all other type III IRESs, is able to directly and independently bind the 40S subunit, thereby bypassing the need for canonical eIFs 4A, 4B, 4F, 1 and 1A. Hepatitis C virus (HCV) has been shown to require eIF3 and the eIF2•GTP/Met-tRNA$^{Met}_i$ ternary complex to bind sequentially for translation initiation. However, some type III IRESs, such as the Simian picornavirus type 9 (SPV9) IRES, have been shown to promote Met-tRNA$^{Met}_i$ recruitment to the ribosome independent of eIF2 (de Breyne, et al., 2008). Therefore negating the need for eIF2, which is quite often phosphorylated (i.e. translationally inactivated) during viral infection due to interferon activation of PKR or PERK, which induce subsequent phosphorylation of the eIF2α subunit. Type III IRES-containing viral mRNA has been demonstrated to be more resistant to translation inhibition caused by eIF2α phosphorylation than that of the cap-dependent cellular mRNAs (Pestova, et al., 2008).

3.5. Type IV IRES

Type IV IRESs (fig.2) initiate translation on the intergenic region (IGR) by direct binding of the 40S subunit or to the 80S ribosome. They are represented by the dicistroviruses, particularly the cricket paralysis virus (CrPV), which contain the smallest regions for internal ribosomal entry. Structurally, its IRES consists of 3 distinct domains. Each domain contains a pseudoknot and may or may not contain a hairpin like structure in stem loop 3. Type IV IRESs translation initiation occurs without involving any canonical initiation factors, initiator tRNA, or a proper AUG start codon. In contrast to conventional AUG codon for IRES translation initiation, the start codon of type IV IRESs may be GCU, GCA, GCC or CAA. In fact, studies have shown that translation initiation of CrPV IRES is impaired by the promotion of the eIF2•GTP/Met-tRNA$^{Met}_i$ ternary complex to the 40S subunit. This may be an evolutionary advancement of conditions where the eIF2α is phosphorylated, such as during ER stress and viral infection (Hellen, 2009).

3.6. IRES of Lentiviruses

The HIV IRESs (fig.1) represent yet another new class of IRES, not previously characterized by the four IRES types already described. On one hand, it displays type III IRES properties possessing the ability to directly and indirectly bind to 40s and eIF3 (Locker, et al., 2011). On the other hand, it requires all eIF's except for eIF4E and eIF1, a property of class I and II IRESs (Locker, et al., 2010). The structure of the HIV IRES is highly complex. It contains a

long 5'UTR harboring a Tar stem loop, Poly-A, PBS, DIS, SD and Psi regions (Vallejos, et al., 2011). Interestingly, in contrast to its type I, II and III IRES counterparts, the HIV IRES appears to be resistant to structural mutations which to date have been unable to alter its function (Vallejos, et al., 2011). Also unique is its ability to recruit three initiation complexes to a single RNA molecule (Locker, et al., 2010). The translational requirements of HIV IRESs lend themselves to the notion that, while able to be translated cap-dependently, HIV RNA possesses and indeed utilizes IRESs as part of a tightly regulated and conserved method of cap-independent translation. The redundant ability of HIV to translate through a variety of mechanisms highlights the importance of translation being a key, highly regulated process of the viral lifecycle. The utilization of the HIV IRESs takes place relatively late in the viral life cycle and is regulated by the G2/M phase of the cell cycle, also activated by osmotic stress (Vallejos, et al., 2011). This is particularly interesting given that cap-dependent translation is shut-off during the cell cycle, leading to the notion of a new level of evolutionary complexity exemplified by the ability of HIV to modulate translation between cap-dependent and independent translation based on cell physiology. The HIV IRES also utilizes a subset of ITAFs that are exclusively available during the G2/M phase (Vallejos, et al., 2011). The utilization of its IRES is thought to regulate the transition between translation and encapsidation. The HIV-2 virus is only able to be encapsidated once the cognate form of it is translated, versus HIV-1 that can be either translated or propagated as a genome and encapsidated into virons (Locker, et al., 2010). This is suggestive of a possible role of generation of structural/functional proteins in correlation with its IRES. In fact, the gag polyprotein encoded by the Gag IRES associates with 5' UTR of HIV mRNA, forming a gRNA–Gag complex that inhibits ribosomal scanning, decreases translation and increases encapsidation (Chamond, et al., 2010). The ability to switch from cap-dependent to IRES-dependent translation by HIV is most closely related to that of cellular IRES-containing mRNA, which will be addressed in the next section.

3.7. IRES of Cellular mRNA

While many of the viral IRES-containing mRNAs have been studied quite extensively, much less is known about cellular IRES-containing mRNA. It's estimated that ~10-15% of cellular mRNA possesses the ability to translate via cap-independent mechanisms (Graber, et al., 2009, Johannes, et al., 1999, Qin & Sarnow, 2004). The cellular genes that contain IRESs in their mRNAs generally have been shown to code for proteins that are involved in growth, proliferation, apoptosis, stress response, differentiation and cell cycle regulation (Komar & Hatzoglou, 2011). Cellular IRESs often are found in mRNA containing long 5'UTRs that are rich in GC and have complex secondary structures (Holcik, et al., 2005). Often, in the mRNA structure there are also multiple short modules whose combined effects are IRES activation, as well as pseudoknots, that are believed to be inhibitory in function (Stoneley & Willis, 2004). However, to date there is no consensus structural or conformational motifs that are conserved among cellular IRES that would make them easily identifiable. Unlike their structurally stable viral counterparts, cellular IRESs identified to date follow a pattern of less structure corresponding to enhanced IRES activation (Filbin & Kieft, 2009). Like their viral

counterparts, cellular IRESs are able to initiate translation without many of the canonical translational factors, particularly cap-binding factors such as eIF4E (Hellen & Sarnow, 2001). Cellular IRESs also utilize ITAFs to replace canonical translational factors rendered unavailable. Many of the ITAFs utilized by the cell are also utilized by viruses, including PTB, UNR, poly-(rC)-binding protein 1 (PCBP1), La autoantigen and hnRNPC1/C2, many of which shuttle between the nucleus and cytoplasm (Stoneley & Willis, 2004). Dicistronic cellular mRNA containing IRESs were inactive when introduced directly into the cytoplasm, suggesting the possibility of prerequisite nuclear ITAF-IRES complex formation for IRES activation, at least for apoptotic genes (Spriggs, et al., 2005). Interestingly, much like the highly evolved HIV IRES, the G2/M phase of the cell cycle (where cap-dependent protein synthesis is inhibited) is important for cell cycle regulatory gene's IRES activation as well, including p58^{PITSLRE} (Stoneley & Willis, 2004).

The notion of cellular mRNAs containing IRESs is not without controversy. The viral shut down of host canonical translation machinery results in an overall reduction in global protein synthesis. However, many host cellular stress responsive mRNAs are still actively translated. This led to the hypothesis that certain select cellular mRNAs contain IRESs in their 5′UTRs. Indeed, there are several cellular mRNAs containing IRESs in their 5′UTR (Gilbert, W.V., 2010). The previous methods used to determine the existence of cellular IRESs have been under some scrutiny as to their capability of truly detecting and confirming actual IRES structures within cellular 5′UTRs. Bicistronic reporter assays where the 5′UTR of the suspected mRNA containing IRES was cloned between two reporter genes are subject to false positives via cryptic promoter artifacts (Gilbert, W.V., 2010). Therefore, future work needs to be done to verify if some cellular genes truly contain IRESs in the 5′UTR of their mRNA.

3.8. DNA virus IRES

Much less studied are the DNA viruses, which transcribe mRNA containing an IRES that translates certain proteins independent of the cap structure, much like their cellular IRES counterparts. To date, there are six known DNA viruses known to contain IRESs, four of which belong to the *Herpesviridae* family (http://iresite.org/), particularily the latent gammaherpesviruses (Coleman, et al., 2003). The most well documented DNA viral IRES is that of the Kaposi's sarcoma herpes virus (KSHV) (fig.2) (Bieleski, and Talbot, 2001) while others include Herpes simplex virus (Griffiths, A. and Coen, D. M., 2005) and Marek's disease virus (Tahiri-Alaoui, et al., 2009) to name a few. The KSHV IRES is representative of most IRESs in the *Herpesviridae* family in that it is similar in structure to that of HCV, containing two major stem loops (Beales, et al., 2003). Although most IRESs identified are located in the 5′UTR, the KSHV IRES is found in the coding sequence of the upstream cistron, vCyclin (Bieleski & Talbot, 2001). Interestingly, the KSHV IRES is translational active during viral latency and codes for a viral FLICE (FADD [Fas-associated death domain]-like interleukin-1 beta-converting enzyme)-inhibitory protein, vFLIP (Flice inhibitory protein homolog), which inhibits caspase activation and also promotes proliferation (Bieleski & Talbot, 2001). Again, the trend for IRES involved in cell

growth/proliferation is consistent in DNA viruses as well. While there remains quite a bit yet to be discovered in our understanding of the structure and function of IRES elements in translation initiation, clearly, the stress-induced shift from cap-dependent to IRES-dependent translation is a vital strategy for the cell and virus to survive unfavorable conditions.

*For a comprehensive review of current known IRESs, the reader may refer to http://iresite.org/.

4. Mechanisms of survival: Switching translation initiation from cap-dependent to IRES-dependent

As discussed above, both cells and viruses utilize a strategy for survival by switching translation initiation from cap-dependent to IRES-dependent. During this process, both the canonical translation factors and ITAFs utilized by a given virus are dependent upon IRES structure, as it is highly indicative of function. For example, structural components found in the mRNA of Hepatitus C virus (HCV) IRES are able to mimic the function of certain canonical translational factors. (Sonenberg, et al., 2009). HCV also utilizes litagin and the oncogenes MCT-1/DENR as ITAFs, supplementing the function canonical factors of eIF1, eIF1A, eIF3 and eIF3 (Skabkin, et al., 2010). Picornaviruses and others have demonstrated the capability of influencing the cell and manipulating its translational components, favoring its own translation and replication. Viral translation includes modulating not only canonical eukaryotic initiation factors, but also their binding proteins as well. The eukaryotic translation initiation components modulated during infection are specific to a given virus and can vary quite substantially. On the other hand, host cells utilize highly conserved mechanisms of defense to a variety of stimuli, including viral infection, osmotic shock, toxin, heat shock, etc. Here, we summarize some of the recent advances in our knowledge of the mechanisms utilized by viruses and cells to promote IRES-dependent translation allowing survival during unfavorable conditions.

4.1. Cleavage of translation initiation factors by viral proteases

In order to influence cellular translation, viral proteases often target the cellular canonical translation initiation factors for cleavage. The early identified such factor is eIF4G (later called eIF4GI), which along with eIF4E, constitute critical translational factors targeted during several viral infections. This is evident by the highly specific cleavage of eIF4GI during picornaviral infection, which generates a truncated C-terminal form that is unable to bind eIF4E (Svitkin, et al., 2005). Another translation initiation factor eIF4GII as well as the polyA binding protein (PABP), a protein facilitating the formation of a closed translation initiation loop by interaction of the 5′ and 3′ ends of the mRNA, has been reported to be cleaved by picornaviral 2A (Gradi, et al., 1998, Joachims, et al., 1999). All these cleavages often correspond with a translational shift to IRES-dependent translation (Redondo, et al., 2011 Welnowska, et al., 2011), rendering the eIFs incapable of performing cap-dependent translation. Another group also showed that the shift in translation seen during the later

phase of poliovirus infection is not entirely due to phosphorylation (inactivation) of eIF2α (see discussion in later session), but may also depend upon protease 3C activation and cleavage of another translation initiation factor, eIF5B, to a C-terminal truncated version thought to replace eIF2 during translation (White, et al., 2011). In all these cleavage events, viral protein synthesis was increased during periods of global protein suppression caused by eIF2α phosphorylation, however the mechanism may likely be a combination of both 2A and 3C proteolytic activity. The apparent shift in translation occurs at times during infection when viral proteases are highly expressed. These observations are representative of viral evolution in correspondence to cellular anti-viral mechanisms. Other factors such as FMDV protease 3C mediated specific cleavage of eIF4AI but not eIF4AII highlight the target specificity that has quite often evolved to be viral specific (Li, et al., 2001).

4.2. Cleavage of translation initiation factors by caspases

Like their viral counterparts, the cell utilizes a subset of proteases, the caspases, to cleave some translation initiation factors. The activation of the caspases often corresponds to the induction of apoptosis (Cohen, 1997). It has been demonstrated in cells committed to apoptosis that caspases cleave eIF4E-BP1, which enhances its capability to bind and inhibit eIF4E, thereby inhibiting cap-dependent translation (Tee & Proud, 2002). eIF2 is cleaved at its α subunit by caspase-3, further implicating its critical role in translational control (Satoh, et al., 1999). Caspase-3 was also shown to cleave scaffolding protein eIF4GI, inhibiting its eIF4E binding capabilities, as well as cleaving its homolog DAP5 (death associated protein 5, also called NAT1/p97), both during conditions of apoptosis (Henis-Korenblit, et al., 2000, Marissen, et al., 1998). Perhaps not surprisingly, viral strategies target many of the same canonical translation initiation factors (including all of those mentioned here) and is reflective of similar strategies used by the cell defense system, marking a translational switch to cap-independent translation during stress and promoting translation of apoptotic inducing genes.

4.3. Phosphorylation of eukaryotic initiation factors and co-factors

The cell has multiple signaling mechanisms that it utilizes to influence translation. Phosphorylation is perhaps the one of most common and conserved method utilized by the cell. Protein kinases involved in cellular stress response regulation such as PKR, PERK, GCN2, and HRT (heme-regulated kinases) all conservatively deactivate eIF2 on its α subunit in response to their respective stress stimulus, influencing the shift to cap-independent translation (Sonenberg, et al., 2009). This multi-faceted capability of the cell to redundantly suppress cap-dependent translation initiation through phosphorylation of eIF2α is quite intriguing and spans multiple disease and stress conditions. This highlights the critical importance of translation initiation in cell fate and physiology. eIF4E also is a highly targeted translation factor during viral infection as well as during other conditions of stress, such as heat shock, ER stress, oxidative stress, etc. In fact, eIF4E and its regulatory protein eIF4E-BP have been utilized as predictive biomarkers in breast cancer (Coleman, et al., 2009). This is because it functions as the cap binding translation initiation factor thought to

be the rate-limiting step of translation and therefore is a key component to cap-dependent translation (Gingras, et al., 1999). The availability of eIF4E (which is highly cytoplasmic) to participate in cap-dependent translation is regulated by several factors, the most apparent being 4E-BP, which binds eIF4E and is involved in its localization to the nucleus and in stress granules, rendering it inactive (Sukarieh, et al., 2009). 4E-BP is regulated by phosphorylation by the highly conserved serine/threonine kinase (mammalian target of rapamycin (mTOR)), which decreases its affinity to eIF4E (Kimball & Jefferson, 2004), thus resulting in increased levels of protein translated cap-dependently due to increased availability of cap binding protein eIF4E. However, hypophosphorylated 4E-BPs binds strongly to eIF4E and thus attenuates cap-dependent translation. Similarly, eIF4G has been shown to be phosphorylated by protein kinase C (PKCα) through the Ras-ERK pathway, resulting in increase affinity for eIF4E binding and enhanced eIF4E-mnk1 modulating capabilities (Dobrikov, et al., 2011). Therefore, phosphorylation modulated by stress stimulus (i.e. heat shock, osmotic stress, ER stress, viral infection) results in stress pathway activation (ERK, MAPK, PKR, etc.) and subsequent phosphorylation of a translation initiation component (i.e. eIF4E, eIF4G, eIF2, 4E-BP) which represses or enhances its function and contributes to the translational switch between IRES and cap-dependent modes.

4.4. eIF4E-binding Proteins and other associated proteins compete with eIF4E to inhibit cap-dependent translation

Another similar mechanism for controlling the shift of translation initiation is the up-regulation of 4E-BP production, which affects the mRNA 5'-cap recognition process of eIF4F. In cap-dependent translation, eIF4E forms the eIF4F complex along with translation initiation factors eIF4A, eIF4B and eIF4G (Merrick, 1992). The interaction between eIF4G and eIF4E in the eIF4F complex is inhibited by 4E-BPs (also called eIF4E homolog). Recently, it was reported that Argonaut (Ago) protein, a core component of RISC, binds directly to the cap structure and that this binding competes with eIF4E and results in inhibition of cap-dependent translation initiation (Kiriakidou, et al., 2007). The central domain of Ago exhibits limited sequence homology to the eIF4E and contains two aromatic residues that could function in a similar manner to those in eIF4E in interaction with the cap structure. However, this conclusion has been questioned by another study (Eulalio, et al., 2008). Another factor eIF6 has been reported to associate with Ago protein and the large ribosomal subunits (Chendrimada, et al., 2007). By binding to the large ribosomal subunit, eIF6 prevents this subunit from prematurely joining with the small ribosomal subunit. Thus, if Ago2 recruits eIF6, then the large and small ribosomal subunits might not be able to associate, causing translation to be repressed (Chendrimada, et al., 2007). Drosophila Cup also suppresses cap dependent translation by binding eIF4E at the same conserved sequence utilized by 4E-BPs (Nakamura, et al., 2004).

4.5. The Role of microRNAs (miRNA) in translational control

Many viruses also indirectly influence the availability of cellular translational components. miRNAs are small (~20-24 nts) non-coding RNAs that bind partially complimentary mRNA

sequences (mostly in the 3'UTR and less so in the 5'UTR and coding regions) resulting in translational repression and mRNA degradation or (in instances of cellular quiescence) translational activation (Fabian, et al., Sonenberg, et al., 2009). They are loaded onto target mRNA sequences by an RNA induced silencing complex (RISC), whose major component proteins are the Ago protein family (Sonenberg, et al., 2009). It was recently shown that Ago proteins are required for miR-122 activated translation during HCV infection (Roberts, et al., 2011). In addition, as mention earlier, Ago binds competitively to the cap structure of mRNA to inhibit cap-dependent initiation of translation. It is not surprising that miRNA mediated repression has been shown to be specific to a given mRNA containing both a cap structure and poly-(A) tail, in fact mRNA without a cap structure or poly-(A) tail were resistant to miRNA-mediated repression (Humphreys, et al., 2005). miRNA modulated repression takes place in processing (P)-bodies that contain decapping enzymes (see discussion in a later section), further supporting the role of miRNA in suppressing cap-dependent translation initiation (Sonenberg, et al., 2009). Viruses have been shown to influence the expression of select miRNAs (Ho, et al., 2011, Humphreys, et al., 2005, Lei, et al., 2010), which are often involved in the inhibition of cap-dependent translation (Humphreys, et al., 2005, Walters, et al., 2009) lending to a virally influenced shift to IRES-mediated translation. In the early study of the mechanism of translation suppression using an artificial miRNA targeting CXCR4, the cap/4E-BP and the poly-(A) tail of mRNA were all found to play an important role because they are each necessary but not sufficient for full miRNA-mediated repression of translation. Replacing the cap with a viral IRES impairs miRNA-mediated suppression. These results suggest that miRNAs interfere with the initiation step of translation and implicate 4E-BP as a molecular target (Humphreys, et al., 2005). This finding was further solidified by a recent study, which demonstrated that enterovirus 71 (EV71) infection up-regulated miR-141 expression and resulted in a shift from cap-dependent to cap-independent translation initiation by targeting 4E-BP. As EV71 RNA translates through a cap-independent, IRES mechanism, this targeting enhanced EV71 replication (Ho, et al., 2011). Another miRNA, miR-2, has also been reported to utilize a similar mechanism to target the cap structure (Zdanowicz, et al., 2009). This study screened a library of chemical m^{7}GpppN cap structures and identified defined modifications of the triphosphate backbone that augment miRNA-mediated inhibition of translation but are "neutral" toward to general cap-dependent translation. Interestingly, these caps also augment inhibition by 4E-BP, suggesting that miR-2's cap targeting is through a mechanism related to the 4E-BP class of translation regulators (Zdanowicz, et al., 2009).

The above studies clearly support the notion of a virally influenced translational shift favoring cap-independent translation. This is achieved through several mechanisms including indirectly, such as up-regulating the expression of certain miRNAs that repress cap-binding canonical translation initiation factors in the eI4F complex (Mathonnet, et al., 2007). Here, it is worth mentioning that viruses with a nuclear DNA phase, including HIV and Herpesviruses, may generate virally derived miRNAs during the infection cycle (Griffiths-Jones, et al., 2008, Pilakka-Kanthikeel, et al., 2011), however, whether HIV generates miRNAs is still contentious as other labs have not been able to verify them experimentally (Lin., Cullen, 2007, Pfeffer, et al., 2005). Intriguingly, the cytoplasmic RNA

tick-borne encephalitis virus (TBEV), a member of the *Flaviviridae* family, has been shown to encode its own viral miRNA when a heterologous miRNA-precursor stem-loop was artificially introduced into the RNA viral genome (Rouha, et al., 2010). This opens up the possibility of other cytoplasmic RNA viruses to have similar capabilities. It may be possible to artificially introduce miRNAs into viral genomes, which may in turn be able to shut down viral replication by targeting mRNAs of specific translation initiation factors required by the virus, which generate a new avenue for generating vaccines and attenuating viral replication. Clearly miRNAs represent an exciting and newly emerging dimension to our study and understanding of viruses and their ability to manipulate cellular translation during infection and other conditions of stress.

4.6. Activation of decapping enzymes

Decapping of mRNA by decapping enzymes represents another modality by which cap-dependent translation is suppressed by the cell. To date, two decapping enzymes have been identified: Dcp2 which cleaves mRNA at the cap site and the scavenger decapping enzyme (DcpS) that hydrolyzes the cap structure, both function to facilitate the subsequent degradation of target cap-dependent mRNA (Li & Kiledjian, 2011). Enzymatic decapping of select mRNAs is influenced by miRNA. As mentioned above, miRNA mediated repression occurs in P-bodies where Ago proteins have been shown to co-immunoprecipitate with decapping enzymes, suggesting their close association (Parker & Sheth, 2007). P bodies also contain other proteins including, GW182, the CAF1-CCR4-NOT deadenylase complex, the decapping activators (e.g., DCP1, EDC3, Ge-1), and the RNA helicase RCK/p54, all of which have been implicated in miRNA function (Eulalio, et al., 2007, Parker, et al., 2007). Decapping enzymes functions may also be modulated by cell signaling pathways and are also found in stress granules. Indeed, the phosphorylation of the decapping enzyme DCP2 has been shown to influence stress granule formation and its availability in P-bodies (Yoon, et al., 2010). HCV has been shown to selectively disrupt P-body components during infection leaving the decapping enzyme DCP2, active and functioning to highjack other translational machinery for the enhancement of its own translation (Ariumi, et al., 2011). Therefore, not surprisingly, viruses modulate decapping enzyme activity to favor their translation.

5. Conclusions and perspectives

It is evident that more and more newly discovered cellular mRNAs contain IRESs and can participate in a shift in translation from global, cap-dependent to IRES-driven initiation during ER stress. One of the most well studied causes of ER stress is viral infection, which can globally shut down cap-dependent translation initiation by different mechanisms. To adapt to unfavorable stress conditions, both cell and virus (e.g., HIV) need to adjust their mode of translation initiation by switching from the cap-dependent to cap-independent mechanism. As picornaviruses do not have a cap structure, its RNA translation will not be inhibited; instead it will be enhanced because more translational machinery is available due to the shutoff of global cap-dependent translation, achieved by a number of mechanisms.

During transient ER stress, the IRES-containing cellular mRNAs that are responsible for cell survival/growth, such as BiP and Bcl-2, will be selectively translated by an IRES-dependent mechanism, utilizing ITAFs in place of inhibited canonical translational factors. This mechanism allows cells to respond rapidly to the transient changes in growth conditions and to delay apoptosis. Once the stress is removed, cellular homeostasis is restored. However, during prolonged or severe stress, such as in persistent infection of picornaviruses, the pro-death genes, such as Apaf1, DAP5, CHOP, p53, etc., are also selectively translated by the same IRES-driven mechanism, allowing the cells to fine-tune their responses to cellular stress and, if conditions for cell survival are not restored, to proceed with final execution of apoptosis (Fig. 2).
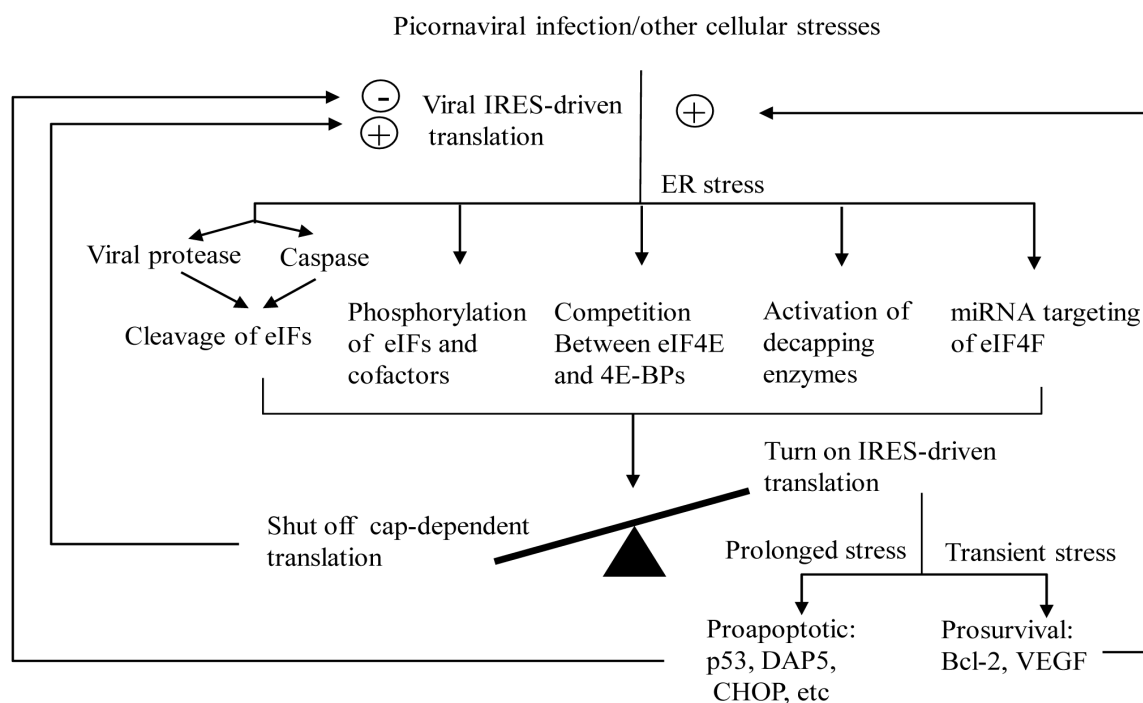

Figure 2. The proposed model for the switch of translation initiation from cap-dependent to IRES-dependent during picornaviral infection or other cellular stresses. Positive and negative feedback loops are indicated by plus and minus signs, respectively.

Although some mechanisms on the switch of the translation initiation and subsequent selective translation have been described, many questions are still unanswered: for example, what are the regulators for selecting the pro-survival or proapoptotic genes? In other words, do these genes contain different binding sequences for their specific regulators? Previous studies using a polysome system predicted that approximately 10-15% of the cellular mRNAs contain IRESs (Carter, 2000, Graber, et al., Qin, et al., 2004); thus, more IRES-containing cellular mRNAs will need to be discovered to fully understand the underlying mechanisms of IRES-dependent translational control. In the shutoff of global cap-dependent translation, cleavages of cellular proteins are known to play an important role. In this regard, besides the viral proteases and the activated cellular caspases, other cellular proteases responsible for the cleavage of translation initiation factors need to be identified.

In addition, efforts to discover other cellular target proteins that are specifically cleaved during cellular stress are another future area of research. Identification of these target proteins may uncover the linkage between translational control and pathogenesis. Recently, miRNAs, as a group of new regulators of gene expression, were found to be involved in regulation of the shift of translation initiation. However, the research in this direction is just emerging. More studies on the interactions between miRNAs and their target mRNAs encoding translation initiation factors need to be carried out. Indeed, the biological implications of the selective translation of specific genes are clearly important. Since the IRES-mediated translation initiation links with many pathophysiological conditions, such as hypoxia, heat shock, toxin, metabolic disorder, viral infection, etc., the failure of maintaining the balance between the cap-dependent and cap-independent translation initiation may cause human diseases, such as heart disease, stroke, diabetes, and viral induced diseases. Similarly, dysregulated apoptosis has been associated with many human disorders, ranging from autoimmune diseases, neurodegeneration to a variety of cancers. Therefore, better understanding how the translational control determines the cellular response to stress will provide novel insights into the molecular pathogenesis of human disorders and will likely eventually lead to the development of effective therapeutics.

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