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Identifying the determinants for IRES-mediated translation of a voltage-gated potassium channel mRNA

DISSERTATION

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in Biomedical Science

by

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ABSTRACT OF THE DISSERTATION

Identifying the determinants for IRES-mediated translation of a voltage-gated potassium channel mRNA

By

Eric L. Baggs

Doctor of Philosophy in in Biomedical Science

University of California, Irvine, 2018

Professor Bert L. Semler, Chair

Internal ribosome entry sites (IRESs) are regions of mRNA that facilitate direct binding of components of the initiation complex for protein synthesis independent of the 5'-terminus. While first discovered in viruses, there are many cellular mRNAs that harbor these elements. The current study is evaluating the requirements (both canonical and non-canonical) for translation initiation on the voltage-gated potassium-channel (Kv1.4) IRES. Kv1.4 is a shaker related family member which contributes to the repolarizing phase of the cardiac action potential. Kv1.4 has a highly tissue-specific expression, despite its relatively non-specific promoter, and is regulated at the post-transcriptional level. Using both a genetic (yeast 3-hybrid screen) and a biochemical approach (biotinylated-RNA capture), we probed for novel interacting proteins that might modulate the activity of these IRES elements. Parallel screens reveal a diversity of interacting proteins, which may represent trans-acting factors for translation initiation. Gene set enrichment analysis as well as manual cross-reference identify a high abundance of RNA and translation associated

proteins with and without ascribed functions in IRES-mediated translation. Characterizations of a select group of these interactions indicate roles in translation initiation and allude to tissue specific mechanisms of post-transcriptional regulation.

Chapter 1:

Mechanisms and regulation of protein translation

Eukaryotic gene regulation

Gene expression is regulated by a multitude of processes and at all conceivable stages of protein biosynthesis, from pre-transcriptional chromatin composition to post-translational modifications and degradation. Epigenetic modifications, transcriptional activators, and repressors all modulate RNA synthesis. Additionally, co- and post-transcription factors alter nascent precursor messenger RNA (mRNA) through alternative splicing, 5'-end capping, and poly-adenylation. In addition to controlling the identity of final RNA and protein products, these processes also determine the presence of cis-acting elements within the mRNA able to alter expression though localization, stability, or translation. Mature RNAs are then selectively exported from the nucleus via the RNA export pathway.

Once an RNA has reached the cytoplasm, it is then subjected to regulation by micro-RNAs (miRNA), an array of trans-acting factors and the RNA decay machinery. This can result in immediate translation, degradation, or RNAs may be sequestered into ribonucleoprotein (RNP) aggregates such as stress-granules or p-bodies (Khong et al., 2017). This represents a major checkpoint in gene regulation, as once translation initiates, subsequent elongation and termination are not thought to be rate-limiting for protein synthesis (Hershey et al., 2012).

Post-translationally, the repertoire of potential gene products is further expanded by protein modification. These processes include, but are not limited to, phosphorylation, glycosylation, ubiquitination, methylation, acetylation, lipidation and proteolysis. Many

proteins are modified shortly after translation to mediate proper protein folding and stability or to direct the nascent protein to distinct cellular compartments (e.g., nucleus, membrane). Other protein modifications occur later to activate or inactivate catalytic activity or to otherwise influence the biological activity of a protein. This can result in the assembly or disassembly of functional complexes, which further exemplifies the complexity and extent of gene expression and regulation. As many of these mechanisms of gene regulation are characterized with ever-expanding clarity, this invites an ever-evolving understanding of biological mechanisms and new diversions of biology to explore.

Translation

Initiation

The initiation of protein translation is the primary checkpoint in translational regulation, and the primary mechanism by which this occurs has been well outlined in the ribosome scanning model proposed by Kozak (Kozak, 1979, 1984, 1989). This model dictates that translation is initiated by the recognition of the 5' terminal 7-methyl guanosine "cap" (m^7G-cap) on the mRNA by a large complex of eukaryotic initiation factors (eIF) known as eIF4F (Gingras et al., 1999)(Figure 1.1). This ribonucleoprotein (RNP) complex then recruits the 43S pre-initiation complex composed of the 40S ribosomal subunit, the eIF2·GTP·$tRNA_i^{met}$ ternary complex, eIF3, eIF5, eIF1, and eIF1A (Pain, 1996).

The recruitment of the 40S ribosomal complex to the 5'end of the mRNA does not position it at the translational start site, which is usually 50-100 nucleotides downstream. The initiation complex then scans through the 5' non-coding region until the start codon is located. This "scanning" process is ATP dependent, although it is unclear whether ATP

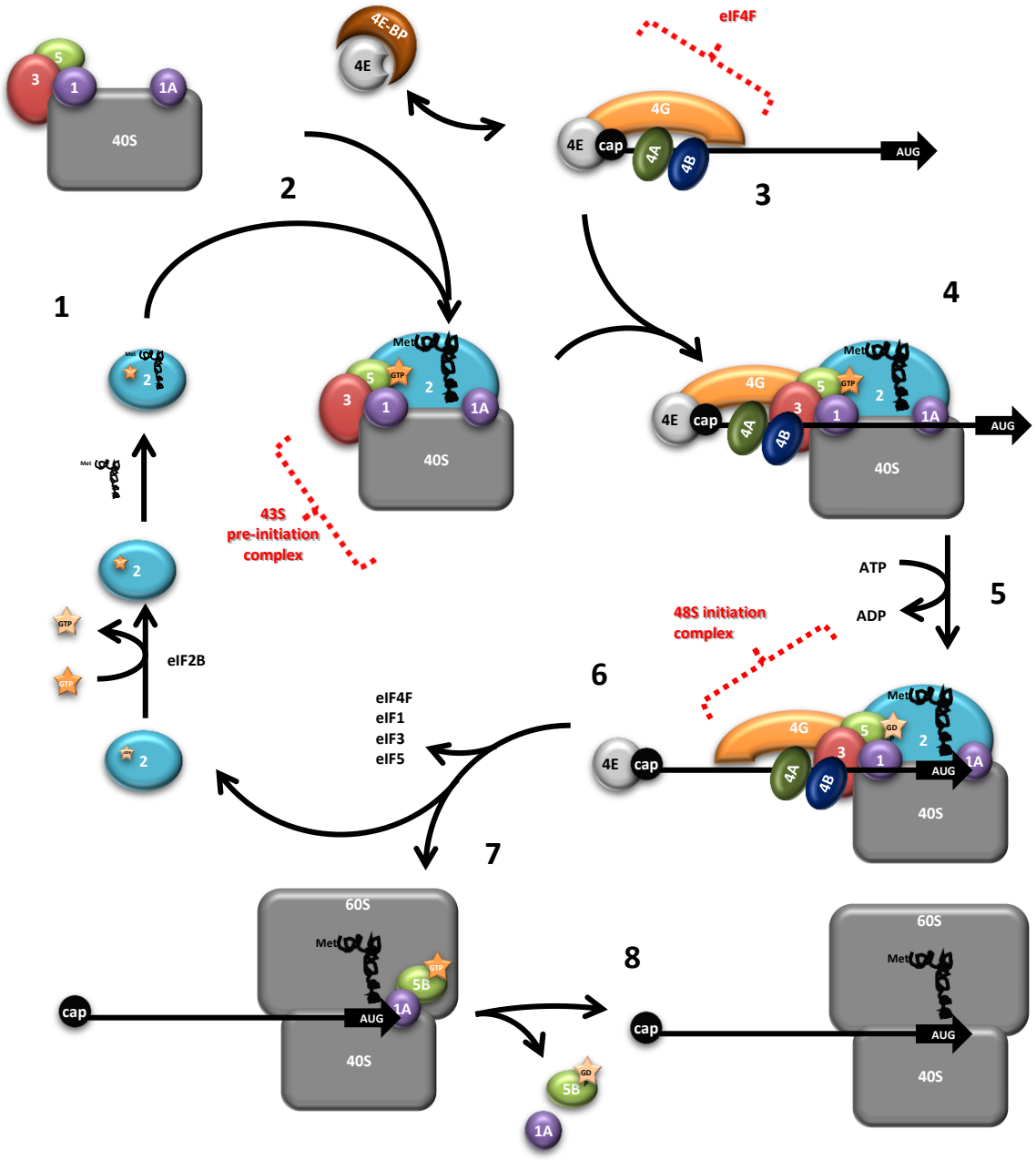

4E-BP
4E
eIF4F
4G
4E
cap
4A
4B
AUG
3
2
1
4
40S
Met
GTP
43S
pre-initiation
complex
ATP
ADP
5
48S initiation
complex
6
eIF2B
eIF4F
eIF1
eIF3
eIF5
7
60S
5B
GD
P
8

Figure 1.1. The canonical pathway of eukaryotic translation initiation. (1) Eukaryotic initiation factor 2 (eIF2)–GTP–Met-tRNAMeti ternary complex formation; (2) formation of a 43S preinitiation complex comprising a 40S subunit, eIF1, eIF1A, eIF3, eIF2–GTP–Met-tRNAMeti and probably eIF5; (3) mRNA activation, during which the mRNA cap-proximal region is unwound in an ATP-dependent manner by eIF4F; (4) 43S complex binding to eIF4F and mRNA 5′ end; (5) scanning of the 5′ UTR in a 5′ to 3′ direction by 43S complexes; (6) recognition of the initiation codon and 48S initiation complex formation, leading to eIF5-mediated hydrolysis of eIF2-bound GTP; (7) joining of 60S subunits to 48S complexes and concomitant displacement of eIF2–GDP and other factors (eIF1, eIF3, eIF4B, eIF4F and eIF5) mediated by eIF5B (8); and GTP hydrolysis by eIF5B and release of eIF1A and GDP-bound eIF5B from assembled elongation-competent 80S ribosomes (9). Translation is a cyclical process, in which termination follows elongation and leads to recycling (1), which generates separated ribosomal subunits.

hydrolysis is required for ribosomal movement or to unwind secondary structure in the mRNA (Pestova et al., 2001). The initiation codon is typically the first AUG encountered, but the composition of the surrounding bases is also important (Kozak, 1984). Careful analysis of hundreds of RNAs found that the optimal start codon context was GCCRCC(AUG)G, with the guanosine at +4 and a purine in position -3 being the most conserved (Kozak, 1987). Occasionally, other non-AUG codons are used, such as CUG, but these are rare and very context dependent. Several initiation factors are directly involved in scanning and selecting the appropriate start codon. The factor eIF1A acts very closely with another factor called eIF1 to synergistically enable scanning of the 43S pre-initiation complex to the initiation codon. Any complexes that form on the mRNA which are not competent for scanning are dissociated by eIF1 and eIF1A, and then rebind the mRNA in a competent state (Pestova et al., 1998).

Once the initiation codon is reached, eIF5 catalyzes the release of initiation factors from the complex. eIF5 specifically binds to eIF3 and eIF2, and promotes the hydrolysis of eIF2-bound GTP, which is thought to be induced by base pairing between the initiator tRNA ($tRNA_i^{met}$) and the initiation codon (Huang et al., 1997; Pestova et al., 2001). GTP hydrolysis results in dissociation of eIF1, eIF2-GDP, eIF3, eIF4F, and eIF5, and leaves the initiator tRNA in the ribosome P site (Chakrabarti and Maitra, 1991). The joining of the 60S subunit is dependent on eIF5B, a homologue of the prokaryotic initiation factor IF2, which has a ribosomal dependent GTPase activity that is essential for its function. Binding and hydrolysis of GTP could be required for it to adopt an active structure or for the release of it or other factors from assembled 80S ribosomes. The 80S ribosome then begins the

elongation stage of translation by incrementally adding amino-acids to the growing polypeptide chain.

The model described above and in Figure 1.1 represents a somewhat superficial description of the canonical mechanism by which the majority of mRNAs are translated, with many ancillary interactions modulating each of these steps (see *Regulation of translation initiation* below). Additionally, several major deviations in this process have been described for specific RNAs. These alternative mechanisms rely in large part on the canonical scanning pathway/machinery but differ in key ways, typically involving cis-acting features in the mRNA. These processes range from the relatively simple (mechanistically) use of leaky scanning, to the more complex use of Cap-Independent Translation Elements (CITE) and Internal Ribosome Entry Sites (IRES)(see **Internal Ribosome Entry Sites** below).

Elongation

Once 80S ribosome formation has occurred, the elongation step of translation begins. This is an iterative procedure in which each codon in the mRNA sequence is recognized by a specific tRNA, which adds one additional amino-acid to the growing peptide. Briefly, an eEF1A·GTP·aminoacyl-tRNA ternary complex binds the aminoacyl-tRNA to the 80S ribosome with the anticodon loop of the tRNA in contact with the mRNA in the A site of the small subunit. Following release of eEF1A·GDP, the aminoacyl-tRNA is accommodated into the A site, and the eEF1A·GDP is recycled to eEF1A·GTP by the exchange factor eEF1B. Peptide bond formation is accompanied by transition of the A- and P-site tRNAs into hybrid states with the acceptor ends of the tRNAs moving to the P and E

sites, respectively. Binding of eEF2·GTP promotes translocation of the tRNAs into the canonical P and E sites and is followed by release of eEF2·GDP. Unlike eEF1A, eEF2 does not appear to require an exchange factor (Sivan et al., 2011). The ribosome is now ready for the next cycle of elongation with release of the deacylated tRNA from the E site and binding of the appropriate eEF1A·GTP·aminoacyl-tRNA to the A site (reviewed in Dever and Green, 2012).

Termination

Translation termination is encoded by three stop codons UAA, UAG, and UGA. Unlike recognition of sense codons, stop-codon recognition does not depend on tRNAs. Instead, termination in eukaryotes is catalyzed by two protein factors, eRF1 and eRF3, that appear to collaborate in the process (Zhouravleva et al., 1995). In eukaryotes, recognition of all three stop codons is ascribed to the class I release factor, eRF1. A ternary complex of eRF1 with the class II release factor eRF3 bound to GTP engages the ribosome, GTP is hydrolyzed, and eRF3·GDP is released (Frolova et al., 1996). Some form of accommodation takes place wherein the M domain of eRF1 swings into the catalytic center of the large ribosomal subunit catalyzing hydrolysis of the terminal peptidyl-tRNA, and nascent polypeptide is released. Finally, subunit dissociation can be mediated by initiation factors eIF3, eIF1, and eIF1A, and is likely stimulated by an ATP-independent activity of ABCE1, a member of the ATP-binding cassette (ABC) family of proteins (Korostelev, 2011; Pisarev et al., 2010).

Regulation

Mechanisms that affect global protein synthesis have been intensely studied and underlie important cellular responses to specific stimuli. Changes in the activity and availability of canonical translation initiation factors, elongation factors, and tRNA modulate global translation in response to specific stimuli and during changes in environmental and cellular conditions. Additionally, mRNA specific mechanisms of translation regulation have since been revealed. Individual mRNAs can be regulated through interactions between cis-encoded elements, canonical translation factors, and non-canonical trans-acting factors. The topic of translation initiation regulation is discussed in the next section (*Regulation of translation initiation*).

The rate of elongation is thought to be maximal under most conditions, but can be inhibited by specific mechanisms (Ingolia et al., 2011). Such inhibition is not thought to be sufficient to make elongation rate-limiting, affecting the rate of bulk protein synthesis (Hershey et al., 2012). Notably, correspondence between tRNA concentration and translation elongation speed have been observed (Varenne et al, 1984). This is the basis behind the concept of codon usage bias, where mRNA codon usage may be optimized or deoptimized in a particular cell type to "tune" protein synthesis (Novoa and Ribas de Pouplana, 2012). More subtle are the cases in which only specific regions within a gene might be strategically selected to feature slow codons. For example, choice of slow codons was suggested to affect co-translational folding [reviewed in (Tsai et al., 2008)]. A simple model suggests that the strategic usage of rare codons provides a pause during translation, during which an already translated segment of a protein may be folded in the absence of an otherwise potentially interfering segment that is not yet translated (Komar et al, 1999; Tsai

et al, 2008). There is evidence for the widespread presence of ribosomal pauses throughout the transcriptome (Ingolia et al., 2011).

Elongation factor eEF2 may also be targeted for regulation of translation, although the mechanism by which this occurs is not entirely clear. A conserved His residue of eEF2 has been shown to be post-translationally modified to diphthamide; however, this modification is nonessential for cell viability (Su et al., 2013). Knockout mice lacking DPH1, DPH3, or DPH4 (for diphthamide biosynthesis) were either embryonic lethal or showed severe developmental defects, suggesting a critical role for diphthamide during development (Webb et al., 2008). In addition to the constitutive modification by diphthamide, eEF2 can also be phosphorylated by a novel Ca^{2+}-activated protein kinase eEF2K. Phosphorylation blocks translation by impairing eEF2 binding to the ribosome (Carlberg et al. 1990). Interestingly, despite its apparent role in blocking total protein synthesis, eEF2 phosphorylation in neurons has been linked to enhanced localized translation of specific transcripts (Park et al., 2008). Although the mechanism for this enhancement has not been determined, this result bears resemblance to previous reports of enhanced translation of a subset of mRNAs in cells treated with the translation elongation inhibitor cycloheximide (Walden and Thach, 1986). In this case it was proposed that inhibition of translation in general enhances the translation of the repressed mRNAs by freeing up a limiting factor required for their translation and overcoming repressive signals.

The termination phase also may be regulated, although, under most circumstances, termination is not considered rate-limiting for protein synthesis. This is based on the observation that ribosomes are not found stacked up at the ends of mRNAs (reviewed in

(Dinman and Berry, 2007)). However, suppression of termination can modulate either frame-shifting or ribosomal read-through on select mRNAs (Adamski et al., 1993).

Regulation of translation initiation

Cells have evolved a multitude of ways to tune the efficiency of translation of different genes to different desired levels. Some gene products are needed in higher amounts than others, while the expression of others tends to be low, and, in some instances, expression must be modulated in different conditions (Ingolia et al., 2011; Takagi et al., 2005)). Initiation is the rate-limiting step of protein translation and thus serves as a key point of regulation. The classical ribosome scanning model highlights several targets of regulation by alteration of initiation factor activity affecting total protein synthesis in the cell. This allows rapid changes in protein synthesis to occur in response to cellular conditions. Translation is promoted in situations of cell growth and inhibited in conditions of cell stress such as heat shock, serum deprivation, mitosis, and viral infection (Feigenblum and Schneider, 1996).

The initiation step is regulated by a number of RNA structural elements and sequence motifs (Kozak, 2005; Jackson et al, 2010). Such structural elements include the 5'-7-methylguanosine cap and the 3'-poly-(A) tail present on all Pol II transcribed RNAs, which synergistically enhance translation-initiation efficiency (Gallie, 1991). This phenomenon has been attributed to eIF4G interaction with poly(A) binding protein (PABP), which in turn binds the 3'-poly-(A) tail leading to the current model of mRNA circularization (Kahvejian et al., 2005; Tarun et al., 1997; Wells et al., 1998). In addition,

binding and assembly of the ribosome for translation is governed by the sequence and the mRNA secondary structure in the 5'NCR and in the vicinity of the start codon.

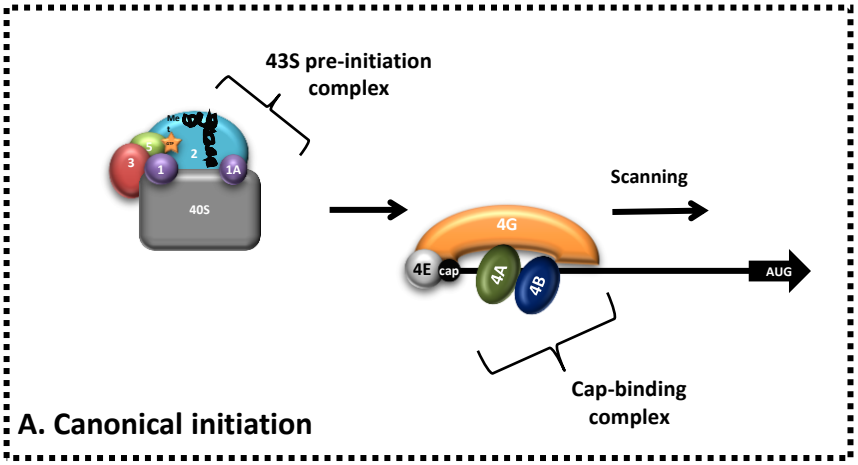
An important control point for translation initiation is in the formation of the ternary complex. This is controlled through changes in the activity and binding properties of eIF2 and eIF2B (Figure 1.1). As mentioned above, eIF2 can be phosphorylated at serine 51 on its α subunit. Phosphorylation increases the affinity of eIF2 for eIF2B and prevents eIF2B from carrying out its GDP/GTP exchange function (Price et al., 1996). This is an essential process, as eIF2 mediates the binding of $tRNA_i^{Met}$ to the ribosome in a GTP-dependent manner. eIF2 phosphorylation is carried out in response to cellular stress by four kinases, HCR (Hemecontrolled repressor), PKR (Protein Kinase activated by double-stranded RNA), GCN2 (general control non-derepressible) and PERK (PKR-like ER kinase) (Krishnamoorthy et al., 2001). These kinases increase eIF2α phosphorylation under conditions of heme deficiency, during viral infection, in amino acid and serum starvation, and following heat shock and ER stress, respectively, leading to a decrease in cap-dependent translation (Pain, 1996). eIF2a phosphorylation also occurs during apoptosis. eIF2B can also be phosphorylated at 5 different sites on its catalytic subunit (ε) by glycogen synthase kinase-3 (GSK3), and casein kinases I and II (CKI and II) (Singh et al., 1994; Wang et al., 2001; Welsh et al., 1998). GSK3 phosphorylation of eIF2Bε inhibits guanine exchange; however, stimuli such as insulin and growth factor treatment which inactivate GSK3 lead to dephosphorylation of eIF2B and increases its activity. On the other hand, the effect of phosphorylation by CKI and II is not thought to be important in control of eIF2B activity (Wang et al., 2001).

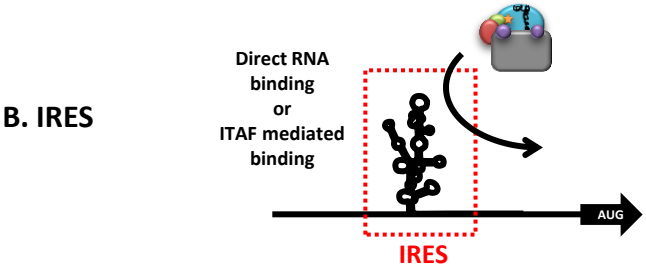
Another important control point in initiation is recognition of the m^7G-cap of an mRNA mediated by the cytoplasmic cap-binding protein eIF4E (Figure 1.1). Translation initiation can be tuned through abundance of eIF4E, phosphorylation of eIF4E, or through the action of eIF4E binding proteins which themselves are phosphorylated to control their activity. eIF4E can be phosphorylated at Ser209 and this phosphorylation results in enhanced translation, although the mechanism of enhancement remains unclear (Kaspar et al., 1990; Pyronnet et al., 1999; Scheper et al., 2002). Phosphorylation is carried out by MNK1 (MAP kinase interacting protein kinase-1) and MNK2 and is mitogen-induced (Waskiewicz et al., 1999). MNK1 and MNK2 both bind eIF4G in the vicinity of the eIF4E interaction domain. The importance of these interactions was demonstrated by showing that mutations in the eIF4E binding site of eIF4G decreased eIF4E phosphorylation (Pyronnet et al., 1999). eIF4E is further regulated by three eIF4E binding proteins (4E-BP 1-3) (Poulin et al., 1998). These small proteins bind eIF4E and block interaction with eIF4G. The 4E-BPs are controlled by hyperphosphorylation in response to cell growth, serum and hormones, and hypophosphorylation during picornavirus infection, nutrient deprivation and mitosis (Gingras et al., 2001; Pyronnet et al., 1999).

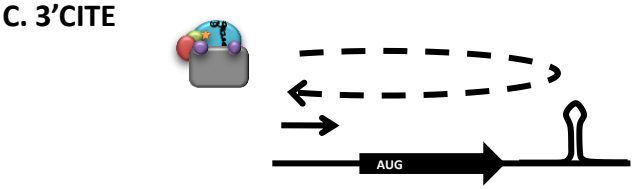
The role of other initiation factors in translational control is not fully understood, although there have been reports of phosphorylation or cleavage of different factors under certain conditions (Bushell et al., 2000; Gingras et al., 1999). eIF3 has been suggested to be regulated by the protein p56, which is induced by interferon and dsRNA, and binds eIF3 and inhibits translation *in vitro* and *in vivo* (Guo et al., 2000). Translational control may also be achieved through phosphorylation of ribosomal protein S6, although the biochemical

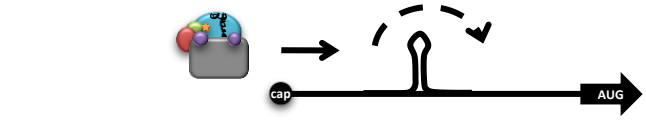
consequence of rpS6 phosphorylation remains poorly understood (Magnuson et al., 2012; Rhoads, 1999; Sturgill and Wu, 1991).

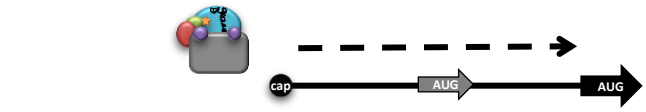
While generally the above-described mechanisms will have wide ranging effects on bulk protein synthesis, there are mRNA intrinsic features that can modulate gene expression. These cis-acting RNA elements may tune the efficiency of canonical translation or drive an entirely separate mode of initiation (Figure 1.2). In addition to the enhancement of translation by the m^7G-cap and poly(A) tail mentioned above, the sequence and structural elements in the 5' and 3'NCR have been shown to regulate initiation. For example, sequence specific control elements have been identified in the 3'NCR of particular mRNAs and are implicated in gene regulation during development (Ostareck et al., 1997). The first such example of 3'NCR mediated control was the repression of Fos proto-oncogene (FOS; c-fos) and c-myc proto-oncogene (MYC; c-myc) by targeted shortening of the poly(A) tail. The major determinant of the rapid turnover of these mRNAs as well as lymphokine mRNAs is the multiple copies of AU-rich motifs (Jackson and Standart, 1990). In contrast, a 3'NCR sequence known as a differentiation control element (DICE) in the 15-lipoxygenase (ALOX15) mRNA, a gene critical for erythrocyte maturation, can stabilize mRNA while repressing translation initiation. The binding of heterogeneous nuclear ribonucleoprotein K (HNRNPK; hnRNP K) and poly(rC)-binding protein 1 (PCBP1) as well as DEAD-box helicase 6 (DDX6) act to translationally silence the mRNA by repressing 80S formation and targeting mRNA to stress granules (Ostareck et al., 1997). Notably, c-myc translation is stimulated by the binding of hnRNP K, PCBP1, and PCBP2 to the IRES element present in the ~400 nt long, structured 5'NCR (see **Internal Ribosome Entry Sites**)(Evans et al., 2003).


43S pre-initiation complex
40S
Scanning
4G
4E
cap
4A
4B
AUG
Cap-binding complex
A. Canonical initiation


B. IRES
Direct RNA binding or ITAF mediated binding
AUG
IRES


C. 3'CITE
AUG


D. Shunting
cap
AUG


E. Leaky scanning
cap
AUG
AUG

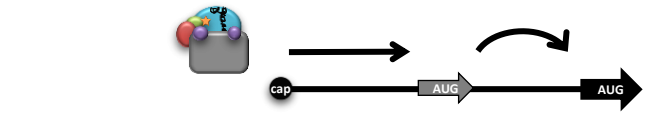

F. Reinitiation
cap
AUG
AUG

Figure 1.2. Alternative mechanisms of translation initiation. A. The paradigm for cap-dependent involves recognition of the eIF4F cap binding complex by the 43S pre-initiation complex and linear scanning to the first cognate AUG. Cis-acting RNA structures drive alternative modes of initiation dependent of independent of the 5'-terminus and/or cap. **B**. Internal ribosome entry sites recruit pre-initiation complexes to translation initiation sites independently of the 5'-end and cap. **C**. Cap-independent translation elements drive cap-independent, but 5'-dependent translation. **D**. Ribosome shunting involves canonical ribosome recruitment, with non-linear scanning wherein a portion of the 5'NCR is skipped. **E**. Leaky scanning occurs when one potential start codon is bypassed for initiation at a downstream site. **F**. Translation-reinitiation involves translation of a downstream cistron following translation of an upstream cistron without 40S ribosome dissociation.

Since scanning is linear, and ATP-dependent, it is no surprise that the majority of mRNAs have fairly short 5'NCRs (typically 50-100 nt)(Berthelot et al., 2004; Kozak, 1987). There are notable exceptions with 5'NCRs ranging up to 2000 nt which can form highly complex structures. Nonetheless, most mRNA molecules will assume some degree of secondary and tertiary structure in their 5'NCR that might be highly structured for some RNAs, and less so for others, which must be scanned through by the 40S ribosome. This offers an mRNA specific mechanism of translation regulation; by virtue of their structure, ribosome binding and the rate of its scanning might be controlled. Early evidence showed that stable secondary structures along the 5'NCR, and particularly proximal to the translation start site, reduce translation efficiency, presumably by obstructing ribosome binding (Wang and Wessler, 2001). More recent studies with libraries of synthetic genes found that secondary structure at the 5' end of the mRNA inhibits translation, whereas relatively unstructured domains promote translation (Kudla et al., 2009).

In addition to cis-acting RNA structures, RNA sequence in the 5'NCR specifically regulate canonical translation initiation or in some cases, determine alternative methods of initiation. For example, leaky-scanning is a process which involves a weak initiation codon (Kozak consensus sequence) on mRNA which is sometimes skipped by the ribosome in translation initiation. The 40S ribosomal subunit continues scanning to a downstream initiation codon. This process has been described in viral RNA translation as a means of regulating expression of a poly-cistronic RNA (Fütterer et al., 1997; Herzog et al., 1995). In eukaryotes, this process has been shown to be modulated under certain conditions where the uORF can be selectively activated, leading to programmed ribosomal arrest, and

preventing leaky-scanning to the downstream ORF (Hayashi et al., 2017; Wang and Sachs, 1997; Wang et al., 1999).

Another closely related deviation from the canonical process is so-called termination re-initiation (REI) wherein translation of an REI-permissive short upstream ORF (uORF) terminates, only the large 60S subunit is recycled, and the post-termination 40S subunit is retained on the mRNA to allow REI downstream (Barbosa et al., 2013; Hronová et al., 2017; Janich et al., 2015). It has been shown in yeast that the efficiency depends on cis-acting features occurring in sequences flanking REI-permissive uORFs. For example, the first two uORFs from the reinitiation-regulated yeast GCN4 mRNA leader carry specific structural elements in their 5' sequences that interact with the translation initiation factor eIF3 to prevent full ribosomal recycling post translation termination.

Another example of non-canonical initiation is the process known as ribosome shunting wherein bound 40S ribosome is able to bypass (without scanning) a portion of the RNA and bind downstream to initiate translation (Dominguez et al., 1998; Fütterer et al., 1993). As with many of the non-canonical mechanisms described, this process was first identified as a viral mechanism for translation initiation. However, typically these processes are later shown to occur for host cell processes as well (Koh et al., 2013; Rogers et al., 2004). The most prominent examples of this mechanism are for the adenovirus tripartite leader 5’NCR and the heat shock protein Hsp70 5’NCR, which can direct shunting over scanning when eIF4F availability or activity is reduced. Notably, this behavior is dependent on complementarity of cis elements in the mRNA to 18S rRNA (Yueh and Schneider, 2000).

Finally, two classes of relatively complex cis-acting elements are able to direct translation initiation in a cap-independent manner. The first of these is the Cap-Independent Translation Elements (CITE), a feature present in some plant virus RNAs. These features, which are typically in the 3'NCR (but have been shown to function when repositioned to the 5'NCR), can directly bind eIF4F and promote initiation independent of a cap, but in a 5'-end and scanning dependent fashion (Danthinne et al., 1993; Miller et al., 2007; Timmer et al., 1993). An Internal Ribosome Entry Site (IRES) on the other hand, is able to direct translation initiation independent of both the cap and the 5'-end (Jang et al., 1988; Pelletier and Sonenberg, 1988). This represents a mechanism first described for picornaviruses that drives translation initiation by a typically long and highly complex 5'NCR which is able to bind various initiation factors. These elements are able to drive translation of genes considered inaccessible to scanning mediated translation. This includes transcripts lacking a 5'-cap, with highly structured 5'NCRs which would inhibit ribosome scanning, and sites well removed from the 5'-terminus. The most common test for such elements is a dicistronic reporter assay, where an RNA has two tandem reporter genes separated by an intergenic NCR. Insertion of an IRES element can drive downstream gene expression in an orientation, sequence, and structure specific manner far from the 5'-end. The 5'-terminus independence has been further solidified by demonstrating that initiation by an IRES mechanism can occur on circular RNAs (Chen and Sarnow, 1995).

Internal Ribosome Entry Sites

Originally described for picornaviruses, IRESs recruit the 40S ribosome to a site distal to, and not strictly dependent on, the 5' end. Since their initial discovery, IRES

mediated translation has been described for a number of other viruses and in the mRNAs of eukaryotic cellular genes (Jang et al., 1988; Macejak and Sarnow, 1991; Negulescu et al., 1998; Pelletier and Sonenberg, 1988). First described for BiP (a molecular chaperone regulating protein folding), these cellular IRES elements have been identified in many mRNAs important for regulating cell growth, apoptosis, and stress responses (Bert et al., 2006; Hellen and Sarnow, 2001; Jackson, 2005). It is thought that IRESs may play an important role by perpetrating continued gene expression of these crucial cell regulators under conditions not conducive to global protein synthesis. For example, when a cell is subjected to pathogenic or physical stressors, cap-dependent translation is down regulated by cleavage or covalent modification of important initiation factors. However, under these conditions, several mRNAs important for regulating the stress responses are still translated by virtue of IRES elements (Gu et al., 2009; López-Lastra et al., 2005). Although most eukaryotic mRNAs are translated by a cap-dependent mechanism, it is approximated that upwards of 10% of cellular mRNAs may utilize IRES-dependent initiation (Spriggs et al., 2010). For example, amino acid starvation generally inhibits translation, but stimulates amino acid transport systems (McGivan and Pastor-Anglada, 1994). It has been shown that two members of this pathway, the cat-1 Arg/Lys transporter (SLC7A1) and the sodium-coupled neutral amino acid transporter (SLC38A2), contain IRESs important for nutrient dependent control ((Fernandez et al., 2005; Gaccioli et al., 2006). Not only is IRES-mediated translation important for cellular survival under stress, it also is important for cell proliferation, angiogenesis, and in many disease states, such as autoimmune diseases, neurodegeneration and cancer. Notable examples of these IRES containing genes include BAG1, XIAP, BCL2, c-IAP1 (BIRC2), FGF2 , c-myc (MYC), n-myc (MYCN), PDGF2, IGF2,

CDKN1B, p53 (TP53), PITSLRE, VEGFA, and HIF1α (HIF1A) (Andreucci et al., 2017; Bastide et al., 2008; Bernstein et al., 1997; Candeias et al., 2006; Dai et al., 2011; Dobbyn et al., 2008; Graber et al., 2010; Holcik and Korneluk, 2000; Holmes et al., 2016; Morfoisse et al., 2013; Tinton et al., 2005; Topisirovic and Sonenberg, 2011; Willimott and Wagner, 2010; Yoon et al., 2006). In a recent review of the literature describing IRES elements throughout the genome, IRES elements have been attributed to nearly every functional class of protein-encoding genes. Interestingly, the plurality of genes harboring IRES elements appear to be transporters, receptors, and channels (22%)(Lacerda et al., 2017). One such example is the murine voltage-gated potassium channel Kv1.4 (KCNA4) that has been shown to translate in an IRES dependent mechanism. Since transporters, receptors and channels are the main vehicle for cell-cell communication and signal transduction, these genes represent fundamental elements in cellular homeostasis. Post-transcriptional regulation may be necessary for example, to maintain the number of functional channels in various tissues, since overexpression would likely reduce the excitatory effects of depolarizing currents, whereas a deficit would likely enhance excitability.

Investigation of picornaviral IRESs has shown that there is very little sequence homology within the virus groups, but a strong conservation of secondary structure (Jackson et al., 1990). These IRES regions were found to consist of a pattern of stem loops organized into structural domains which have been classified into 4 types (for picornaviruses). The type I enterovirus and rhinovirus IRESs have been shown to consist of a series of 5 stem-loop structures while the type II IRESs of cardioviruses contain 9 stem-loop structures, followed by a short poly-pyrimidine tract (8 to 10 nt)(Duke et al., 1992). For type II IRESs the initiation AUG immediately follows the poly-pyrimidine tract, whereas

for type I IRESs the initiation codon is located 30-150 nucleotides downstream, likely requiring some scanning or shunting mechanism to initiate (Haller and Semler, 1992; Jackson et al., 1990). Other common features have also been identified, including GNRA motifs identified in stem loop IV of type I IRESs and stem loop I of type II IRESs, and A/C rich sequences in stem loops IV and V of type I IRESs and stem loop I of type II IRESs (Justine and Franck, 2018). Mutations in these GNRA motifs in EMCV and PV IRESs have been shown to inhibit IRES activity (Robertson et al., 1999). Outside picornaviruses, the mechanism of internal ribosome entry for other viruses and eukaryotic genes appears to be diverse and wide ranging. For example, dicistroviruses harbor an IRES in an intergenic region of their mRNA which is so refined that it can directly recruit 40S ribosome and position it for initiation without other initiation factors (Kanamori and Nakashima, 2001; Sasaki and Nakashima, 2000; Wilson et al., 2000). In contrast, most other IRES elements depend on different sets of canonical initiation factors as well as non-canonical factors collectively described as IRES trans-acting factors (ITAFs)(Discussed below in **IRES trans-acting factors**).

Unlike many viral mRNAs, all cytoplasmic mRNAs are capped and predestined to attract ribosomes to the 5'-terminus. This provides a quandary as to whether mRNA translation is entirely driven by IRES-mediated translation, or whether the 5'-end and -cap play a significant role. Dicistronic assays, the most common test for IRES-mediated translation, indicate that translation is mildly attenuated compared to a monocistronic context (Andreev et al., 2009; Bert et al., 2006). Nevertheless, most of these IRESs do drive translation in dicistronic context, and it may very well be that in their natural context the

5'-cap stimulates IRES mediated translation by enhanced recruitment of initiation factors, with the IRES acting as a buffer to translation shutoff in certain contexts.

Kv1.4

The number of known potassium channel genes is relatively large (~40); however, the diversity of potassium current phenotypes observed from various excitable cells is much greater. Alternative splicing, posttranslational modification, and heterologous assembly of pore-forming subunits all expand the functional diversity of K^+ channel gene products. Even greater diversity can be achieved through interactions between K^+ channel proteins and accessory proteins or subunits. Voltage gated potassium channels (Kv1-Kv4 in mammals) modulate excitability of cells by opening and closing a potassium-selective pore in response to voltage (reviewed in Bezanilla and Perozo, 2003). The Kv1 superfamily of channels (also known as the Shaker superfamily) consists of four α subunits arranged around a central axis that is the ion conduction pathway (Doyle et al., 1998; Long et al., 2005). The α subunits share structural features including six membrane-spanning segments (S1–S6) cytoplasmic N- and C-termini. The ion conduction pore is formed by S6 segment and the connecting loop between S5 and S6, which contains a conserved sequence (gly-tyr-gly, or gly-phe-gly) which imparts K^+ selectivity to the pore. Within the S4 segment are positively charged amino acids (arginine or lysine) at every third position which act as the voltage sensor responsible for voltage-dependent gating.

Initial characterization of the Kv1 family genes revealed unusually long transcripts due to the presence of extensive and highly conserved 5' and 3'NCRs (Stuhmer et al., 1989; Tamkun et al., 1991; Wymore et al., 1994). For example, Kv1.4, which is a channel specific

to the heart and brain, has two transcript isoforms of 3.5 and 4.5 kb. These two are identical except for the 3′-NCR, with the longer transcript terminating at an alternative polyadenylation site. The 5'NCR of this gene is unusually long (1.2 kb) and has multiple AUGs upstream of the initiation codon (Wymore et al., 1996). It is now known that the 5'NCR of Kv1.4 plays an important role in regulating channel expression through an IRES mechanism (Negulescu et al., 1998).

The Kv1.4 gene encodes a rapidly inactivating, 4-aminopyridine-sensitive K^+ channel with electrophysiological properties resembling the neuronal after-hyperpolarization inducing current, and the cardiac slow recovery from inactivation component of the transient outward current $I_{to,s}$ (Guo et al., 1999; Xu et al., 1996, 1999). The promoter for this gene has little if any tissue specificity, is located in a GC-island containing three SP1 binding sites (CCGCCC), and is modulated by at least one region containing enhancer activity. It is known that Kv1.4 is under tight regulation, but the mechanisms responsible for tissue-specific expression of this gene have yet to be determined.

The tight transcriptional and post-transcriptional regulation of Kv1.4 reflects the need to maintain closely defined numbers of functional channels in relevant tissues. Dysregulation of a rapidly inactivating K^+ current like Kv1.4 by overrepresentation may lower the threshold range of excitation, likely reducing the excitatory effects of depolarizing currents on neurons, whereas a deficit of Kv1.4 current might enhance neural excitability (Ruppersberg et al., 1991). Alteration of transcriptional or post-transcriptional processes is likely to be associated with pathological states, possibly contributing, for

example, to the enhanced Kv1.4 mRNA expression seen in cardiac hypertrophy (Matsubara et al., 1993).

Previous work by Gutman, Semler, and colleagues has shown that IRES element(s) are present in the 5'NCR of the murine voltage-gated potassium channel Kv1.4 (Jang et al., 2004; Negulescu et al., 1998). This mRNA displays hallmarks of IRES-mediated translation: long and highly structured 5'NCR, multiple upstream AUGs, and unexpected translation profiles (Stuhmer et al., 1989). Furthermore, the Kv1.4 mRNA has been shown to harbor bona fide IRES elements by dicistronic reporter assays as well as through *in vitro* experimentation. As noted above, Kv1.4 has a highly tissue-specific expression (heart and brain) despite the apparently nonspecific promoter, pointing to a possible post-transcriptional mechanism of regulation. Indeed, wide variation was observed in expression from the Kv1.4 IRES reporter RNA depending on the cell type used (Jang et al., 2004; Negulescu et al., 1998). This phenomenon has been described for other reported cellular IRESs and suggests that IRES-mediated translation may confer regulation of gene expression in a cell-type specific manner (possibly through the protein species that are involved in non-canonical translation in combination with their relative abundances in different cell types).

IRES trans-acting factors

Translation initiation, whether by scanning or internal entry, is highly dependent upon RNA binding proteins. Those proteins involved in ribosome scanning are well-studied and many, including the cap binding proteins and proteins which relax RNA secondary structure, have been studied genetically as well as functionally. Many of the proteins

required for scanning are certainly also involved in translation initiation by internal ribosome entry, with a few notable exceptions (dicistroviruses).

Considerable work has been focused on the trans-acting factor requirements for picornaviral IRES translation. From this work it was found that almost the same set of factors that are important for cap-dependent translation are also important for cap-independent translation, with the IRES granting independence from only a few key factors (Hunt et al., 1999). The EMCV IRES was found to require the factors eIF2, eIF3, eIF4A and the central domain of eIF4G for 48S complex formation in vitro (Lomakin et al., 2000; Pestova et al., 1996). As well as initiation factors, EMCV IRES translation also required $tRNA_i^{Met}$, GTP and ATP, suggesting that the helicase function of eIF4A is important. eIF4A may act to unwind mRNA structure or have some function in structural IRES rearrangements for ribosome binding (Hellen and Sarnow, 2001). The FMDV IRES has been found to have the same requirements for eIF2, eIF3, eIF4A, eIF4B and eIF4F and slightly different requirement for non-canonical factors (Pilipenko et al., 2000). Again, an interaction of eIF4G and eIF4A is required, and the central domain of eIF4G was found to bind the J-K domain of the IRES.

The type II picornaviral IRESs were found to be very efficient in *in vitro* translation systems such as rabbit reticulocyte lysate (RRL), whereas the type I IRES of poliovirus needed HeLa cell lysate for efficient translation. This suggested that type I IRESs had a requirement for additional protein factors not present or present in low abundance in reticulocyte lysate and prompted investigation into these additional protein factors (Jackson and Kaminski, 1995). One of the first IRES trans-acting factors (ITAFs) identified was a 57 kDa protein, later identified as a nuclear RNA binding protein called

polypyrimidine tract binding protein (PTB), that UV cross-linked to stem loop H of the EMCV IRES, and was required for IRES mediated translation (Borman et al., 1993; Jang and Wimmer, 1990). PTB was also identified separately as an activity that enhanced HRV and poliovirus type I IRES translation *in vitro* (Hellen et al., 1993; Hunt and Jackson, 1999).

PTB functions in the uninfected cell as a negative regulator of alternative splicing, in 3' end processing and RNA localization. PTB, also known as heterogeneous nuclear ribonucleoprotein I, contains 4 RNA recognition motifs used for interaction with short pyrimidine motifs (Yuan et al., 2002). A number of other cellular trans-acting factors have since been discovered and the requirement for these factors varies considerably between different viral IRESs. A likely incomplete list of ITAFs effecting IRES-mediated translation for viruses is as follows: [adapted from (Lee et al., 2017)]

Table 1.1. IRES trans-acting factors (ITAFs) of viral IRES-dependent translation

Official full name	Also known as	Viruses	Regulation sites (5' UTR)	IRES activity
Heterogeneous nuclear ribonucleoprotein D	hnRNP D, AUF1	PV, EV-A71, HRV2, HCV	SL-II (EV-A71)	Inhibition/ Enhancement
Polypyrimidine tract binding protein 1	PTBP1	PV, CVB3, EMCV, FMDV, HRV2, HAV	Domain V (PV)	Enhancement
Poly(rC) binding protein 2	PCBP2	PV, CVB3, HAV, HRV	Cloverleaf and domain IV (PV), SL-I and IV(CVB3)	Enhancement
Sjogren syndrome antigen B	SSB, La	PV, CVB3, EMCV, HCV, HAV	-	Enhancement/ Inhibition
Cold-shock domain containing E1	CSDE1, Unr	PV, HRV	-	Enhancement
Glycyl-tRNA synthetase	GARS	PV	Domain V	Enhancement
Gem nuclear organelle associated protein 5	Gemin5	HCV FMDV	Domain V (FMDV)	Inhibition
Heterogeneous nuclear ribonucleoprotein L	hnRNP L	HCV	Between domains I and II	Enhancement
Synaptotagmin binding cytoplasmic RNA interacting protein	SYNCRIP, NSAP1, hnRNP Q	HCV	-	Enhancement

Nucleolin	NCL, C23	PV, HRV	-	Enhancement
Serine- and arginine- rich splicing factor 3	SRSF3, SRp20	PV, HRV, CVB3	-	Enhancement
KH RNA binding domain containing, signal transduction associated 1	KHDRBS1, Sam68	PV, EV-A71, FMDV	Domains III and IV (FMDV)	Enhancement
Far upstream element-binding protein 1	FUBP1, FBP1	EV-A71	Spacer region	Enhancement
KH-type splicing regulatory protein	KHSRP, FBP2	EV-A71	SL-I to II, SL-II to III, SL-V to VI, and the spacer region	Inhibition
Heterogeneous nuclear ribonucleoprotein K	hnRNP K	EV-A71	SL-II and IV	Enhancement
Heterogeneous nuclear ribonucleoprotein A1	hnRNP A1	EV-A71, SV, HCV	SL-II and VI (EV-A71)	Enhancement
ELAV-like RNA-binding protein 1	ELAVL1, HuR	EV-A71	SL-II	Enhancement
Argonaute 2, RISC catalytic component	AGO2	EV-A71	SL-II	Enhancement
Proliferation-associated 2G4	PA2G4, Ebp1, ITAF45	FMDV	Domains II to V	Enhancement
G3BP stress granule assembly factor 1	G3BP1	CVB3	-	Inhibition
Eukaryotic translation initiation factor 4 gamma 2	EIF4G2, DAP5	CVB3	-	Enhancement

While some of these factors act for many viruses (e.g., PTB and PV, CVB3, EMCV, FMDV, HRV2, HAV), some of these factors have only been shown to affect particular IRESs (e.g. HuR and EV-A71).

ITAFs are considered to aid IRES elements in adopting and maintain the correct structure for initiation factor and 40S ribosome binding. Many of these IRES trans-acting factors have multiple RNA binding domains, and may directly bind the IRES to stabilize the secondary and tertiary structure, or direct initiation factor recruitment (Hellen and Sarnow, 2001). It is noteworthy that many of these factors are strongly nuclear proteins, justifying the disruption of nucleocytoplasmic trafficking observed during infection (Flather and Semler, 2015; Vagner et al., 2001).

Several lines of evidence suggest that the trans-acting factor requirements are just as diverse for cellular IRESs as those for viruses. Frequently, eukaryotic IRESs display cell tropism; e.g., the c-myc and n-myc IRESs have comparable activity in HeLa cells, yet n-myc IRES-mediated translation is increased sevenfold compared to c-Myc IRES in neuronal cells (Jopling and Willis, 2001). While both the fibroblast growth factor 2 (FGF2) and c-Myc IRESs are active in developing embryos, only the FGF2 IRES activity is apparent in adult brain, suggesting that certain ITAFs are not present in the fully differentiated cell types (Creancier et al., 2000, 2001). Finally, the trans-acting factors for eukaryotic cellular IRESs that have been studied so far have been shown to differ. For example, the XIAP IRES requires La and hnRNP C, while the Apaf-1 IRES requires Unr and polypyrimidine tract binding protein/neuronal polypyrimidine tract binding protein (PTB/nPTB) for function (Holcik and Korneluk, 2000; Holcík et al., 2003; Mitchell et al., 2001, 2003).

The dicistronic assay for identifying IRES elements involves either transfection of *in vitro* transcribed and capped RNA, or transfection of plasmid DNA. For a number of cellular IRESs, there has been an observed discrepancy in the reporter expression using these two methods, relative to RNA levels. This was a major point raised early on in questioning the existence of cellular IRESs (Bert et al., 2006; Hellen and Sarnow, 2001; Kozak, 2005; Van Eden et al., 2004). It has been suggested that this could be due to the presence of cryptic splice sites in the reporter plasmid or cryptic promoter sequences generating truncated reporter transcripts. However, for many such IRESs, like the IRES for lymphoid enhancer binding factor 1 (LEF1), no such truncated transcripts have been detected by Northern-blot analysis, and cryptic promoter activity is not apparent when the SV40 promoter sequence is removed. One possibility is that this observed discrepancy is due to the so called

"nuclear-experience" (or lack thereof), and not cryptic promoters or splice sites (Figure 1.3). When RNA is transcribed within the nucleus and is exported to the cytoplasm to be translated (as during plasmid transfection), it is exposed to an entirely unique cadre of proteins that may be required for maximal translation efficiency (Semler and Waterman, 2008). The proteins that interact with the RNA produced in the nucleus may not be present/available for binding when RNA is directly introduced into the cytoplasm of cells (e.g., the many hnRNPs described as ITAFs).

By utilizing a two-pronged approach to screen for IRES interacting proteins, the goal of this dissertation research was to identify these factors and demonstrate that enhanced translation from the Kv1.4 IRES element can be recapitulated in the presence of nuclear trans-acting factors. The first prong of this approach involved a yeast hybrid genetic screen, and the second prong was biochemical purification of these factors by RNA-affinity. The ensuing chapters of this thesis will describe both of these approaches, the candidate proteins they uncovered, and the functional significance of these putative IRES trans-acting factors in directing the synthesis of one family of ion channel proteins.

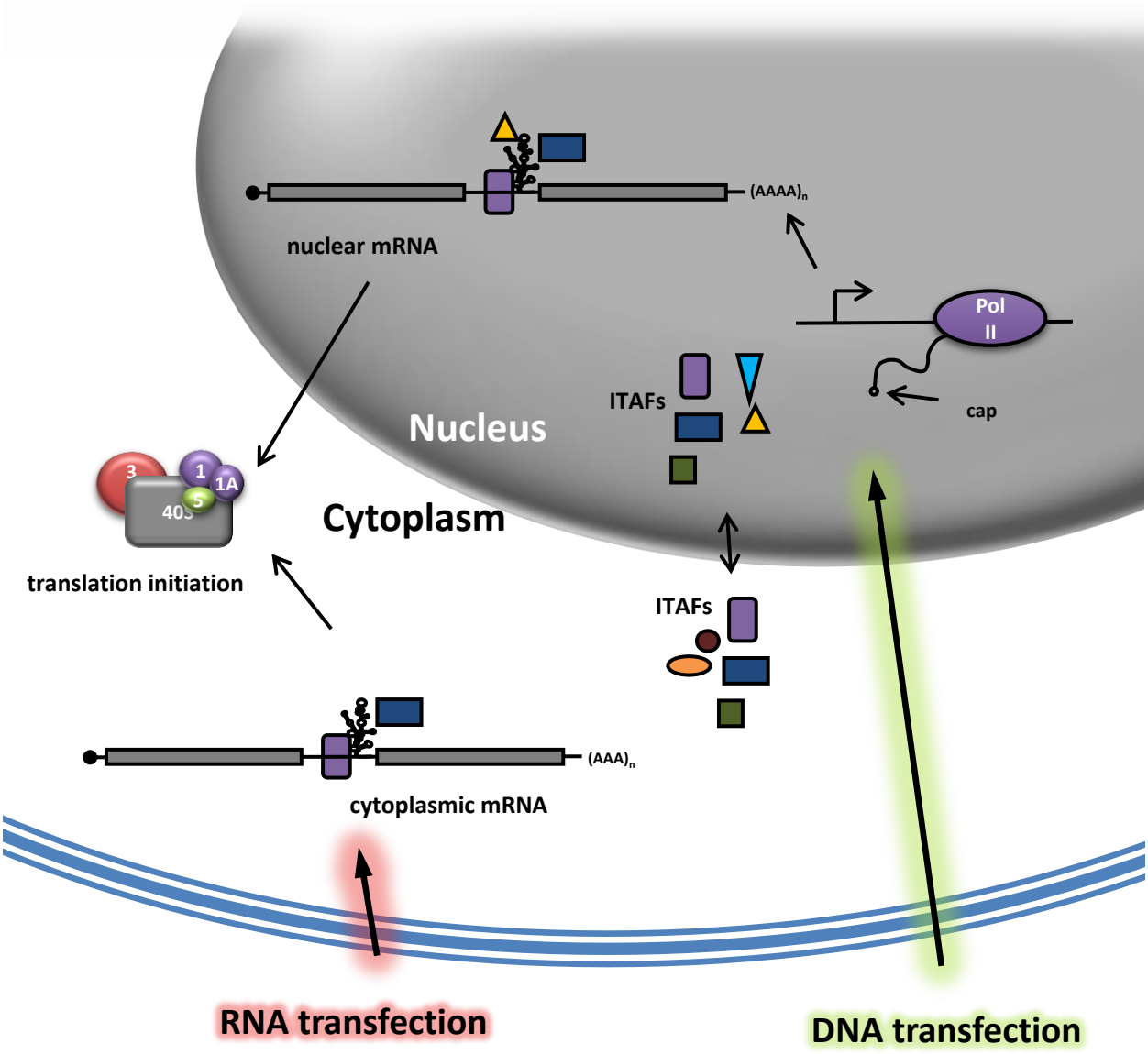


Figure 1.3. The nuclear experience. RNA produced from a transfected DNA plasmid is transcribed in the nucleus and exposed to a unique cadre of RNA binding proteins before export that RNA directly transfected into a cell would not. A newly transcribed RNA will be decorated with spicing proteins and RNA export factors before being transported into the cytoplasm. These proteins may include ITAFs important for maximal translation directed by IRES elements. This nuclear experience will impart greater apparent IRES activity to RNAs derived from plasmid transfection.

Chapter 2:

Parallel screening for IRES trans-acting factors

Introduction

The mechanism of IRES-mediated translation for any given mRNA is most readily addressed by determining the initiation factors and ITAFs necessary for their function. While some IRES elements are able to potentiate translation independently of most initiation factors, the majority of IRESs require the interaction of several factors to recruit the ribosome. It has been hypothesized that tissue and cell specific factors may play a role in determining translation from a given IRES as a mechanism for post-transcriptional regulation (Kondrashov et al., 2011; Pilipenko et al., 2000; Xue et al., 2015). As noted in the previous chapter, Kv1.4 demonstrates a very tissue specific expression pattern, despite its non-specific promoter, and investigation of the IRES element indicated cell line specific variation in translation. These observations lead to the hypothesis that Kv1.4 is regulated post-transcriptionally, which may be contingent upon tissue/cell specific IRES trans-acting factors. Indeed, context dependent mechanisms are the paradigm for post-transcriptional regulation (see **Chapter 1:** *Regulation of translation initiation*).

The aim of the experiments described in this chapter was to investigate the protein factor requirements of the Kv1.4 IRES. This was achieved by identifying novel trans-acting factors by performing a yeast three-hybrid screen in parallel with *in vitro* affinity purification and mass spectrometry analysis. Yeast 3-hybrid screen provides a sensitive approach to identifying specific RNA binding proteins, particularly those at low abundance, with the caveat that ternary interactions cannot be detected. IRES elements likely function by recruiting a functional complex to the initiation start site and this logically would

involve protein-protein interactions which may or may not be dependent on the RNA context. For example the poliovirus ITAF SRp20 (SRSF3) does not bind the IRES, but instead acts as a complex with PCBP2 to recruit ribosomes (Fitzgerald and Semler, 2011). Additionally, Y3H relies on interactions between chimeric proteins and RNA, which is necessarily truncated compared to the full IRES element (Bernstein et al., 2002). By using a biochemical approach, we are able to use full length IRES sequences which can bind native proteins directly, or as a complex. This approach favors high affinity interactions and is biased against transient interactions, which may still be important for IRES function.

Yeast 3-hybrid screen

The yeast 2-hybrid assay is a powerful genetic technique that has been extensively used to characterize protein-protein interactions (Fields and Sternglanz, 1994). Importantly, this technique can be used as an unbiased screen for protein-protein interactions from a cDNA library. Here we employed an extension of this technology, the yeast 3-hybrid assay (Y3H), to identify IRES trans-acting factors (SenGupta et al., 1996). This approach allowed us to screen the entire transcriptome for protein-RNA interactions (Bernstein et al., 2002; SenGupta et al., 1996). Briefly, a bi-functional "bait RNA" representing the IRES region of interest fused to tandem MS2 RNA hairpins (RNA structures found within the MS2 bacteriophage which are bound with high affinity by the MS2 coat protein)(Figure 2.1). This RNA can then interact with two hybrid proteins to activate reporter gene expression in yeast. The first hybrid protein is a LexA DNA binding domain fused to the MS2 bacteriophage coat protein, which binds the MS2 RNA hairpins of the bait RNA and presents the IRES region of interest to binding by other proteins. A cDNA

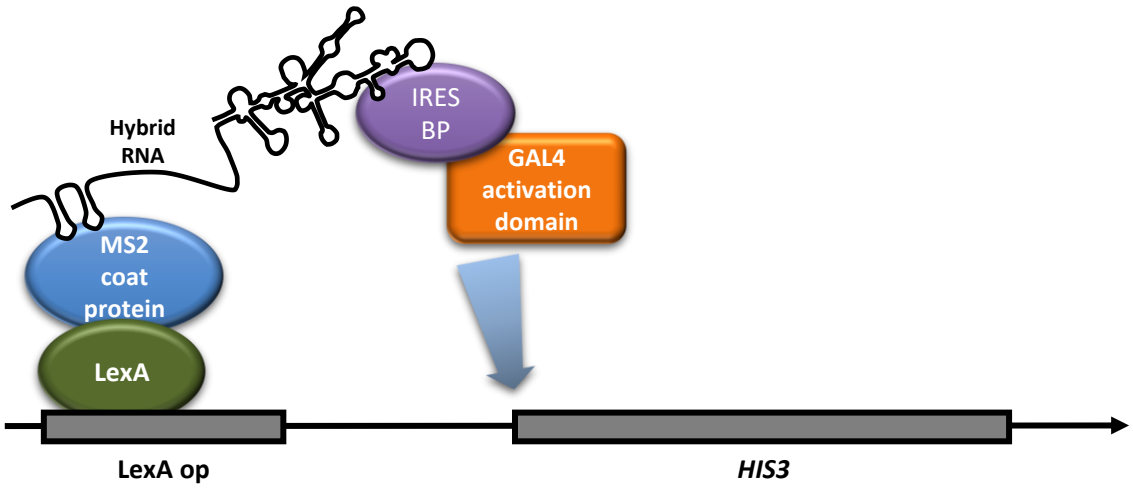


Figure 2.1. Schematic of three-hybrid interaction strategy for detection of RNA-protein interactions. Both LacZ and HIS3 are present in strains YBZ1, and are under control of the LexA operator. YBZ1 also expresses a LexA BD/MS2 CP fusion for binding to the LexA operon and interaction with a hybrid RNA which contains MS2 coat protein binding sites and the experimental "bait" RNA. Interaction with exogenous binding protein as a fusion with the Gal4 activation domain activates LexA reporter genes permitting growth on histidine free media. LexA operon also controls β-galactosidase expression, allowing for blue white screening for 3-hybrid interactions.

library fusion with a Gal4 activation domain is subsequently introduced and if the library protein domain interacts, the Gal4 activation domain will activate HIS3 (imidazoleglycerol-phosphate dehydratase) gene expression. Since the yeast used in this assay are auxotrophic for histidine, HIS3 activation will allow for growth on media lacking histidine (histidine drop-out media). Sequence results revealed a number of known trans-acting factors, initiation factors, ribosomal proteins in addition to novel factors which may be important for IRES-mediated translation.

RNA affinity chromatography

A more standard approach to identifying important RNP complexes is direct purification from cell lysates. A number of approaches have been taken, each with their advantages and disadvantages, from sucrose gradient centrifugation to immunoprecipitation (IP) and RNA-affinity isolation. These methods have the advantage of purifying native proteins from cell lysates. In the present study, we used an *in vitro* transcribed and biotinylated RNA of the full Kv1.4 5'NCR to affinity purify RNP associated proteins. This approach is frequently employed to identify unknown RNA binding proteins and has been used to identify functional ITAFs (Hunt and Jackson, 1999). By incorporating biotinylated-nucleotides in *in vitro* transcribed RNA, RNA can be bound with high affinity to a streptavidin matrix. By allowing the formation of RNP complexes in cell extract prior to streptavidin chromatography, protein components can then be selectively isolated by sodium chloride gradient elution. Two different tactics were taken to this approach, the first a more stringent approach of tandem purification using gradient elution, and the second a less stringent approach involving one step side-by-side purifications and

comparative, quantitative mass spectrometry. Relevant to this experimental approach was an *in vitro* stimulation of translation assay to test for functional fractions. This assay was similar to the assay used by Hunt and Jackson for identifying PTB as an HRV ITAF and involved supplementation of cell-free *in vitro* translation reactions with lysates/fractions. Later disuse of this assay is detailed in the discussion(Hunt and Jackson, 1999).

Results

Yeast 3-hybrid screen

To identify proteins that might modulate IRES mediated translation of Kv1.4, we conducted a yeast three-hybrid screen for proteins that could interact with the IRES RNA. *Saccharomyces cerevisiae* strain YBZ1 is auxotrophic for uracil, leucine, adenine, and histidine and has the *HIS3* and *LacZ* genes under control of the *lexA* operator. This strain also has a gene encoding a LexA-MS2 fusion protein integrated into the genome, a fusion of the LexA DNA binding domain and a modified MS2 bacteriophage coat protein. Two coat proteins are placed in tandem for intramolecular dimerization, and a point mutation in each lowers the K_d of interaction with MS2-RNA hairpins 10-fold. YBZ1 was transformed with different hybrid RNA constructs cloned into the pIIIA/MS2 vector, which carries a *URA3* marker and produces hybrid RNAs from an RNA polymerase III promoter (RPR1) promoter. Transcripts produced from this promoter do not enter the pre-mRNA processing pathway and are retained in the nucleus.

To validate the functionality of the Y3H system, we performed a validation assay by co-transforming YBZ1 yeast with pIIIA/IRE-MS2 plasmid, which expresses the 5' iron response element in fusion with MS2 hairpins, and pAD-IRP which expresses the iron

regulatory protein 1 (IRP1) in fusion with the GAL4 activation domain (AD). The IRE is a stem-loop structure found in the 5'NCR of mRNAs encoding certain proteins involved in iron utilization and binds specifically to IRP1. HIS3 activation was readily observed at 5 mM 3-amino-1,2,4-triazole (3-AT) concentration and transcription activation of LacZ was verified by colony color assay. 3-AT is a specific inhibitor of the HIS3 gene and increasing concentrations signify the strength of the 3-hybrid interaction.

The first step in the screening process was generating the hybrid-RNAs to be screened. A major limitation of this approach is that the maximum length of RNA which can be efficiently transcribed by RNA Pol III using the hybrid-RNA plasmid is approximately 400nt (Bernstein et al., 2002). This drawback necessitated the production of multiple hybrid-RNAs which represent segments of the 5'NCR. In so doing, such truncations may disrupt essential RNA secondary or tertiary structures necessary for the protein interaction. With this in mind, we predicted (*in silico*) the top ten minimum free energy secondary structures of the 5'NCR for the Kv1.4 mRNA using RNAstructure™, a secondary structure prediction software, and identified regions which are consistently predicted to form stable, independently folded domains (Figure 2.2.A) (Reuter and Mathews, 2010). Notably, the 200 bp region proximal to the initiation codon, which has been previously shown to independently fold and drive translation at a diminished level compared to full length 5'NCR, was predicted to fold independently in this analysis (Jang et al., 2004). Segments of the 5'NCR which preserve these regions were selected for generation of hybrid RNAs. In addition, we selected segments that overlap these regions to account for important structures or sequence stretches which would not be present in our initial

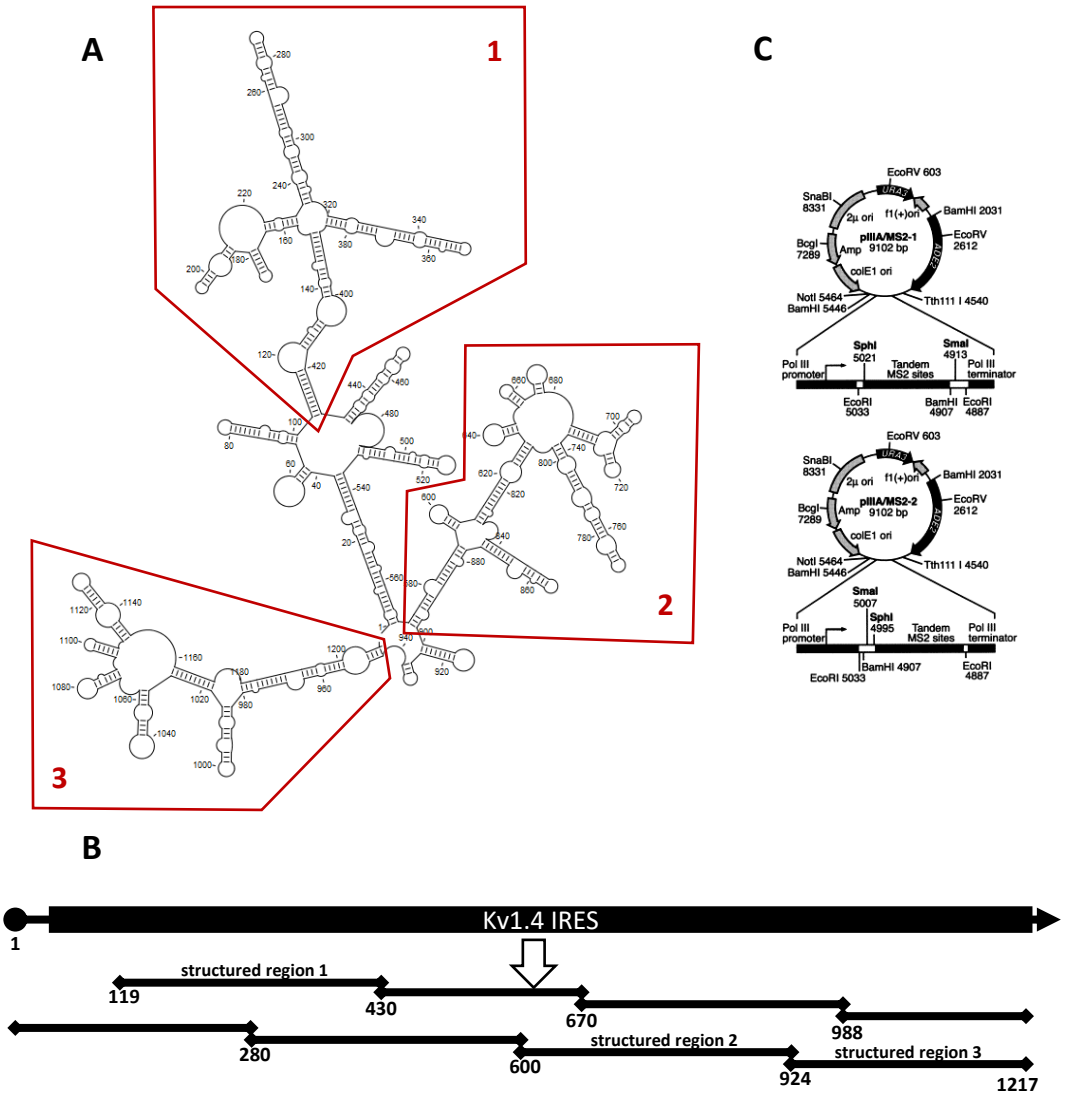


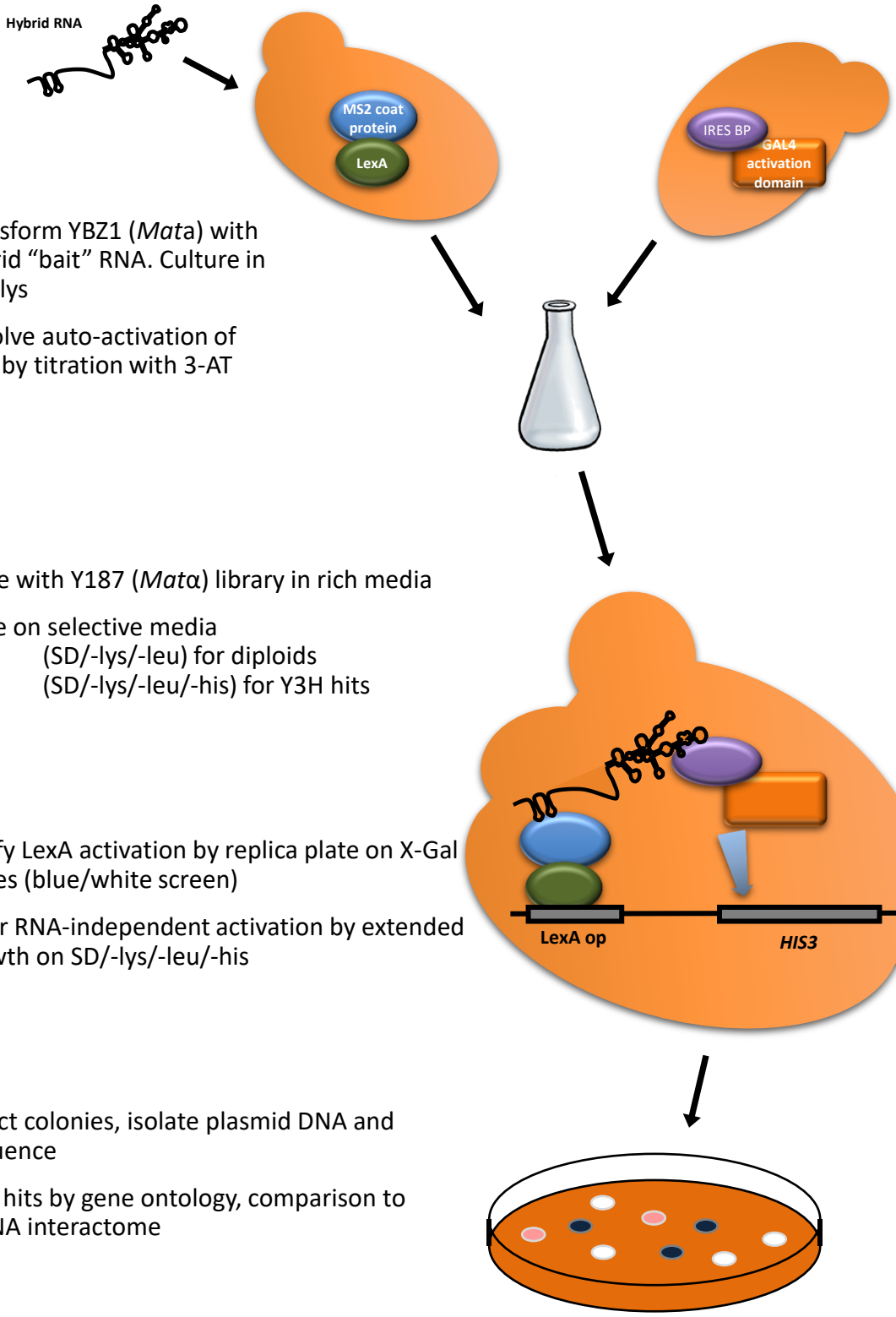
Figure 2.2. RNAstructure™ *in silico* secondary structure prediction and IRES bait design. **A**. Top ten minimum free energy (MFE) structure predictions using RNAstructure™ were evaluated, revealing 3 regions of the 5'NCR that are likely independently folded structures (one representative MFE structure prediction is shown). **B**. RNAstructure™ predictions informed the selection of 5'NCR regions which were cloned into pIIIA/MS2 vectors (**C**) and used for yeast 3-hybrid analysis.

selection. This strategy proffered 8 different RNA segments for the Kv1.4 5'NCR, which were then screened via the Y3H approach (Figure 2.2.C).

Using multiple cloning methods, these sequence segments were inserted into two different vectors to produce the hybrid RNAs, pIIIA/MS2-1 and -2 (K1-8), which differ in the MS2 RNA hairpin placement; either 5' or 3' of the RNA fragment of interest, respectively. The DNA was introduced into yeast strain YBZ1. Auto-activation of HIS3 by each hybrid-RNA was variable and titrated out using the inhibitor of the HIS3 gene product imidazoleglycerol-phosphate dehydratase, 3-AT (0-3 mM).

In the present study, only hybrid-RNAs of the pIIIA/MS2-1 variety were screened against a normalized HeLa cDNA library cloned into a GAL4 AD vector and transformed into yeast strain Y187 (Mate & Plate™ Library - HeLa S3 (Normalized)). Normalization selectively removes highly abundant transcripts from the libraries to enhance the representations of low-abundance and rare cDNAs. pIIIA/MS2-1(K1-8) constructs were first transformed into the YBZ1 yeast strain and selected for lysine and uridine auxotrophy (Figure 2.3). YBZ1 transformants were then individually screened against a cDNA library by mating and positive interactions, as well as titers for mating efficiency, were detected by nutrient selection. Efficiency of mating was determined for each screen by titration onto SD/-lys, SD/-leu, and SD/-lys/-leu plates and ranged from 30% to 76%.

Following selection for HIS3, colonies were tested for spurious activation of HIS3 by replica plating on X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates to test for LacZ activation. Doubly positive colonies were further tested for RNA-independent activation by extended growth on plates with uridine (selection marker for pIIIA/MS2 plasmid), but lacking histidine. RNA independent activation results in loss of the pIIIA



1

- Transform YBZ1 (*Mat*a) with hybrid "bait" RNA. Culture in SD/-lys
- Resolve auto-activation of HIS3by titration with 3-AT

2

- Mate with Y187 (*Mat*α) library in rich media
- Plate on selective media
  (SD/-lys/-leu) for diploids
  (SD/-lys/-leu/-his) for Y3H hits

3

- Verify LexA activation by replica plate on X-Gal plates (blue/white screen)
- Filter RNA-independent activation by extended growth on SD/-lys/-leu/-his

4

- Select colonies, isolate plasmid DNA and sequence
- Sort hits by gene ontology, comparison to mRNA interactome

Figure 2.3. Y3H cDNA library screening workflow. Screening of a cDNA library involved the following: (1) transformation of LexA-MS2 CP expressing YBZ1 cells with a pIIIA/MS2 1 hybrid RNA plasmid and titration of leaky HIS3 expression, (2) Mating of these clones with Y187 pretransformed cDNA library yeast and selection for expression of the HIS3 auxotrophic marker, (3) confirmation of LexA operon activation by blue/white screening and control for pIIIA-independent activation by red/white screening, (4) selection/picking of positive colonies, plasmid isolation and sequencing to identify interacting proteins/cDNA.

plasmid and subsequent development of a pink color, due to the loss of the ADE2 gene and the build-up of the intermediate metabolite aminoimidazole ribotide, which is converted to form a red pigment. Colonies which turned pink indicated RNA-independent activation and were discarded. Plasmids encoding cDNA/AD fusion proteins were recovered from the doubly positive yeast clones by mini-prep, mechanical and enzymatic disruption of yeast cell walls, followed by SDS/alkaline lysis and purification. Plasmid DNA was then transformed into *E. coli* for scaled-up plasmid preparation and sequencing to identify cDNAs directing IRES recognition.

From our Y3H screens, we identified 256 unique hits which specifically bound to Kv1.4 IRES sequences and were dependent on both the IRES sequence and the activation domain fusion protein for reporter expression. These screens produced a wide variation in the number of positive interactions identified for a given RNA, from 0 to 117 hits. BLAST analysis of sequence data was used to identify cDNAs, and gene information was compiled using the DAVID Bioinformatics Resource (Appendix A). Enrichr analysis was conducted on the gene list, which sorts gene ontology (GO) terms for a dataset based on enrichment compared to the expected rank for each term in many gene-set libraries (Combined Score)(Figure 2.4). The combined score is computed by taking the log of the p-value from the Fisher exact test and multiplying that by the z-score of the deviation from the expected rank. As shown in Figure 2.4, Enrichr analysis indicates strong enrichment for ss- and dsRNA binding proteins, with other regulatory and RNA binding terms also enriched.

These putative ITAFs were similarly categorized by the PANTHER (Protein analysis through evolutionary relationships) Classification System (Figure 2.5). This database was used to determine the types and general function of putative ITAFs and they were sorted

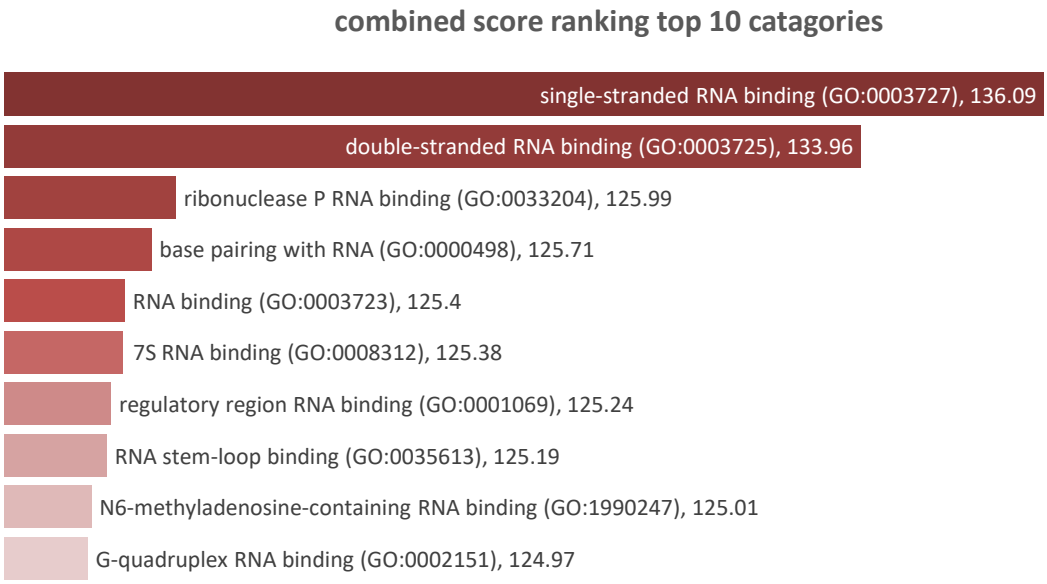


Figure 2.4. Gene set enrichment analysis of Y3H interactions. Y3H interaction gene set was uploaded to the online Enrichr tool and analyzed for GO molecular function and sorted by combined score (Enrichr). The combined score is the $\log_{10}$ of the p-value from the Fisher exact test and multiplying that by the z-score of the deviation from the expected rank. Greatest enrichment was of single stranded RNA binding terms with a combined score of 136, with the next 10 most enriched terms following.

by PANTHER GO-slim analysis based on PANTHER Protein Class, which is an adaptation of molecular function ontology, and includes commonly used classes of protein functions, many of which are not covered by GO molecular function. PANTHER analysis indicated a strong selection for nucleic acid binding proteins, which represented the preponderance of hits (Figure 2.5). Importantly, PANTHER categorization groups proteins into classes, and necessarily separates genes with overlapping annotated GO terms (i.e. GO:0003676 nucleic acid binding). We therefore annotated genes based on PANTHER Protein Class, GO: Molecular Function- nucleic acid binding, and identification in an mRNA interactome screen (Castello et al., 2012). By further limiting the results to those which are annotated in 2 or 3 categories, we were able to winnow our list of potential, non-canonical ITAFs to 59 genes (Table 2.1; Figure 2.5).

Table 2.1. Top 59 yeast 3-hybrid hits by representation in 2 or 3 cross-referenced gene sets

GENE SYMBOL	Gene Name
BOP1	block of proliferation 1
DHX15	DEAH-box helicase 15
DIS3	DIS3 homolog, exosome endoribonuclease and 3'-5' exoribonuclease
DROSHA	drosha ribonuclease III
EIF2S2	eukaryotic translation initiation factor 2 subunit beta
EIF3G	eukaryotic translation initiation factor 3 subunit G
EIF4G2	eukaryotic translation initiation factor 4 gamma 2
HNRNPK	heterogeneous nuclear ribonucleoprotein K
HMGB3	high mobility group box 3
IGF2BP3	insulin like growth factor 2 mRNA binding protein 3
LARP7	La ribonucleoprotein domain family member 7
PUM2	pumilio RNA binding family member 2
RPL27	ribosomal protein L27
RPL36A	ribosomal protein L36a
RPF2	ribosome production factor 2 homolog
SRSF3	serine and arginine rich splicing factor 3
SSBP1	single stranded DNA binding protein 1
continued	
GNL2	G protein nucleolar 2
HNRNPA0	heterogeneous nuclear ribonucleoprotein A0
KLF10	Kruppel like factor 10
KLF5	Kruppel like factor 5
LAS1L	LAS1 like, ribosome biogenesis factor
LTA4H	leukotriene A4 hydrolase
MRPL4	mitochondrial ribosomal protein L4
MYSM1	Myb like, SWIRM and MPN domains 1
NGDN	neuroguidin
NFIB	nuclear factor I B
FARSB	phenylalanyl-tRNA synthetase beta subunit
PNN	pinin, desmosome associated protein
PCSK9	proprotein convertase subtilisin/kexin type 9
RFC3	replication factor C subunit 3
RPA1	replication protein A1
RTN4	reticulon 4
RPL9	ribosomal protein L9

SMG1	SMG1, nonsense mediated mRNA decay associated PI3K related kinase
XRCC6	X-ray repair cross complementing 6
ANXA2	annexin A2
RSRC2	arginine and serine rich coiled-coil 2
BMS1	BMS1, ribosome biogenesis factor
CASC3	cancer susceptibility candidate 3
CCT6A	chaperonin containing TCP1 subunit 6A
DDX18	DEAD-box helicase 18
DDX23	DEAD-box helicase 23
DUT	deoxyuridine triphosphatase
E2F3	E2F transcription factor 3
EIF3H	eukaryotic translation initiation factor 3 subunit H
SARNP	SAP domain containing ribonucleoprotein
SEC63	SEC63 homolog, protein translocation regulator
STRAP	serine/threonine kinase receptor associated protein
STAT1	signal transducer and activator of transcription 1
SKIV2L2	Ski2 like RNA helicase 2
SUMO1	small ubiquitin-like modifier 1
SP1	Sp1 transcription factor
TBRG4	transforming growth factor beta regulator 4
TPR	translocated promoter region, nuclear basket protein
TRMT1L	tRNA methyltransferase 1 like
UCHL5	ubiquitin C-terminal hydrolase L5
UPF2	UPF2 regulator of nonsense transcripts homolog
ZNF638	zinc finger protein 638

Of these 59 putative ITAFs, several proteins have been shown to directly be involved in translation, and of particular interest, several were previously identified ITAFs. These proteins are Annexin A2 (ANXA2), serine and arginine rich splicing factor 3 (SRSF3, aka SRp20), heterogeneous nuclear ribonucleoprotein K (HNRNPK), and eukaryotic translation initiation factor 4 gamma 2 (EIF4G2, aka DAP5). Canonical initiation factors subunits were also identified, notably eIF2 and eIF3 which are components of the 43S pre-initiation complex. eIF4G2 is a paralog of eIF4G1and eIF4G3 lacking the N-terminal region responsible for eIF4E binding, but with a homologous C-terminal region that retains the binding sites for eIF4A and eIF3.

RNA affinity chromatography 1.0

To address the caveats of the genetic yeast 3-hybrid approach, we carried out RNA-affinity purifications of Kv1.4 IRES binding proteins. This approach was first validated

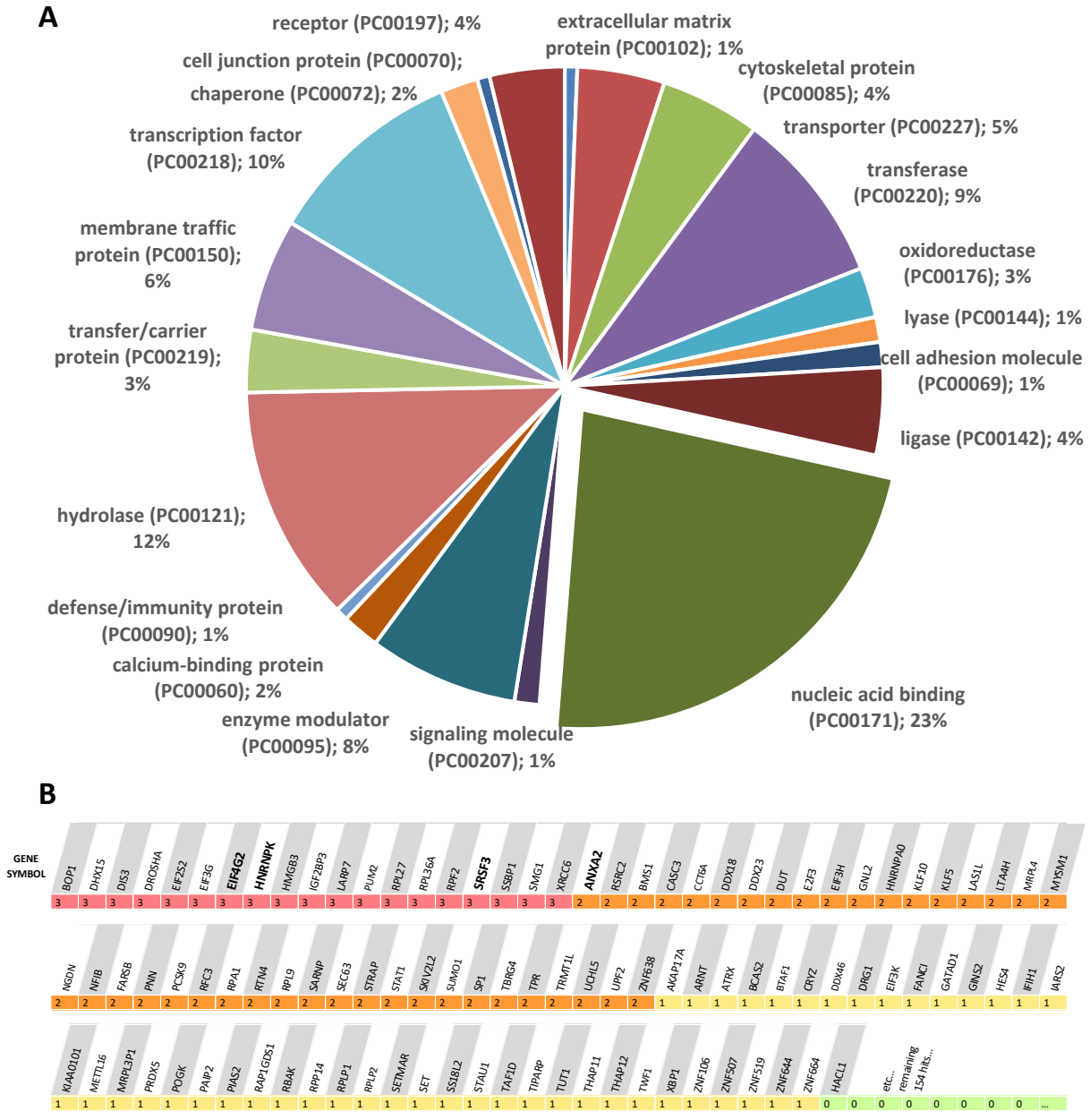


Figure 2.5. Y3H hits categorized and sorted. **A**. Categorization of Y3H dataset by PANTHER™ Protein Class analysis bin the plurality of Y3H hits into 'nucleic acid binding' category. **B**. Cross-reference of Y3H hits with 'nucleic acid binding' GO term (0003767), 'nucleic acid binding' PANTHER™ Protein Class term (00171), and identification in mRNA interactome data (Castello et al., 2013). Sorted by score and displaying only genes present in ≥1 cross-referenced dataset. Previously identified ITAFs are displayed in bold.

using the known interaction between the ITAF PCBP2 and poliovirus stem-loop IV RNA, a major domain within the poliovirus IRES (Blyn et al., 1997). Using biotinylated *in vitro* transcribed poliovirus stem-loop IV RNA, we bound proteins from the high-speed supernatant of a HeLa whole cell lysate (S100) in low salt conditions. These RNP complexes were then affinity purified using the AKTA™ FPLC system with a streptavidin sepharose column. Proteins were eluted from the column in two steps, using 1 M then 2 M NaCl buffer, and collected in multiple fractions. The stable PCBP2/PV-SL IV interaction was confirmed by Western blot analysis (Figure 2.6).

After confirming the viability of this approach, affinity purification was performed using biotinylated RNA corresponding to the Kv1.4 IRES. This first of two approaches involved tandem chromatography; first by biotin-RNA affinity chromatography, followed by a heparin chromatography method (Figure 2.7). Heparins are negatively charged linear polysaccharides which have the ability to bind a wide range of biomolecules including DNA- and -RNA binding proteins, lipoproteins, protein synthesis factors, enzymes, growth factors, and extracellular matrix proteins. In this chromatography, heparin is not only an affinity ligand but also an ion exchanger with high charge density and distribution. *In vitro* transcribed, biotinylated RNA was preincubated with homogenate from whole mouse hearts. Since this gene is highly expressed mouse heart, brain, and skeletal muscle, this protein source likely contains critical trans-acting factors. Incubated mixtures were then injected onto a HiTrap™ Streptavidin HP is a column and washed extensively with low salt buffer. Associated proteins were then eluted from the column using a sodium chloride gradient.

In a since abandoned *in vitro* assay, we tested for stimulation of translation in HeLa

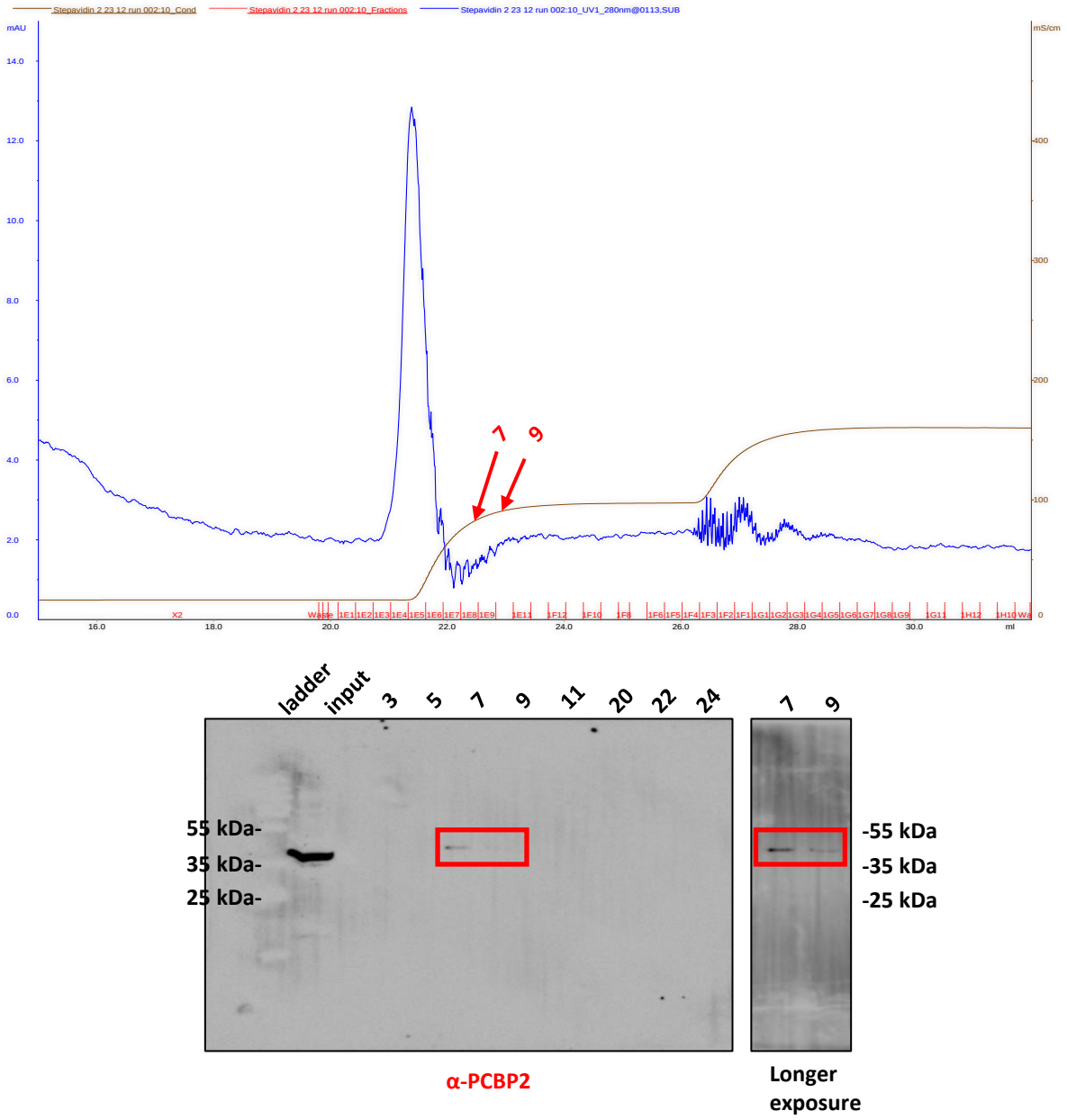
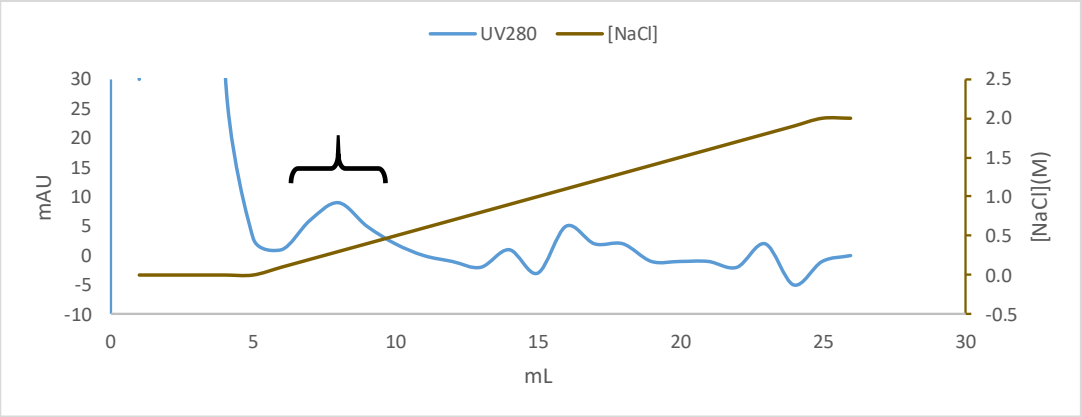


Figure 2.6. Proof-of-principle for biochemical approach to ITAF discovery. Biotinylated poliovirus stem-loop IV RNA (major domain of the poliovirus IRES) was used to capture IRES interacting proteins. These complexes were injected into a HiTrap streptavidin column using an AKTA FPLC, and proteins were eluted using a two-step NaCl elution. Eluate was monitored for Abs_{280nm} and conductivity and collected in 500 µl fractions. Western-blot analysis of fractions indicate that PCBP2 (known to bind stem-loop IV RNA) was eluted between fractions 7 and 9.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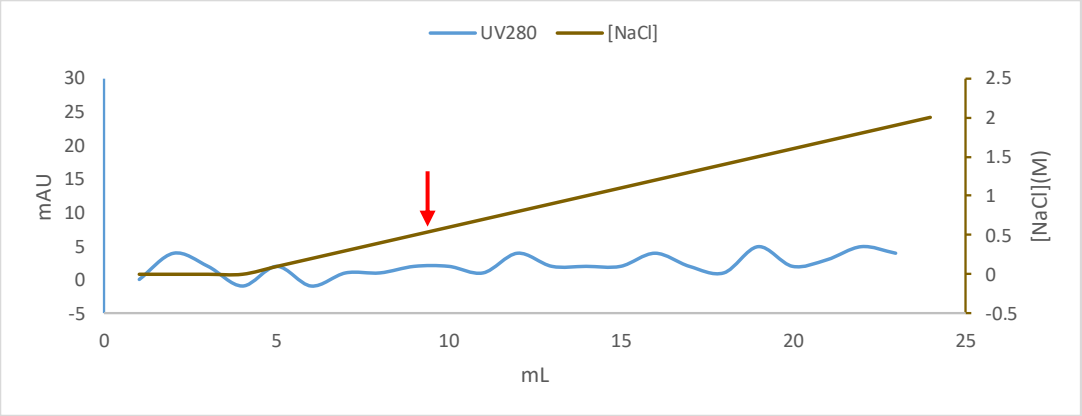
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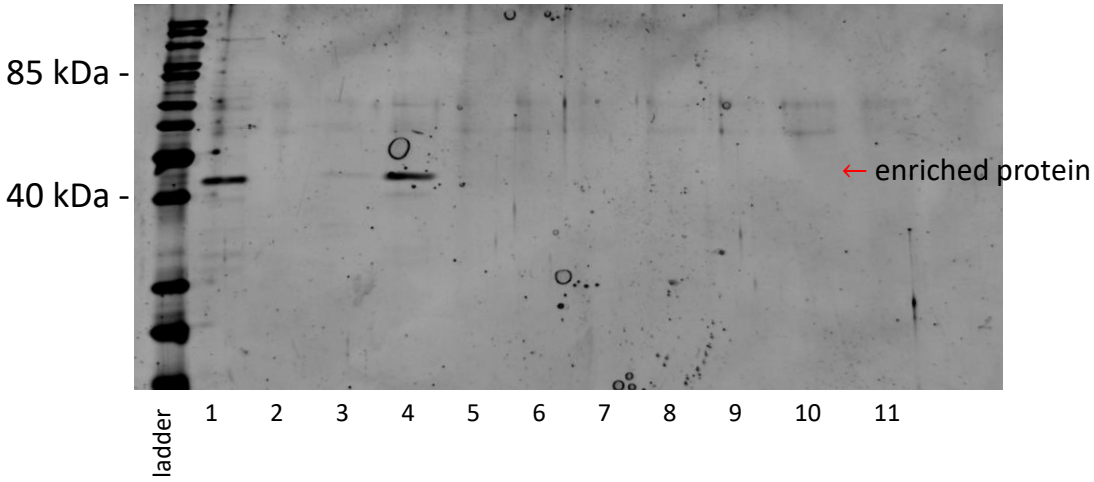
85 kDa -
40 kDa -
← enriched protein
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9
10
11

Figure 2.7. RNA affinity chromatography 1.0: tandem purification. RNP complexes were formed by incubating *in vitro* transcribed and biotinylated Kv1.4 5'NCR RNA and mouse heart homogenate. Binding mixtures were subjected to streptavidin chromatography. Early fractions were pooled, dialyzed and subjected to heparin affinity chromatography. SDS-PAGE and Sypro ruby stain analysis of proteins from heparin elution fractions identify enriched protein species in lane 4 (9 mL elution volume). Fraction 9 was subjected to mass spectrometry analysis by our collaborator Dr. Paul Gershon.

cell-free *in vitro* translation extracts and chose an early peak of apparent activity for subsequent purification by heparin affinity chromatography (Walter et al., 2002). Heparin chromatography was similarly conducted using a linear salt gradient. Fractions were also assayed for *in vitro* stimulatory activity, with no fractions stimulating translation over buffer-only control. Re-addition experiments with each fraction were unable to rescue *in vitro* translation stimulation. Nevertheless, SDS-PAGE and Sypro Ruby staining revealed enriched protein in fraction A9 of approximately 40 kDa.

Purified fraction A9 was digested with trypsin and peptides were identified by mass spectrometry by our collaborator Dr. Paul Gershon. Results from mass spectrometry indicate that the ~40 kDa band observed belonged to Annexin A2, the previously identified Y3H candidate, and previously characterized ITAF. Additional low abundance proteins were identified by mass spectrometry and include additional known ITAFs hnRNP A1 and NONO (Table 2.2).

Table 2.2 Tandem chromatography and mass spectrometry results

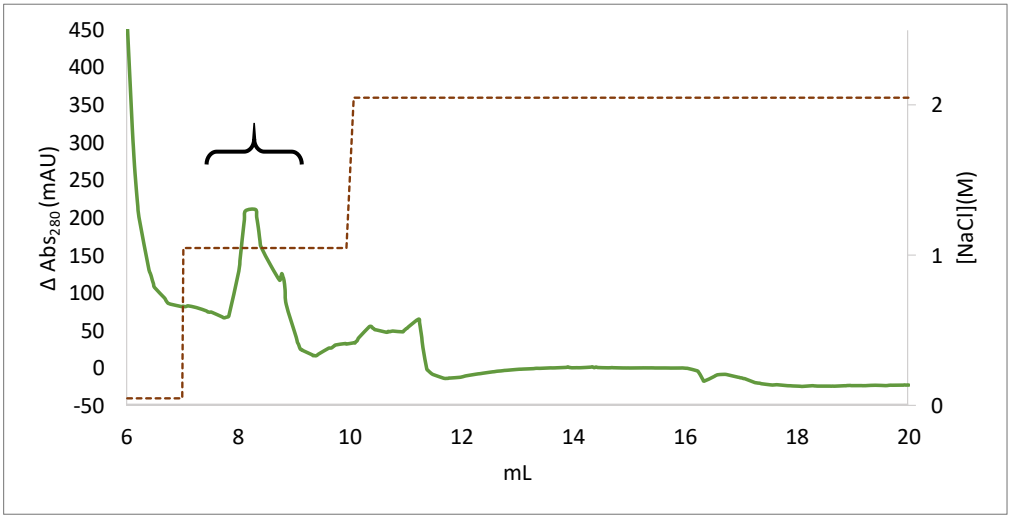
Gene ID	Gene Symbol	Mass	Unique peptides
302	ANXA2	38937	GVDEVTIVNILTNR, GLGTDEDSLIEIICSR, GDLENAFLNLVQCIQNKPLYFADR
3181	HNRNPA2B1	37437	LFIGGLSFETTEESLR, KLFIGGLSFETTEESLR
1915	EEF1A1	50424	THINIVVIGHVDSGK
4841	NONO	54620	NLPQYVSNELLEEAFSVFGQVER
22948	CCT5	60042	SQDDEIGDGTTGVVVLAGALLEEAEQLLDR
3178	HNRNPA1	34289	LFIGGLSFETTDESLR, KLFIGGLSFETTDESLR
2023	ENO1	47453	VNQIGSVTESLQACK, AAVPSGASTGIYEALELR
2026	ENO2	47609	AAVPSGASTGIYEALELR
9138	ARHGEF1	103368	LLLKSHSR
506	ATP5B	56265	GFQQILAGEYDHLPEQAFYMVGPIEEAVAK
4869	NPM1	32711	TVSLGAGAKDELHIVEAEAMNYEGSPIK
3187	HNRNPH1	49454	YVELFLNSTAGASGGAYEHR
389434	IYD	32964	KVHYYNEISVSIACGLLLAALQNAGLVTVTTTPLNCGPR
55215	FANCI	150999	VNLMQHMKLSTSR

RNA affinity chromatography 2.0

Loss of *in vitro* stimulatory activity using two-step purification may have been due to separation of an important functional complex; however, on recombining of fractions, stimulatory activity could not be restored. We now consider the assay to be sensitive to addition of: 1) bulk translation resources in the case of strong stimulation by addition of crude fractions of rabbit reticulocyte lysate, and 2) generic protein concentration for more purified components as evidenced by the equivalent stimulation by recombinant hnRNP K, heat denatured hnRNP K, and bovine serum albumin (Appendix Figure A.1).

Since two-step purification of IRES binding proteins did not lead to purification of an enriched fraction capable of stimulated Kv1.4 IRES activity *in vitro*, activity, possibly due to dissociation of critical functional complexes, we attempted to purify IRES binding proteins using a less stringent one-step approach. Since single step RNA affinity chromatography results in complex mixtures compared to purified components, and these mixtures may contain high degree of background/non-specific binding, we performed side by side purifications using biotin-tagged and untagged Kv1.4 5'NCR RNA. Paired fractions were then subjected to comparative and quantitative mass spectrometry to analyze enrichment of components. For these experiments, RNA was preincubated with rabbit reticulocyte lysate (RRL) for RNP formation and subjected to streptavidin affinity chromatography (HiTrap™ Streptavidin HP and AKTA™ FPLC). RRL supports high levels of translation *in vitro*, potentially representing an abundance of trans-acting factors necessary for Kv1.4 IRES-mediated translation. Comparison of the chromatograms reveals fractions with different protein concentrations, indicating dissimilar purification of components (Figure 2.8). The greatest dissimilarity came in fraction A6, which was selected for mass-

A


450
400
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Δ Abs280 (mAU)
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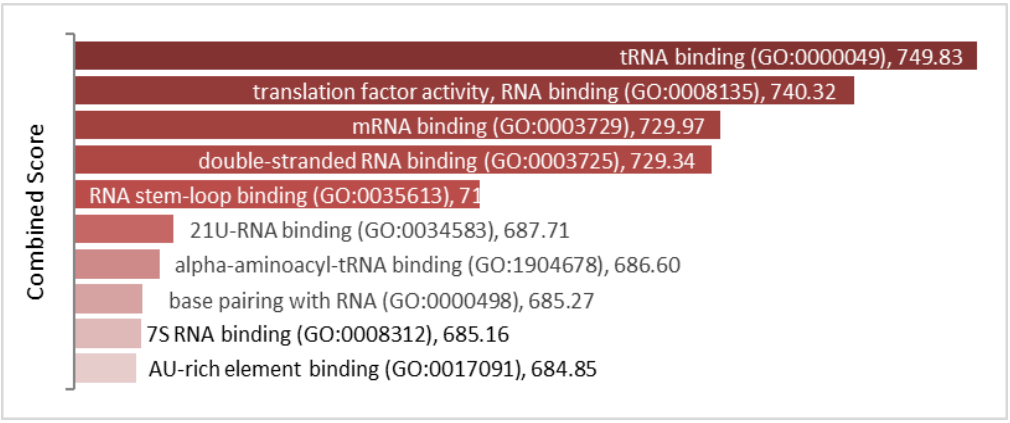
B


Combined Score
tRNA binding (GO:0000049), 749.83
translation factor activity, RNA binding (GO:0008135), 740.32
mRNA binding (GO:0003729), 729.97
double-stranded RNA binding (GO:0003725), 729.34
RNA stem-loop binding (GO:0035613), 71
21U-RNA binding (GO:0034583), 687.71
alpha-aminoacyl-tRNA binding (GO:1904678), 686.60
base pairing with RNA (GO:0000498), 685.27
7S RNA binding (GO:0008312), 685.16
AU-rich element binding (GO:0017091), 684.85

C

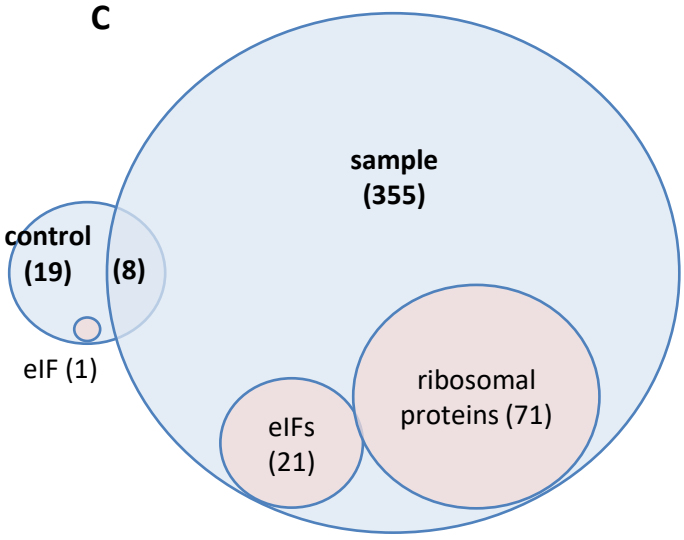

sample
(355)
control
(19)
(8)
eIF (1)
eIFs
(21)
ribosomal
proteins (71)

Figure 2.8. Parallel chromatography and quantitative mass spectrometry. A. Rabbit reticulocyte lysate was bound to either biotinylated or non-biotinylated Kv1.4 5'NCR RNA and subjected to side-by-side streptavidin affinity chromatography. Results were analyzed by adjusting retention to matching volume and subtracting control signal (Abs_{280nm}) from sample signal. Peak from 8-9 mL retention was analyzed by mass spectrometry. **B**. Enrichr gene enrichment analysis by GO molecular function sorted by combined score of proteins identified by mass spectrometry . The combined score is the $\log_{10}$ of the p-value from the Fisher exact test multiplied by the z-score of the deviation from the expected rank. tRNA binding represents the most enriched GO term by this criteria with a combined score of 750. The top 10 terms by enrichment are shown. **C**. Venn diagram of proteins identified in control fractions and sample fractions. Bubbles representing canonical translation factors and ribosomal proteins show relative representation.

spectrometry analysis. As expected mass-spectrometry shows low background binding in untagged sample, with partial overlap with signal from tagged sample (Figure 2.8). Tagged RNA purification resulted in substantial isolation of ribosomal proteins (71) and initiation factors (21), with 63 non-ribosomal and non-initiation factor hits with a protein score >75. Protein score is a statistical indicator for how well the experimental data match the Mascot database sequence, and 75 was chosen to represent approximately the highest confidence half of the data set. Of these are several known ITAFs: YBX1, hnRNP K, for cellular IRESs, and PA2G4, G3BP1 for viral IRESs (bold in Table 2.3). Several additional known ITAFs were present below our protein score >75 cutoff, namely hnRNP Q (cellular; 47), hnRNP D (viral; 42), and hnRNP A1 (viral; 44).

Table 2.3. Top 63 hits from comparative mass spectrometry by protein score (>75)

Protein score	Gene name	Description	continued	Gene name	Description
690	HSPA8	Heat shock 70kDa protein 8	109	PFN1	Profilin 1
676	**PA2G4**	**Proliferation-associated 2G4, 38kDa**	108	RUVBL2	RuvB-like 2 (E. coli)
503	HSP90AA1	Heat shock protein 90kDa alpha (cytosolic), class A member 1	108	PSMA7	Proteasome (prosome, macropain) subunit, alpha type, 7
441	NACA2	Nascent polypeptide-associated complex alpha subunit 2	103	NMT1	N-myristoyltransferase 1
257	PSMB5	Proteasome (prosome, macropain) subunit, beta type, 5	103	PSMB3	Proteasome (prosome, macropain) subunit, beta type, 3
250	PCMT1	protein-L-isoaspartate (D-aspartate) O-methyltransferase	100	HNRNPAB	Heterogeneous nuclear ribonucleoprotein A/B
244	TF	Transferrin	98	SRP14	Signal recognition particle 14kDa
238	CLTC	Clathrin, heavy chain (Hc)	97	EEF1A1	Eukaryotic translation elongation factor 1 alpha 1
228	PDAP1	PDGFA associated protein 1	96	TPT1	Tumor protein, translationally-controlled 1
206	CAT	Catalase	96	PFKL	Phosphofructokinase, liver
203	SND1	Staphylococcal nuclease and tudor domain containing 1	96	NAP1L1	Nucleosome assembly protein 1-like 1
196	PSMB4	Proteasome (prosome, macropain) subunit, beta type, 4	94	PABPC1	poly(A) binding protein, cytoplasmic 1
190	PPIA	Peptidylprolyl isomerase A (cyclophilin A)	93	GSTP1	Glutathione S-transferase pi 1
189	**G3BP1**	**GTPase activating protein (SH3 domain) binding protein 1**	90	ABCE1	ATP-binding cassette, sub-family E (OABP), member 1
186	YARS	tyrosyl-tRNA synthetase	90	FARSB	phenylalanyl-tRNA synthetase, beta subunit
168	HNRNPF	Heterogeneous nuclear ribonucleoprotein F	89	CAPRIN1	Cell cycle associated protein 1
147	**YBX1**	**Y box binding protein 1**	**87**	**HNRNPK**	**Heterogeneous nuclear ribonucleoprotein K**

147	SERBP1	SERPINE1 mRNA binding protein 1
144	CCT5	Chaperonin containing TCP1, subunit 5 (epsilon)
139	PFDN5	Prefoldin subunit 5
139	HAGH	Hydroxyacylglutathione hydrolase
138	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
138	NSFL1C	NSFL1 (p97) cofactor (p47)
137	YWHAE	Tyrosine 3-monooxygenase /tryptophan 5-monooxygenase activation protein, ε polypeptide
133	VCP	Valosin containing protein
129	PRDX2	Peroxiredoxin 2
129	ACLY	ATP citrate lyase
119	EEF1G	Eukaryotic translation elongation factor 1 gamma
114	CBR3	Carbonyl reductase 3
111	SLFN14	Schlafen family member 14
109	WDR1	WD repeat domain 1
109	EEF1B2	Eukaryotic translation elongation factor 1 beta 2
87	PSME1	Proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)
87	PSMB2	Proteasome (prosome, macropain) subunit, beta type, 2
84	GLUL	Glutamate-ammonia ligase
83	TMEM17	Transmembrane protein 17
83	HSPA4	Heat shock 70kDa protein 4 [a.k.a. APG2, hsp70RY, hsp70]
83	LSM2	LSM2 homolog, U6 small nuclear RNA associated (S. cerevisiae)
82	PSMA3	Proteasome (prosome, macropain) subunit, alpha type, 3
82	GPX1	Glutathione peroxidase 1
80	STIP1	Stress-induced-phosphoprotein 1
80	CHMP4B	Charged multivesicular body protein 4B
79	PSMA6	Proteasome (prosome, macropain) subunit, alpha type, 6
79	PGK1	Phosphoglycerate kinase 1
77	VARS	valyl-tRNA synthetase [a.k.a. G7A, VARS2, VARS1,Valine-tRNA ligase]
76	CCT8	Chaperonin containing TCP1, subunit 8 (theta)

Discussion

Translation initiation regulation appears to operate in two modes, the non-specific regulation of bulk translation, typically through the modulation of activity of one or more initiation factors, or specifically through cis- and trans-activities of particular RNAs. In both cases, RNA-protein interactions critically influence gene expression patterns and the identification of these ribonucleoprotein complexes is essential for understanding these regulatory mechanisms. In considering IRES-mediated translation, there is diversity in the use of initiation factors normally required for canonical scanning mediated translation. Additionally, there appears to be little consensus among different IRES elements with regard to non-canonical factors necessary for their translation. Some degree of regularity can be found in the IRES elements of different viruses, but even those will frequently have disparate usage of ITAFs. Only with greater knowledge of all the extant IRES elements and

their factor requirements for initiation may we develop a complete understanding of this process.

Much work has been focused on describing IRES-mediated translation for a select set of RNAs such as viral RNAs and those of oncogenes, where the purpose of such elements is readily explained. Here we have analyzed the IRES element for the voltage-gated potassium channel Kv1.4, a gene critical for the cardiac action potential. Although to date Kv1.4 represents the only Kv channel for which an IRES has been described, it is notable that other Kv channels similarly have extensive 5'NCRs and upstream AUGs, which might inhibit scanning-dependent initiation. The "purpose" of IRES elements in ion channel RNAs has been previously ascribed to general necessity for cell homeostasis during cellular stress response. However, the tissue specific expression of Kv1.4 coupled with the non-specific nature of its promoter, points to a possible IRES mechanism of tissue specificity. Any such mechanism would likely involve tissue specific IRES trans-acting factors. Understanding RNP composition of Kv1.4 mRNA is critical to understanding ITAF requirements for post-transcriptional regulation.

Protein-centric methods of RNP characterization such as the various kinds of immunoprecipitation (IP) are particularly advantageous as they can isolate endogenous, unmodified RNA from an *in vivo* context. However, this necessitates prior knowledge of at least one RNA-protein interaction and may limit isolation of distinct complexes on a given RNA. To gain an unbiased understanding of all RNA binding interactions, an RNA-centric approach must be taken. A number of genetic and biochemical methods have been developed for comprehensive identification of RNA-binding proteins; however, each of these methods present their own challenges. The most conventional approach has been *in*

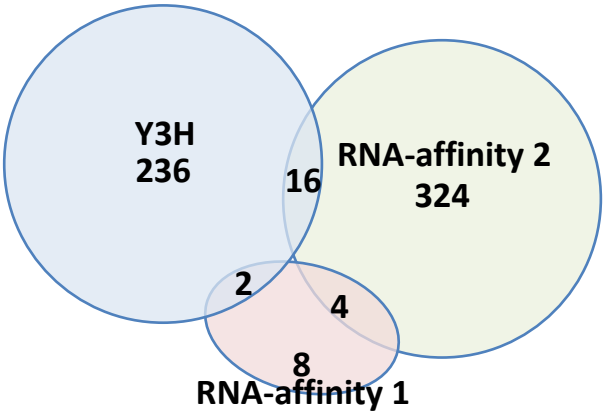
vitro purification using RNA tagged by different strategies, such as covalent linking, biotin labeling, or the introduction of artificial aptamers. Typically, a bait RNA is used to form RNP complexes either *in vitro* or *in vivo*, and RNA is immobilized to a chromatographic matrix. Non-specific proteins interactions are removed by extensive washing, and RNP complexes are eluted from the matrix for mass spectrometry analysis. This technique has proven effective for isolating RNPs, although with several major caveats. The most typical method involving *in vitro* transcription of biotin (or other) -tagged RNA, has the benefit of high specificity of binding to the matrix, allowing greater stringency of selection. However, RNA must be refolded *in vitro* introducing the distinct possibility that RNA tertiary structure will not be faithful to the native cellular conformation. Additionally, RNP complex formation is similarly accomplished *in vitro*, typically with crude extract in which many highly abundant proteins that bind non-specifically to RNA might mask the isolation of potentially specific and low-abundance proteins. Methods to circumvent this *in vitro* RNP formation involve aptamer-tagged RNA which can be generated in the cell. These approaches are limiting due to lower abundance of the RNA substrate for binding, as well as lower affinities for their respective matrices than *in vitro* transcribed and biotinylated RNA. These difficulties have prompted investigators to develop new approaches to identifying RNPs, as well as continuing to improve existing strategies by optimizing aptamer structures for binding and using quantitative proteomics.

Any biochemical purification approach is inherently confounded by highly abundant proteins with low specificity masking the interactions of low abundance proteins. This problem invites genetic library-based approaches where the proteome might be screened incrementally. By modifying the powerful yeast-hybrid technology to assay for RNA

binding, the greater diversity of RNA-protein interactions can be unlocked. Yeast 3-hybrid screens also have caveats, particularly the use of shortened RNA regions as well as screening against fusion proteins generated from cDNA. This leaves open the possibility that RNA protein interactions detected in yeast may not be truly representative of interactions as they naturally occur *in vivo*. Size constrained RNA presents possible binding motifs to RBPs but ignores the requirement for greater RNA superstructures for the function of IRES elements. Library fusion protein may not fold into completely natural conformations when linked as a fusion protein to the Gal4 AD, possibly disrupting critical binding domains.

We have presented here a two-pronged approach to identifying IRES trans-acting factors via their presumed association with IRES RNA. By simultaneously screening for IRES binding proteins through a genetic approach and through a biochemical approach, we attempted to circumvent the limitations of either approach alone and gain a more comprehensive understanding of RNP complexes assembled on the Kv1.4 IRES, and potentially uncover novel functional interactions. For example, a major limitation of RNA-affinity purification is the overrepresentation of highly abundant proteins such as hnRNPs. We in fact isolated several hnRNPs in both RNA-affinity screens, (HNRNPA2B1, HNRNPA1, HNRNPH1, HNRNPAB, HNRNPD, HNRNPF, HNRNPK); however, hnRNPs were also identified by yeast 3-hybrid screen (HNRNPA0, HNRNPK) and represent a class of protein frequently implicated in IRES-mediated translation (Figure 2.9). Whether these highly abundant proteins represent genuine interactions of significance, or whether Y3H results represent an effective filter for such interactions remains to be seen. Notably, experiments interrogating hnRNP K on IRES activity do suggest a functional role for this hnRNP (see

A



B

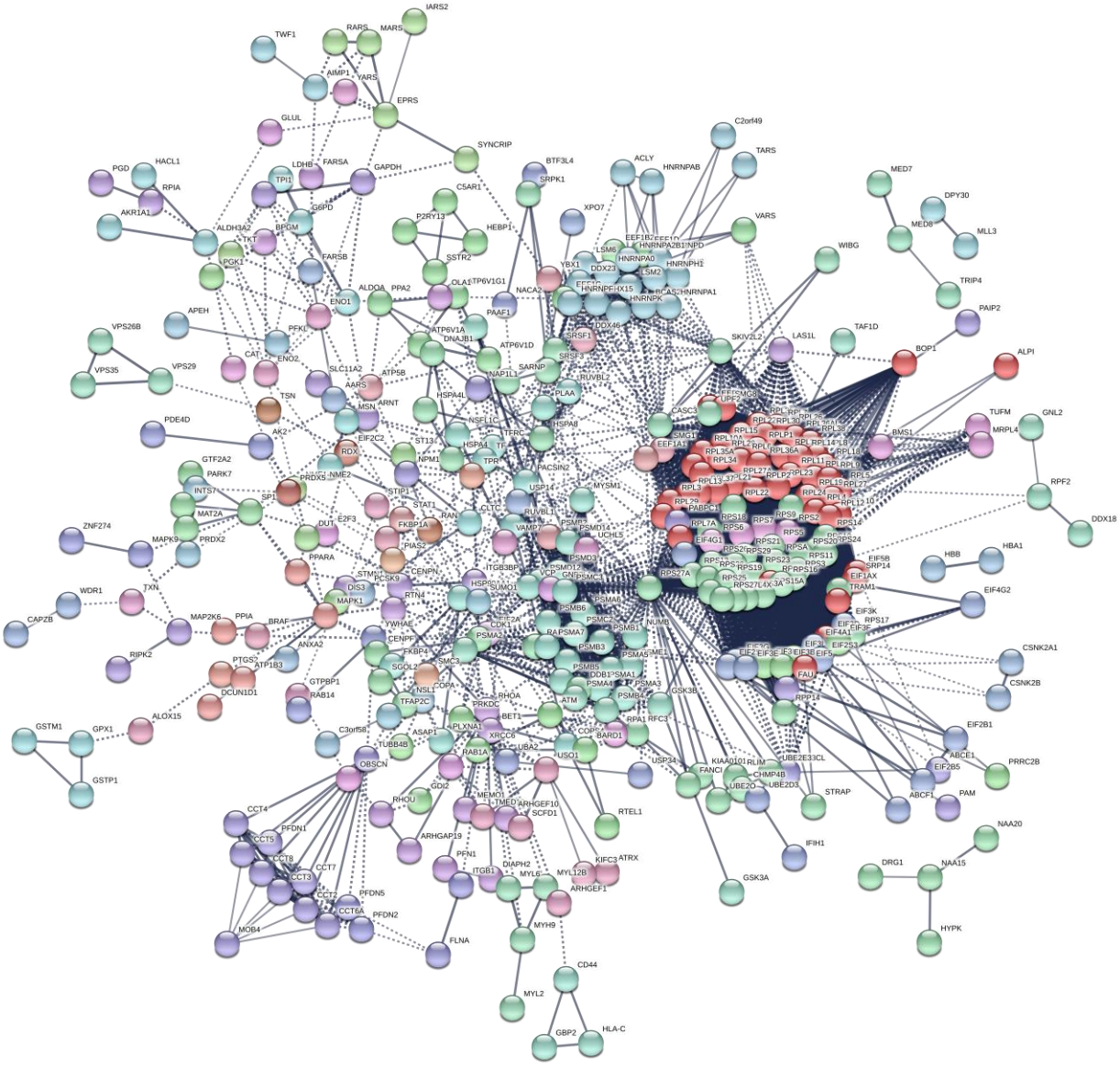


Figure 2.9. Analysis of pooled gene sets from genetic and biochemical screens. **A**. Homologous genes for Homo Sapiens for each screen were cross-referenced and overlapping hits from each screen are displayed as a Venn diagram. **B**. Lists were pooled, and String analysis was performed, selecting for high confidence interactions relying exclusively on experimental and database active interaction sources. Network was clustered and color coded using Markov Cluster algorithm (MCL) with inflation parameter of 10 to identify nodes of biological processes. The most obvious node represents the protein translation apparatus (red for 60S ribosome, green for 40S).

Chapter 3). Additionally, starting material for affinity purifications were chosen for their likelihood of having relevant ITAFs, based on the high degree of IRES-mediated translation they support (RRL), or their tissue relevance (heart lysate). By using two different starting materials for biochemical purification, we were able to pull from unique pools of putative ITAFs. For example, using two-step purification from heart lysate, the most prevalent result was a protein Annexin A2 (ANXA2). This interaction was also observed in our Y3H analysis, but not in the second, less stringent RNA-affinity purification (Figure 2.9). This is likely due to the conspicuous absence of ANXA2 in erythrocytes (see **Chapter 3**)(Waisman, 1995). The results from comparative RNA-affinity purification are a further example of bias toward high abundance proteins, as ribosomal proteins represented 71 of the total 355 unique proteins identified from RRL. This may represent background from the highly concentrated translation machinery in RRL, although lack of these proteins in control purifications indicates RNA-specific binding. It is likely this represents ribosome binding to the Kv1.4 IRES, and the large numbers of large subunit proteins suggests even 80S ribosome formation. While ribosome binding may be driven by canonical mechanisms, or through the action of other trans-acting factors, there may be IRES-specific interactions of one or more of these proteins. Notably, RPS25 has been previously characterized as an IRES regulating component of the 40S subunit which is dispensable for cap-dependent scanning but used for recognition and function of HCV IRES and CrPV IGR IRES (Landry et al., 2009).

In addition to the ANXA2 and hnRNP K noted above, many additional hits are known IRES trans-acting factors such as SRp20 and DAP5 (EIF4G2) for Y3H screens, hnRNP A1 and NONO for two-step RNA-affinity, and YBX1 and PA2G4 for comparative RNA-affinity.

Additionally, STRING analysis (Search Tool for the Retrieval of Interacting Genes/Proteins) of the pooled genes from all three screens, presents a translation specific pattern of interactions (Figure 2.9). A large node representing the ribosome and directly associating proteins dominates the network, with several additional clusters representing the proteasome, the spliceosome, and protein folding chaperones. Enrichr gene set enrichment analysis of both Y3H and comparative RNA-affinity results indicate a strong enrichment for RNA binding proteins and translation factors (refer to Figures 2.4 and 2.8). Each of these results indicates that a diverse set of factors may be involved with RNP formation on the Kv1.4 IRES, with an understandable focus on RNA binding proteins and those related to the translation apparatus.

Another class of proteins which was notably enriched in these gene sets included helicases. Helicases bind to and unwind duplexed nucleic acid, such as the structured RNA found in internal ribosome entry sites. These may represent important IRES interacting proteins by enabling partial RNA unwinding to allow ribosome association. In fact, the initiation factor eIF4A, which is the RNA helicase critical for ribosome scanning, was identified from RNA affinity purification. Along with other RNA helicases, these may represent important trans-acting factors which may be necessary to the function of these long and highly structured RNAs.

Finally, the screens described here only select for IRES binding proteins with no consideration for possible functional roles. In the next chapter we describe experiments to discern the possible functional role of select hits (hnRNP K, PCBP2, DHX15, and ANXA2) on IRES mediated translation of Kv1.4. Overall, by using a biochemical purification approach in parallel with a yeast 3-hybrid approach, we were able to gain a more comprehensive

understanding of RNA binding proteins, and potentially uncover novel functional interactions.

Materials and Methods

Yeast strain construction

YBZI *S. cerevisiae (MATa, ura3-52, leu2-3, 112, his3-200, trp1-1, ade2, LYS2 :: (LexAop)-HIS3, ura3 :: (lexA-op)-lacZ, LexA-MS2 coat (N55K)),* which harbor the LexA-MS2 coat protein fusion, were obtained from Dr. Marvin Wickens (University of Wisconsin) along with plasmids for generating hybrid RNAs pIIIA/MS2-1 and -2. Select regions of the Kv1.4 5'NCR were cloned into each pIIIA plasmid by either iterative restriction digestion of pT7-Kv1.4-luc, gel purification, Klenow treatment and blunt end ligation at the SmaI site, or by Sequence and Ligation Independent Cloning (SLIC) using insert specific primers:

K1-5F <TGTCTGCAGGTCGACTCTAGAGGATCGCCCCGACTCACTATAGGGAGACAAG>

K1-3R <GGAATTCCGGCTAGAACTAGTGGATCCCCCGGTTTGGCAGGTGGAGGAT>

K6-5F <TGTCTGCAGGTCGACTCTAGAGGATCGCCCATTTTTATTTTTCGCACTTG>

K6-3R <GGAATTCCGGCTAGAACTAGTGGATCCCCC TGCCCAGCCATTGCTGCTTAGT>

K7-5F <TGTCTGCAGGTCGACTCTAGAGGATCGCCCCTAAGCAGCAATGGCTGGGCATAT>

K7-3R <GGAATTCCGGCTAGAACTAGTGGATCCCCCCCAGAGCTTGCCTCGTCTAGACAA>

(Li and Elledge, 2007). These clones were transformed into YBZ1 *S. cerevisiae* by the LiAc method and selected for lysine and uridine auxotrophy by culturing on synthetic drop out plates lacking lysine and uridine (SD/-Lys/-Ura)(Gietz et al., 1992). Briefly, YBZ1 was cultured in 300 mL YPDA at 30 °C till OD_{600}~0.5. Yeast were pelleted, washed with TE buffer (10 mM Tris HCl, 1 mM EDTA pH 8.0) and resuspend in 1.5 mL TE/ 1 mM LiAc.

Aliquots of 100μL were transformed with 0.1 μg plasmid in the presence of 0.1 mg sheared salmon sperm carrier DNA, 600 μL 40% PEG4000 and 70 μL DMSO by heat shock (42 °C/15min). Transformants were screened by colony PCR, confirmed by sequencing, aliquoted and stored at -80 °C.

Auto-activation of HIS3 gene expression by the pIIIA plasmid was titrated by culturing on SD/-His/-Lys/-Ura with increasing concentrations of 3-aminotriazole (3-AT). Minimal 3-AT concentrations necessary to prevent transformed YBZ1 growth on SD/-His/-Lys/-Ura ranged from 0-3 mM and was used in Y3H selection plates.

Yeast 3-hybrid screen

Diploid yeast forming a stable three-hybrid association were selected for HIS3 expression by plating liquid mating culture onto SD/-His/-Lys/-Leu plates. 3-aminotriazole (3-AT) was added to selection media at 0-3 mM concentrations for suppression of auto-activation by the pIIIA plasmid. Mating mixtures were allowed to grow for 5-7 days at 30 °C. Three-hybrid associations also activate β-galactosidase (X-gal) activity which was verified by replica plating onto plates containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (colony color assay). Colonies were grown for an additional 7 days for detection of RNA independent activation. Without selective pressure by uridine, extended growth results in loss of the pIIIA plasmid (and ADE2 gene) resulting in development of a pink color, due to the build-up of the intermediate metabolite aminoimidazole ribotide, which is converted to form a red pigment. These colonies were dismissed. Plasmids were rescued from each of the surviving colonies by mini-prep and re-transformation into

DH10β for scaled up plasmid prep and sequencing. cDNA-AD fusions were identified using BLAST.

Biotin-RNA/streptavidin chromatography (tandem)

Transcription template corresponding to RNA of the Kv1.4 5'NCR was generated by linearizing pT7-Kv1.4-luc with NcoI. Transcription template corresponding to poliovirus stem-loop IV was generated by linearizing pT220-460 with HindIII. RNA transcription was performed using the MEGAscript T7 transcription kit (Ambion), with only 3.75 mM CTP and added 2 mM Biotin-14-CTP (ThermoFisher). Following transcription, RNA was purified using the RNeasy Mini kit (Qiagen). The yield of RNA was quantified by absorbance at 260 nm using a NanoDrop™ UV-Vis spectrophotometer.

Homogenate of mouse heart was generated from 27 C57BL/6 hearts (4.95 g) by using a cryomill. Whole hearts were flash frozen in liquid nitrogen, diced, then milled at 25Hz for 3 cycles of 1 min milling with 30s cooling (Retsch). Cryogenic homogenate was collected and stored at -80 °C. 250 mg was resuspended using 1 mL post-lysis buffer*, centrifuged at 550 rcf for 15 min to pellet large cellular debris, then at 13,750 rcf to produce S10 lysate. RNP complexes were assembled at 4 °C using 1 mL mouse heart S10 with 100 µg biotinylated RNA and 100 µg tRNA, slowly rotating at 4°C for 12 hrs.

Column chromatography was carried out at 4 °C using binding buffer containing 50 mM KCl, 5% glycerol, 1 mM DTT, 0.5 mM EDTA, 25 µg/ml tRNA, and elution buffer containing additional 2M NaCl. RNP complexes were injected into a HiTrap streptavidin HP column (GE Healthcare) and proteins were eluted by NaCl linear gradient. Select fractions were subsequently buffer exchanged back to binding buffer and subjected to heparin

chromatography using a HiTrap heparin HP column (GE Healthcare) and again eluted by NaCl linear gradient. For i*n vitro* stimulation of translation assays, individual fractions were buffer exchanged into the post-lysis buffer for HeLa S10 cell-free extract (20 mM HEPES pH 7.4, 120 mM KAcO,4 mM MgAcO, 5 mM DTT) either by dialysis or using a Sephadex G-25 resin desalting column. 1, 2, or 3 µL of salt-adjusted fractions were added to HeLa S10 translation reactions comprised of 40% S10, 250 ng Kv1.4 IRES/ firefly luciferase reporter RNA, and "All 4 buffer" (30 mM creatine phosphate, 60 mM KAcO, 0.4 mg/mL creatine kinase, 15 µM HEPES-KOH pH 7.4, 1 mM ATP, 0.25 mM CTP, 0.25 mM GTP, 0.25 mM UTP) in 20 µL reactions.

For mass spectrometry analysis, eluted samples were digested with trypsin and subjected to nano-liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) by our collaborator Dr. Paul Gershon (University of California, Irvine). Ions were identified using the Mascot 2.5.1 search engine.

Biotin-RNA/streptavidin chromatography (comparative)

Biotinylated RNA was transcribed as described above. Non-biotinylated RNA was transcribed using the MEGAscript T7 transcription kit (Ambion) without modification, followed by RNeasy Mini kit (Qiagen) purification. RNP complexes were formed *in vitro* by incubating 3 mL rabbit reticulocyte lysate with either 300 µg biotinylated or non-biotinylated RNA and 300 µg tRNA, slowly rotating at 4°C for 12 hrs. Mixtures were diluted with 2x binding buffer (50 mM KCl, 5% glycerol, 1 mM DTT, 0.5 mM EDTA, 25 µg/ml tRNA) and injected into a HiTrap streptavidin HP column (GE Healthcare). Elution was performed

in steps with 1 and 2 M NaCl elution buffer. Resultant chromatograms were aligned, base-line adjusted and subtracted.

Gene analysis of hits

PANTHER (Protein analysis through evolutionary relationships) Classification System is a database of annotated gene and gene functions based on their evolutionary relationships. This database was used to categorize the types and general function of the identified proteins (Mi et al., 2017). Gene IDs were submitted to the database and sorted by GO molecular function or PANTHER Protein Class. The STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database displays predicted protein-protein interactions based on various sources (genomic context, high-throughput experiments, co-expression, and literature)(Szklarczyk et al., 2015). To illustrate the possible interactions between the identified factors, homologous genes for Homo Sapiens from each screen were cross referenced, lists were pooled, and string analysis was performed with filtering for only high confidence interaction scores (>0.7) and using only experimental and database interaction sources. Results were clustered by Markov clustering algorithm (inflation parameter = 10). Enrichr gene set enrichment analysis tool was used to analyze for enrichment based on 129 gene set libraries (Kuleshov et al., 2016).

Chapter 3:

Functional exploration of putative IRES trans-acting factors for Kv1.4 translation

Introduction

The importance of post-transcriptional regulation on phenotype has become increasingly apparent. What might be considered a nexus for post-transcriptional regulation is the process of translation and, notably, the most tightly controlled point in that process is initiation. The focus of this work has been better understanding the mechanism of initiation as directed by IRES elements, in particular the IRES element present in the 5'NCR of Kv1.4 mRNA. The IRES elements present in different viral RNAs are notable for their conserved structures and trans-acting factors, at least among related viruses. This does not appear to hold true for IRES elements presently described in the mRNAs of eukaryotic genes. Importantly, the IRES trans-acting factors that drive translation initiation frequently determine important regulatory paradigms for these genes. Thus, it is critical to our understanding of gene regulation to gain a more comprehensive view of these proteins/interactions.

Since there is no consensus set of initiation factors required for cellular IRES elements, we set out to dissect the initiation factor requirement for the Kv1.4 IRES. In Chapter 2, we described a parallel approach to identifying IRES binding proteins by combining both a biochemical and genetic method, which each have their own set of caveats, to develop an unbiased data set. Many of the proteins identified in Chapter 2 are particularly intriguing in the context of IRES mediated translation, either due to their previous identification as ITAFs (hnRNP K, Annexin A2, eIF4G2 (DAP5), SRSF3 (SRp20),

PA2G4, etc.), their relevant enzymatic activity (DHX15, DDX18, G3BP1), or their role in cap-dependent translation. Several proteins in the cap-dependent pathway of initiation have been shown to have a disproportionate effect on different IRESs, particularly the small ribosomal subunit protein RPS25 and the DEAD-box helicase eIF4A (Landry et al., 2009; Spriggs et al., 2010).

Screens for IRES interacting proteins only delineate RNA-protein interactions, with no readout for functionality. To assess the relevance of these IRES interacting proteins we selected several candidates for downstream analysis. With the ultimate goal of this study being identification of IRES trans-acting factors, an *in vitro* functional assay was first employed. Previously, ITAFs have been identified using an *in vitro* translation assay wherein the IRES containing reporter RNA is translated weakly in one cellular extract, and that translation is rescued by the addition of fractions from another cell extract (Hunt and Jackson, 1999). Studies on the human rhinovirus IRES were able to take advantage of its poor translation efficiency and fidelity in rabbit reticulocyte lysate (RRL) by adding purified components from HeLa cells to subsequently identify the ITAF PTB. Using the rationale behind this approach, we assayed the *in vitro* translation directed by the Kv1.4 IRES element in different cell free extracts and found that translation was very low in HeLa S10 and that translation could be rescued by the addition of RRL. Additionally, we found that this stimulatory activity could be separated by high speed centrifugation. Therefore, *in vitro* translation using HeLa S10 cell-free extract originally served as the basis of our functional assay and fractions generated via biochemical purification, or recombinant proteins identified via Y3H screening, were tested by this approach (see **Chapter 2**, *RNA affinity chromatography*). This served as the initial test for IRES-binding proteins. However,

we found this test unreliable, highly contingent on the S10, and responsive more to the addition of bulk protein than specific recombinant proteins or purified fractions. In this chapter, we explore the functional consequences of a select few putative IRES trans-acting factors. We endeavored to verify binding interactions *in vitro* through a range of approaches and dissect the functional consequences of those interactions in cells.

Rationale and Results

Poly(rC)-binding proteins (hnRNP K and PCBP2)

Heterogeneous nuclear RNPs (hnRNPs) classically participate in pre-mRNA processing and export of mature mRNAs from the nucleus (Dreyfuss et al., 1993; Ostareck-lederer et al., 1998). More recently it has been shown that certain hnRNPs may also play a role in translational regulation, either by recruitment of initiation complexes or by mRNA stabilization (Ertel et al., 2010; Evans et al., 2003; Holcik and Korneluk, 2000; Ostareck et al., 1997). One particular family of hnRNPs, the poly(rC)-binding proteins, are of particular relevance since different members have been shown to regulate translation of specific target mRNAs. The five members of this family (hnRNP K, PCBP1, 2, 3, and 4 (HNRNPE1-4)) all bear three hnRNP K-homology (KH) domains that enable them to interact with pyrimidine-rich binding sequences in target RNAs. The presence of a pyrimidine-rich stretch proximal to the start codon for Kv1.4 offers a possible motif for interaction. KH domains were originally described for hnRNP K and are conserved regions of 65-70 amino acid residues that bind single-stranded C-rich DNA or RNA. KH domains are present in numerous other proteins including vigilin (HDLBP) which has 15 KH domains, nova which also has three KH domains, and FMR1 which has two KH domains (Buckanovich et al.,

1993; Musco et al., 1996; Siomi et al., 1994). In the poly(rC)-binding proteins, the three KH domains are arranged similarly, with two KH domains near the N-terminus and and the third near the C-terminus. hnRNP K contains other important sequence motifs in the region between KH-domains 2 and 3. The first of these is the K-protein-interactive (KI) region, which is not found in the other PCBPs. This region contains five RGG boxes, which are apparently dispensable for poly-(rC) binding, followed by proline-rich SH3-domain-interaction motifs (Bomsztyk et al., 2004; Paziewska et al., 2004). The proline-rich motifs bind to proteins with SH3 domains, most notably Src-family kinases. The KI region is followed by a specific KNS (hnRNP K nuclear shuttling) domain.

hnRNP K and PCBP1 and 2 have been shown to function as regulators of cytoplasmic mRNAs through various mechanisms. For example, A CU-rich repetitive sequence (known as the differentiation-control element (DICE)) in the 3'NCR of 15-lipoxygenase (ALOX15) mRNA mediates translational silencing. ALOX15 is a key enzyme in erythroid-cell differentiation; by catalyzing the dioxygenation of phospholipids in mitochondrial membranes resulting in their breakdown, ALOX15 mediates the final steps of erythrocyte maturation. This silencing has been shown to involve cooperative binding of hnRNP K and PCBP1 to the DICE and resultant targeting to stress-granules (Ostareck et al., 1997). In contrast, hnRNP K, PCBP1, and PCBP2 all function as stimulatory ITAFs for c-myc translation (Evans et al., 2003). hnRNP K, PCBP1, and PCBP2 not only affect the fate of cellular mRNAs but have also been implicated in the replication and translation of virus RNAs through distinct mechanisms. For example, PCBP2 binds to the 5'-terminal clover-leaf structure of poliovirus RNA and facilitates the interaction with the viral protein 3CD, a precursor of proteinase 3C and RNA polymerase 3D, promoting viral RNA replication

(Gamarnik and Andino, 1997; Parsley et al., 1997; Walter et al., 2002). Additionally, PCBP2 binds to stem-loop IV of the IRES downstream of the clover-leaf structure and drives IRES-mediated translation (Blyn et al., 1997). More recent work has implicated hnRNP K in viral replication, with a role in limiting hepatitis C virus (HCV) production independently of translation or RNA replication, possibly by binding to and limiting the availability of viral RNA for incorporation into virions (Poenisch et al., 2015).

hnRNPK regulates IRES translation in HeLa cells

As discussed in **Chapter 2**, hnRNP K was identified as a Kv1.4 IRES interacting protein in both a genetic yeast 3-hybrid screen as well as a biochemical RNA-affinity screen. Previously described roles for hnRNP K regulation of mRNA translation make this a compelling interaction for follow-up studies. Using the RBPmap computational tool to predict RNA binding sites, hnRNP K was predicted to bind at 8 possible sites with the motif CCAWMCC, all within the 5'NCR of the Kv1.4 RNA ($p<0.05$). With the aim of understanding the functional consequences of this interaction, we began by assaying the effect of recombinant purified hnRNP K in an *in vitro* translation assay, which has since been discarded (see *Introduction*). Results indicated that addition of purified hnRNP K only modestly stimulated translation of a monocistronic Kv1.4 IRES luciferase reporter RNA. Similar levels of stimulation were observed with addition of denatured hnRNP K and bovine serum albumin. Additionally, endogenous hnRNP K was readily observed in S10 extracts, suggesting that hnRNP K may not be limiting in this context. To understand hnRNP K consequences on IRES-mediated translation, we made use of the dicistronic reporter assay. Dicistronic reporter plasmid (pRst1209F) produces RNA with a 5' Renilla

luciferase coding region followed by an inverted repeat within an intercistronic sequence, followed by the firefly luciferase coding region. Full length Kv1.4 IRES is inserted in the intergenic region and drives firefly luciferase (Fluc) expression (Figure 3.1.A). This construct was transiently transfected into HeLa cells along with plasmids for the overexpression of hnRNP K, or short hairpin RNA (shRNA) against hnRNP K for gene silencing. Following transfection, cells were lysed and IRES activity was measured as the ratio between Fluc and Rluc by dual-luciferase assay. As a control for exogenous protein expression, plasmid encoding green fluorescent protein (GFP), or empty vector was used. For hnRNP K, 7-fold stimulation of IRES-mediated translation was observed in Hela cells compared to GFP control vector (Figure 3.1.B). This indicates that hnRNP K, and not GFP or empty vector, is able to specifically stimulate IRES translation in HeLa cells. Additionally, cotransfection with GFP, and hnRNP K partially recovered Fluc/Rluc ratios, although to levels of empty vector control.

Previous studies characterizing ITAFs has indicated that HeLa cells may not be amenable to knockdown of another poly-(rC) binding protein, PCBP2 (Gamarnik and Andino, 1997; unpublished observations). To assay the effect of hnRNP K silencing on Kv1.4 IRES activity, we elected to develop this experiment in AC16 cells, a transformed human ventricular cardiomyocyte cell line which provides greater biological relevance to a gene expressed primarily in the heart. Over expression of hnRNP K in these cells produced a moderate but non-significant increase in luciferase ratios compared to GFP and empty vector controls. It may be that endogenous levels of hnRNP K are sufficient to drive maximal translation from the IRES, under these conditions. Transient knock-down

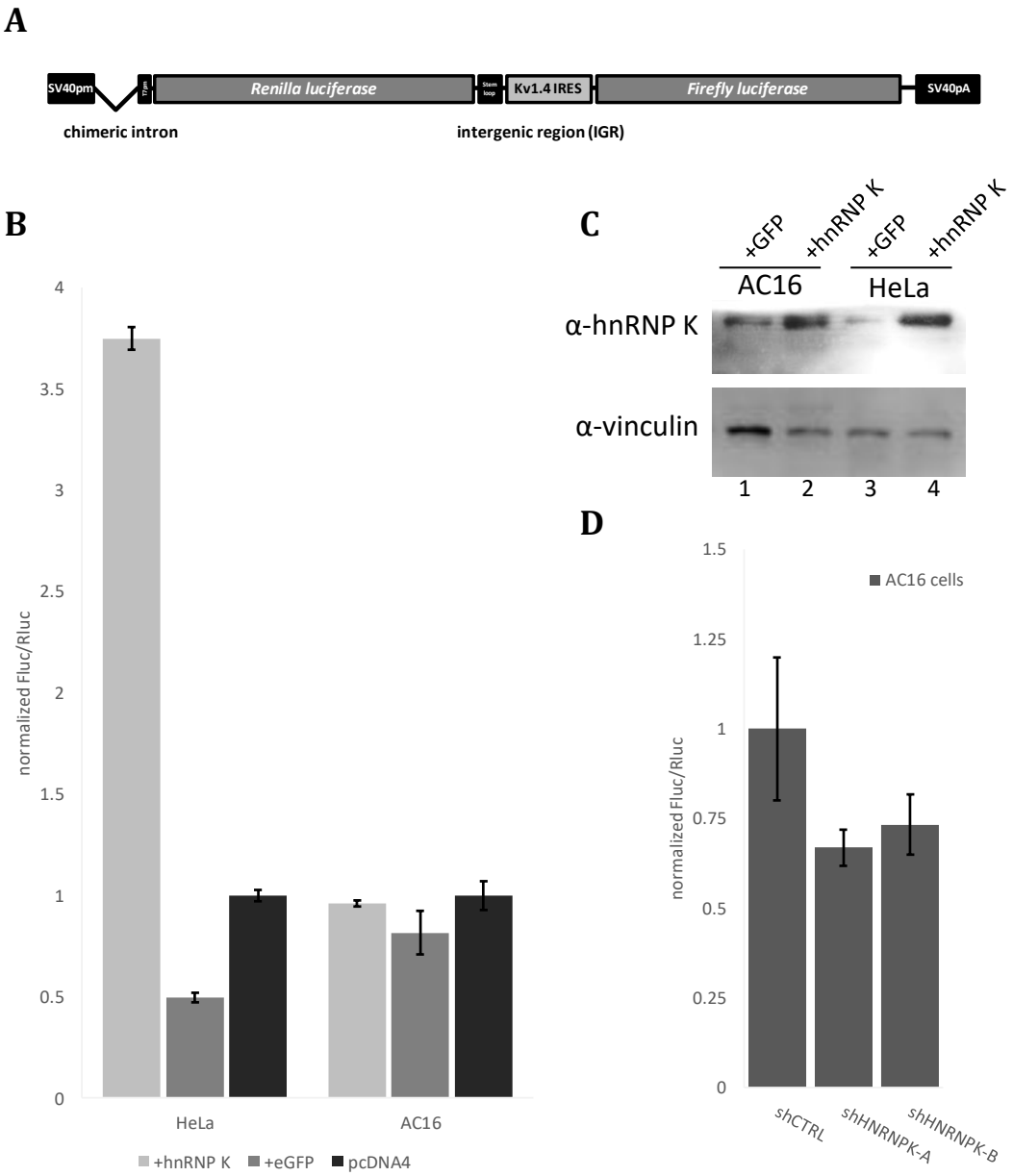


Figure 3.1. hnRNPK positively regulates IRES-mediated translation. A. Schematic of dicistronic reporter construct for cotransfection into cells. **B**. Overexpression of hnRNP K in HeLa cells boosts IRES translation compared to GFP control. Over expression in AC16 cardiomyocytes resulted in no specific effect. **C**. Western blot analysis of hnRNP K overexpression in HeLa and AC16 cells show elevated levels compared to GFP control (lane 1 vs. 2 and 3 vs. 4). **D**. transient shRNA knockdown of hnRNP K using two different shRNAs negatively regulates IRES translation in AC16 cells.

however did result in significant reduction in IRES activity with two different shRNAs (Figure 3.1.D). These results suggest a cell type dependent role for hnRNP K in Kv1.4 IRES-mediated translation. Attempts to UV crosslink recombinant hnRNP K with Kv1.4 IRES have so far been unsuccessful.

PCBP2 binds Kv1.4 IRES RNA in vitro

A closely related protein to hnRNP K, PCBP2, plays a key role in IRES-mediated translation of poliovirus and binds specifically in the PV IRES at SL-IV. Although not detected in RNA-affinity or yeast 3-hybrid screens, *in vitro* binding experiments to evaluate other putative ITAFs used the PCBP and PV SL-IV interaction as a control for specificity, and unexpectedly demonstrated binding to Kv1.4 IRES. Using an *in vitro* UV cross-linking assay with ^{32}P-labeled RNA and recombinant protein, PCBP2 was found to bind to Kv1.4 IRES RNA (Figure 3.2.A). Specificity was evaluated by addition of excess tRNA and titration with unlabeled Kv1.4 IRES competitor RNA, showing that the unlabeled competitor was capable of efficiently competing with the ^{32}P-labeled probe for binding with PCBP2. Additionally, PCBP2 was pulled down from extracts of AC16 cells using a biotinylated Kv1.4 IRES RNA bait. In the presence of excess tRNA and BSA, PCBP2 was pulled out of extract by both Kv1.4 IRES RNA and PV SL-IV RNA, but not with Rluc RNA (Figure 3.2.B.). Although PCBP2 and hnRNP K are closely related and can bind poly-(rC) readily, they do not share consensus binding motifs. Several predicted binding sites for PCBP2 are present in the Kv1.4 IRES, as predicted by RBPmap analysis. It is perhaps less of a surprise that PCBP2 binds Kv1.4 IRES and more that this interaction was not detected in any of the screens.

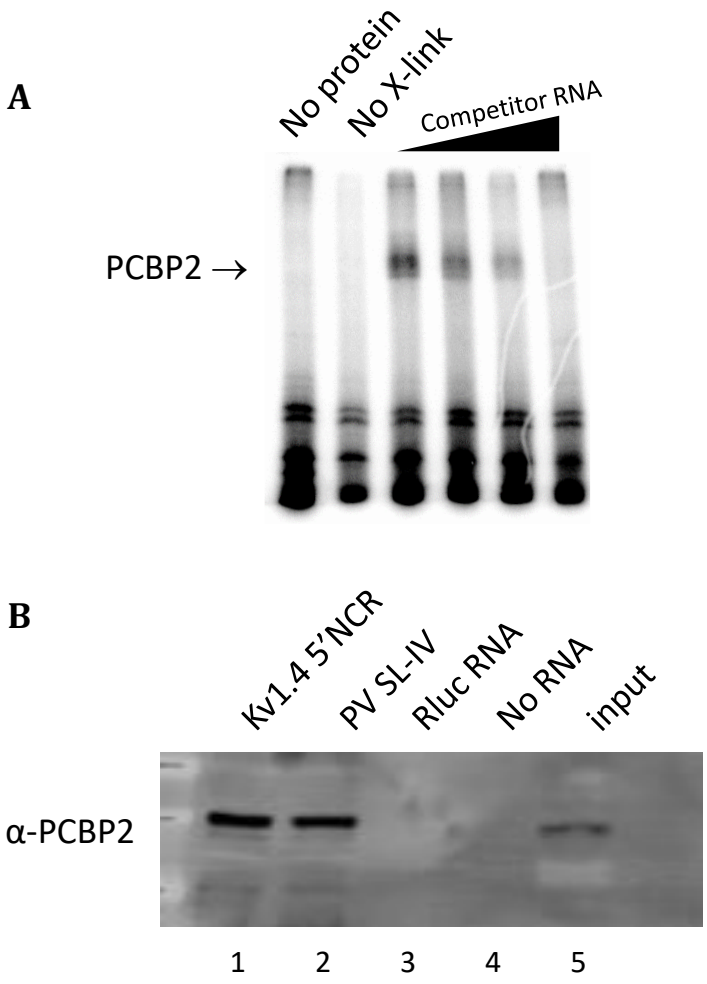


Figure 3.2. PCBP2 binds Kv1.4 5'NCR RNA. A. UV cross-linking assay with ^{32}P-labeled Kv1.4 5'NCR RNA and PCBP2 with increasing concentrations of unlabeled competitor RNA. Binding mixtures were incubated at 30 °C prior to crosslinking with 254 nm light, followed by RNase digestion and SDS-PAGE **B**. Biotinylated RNA pulldown of PCBP2 from AC16 cell lysate using Kv1.4 5'NCR RNA (1), poliovirus stem-loop IV RNA (2), or renilla luciferase mRNA (3). Streptavidin-agarose beads were used to pull-down RNP complexes, washed extensively and proteins were eluted with Laemmli sample buffer for Western blot analysis.

Nevertheless, PCBP2 represents another putative Kv1.4 ITAF, whose function warrants further exploration.

DEAH-box helicase 15 (DHX15)

The human genome encodes 95 non-redundant helicase proteins, of which 64 are RNA helicases and 16 are DExH-box helicases (Suthar et al., 2016; Umate et al., 2011). These RNA helicases play many roles in RNA metabolism, from transcription and pre-mRNA processing, RNA folding and nuclear transport, to translation and RNA degradation (Patel and Donmez, 2006). RNA helicases have been shown to be involved in many biological processes, including cellular differentiation, immune response, and apoptosis (Jiang and Wu, 1999; Mosallanejad et al, 2014; Wang et al, 2015). Focusing on translation, several DExD/H-box helicase proteins have been demonstrated to have critical roles, most notably the ATP-dependent RNA helicase eIF4A, a subunit of the eIF4F complex, which is thought to facilitate unwinding of RNA during 40S ribosome scanning. Recent evidence demonstrates a potential role for DHX33 in mRNA translation initiation by promoting elongation-competent 80S assembly (Zhang et al., 2015). DDX3, the mammalian homolog of Ded1 in yeast, as well as DHX29, have both been shown to facilitate the translation of mRNAs containing particularly long or structured 5'-NCRs (Lai et al., 2008; Pisareva et al., 2008; Soto-Rifo et al., 2012). Finally, DHX9 (RNA helicase A, RHA) has recently been shown to act as an ITAF by positively regulating p53 IRES activity (Halaby et al., 2015).

DHX15 is primarily a nuclear protein and is required for the release of the lariat intron from the spliceosome, as well as for early steps of pre-rRNA processing into mature rRNAs (Lebaron et al., 2005). Additionally, DHX15 has been shown to regulate antiviral

innate immunity to EMCV by binding the NOD like receptor Nlrp6 and act as a viral RNA sensor to activate NF-κB, indicating a cytoplasmic role for this protein (Wang et al., 2015). Notably, DHX15 has been shown to specifically interact with La, an important ITAF for not only the viral IRESs of PV and CVB3, but also the cellular IRESs of both BiP and XIAP (Fouraux et al., 2002).

DHX15 binds Kv1.4 RNA in vitro

DHX15 was detected in our yeast 3-hyrid screen, although it has no previously reported role in translation. To verify DHX15 interaction with full length Kv1.4, recombinant DHX15 was purified and incubated with ^{32}P-labled Kv1.4 5'NCR RNA. UV crosslinking and RNase digestion reveals interaction with Kv1.4 IRES, albeit weak compared to the PV SL-IV/PCBP2 interaction (Figure 3.3).

Since DHX15 is able to bind Kv1.4 IRES, we next determined what effect overexpression of DHX15 had on IRES activity in cells. Dual luciferase assays in HeLa cells showed 7-fold stimulation of IRES-mediated translation, relative to GFP overexpression control. This alteration of dual luciferase expression might be a feature of HeLa cells, because over-expression experiments in AC16 cells displayed no significant effect on IRES activity. These findings indicate DHX15 is able to bind Kv1.4 IRES, and that this interaction may stimulate its translation in HeLa cells. This effect was not observed for experiments in other cell types, as well as *in vitro*, where introduction of recombinant DHX15 into translation reactions inhibited translation of monocistronic reporter. Although this assay may not be reliable, this result was notable in that even small amounts of added DHX15 inhibited translation in a dose dependent fashion (Appendix Figure A.2).

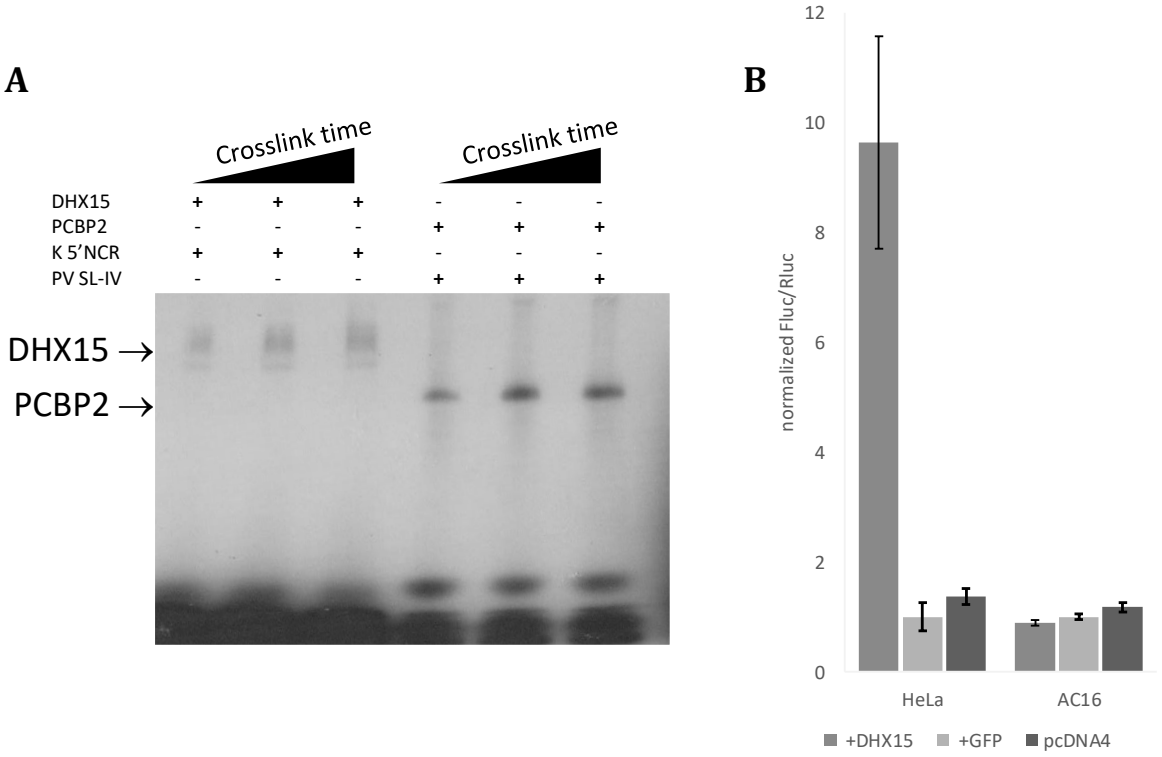


Figure 3.3. DHX15 binds Kv1.4 IRES and overexpression stimulates translation in HeLa cells. **A**. Recombinant DHX15 binds to ^{32}P-labeled Kv1.4 5'NCR RNA *in vitro* by UV crosslinking assay. The known PCBP2 and poliovirus SL-IV RNA interaction serves as a positive control. **B**. Cotransfection of HeLa and AC16 cells with dual-luciferase Kv1.4 IRES reporter construct and plasmids for overexpression of DHX15, GFP, or empty plasmid indicate stimulation of IRES-mediated translation by exogenous DHX15 in HeLa cells. Firefly to renilla luciferase ratios (Fluc/Rluc) for each experiment were normalized to GFP control.

Annexin A2 (ANXA2)

Annexins are a Ca^{2+}-dependent phospholipid binding family of proteins which regulate membrane trafficking events such as exocytosis, endocytosis and cell-cell adhesion (Waisman, 1995). Annexins have also been suggested to have inherent channel activity and have also been implicated in regulating the expression of other ion channels. Annexin A7 has intrinsic calcium channel activity, whereas Annexin A2 has been shown to alter the activity of osmotically-regulated chloride channels in endothelial cells (Nilius et al., 1996; Okuse et al., 2002; Pollard and Rojas, 1988). Additionally, Annexin A2 has been shown to upregulate the voltage-gated sodium channel $Na_V1.8$ through a mechanism the authors suggested was related to increased translocation.

ANXA2 binds Kv1.4 5'NCR

Annexin A2 was detected both by affinity purification from mouse heart and by yeast 3-hybrid screen of a HeLa S3 cDNA library to interact with the Kv1.4 IRES. Annexin A2 was notably absent from our second, less stringent biochemical purification. However, previous description of annexin A2 function has reported lack of expression in erythrocytes, reasonably explaining why it was not identified in our second screen using rabbit reticulocyte lysate (Waisman, 1995). By Western blot analysis of RRL, we in fact do not detect annexin A2, while it is readily apparent in HeLa cells and AC16 cardiomyocytes (Figure 3.4.A). To confirm biochemical association between annexin A2 and the Kv1.4 5'NCR, RNA pulldown of ANXA2 was performed using biotinylated Kv1.4 5'NCR and lysate of AC16 cardiomyocyte cells, which are more relevant to Kv1.4 than HeLa cells. *In vitro* transcribed and biotinylated RNA was incubated in AC16 cell lysate for 30 min, prior to

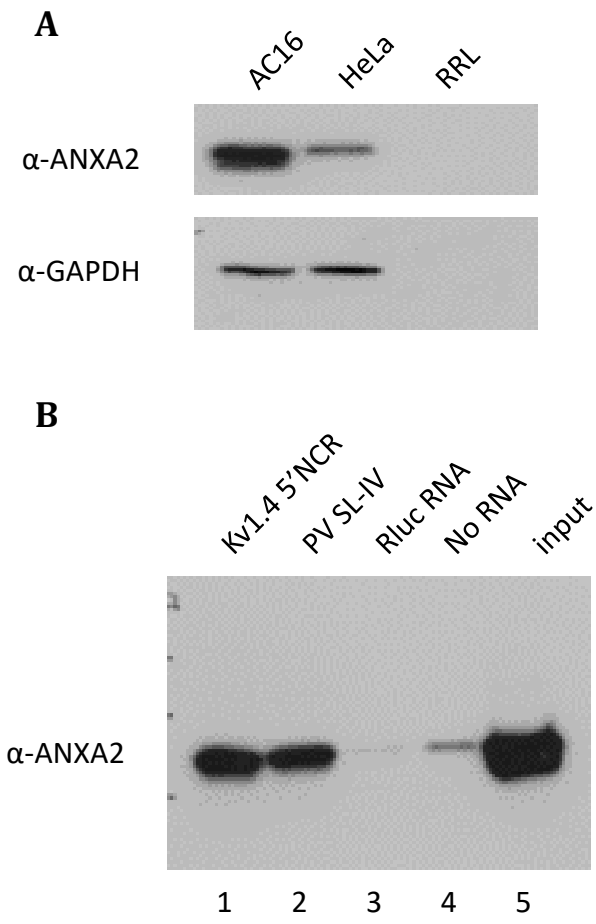


Figure 3.4. ANXA2 binds Kv1.4 IRES in AC16 cells. A. Western blot analysis of AC16 cell lysate, HeLa cell lysate, or RRL for ANXA2 expression. ANXA2 levels are highest in AC16 cell lysate, and is not readily observed in RRL. **B**. Biotinylated RNA pulldown of ANXA2 from AC16 cell lysate using Kv1.4 5'NCR RNA (1), poliovirus stem-loop IV RNA (2), or renilla luciferase mRNA (3). Streptavidin-agarose beads were used to pull-down RNP complexes, washed extensively and proteins were eluted with Laemmli sample buffer for Western blot analysis.

mixing with streptavidin conjugated agarose affinity gel. Complexes were separated by centrifugation and washed extensively prior to elution with Laemmli sample buffer. Western blot analysis of eluate from streptavidin beads shows annexin A2 binding to Kv1.4 IRES (and PV SL-IV RNA), and not renilla luciferase RNA (Figure 3.4.B).

ANXA2 drives IRES-mediated translation of Kv1.4

To assess whether RNA binding serves a functional role of annexin A2 for Kv1.4 IRES activity, dual luciferase assays in cell culture were employed. ANXA2 expression construct and dicistronic reporter construct were cotransfected into different cell lines and dual luciferase assays were performed. As seen in Figure 3.5, addition of exogenous ANXA2 to HeLa cells resulted in greater relative IRES activity, compared to GFP control. Much like experiments with DHX15 and hnRNP K, this effect did not hold for AC16 cells. It is unclear what additional factors may be at play in providing a cell type dependent effect on IRES translation. Possibly endogenous ANXA2 concentrations are sufficient for IRES activity in certain cell types and not others. Notably, ANXA2 is observed at greater relative concentrations for AC16 cells than for HeLa cells.

To further address its functional importance, ANXA2 was silenced by using a pool of three siRNA sequences. As shown in Figure 3.5.B, ANXA2 was effectively knocked down in HeLa cells but to a much lesser extent in AC16 cells. Gene silencing 48 hrs prior to transfection with dicistronic reporter constructs and subsequent dual-luciferase assay shows an inhibitory effect in HeLa cells and not in AC16 cells. Since ANXA2 is inefficiently repressed in AC16 cells by these siRNA, it may be that the levels of ANXA2 remain sufficient to drive IRES translation. To more effectively assess the effects of ANXA2 silencing, in

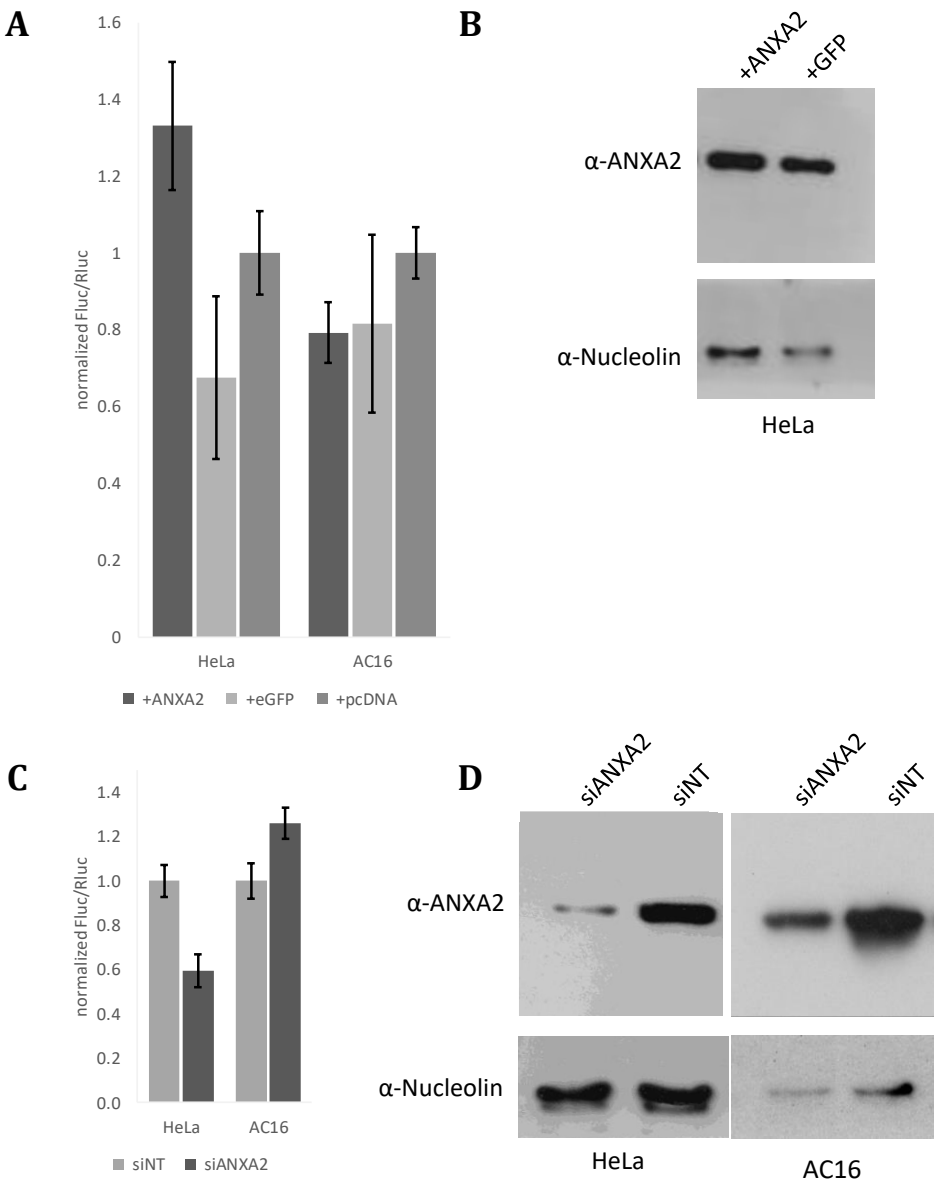


Figure 3.5. Overexpression or knock down of ANXA2 affects Kv1.4 IRES translation. **A**. Cotransfection of HeLa or AC16 cells with expression construct for ANXA2 or GFP with dicistronic reporter. Over expression in AC16 cardiomyocytes resulted in no specific effect, whereas overexpression in HeLa cells stimulated Kv1.4 IRES-mediated translation. **B**. Western blot analysis of cotransfected HeLa cells shows increased ANXA2 vs GFP control. **C**. siRNA-mediated knockdown of ANXA2 in HeLa or AC16 cells indicates repression of IRES-mediated translation in HeLa cells, whereas siRNA treatment of AC16 cells results in a non-significant increase in translation. **D**. Western blot analysis shows efficient gene silencing in HeLa cells and inefficient silencing in AC16 cells.

collaboration with Hung Nguyen we next established a stable AC16 cell line expressing a short hairpin RNA (shRNA) targeting ANXA2 for suppression. These cells displayed greatly reduced levels of ANXA2 by Western blot analysis (Figure 3.6). When these cells were transfected with dicistronic reporter constructs, firefly to renilla luciferase ratios were greatly diminished compared to knock down control cells. These results suggest a role for ANXA2 in Kv1.4 IRES translation in different cell types and that low level ANXA2 may be sufficient to drive maximal translation. Alternatively, the variation in responses of IRES-reporter to ANXA2 manipulation suggests that IRES-mediated translation may be modulated by additional cellular factors that may synergize with ANXA2.

Discussion

The sequence, length, and structure of the 5'NCR of Kv1.4 mRNA all predict a non-canonical process for translation initiation. Under closer inspection it was demonstrated that Kv1.4 5'NCR could direct internal binding of the ribosome to initiate protein synthesis (Negulescu et al., 1998). Kv1.4 is not alone in this category as many notable cellular mRNAs are controlled by this mechanism, including c-myc, XIAP, and VEGF. Unlike the picornavirus IRES elements and their non-canonical mode of translation initiation, cellular IRES containing mRNAs are capped and typically operate alongside scanning-dependent translation. Therefore, the degree to which a cellular IRES mRNA is practically 5' and cap-independent is a matter of debate (Jackson, 2013; Terenin et al., 2017). The trans-acting factor requirements of different IRES elements exists on a spectrum, from requiring practically all the eIFs of cap-dependent initiation, to the use of none of these factors (e.g. the CrPV IRG IRES). Differential dependence on canonical initiation factors is typically

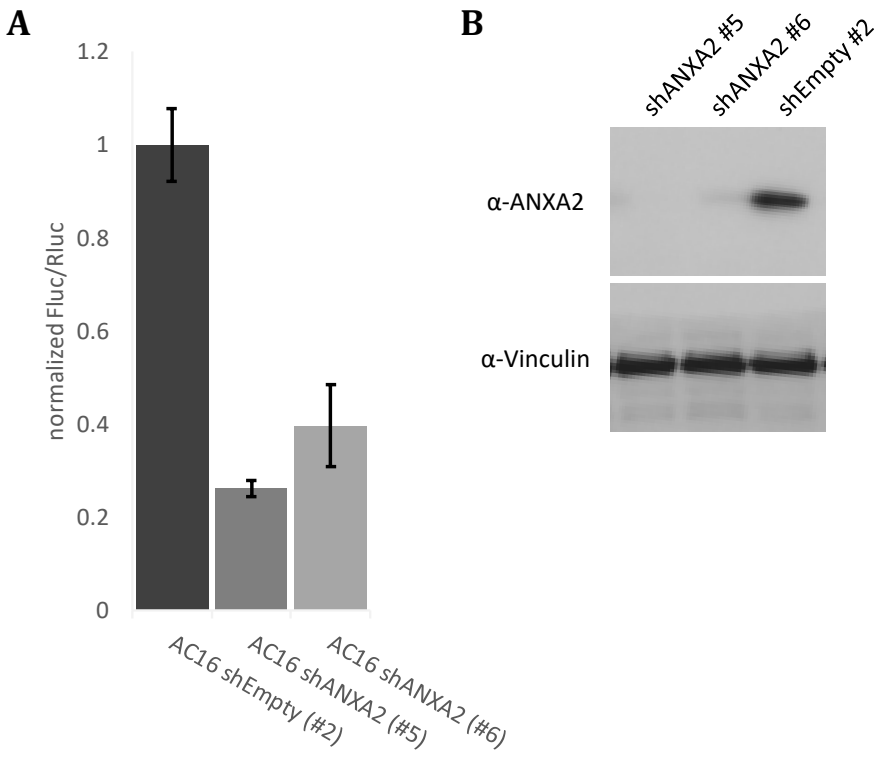


Figure 3.6. AC16 stable ANXA2 knock down cells restrict IRES-mediated translation. A. AC16 cells transduced with shRNA constructs targeting ANXA2 reduce IRES-mediated translation from transfected dicistronic reporter. **B**. Western blot analysis of stable cell lysates shows efficient ANXA2 silencing.

associated with functions of additional non-canonical factors (i.e., ITAFs)(Liberman et al., 2015). To establish the mechanism by which Kv1.4 IRES controls translation, it is necessary to determine which canonical and non-canonical factors participate in initiation. Chapter 2 describes work carried out to identify such factors and revealed a wide range of candidate RNA-binding proteins. Work described in this chapter represents an attempt to characterize these interactions and determine if they represent a trans-activity for translation initiation.

Absent a high-throughput method for functional screening of each of the candidates identified, select proteins were chosen for further analysis based on relevant enzymatic activity (DHX15), relevance to ion channel post-transcriptional regulation (ANXA2), or previous characterization as an ITAF (hnRNP K, ANXA2). There were a number of other hits which fit this description as well including the helicases (DDX18, DDX23, DDX46, IFIH1, and SKIV2L2), and known ITAFs (AUF1, hnRNP A1, SYNCRIP, SRp20, PA2G4, DAP5, YBX1, and NONO).

Members of the poly(rC) binding protein family have been implicated in IRES-mediated translation for a large number of RNAs. hnRNP K was shown to bind Kv1.4 IRES by yeast 3-hybrid assay, as well as by RNA-affinity chromatography. We have also shown that not only does hnRNP K affect IRES translation in cells, but that PCBP2, the closely related poly(rC) binding protein, is also able to bind the IRES *in vitro*. The stimulation of translation observed with co-transfection of hnRNP K, was corroborated by shRNA-mediated knock down of hnRNP K and a subsequent drop in IRES-mediated translation in AC16 cells. It is notable that the cell type used in these experiments had a dramatic impact on the effects seen for dicistronic reporter expression of different stimuli. While exogenous

expression of hnRNP K had a stimulatory effect in HeLa cells, this effect was not consistent in AC16 cells. It may be that the lack of effect in AC16 cells is due to already high expression of endogenous hnRNP K in these cells, or due to limitations imposed by other factors preventing stimulation of IRES translation. Although *in vitro* stimulation of translation has not been observed with addition of hnRNP K or PCBP2, these proteins may act as a higher-order complex of proteins for Kv1.4 function. In fact, previous description of the c-myc IRES has shown that addition of the individual poly(rC) binding proteins did not affect *in vitro* translation, whereas combinations of PCBP2, PCBP1, and hnRNP K with unrip (unr-interacting protein) and unr (upstream of N-ras) were able to stimulate IRES translation 3-fold (Evans et al., 2003). The cell type specific effect we observe for Kv1.4 IRES may be related to relative concentrations of these ITAFs in different cells, dictating the assembly of pre-initiation complexes on the RNA.

Recruitment of pre-initiation complexes to the IRES will almost certainly involve restructuring of the RNA near the initiation site. This region of the Kv1.4 IRES has been shown to form significant secondary structures in direct proximity to the start codon (Jang et al., 2004). While other RNA helicases important for canonical translation initiation such as eIF4A and DDX3 are thought to act by unwinding the 5'NCR for processive ribosome scanning, the process of ribosome recruitment to the IRES is distinct. With internal binding of the 40S ribosome, a region of single stranded RNA must be available for accommodation into the mRNA channel. A mechanism for partial unwinding of secondary structures for internal ribosome binding has been suggested for other 5'NCRs, an activity attributed to trans-acting helicases (Halaby et al., 2015). Yeast 3-hybrid screen against the Kv1.4 IRES identified the DEAD-box helicase DHX15 as a putative IRES interactor. We showed here

that DHX15 is able to bind to IRES RNA *in vitro* and is able to activate the IRES of Kv1.4 in HeLa cells. This effect was even more pronounced than for hnRNP K transfection, though much like hnRNP K, these results did not extend to AC16 cells. Further experiments targeting DHX15 for silencing need to be performed to properly assess its role in both HeLa and AC16 cells. Both DHX15 and hnRNP K are predominantly nuclear proteins with well-established roles for RNA metabolism in the nucleus (Matunis et al., 1992; Michelotti et al., 1996). The possibility that these proteins might bind to nascent Kv1.4 mRNA to be exported as part of the mRNP fits the model of the nuclear experience as necessary for efficient IRES activity (see **Chapter 1**). This could explain the deficiencies observed for IRES-mediated translation of *in vitro* transcribed RNAs, both in transfection experiments in cells, as well as in *in vitro* translation assays.

Lastly, we described the interaction of ANXA2 with the Kv1.4 IRES. ANXA2 was identified both through yeast 3-hybrid screen, as well as by two-step RNA affinity chromatography. Its absence from our second comparative chromatography screen is clarified by the apparent lack of expression in reticulocytes, the starting material used for this purification. A Ca^{2+}-dependent protein, ANXA2 is involved in a wide range of cellular processes including exocytosis, fibrinolysis, and cell motility. Its previously described role in the post-transcriptional regulation of other voltage-gated ion channels, through a mechanism not entirely clear, as well as recent description as an IRES trans-acting factor for p53, suggested to us that this hit was of particular relevance (Okuse et al., 2002; Sharathchandra et al., 2012). By using biotinylated RNA pulldown, we were able to confirm that ANXA2 is able to bind Kv1.4 IRES in extracts from AC16 cardiomyocytes. Additionally, overexpression of ANXA2 in HeLa cells was able to stimulate the IRES-mediated

component of a dicistronic reporter, albeit modestly. Much like experiments with hnRNP K and DHX15, this effect was not observed in AC16 cells. However, interaction of Kv1.4 IRES and ANXA2 were shown not only in extracts of AC16 cells, but also by stringent purification from heart tissue (Figure 2.7), suggesting this interaction is of significance. siRNA mediated knockdown of ANXA2 in HeLa cells reduced the ratio of IRES to cap-dependent translation, whereas siRNA treatment of AC16 cells resulted in incomplete knock down and a modest uptick in relative IRES translation. Western blot analysis of ANXA2 in these cells indicated that knock-down was efficient in HeLa cells but inefficient in AC16 cells. By generating stable shRNA transduced AC16 cell lines, which resulted in an almost complete knock-down of ANXA2, subsequent inhibition of the Kv1.4 IRES was observed. This suggests that in the AC16 cell model, the higher endogenous expression of ANXA2 prevents further stimulation of IRES-mediated translation by exogenous expression, and repression with transient siRNA treatment does not lower the protein expression to the threshold needed to affect Kv1.4 IRES translation. Only with constitutive silencing was ANXA2 expression reduced to levels that affect IRES-mediated translation.

Some cellular IRESs appear to drive translation in response to specific stimuli and are uniquely sensitive to the levels of translation machinery to fine tune their regulation (Bellodi et al., 2010; Dobbyn et al., 2008; Komar and Hatzoglou, 2011). Others are thought to exist to maintain homeostasis and may be tuned to generally resist cell stresses and fluctuations in bulk translation. This would describe a role for ANXA2 in IRES-mediated translation of Kv1.4, since expression of this apparent ITAF in cardiomyocytes is high. Changes in IRES translation may be resistant to fluctuating translation regulation.

Materials and Methods

Recombinant protein purification

pET16b-hnRNP K was obtained from (Ostareck et al., 1997), pET15-DHX15 was obtained from (Carl Ware, Sanford Burnham), pET22b-PCBP2 was produced as previously described ((Parsley et al., 1997). Plasmids were transformed into *Escherichia coli* Rosetta (DE3) cells. The cells were grown at 37 °C to an OD_{600nm} of 0.5 in Luria–Bertani (LB) broth with 25 μg/ml kanamycin. Protein expression was induced with 0.5 or 1 mM IPTG and the temperature was lowered to 25 °C for 4-16 hrs. Cell pellets were resuspended in 20 mM Tris HCl pH 7.0, 250 mM NaCl, 5 mM imidazole, 10%(v/v) glycerol (I_5 buffer). Lysis was performed by sonication and the lysate was then centrifuged at 13,800 rcf for 15 min. Polyethylenimine (PEI) was added at 1% and lysate was centrifuged again to precipitate DNA. The supernatant was filtered through 0.45 μm polyethersulfone (PES) filters and loaded onto a 5 ml Ni–NTA HisTrap chelating column (GE Healthcare) previously equilibrated with I_5 buffer. Tagged proteins were eluted by linear or segmented gradients using a buffer consisting of 20 mM Tris HCl pH 7.0, 250 mM NaCl, 700 mM imidazole, 10%(v/v) glycerol (I_{700} buffer). Relevant fractions were pooled, buffer exchanged and concentrated using Microcon® centrifugal filter devices (Millipore).

UV-crosslinking assays

High-specific activity ^{32}P-labeled RNA probes were synthesized in 20 μL reactions using the MEGAscript T7 transcription kit (Ambion), with only 7.5 mM CTP, 40 μCi [α^{32}P]CTP (PerkinElmer). Following transcription, RNA was purified using the RNeasy Mini

kit (Qiagen). The yield of labeled RNA was quantified using a scintillation counter (Beckman-Coulter).

UV-crosslinking reactions were carried out in 25 mM KCl, 5 mM Hepes pH 7.4, 2 mM $MgCl_2$, 0.1 mM EDTA, 3.8% glycerol, and 2 mM DTT. Typically, 2 µg purified protein was preincubated in a 10 µL reaction at 30 °C for 10 min with 10 µg tRNA competitor, 1 µg bovine serum albumin (BSA), and 10 U RNasin. 10 pmol ^{32}P-labeled RNA probe was prepared by melting and refolding by gradual cooling. RNA was added to the binding mix and allowed to bind of 10 min at 30 °C. Reactions were subsequently placed on ice in a UV Stratalinker 1800 (Stratagene) and irradiated with 254 nm UV light for 10-60 min. Reactions were digested with 10 µg RNaseA (Sigma) and 30 U RNase T1 (Pharmacia) at 37 °C for 30 min and analyzed by 12.5% SDS-PAGE and autoradiography.

Biotinylated RNA pull-down

500 µg of cell lysate from AC16 cells for each pull-down was pre-cleared using 50 µL streptavidin magnetic beads (New England BioLabs) or 50 µL EZview™ Red Streptavidin Affinity Gel (Sigma). A 500µL binding mix was assembled with precleared lysate, 10 µg of biotinylated RNA, 1× protease inhibitor (Peirce), 200U of RNasin® (Promega), in binding buffer (50 mM KCl, 5% glycerol, 1 mM DTT, 0.5 mM EDTA, 25 µg/ml tRNA). Reactions were incubated at room temperature for 30 min before adding 50 µl pre-equilibrated beads and incubation at room temperature for another 30 min. Beads were separated, either using a magnetic stand or via centrifugation, supernatant was removed, and beads were washed 5 times with binding buffer. Beads were then resuspended in 40 µL 2× Laemmli sample buffer (LSB), boiled, and proteins were detected by SDS-PAGE and Western blotting.

Immunoblotting

Following SDS-PAGE, proteins were electroblotted to a PVDF membrane. The membranes were blocked in 5% milk in phosphate buffered saline (PBS) with 0.1% Tween-20 (PBS-T) for 1 h at room temperature. Mouse monoclonal antibody against PCBP2 (1:2000 in PBS-T), rabbit monoclonal antibody against ANXA2 (1:10,000 in PBS-T), rabbit monoclonal antibody against hnRNPK (1:100,000 in PBS-T), or rabbit polyclonal antibody against DHX15 (1:10,000 in PBS-T) was used as a primary antibody with incubation at room temperature for 1 hr. This was followed by incubation with relevant goat anti-mouse or goat anti-rabbit horseradish peroxidase (HRP) secondary antibody (Bethyl) for 1 h. Protein bands were visualized using enhanced chemiluminescence (Thermo Scientific).

Cell culture and DNA constructs

HeLa cells and HEK293 cells were grown as monolayers in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 8% newborn calf serum (NCS) or 10% fetal calf serum (FCS), respectively. AC16 cells were grown as monolayers in Ham's Nutrient Mixture F12 media supplemented 12% FCS. Stable cells were selected with 800 μg/mL Geneticin.

Transfections and dicistronic reporter assays

pCMV-HA-DHX15 eukaryotic expression construct for DHX15 was obtained from Dr. Zhiqiang Zhang (Lu et al., 2014). MigR1-HNRPK-HA eukaryotic expression construct for hnRNP K, and pSuper-retro-Hnrpk shRNA were obtained from Dr. Danilo Perrotti (Notari et al., 2006), pcDNA-AnxA2 eukaryotic expression construct for ANXA2 was obtained from

Dr. T. R. Kannan (Somarajan et al., 2014), pSUPER-AnxA2 shRNA targeting ANXA2 was obtained from Dr. Bob van de Water (Graauw et al., 2008), pcDNA3.1(+) empty vector was from (Invitrogen), pRst1209F Kv1.4 dicistronic IRES reporter was generated as previously described (Jang et al., 2004).

Six-well plates were seeded with 125,000 cells 24 hrs prior to transfection. Plasmids were transfected/cotransfected into cell lines using jetPRIME (Polyplus transfection) using 1 μg total DNA per well. After 24 hrs cells were harvested, and dual-luciferase assays were performed with a Promega dual-luciferase kit. Twenty μL lysate was added to 100 μL firefly luciferase substrate, readings were taken for 10 s, and 100 μL renilla luciferase substrate was added and readings were again measured for 10s. Assays were read using a SIRIUS luminometer (Berthold Detection Systems). Values from each interval of firefly and renilla luciferase were summed and exported to Excel for analysis. siRNA knock-down using 3×27mer RNA duplexes targeting ANXA2 from Origene (SR319345). Cells were seeded as above, then transfected with 10 nM siRNA pool or non-targeting siRNA (siRNA-NT) using jetPRIME transfection reagent. After 48 hrs, cells were transfected with dicistronic reporter as above.

Chapter 4:

Conclusions and significance

In the late 1980s, a novel RNA regulatory element was discovered in the RNA genomes of picornaviruses which allows messages to recruit 40S ribosomal subunits bypassing the canonical m^7G-cap-depedent recruitment of ribosomes (Jang et al., 1988; Pelletier and Sonenberg, 1988). This element came to be known as an internal ribosome entry site (IRES) and is composed of RNA sequences/structures which allow viral mRNA to be translated while the virus simultaneously shuts down host-translation. Since picornavirus RNAs are naturally uncapped and drive translation when scanning-dependent translation is inhibited, the obvious question arises, "what is the mechanism of ribosome recruitment?" Paramount to understanding this mechanism is not only characterization of the RNA which defines the IRES, but also identification of the translation apparatus that the IRES recruits. Significant work has been done to reveal the trans acting factors, both canonical and non-canonical, which participate in picornavirus translation.

Identification of IRES elements in cellular mRNAs shifted the paradigm for IRES-mediated translation from an alternative mechanism used by viruses to subvert their host to one where an alternative mode of initiation is a mechanism of regulation which proceeds alongside canonical translation (Komar and Hatzoglou, 2011; Macejak and Sarnow, 1991). While viral IRESs have varying degrees of conservation in sequence, there is greater conservation of secondary and tertiary structure, as well as the translation factors they employ. This allows broad categorization of viral IRES elements (Justine and Franck, 2018). The search for commonalities among cellular IRESs has been less successful due to their diversity in length, secondary structure, and activity in different cellular contexts. To date,

descriptions of cellular IRESs have focused on translation factor and ITAF requirements for individual IRESs, while broad characterization has remained elusive. To gain a deeper understanding of cellular IRESs, more comprehensive approaches are needed to reveal common regulatory factors and signals. The work described in this dissertation focused on developing a technique to broadly identify IRES-interacting regulatory factors and characterize their role in regulation of IRES translation.

While many IRESs have a clear "purpose" in which the stimulus for translation matches the phenotype of expression (e.g. hypoxia: HIF1α, VEGF; apoptosis: c-myc, XIAP, BCL-2), for others the role that IRES-mediated translation plays is less clear. For these genes, it is thought that the IRES exists to ensure expression when bulk translation is otherwise repressed. For example, the voltage-gated potassium channel Kv1.4 is important for the electrophysiological properties of cardiac cells, and even small variations in expression may perturb cardiac function. This informs a model in which IRES-mediated translation serves to maintain Kv1.4 expression and cellular hemostasis. The Kv1.4 IRES was originally identified based on the extensive and highly structured 5'NCR which harbors many upstream AUGs. Each of these features is generally inhibitory to scanning-dependent translation, and yet expression in certain cell types remains. Notably, extensive 5'NCRs and upstream AUGs are observed for several other Kv ion channels, although it has not yet been determined whether functional IRES elements are present. It may be that translational control by internal ribosome entry is important not only for Kv1.4 but may represent a mechanism more broadly used for regulation of the extended Kv family of genes.

The mechanism of IRES-mediated translation is typically associated with the internal recruitment of trans acting factors, both canonical translation initiation factors and

non-canonical factors. These non-canonical factors are thought to compensate for the processes otherwise driven by recognition of the capped 5'-terminus of the mRNA. To understand regulation of expression of these mRNAs, it is necessary to define the trans-activities which drive internal translation initiation. By identifying the composition of the mRNP formed by an IRES element, we may begin to understand the mechanistic requirements for assembling an initiation complex at the translation start site. By investigating the IRES of Kv1.4, we gain insight into the homeostatic regulation of IRES-containing genes and potentially unlock a subtype of post-transcriptional regulation.

A number of *in vitro* and *in vivo* approaches have been employed to identify RNA-binding proteins including genetic, biochemical, and microscopic assays (Koh and Wickens, 2014; Tacheny et al., 2013; Zielinski et al., 2006). Recently, developments in high throughput technologies, such as RNA affinity purification combined with quantitative mass spectrometry and protein microarrays, have significantly accelerated the analysis of RNP complexes (Sutandy et al., 2016). Perhaps the most widely used method for exploring RNA-protein interactions uses tagged RNA, where RNP complexes are formed *in vitro* or *in vivo*, and RNA is immobilized to a chromatographic matrix. Non-specific protein interactions are removed by extensive washing and RNP complexes are eluted for mass spectrometry analysis. Despite technical optimization, isolation of low-abundance proteins that specifically interact with an RNA of interest is often confounded by complex protein mixtures containing highly abundant proteins that bind non-specifically to RNA. Another major caveat of this approach is that RNA-protein interactions in cells may be highly dynamic, undergoing extensive remodeling. The transient nature of some RNP complexes may not be amenable to purification by chromatography. Use of a genetic approach can

circumvent some of these caveats by screening a cDNA library. In this method, RNA-protein interactions are queried incrementally and selected based on gene activation where even transient interactions can be detected. Use of this approach also has limitations, most importantly the use of fusion proteins and fusion RNAs of limited size for interaction. This raises issues about whether the RNA protein interactions detected in yeast are truly representative of interactions as they occur *in vivo*. The shortened RNA sequences used in this approach may present possible binding motifs to RBPs, but greater RNA superstructures critical for IRES function are not represented.

In this study, we have identified and analyzed the proteins that bind to the complex, highly structured, 1.2 kb IRES of Kv1.4. In **Chapter 2**, we describe a parallel approach of identifying proteins interacting with the Kv1.4 IRES, by both a yeast 3-hybrid screen and by biochemical purification. By using these approaches in parallel, we were able to gain a more comprehensive identification of RNA binding proteins for the Kv1.4 IRES and potentially uncover novel functional interactions. However, these screens only select for IRES binding proteins with no consideration for possible functional roles. In **Chapter 3**, we determined the effect of select interacting proteins (hnRNP K, PCBP2, DHX15, and ANXA2) on IRES mediated translation of Kv1.4 to identify novel IRES trans-acting factors.

By yeast 3-hybrid screening of iterative subsequences of the Kv1.4 5'NCR, stringent chromatographic purification, and comparative quantitative mass spectrometry, we developed a comprehensive list of putative interacting proteins. Grouping of this list into functional categories using PANTHER classification tools, gene ontology terms, and cross-reference to an mRNA interactome indicated a plurality of these hits were primarily nucleic acid binding proteins. Analysis of this list by gene set enrichment indicated strong

enrichment for RNA-binding proteins, helicases, and translation factors. Cross-reference of this list against previously identified IRES trans-acting factors revealed multiple ITAFs, hinting at shared mechanisms between IRES elements.

Subsequent analysis of select proteins indicates that this two-pronged approach correctly identified IRES binding proteins, and that these interactions have a functional consequence to IRES-mediated translation of Kv1.4. Primarily, we showed that Annexin A2 (ANXA2) is a previously unknown regulatory factor for Kv1.4 IRES-mediated expression. We showed that alterations in ANXA2 levels in HeLa and AC16 cells modulated translation of a Kv1,4 5'NCR dicistronic reporter construct. However, the degree to which ANXA2 impacted IRES translation was cell type dependent. Specifically, overexpression of ANXA2 in HeLa cells stimulated IRES-mediated translation and siRNA knockdown was inhibitory; whereas in AC16 cells, neither treatment had an effect. Only with more efficient knockdown achieved by shRNA transduction was IRES-mediated translation inhibited in AC16 cells. Similarly, overexpression of hnRNP K or DHX15 yielded pronounced enhancement of IRES translation in HeLa cells, but not in AC16 cells. However, IRES translation was inhibited in AC16 cells which were transiently knocked-down for hnRNP K.

These data suggest that expression levels of these putative ITAFs are sufficient to drive maximal IRES-mediated translation of Kv1.4 in AC16 cells, but in HeLa cells where expression levels are lower, alterations in abundance of these factors may tune IRES-mediated translation. Our results fit with a model where the Kv1.4 IRES serves to maintain gene expression in cardiomyocytes due to maximal translation by highly abundant ITAFs. Fluctuations in ITAF abundance would have little impact on expression of Kv1.4, thereby maintaining constitutive expression.

hnRNPs are highly abundant proteins and are frequently described as interacting with IRES elements. For many such interactions, no functional consequences have been observed, leading investigators to speculate that these protein interactions may be artifacts of RNA-affinity purification or may bind IRES RNA as placeholders to maintain stability (Spriggs et al., 2005). Work involving hnRNP K has shown a similar lack of functionality for the c-myc IRES; however, with cooperation of other ITAFs, a functional role was revealed (Evans et al., 2003). Here we showed a less complex role for hnRNP K, where overexpression of hnRNP K alone is sufficient to drive IRES-mediated translation in HeLa cells. This may be due to the expression of necessary cooperating factors *in vivo*, whereas experiments involving translation of the c-myc IRES were carried out *in vitro* with cell-free extracts. Although previously discounted, our *in vitro* translation assays using Kv1.4 and recombinant hnRNP K also did not reveal a stimulation of translation. Confusing to our analysis was our inability to directly demonstrate hnRNP K binding to the Kv1.4 IRES *in vitro*. Notably, hnRNP K was identified through yeast 3-hybrid and comparative chromatography screens and not in the more selective tandem affinity purification. It may be that the interaction of hnRNP K with the RNA is low affinity or transient. However, this transient interaction would still apparently be sufficient to drive IRES-mediated translation. A final point of interest involving hnRNP K was the identification of an interacting protein G3BP1 (GTPase Activating Protein (SH3 Domain) Binding Protein 1). G3BP1 has been shown to bind hnRNP K through the SH3-interaction domain (present in hnRNP K and not other poly(rC)-binding proteins), an interaction which inhibits translation by targeting bound mRNA to stress granules (Naarmann et al., 2010). G3BP1 targeting to stress granules has been implicated in the regulation of several IRES

containing mRNAs (Fung et al., 2013; Naarmann et al., 2010). The simultaneous purification of G3BP1 with hnRNP K in comparative purification could represent components of a greater functional complex. This leads us to speculate that hnRNP K may regulate Kv1.4 mRNA by a transient interaction, with possible targeting to stress granules through the action of G3BP1.

Screens described in **Chapter 2** identified a number of helicase proteins as Kv1.4 IRES-interacting proteins, including the DNA helicase G3BP1. A number of RNA helicases were also identified and their relevance to IRES mediated translation was immediately considered. Critical to the scanning mechanism of initiation is the DEAD-box RNA helicase eIF4A which unwinds RNA structure as the 48S ribosome scans the 5'NCR (Spriggs et al., 2010). This protein was also identified as interacting with Kv1.4 IRES, along with a number of other RNA helicases including DHX15. A mechanism for partial unwinding of secondary structures for internal ribosome binding to structured 5'NCRs has been proposed, and this activity has been attributed to helicases that stimulate IRES translation (Halaby et al., 2015). We show in **Chapter 3** that DHX15 is able to bind to Kv1.4 IRES RNA *in vitro* and is able to activate IRES mediated translation in HeLa cells. Although intriguing on its face, further elucidation is needed on the DHX15 binding site and whether the enzymatic activity of this protein is required for IRES stimulation. Additionally, the role that other RNA helicases may play on Kv1.4 translation must be parsed. It is possible that helicases are able to activate translation from specific IRESs or are functionally interchangeable and capable of broad activation of IRES translation.

The binding of ANXA2, a calcium-dependent phospholipid binding protein, to the IRES is not entirely unprecedented (Filipenko et al., 2004; Hollås et al., 2006). Previous

ANXA2-RNA interactions were shown to involve structured RNA in the 3'NCR and are thought to direct localization of cognate mRNA to cytoskeleton bound polysomes, much like the calcium dependent protein localization mechanism previously ascribed to ANXA2. This leads us to consider if ANXA2 binding to the 5'NCR directs localization of the mRNA to the rough endoplasmic reticulum thereby promoting its translation. ANXA2 may then serve an additional role in targeting nascent Kv1.4 channels to the cell membrane, as was demonstrated for another ion channel, Nav1.8 (Swanwick et al., 2010). The involvement of ANXA2 in IRES-mediated translation of Kv1.4 invites speculation about ANXA2 serving as a principal regulator for Kv1.4 expression in cardiac tissues. Previous description of IRES trans-activity of ANXA2 to the p53 IRES was shown to be calcium dependent as well, leading us to speculate on the role that Ca^{2+} may play in Kv1.4 IRES regulation (Hollås et al., 2006; Sharathchandra et al., 2012). Further experiments investigating Ca^{2+} effects and ER stress responses will hopefully shed light onto the mechanism for ANXA2 regulation of Kv1.4 IRES.

Although only a small number of the putative ITAFs identified in our screens could be validated, a great many of the identified proteins invite speculation as to their role in IRES-mediated translation. Some of these proteins are multifunctional nuclear proteins found in several complexes associated with different RNA processes (SFPQ/PSF, NONO/p54nrb) or even with IRES activities (SRp20, DAP5 (eIF4G2), hnRNP A1, YBX1, PA2G4, AUF1, SYNCRIP (aka hnRNP Q)) (Cathcart et al., 2013; Cobbold et al., 2008; Fitzgerald and Semler, 2011; Jo et al., 2008; Liberman et al., 2015; Pacheco et al., 2008). Others are uncharacterized RNA binding proteins and helicases not previously associated with cellular or viral IRESs. Interestingly, several proteins involved in canonical scanning-

dependent translation were identified. These include translation initiation factors (eIF1A, eIF2A, eIF2B, eIF3, eIF4A, eIF4G1, eIF4G2, eIF5, and eIF5B) and ribosomal subunits from both the 40S and 60S ribosome, with known canonical as well as non-canonical functions (Bordeleau et al, 2006; Spriggs et al, 2009; Cuchalova et al, 2010; Campagnoli et al, 2008; Chen & Kastan, 2010). Ribosomal proteins, which would be expected to be associated with translationally competent RNA, may serve non-canonical functions in the context of an IRES. In particular RPS25 has been shown to be dispensable for cap-dependent translation but required for CrPV IGR IRES and HCV IRES-mediated translation. Additionally the DEAD-box helicase eIF4A has been shown to have a disproportionate effect on IRES-mediated translation (CVB3, FMDV, c-myc, n-myc)(Spriggs et al., 2009; Tsai et al., 2011).

It is possible that IRES motifs are not recognized by one or two highly specific ITAFs but rather an assortment of RNA regulatory factors with moderate specificity that collectively function to promote ribosome association and translation. Results from RBP screens reveal a wide array of putative interactions which may collectively coordinate internal initiation. Additionally, results from cell culture assays show that alteration of IRES activity can be cell-type dependent. This suggests that expression of these components in some cells (HeLa) may be at judicious levels, suitable for modulation both up and down, whereas other cell types may be much better primed for translation from a given IRES. This may be a lesson when studying regulation of translation: the most relevant cell type may actually not be preferable, when probing for functional consequences. Together, results from our experiments reveal important factors and mechanistic insight into IRES-mediated translation of Kv1.4. By analyzing the proteins that bind its complex and highly structured

1.2 kb IRES, we may better understand the evolutionary pressures that allow for these RNA elements to persist.

References

Adamski, F.M., Donly, B.C., and Tate, W.P. (1993). Competition between frameshifting, termination and suppression at the frameshift site in the Escherichia coli release factor-2 mRNA. Nucleic Acids Res. *21*, 5074–5078.

Andreev, D.E., Dmitriev, S.E., Terenin, I.M., Prassolov, V.S., Merrick, W.C., and Shatsky, I.N. (2009). Differential contribution of the m7G-cap to the 5' end-dependent translation initiation of mammalian mRNAs. Nucleic Acids Res. *37*, 6135–6147.

Andreucci, E., Bianchini, F., Biagioni, A., Rosso, M.D., Papucci, L., Schiavone, N., and Magnelli, L. (2017). Roles of different IRES-dependent FGF2 isoforms in the acquisition of the major aggressive features of human metastatic melanoma. J. Mol. Med. *95*, 97–108.

Barbosa, C., Peixeiro, I., and Romão, L. (2013). Gene expression regulation by upstream open reading frames and human disease. PLoS Genet. *9*.

Bastide, A., Karaa, Z., Bornes, S., Hieblot, C., Lacazette, E., Prats, H., and Touriol, C. (2008). An upstream open reading frame within an IRES controls expression of a specific VEGF-A isoform. Nucleic Acids Res. *36*, 2434–2445.

Bellodi, C., Kopmar, N., and Ruggero, D. (2010). Deregulation of oncogene-induced senescence and p53 translational control in X-linked dyskeratosis congenita. EMBO J. *29*, 1865–1876.

Bernstein, D.S., Buter, N., Stumpf, C., and Wickens, M. (2002). Analyzing mRNA–protein complexes using a yeast three-hybrid system. Methods *26*, 123–141.

Bernstein, J., Sella, O., Le, S.Y., and Elroy-Stein, O. (1997). PDGF2/c-sis mRNA leader contains a differentiation-linked internal ribosomal entry site (D-IRES). J. Biol. Chem. *272*, 9356–9362.

Bert, A.G., Grépin, R., Vadas, M.A., and Goodall, G.J. (2006). Assessing IRES activity in the HIF-1α and other cellular 5′ UTRs. RNA *12*, 1074–1083.

Berthelot, K., Muldoon, M., Rajkowitsch, L., Hughes, J., and McCarthy, J.E.G. (2004). Dynamics and processivity of 40S ribosome scanning on mRNA in yeast. Mol. Microbiol. *51*, 987–1001.

Bezanilla, F., and Perozo, E. (2003). The voltage sensor and the gate in ion channels. In Advances in Protein Chemistry, (Academic Press), pp. 211–241.

Blyn, L.B., Towner, J.S., Semler, B.L., and Ehrenfeld, E. (1997). Requirement of poly (rC) binding protein 2 for translation of poliovirus RNA. J. Virol. *71*, 6243.

Bomsztyk, K., Denisenko, O., and Ostrowski, J. (2004). hnRNP K: One protein multiple processes. BioEssays *26*, 629–638.

Borman, A., Howell, M.T., Patton, J.G., and Jackson, R.J. (1993). The involvement of a spliceosome component in internal initiation of human rhinovirus RNA translation. J. Gen. Virol. *74 (Pt 9)*, 1775–1788.

Buckanovich, R.J., Posner, J.B., and Darnell, R.B. (1993). Nova, the paraneoplastic Ri antigen, is homologous to an RNA-binding protein and is specifically expressed in the developing motor system. Neuron *11*, 657–672.

Bushell, M., Poncet, D., Marissen, W.E., Flotow, H., Lloyd, R.E., Clemens, M.J., and Morley, S.J. (2000). Cleavage of polypeptide chain initiation factor eIF4GI during apoptosis in lymphoma cells: characterisation of an internal fragment generated by caspase-3-mediated cleavage. Cell Death Differ. *7*, 628–636.

Candeias, M.M., Powell, D.J., Roubalova, E., Apcher, S., Bourougaa, K., Vojtesek, B., Bruzzoni-Giovanelli, H., and Fåhraeus, R. (2006). Expression of p53 and p53/47 are controlled by alternative mechanisms of messenger RNA translation initiation. Oncogene *25*, 6936–6947.

Castello, A., Fischer, B., Eichelbaum, K., Horos, R., Beckmann, B.M., Strein, C., Davey, N.E., Humphreys, D.T., Preiss, T., Steinmetz, L.M., et al. (2012). Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. Cell *149*, 1393–1406.

Castello, A., Horos, R., Strein, C., Fischer, B., Eichelbaum, K., Steinmetz, L.M., Krijgsveld, J., and Hentze, M.W. (2013). System-wide identification of RNA-binding proteins by interactome capture. Nat. Protoc. *8*, 491–500.

Cathcart, A.L., Rozovics, J.M., and Semler, B.L. (2013). Cellular mRNA decay protein AUF1 negatively regulates enterovirus and human rhinovirus infections. J. Virol. *87*, 10423–10434.

Chakrabarti, A., and Maitra, U. (1991). Function of eukaryotic initiation factor 5 in the formation of an 80 S ribosomal polypeptide chain initiation complex. J. Biol. Chem. *266*, 14039–14045.

Chen, C.Y., and Sarnow, P. (1995). Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs. Science *268*, 415–417.

Cobbold, L.C., Spriggs, K.A., Haines, S.J., Dobbyn, H.C., Hayes, C., Moor, C.H. de, Lilley, K.S., Bushell, M., and Willis, A.E. (2008). Identification of Internal Ribosome Entry Segment (IRES)-trans-acting factors for the Myc family of IRESs. Mol. Cell. Biol. *28*, 40–49.

Dai, N., Rapley, J., Angel, M., Yanik, M.F., Blower, M.D., and Avruch, J. (2011). mTOR phosphorylates IMP2 to promote IGF2 mRNA translation by internal ribosomal entry. Genes Dev. *25*, 1159–1172.

Danthinne, X., Seurinck, J., Meulewaeter, F., Montagu, M.V., and Cornelissen, M. (1993). The 3' untranslated region of satellite tobacco necrosis virus RNA stimulates translation in vitro. Mol. Cell. Biol. *13*, 3340–3349.

Dever, T.E., and Green, R. (2012). The elongation, termination, and recycling phases of translation in eukaryotes. Cold Spring Harb. Perspect. Biol. *4*.

Dinman, J.D., and Berry, M.J. (2007). Regulation of termination and recoding. In Translational Control in Biology and Medicine (2007, Volume 48), pp. 625–654.

Dobbyn, H.C., Hill, K., Hamilton, T.L., Spriggs, K.A., Pickering, B.M., Coldwell, M.J., de Moor, C.H., Bushell, M., and Willis, A.E. (2008). Regulation of BAG-1 IRES-mediated translation following chemotoxic stress. Oncogene *27*, 1167–1174.

Dominguez, D.I., Ryabova, L.A., Pooggin, M.M., Schmidt-Puchta, W., Fütterer, J., and Hohn, T. (1998). Ribosome shunting in cauliflower mosaic virus. Identification of an essential and sufficient structural element. J. Biol. Chem. *273*, 3669–3678.

Doyle, D.A., Morais Cabral, J., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T., and MacKinnon, R. (1998). The structure of the potassium channel: molecular basis of K+ conduction and selectivity. Science *280*, 69–77.

Dreyfuss, G., Matunis, M.J., Pinol-Roma, S., and Burd, C.G. (1993). hnRNP Proteins and the Biogenesis of mRNA. Annu. Rev. Biochem. *62*, 289–321.

Duke, G.M., Hoffman, M.A., and Palmenberg, A.C. (1992). Sequence and structural elements that contribute to efficient encephalomyocarditis virus RNA translation. J. Virol. *66*, 1602–1609.

Ertel, K.J., Brunner, J.E., and Semler, B.L. (2010). Mechanistic consequences of hnRNP C binding to both RNA termini of poliovirus negative-strand RNA intermediates. J. Virol. *84*, 4229–4242.

Evans, J.R., Mitchell, S.A., Spriggs, K.A., Ostrowski, J., Bomsztyk, K., Ostarek, D., and Willis, A.E. (2003). Members of the poly (rC) binding protein family stimulate the activity of the c-myc internal ribosome entry segment in vitro and in vivo. Oncogene *22*, 8012–8020.

Feigenblum, D., and Schneider, R.J. (1996). Cap-binding protein (eukaryotic initiation factor 4E) and 4E-inactivating protein BP-1 independently regulate cap-dependent translation. Mol. Cell. Biol. *16*, 5450–5457.

Fernandez, J., Yaman, I., Huang, C., Liu, H., Lopez, A.B., Komar, A.A., Caprara, M.G., Merrick, W.C., Snider, M.D., Kaufman, R.J., et al. (2005). Ribosome stalling regulates IRES-mediated translation in eukaryotes, a parallel to prokaryotic attenuation. Mol. Cell *17*, 405–416.

Fields, S., and Sternglanz, R. (1994). The two-hybrid system: an assay for protein-protein interactions. Trends Genet. TIG *10*, 286–292.

Filipenko, N.R., MacLeod, T.J., Yoon, C.-S., and Waisman, D.M. (2004). Annexin A2 is a novel RNA-binding protein. J. Biol. Chem. *279*, 8723–8731.

Fitzgerald, K.D., and Semler, B.L. (2011). Re-localization of cellular protein SRp20 during poliovirus infection: bridging a viral IRES to the host cell translation apparatus. PLoS Pathog. *7*.

Flather, D., and Semler, B.L. (2015). Picornaviruses and nuclear functions: targeting a cellular compartment distinct from the replication site of a positive-strand RNA virus. Front. Microbiol. *6*, 594.

Fouraux, M.A., Kolkman, M.J.M., Van der Heijden, A., De Jong, A.S., Van Venrooij, W.J., and Pruijn, G.J.M. (2002). The human La (SS-B) autoantigen interacts with DDX15/hPrp43, a putative DEAH-box RNA helicase. RNA N. Y. N *8*, 1428–1443.

Frolova, L., Le Goff, X., Zhouravleva, G., Davydova, E., Philippe, M., and Kisselev, L. (1996). Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent guanosine triphosphatase. RNA N. Y. N *2*, 334–341.

Fung, G., Ng, C.S., Zhang, J., Shi, J., Wong, J., Piesik, P., Han, L., Chu, F., Jagdeo, J., Jan, E., et al. (2013). Production of a dominant-negative fragment due to G3BP1 cleavage contributes to the disruption of mitochondria-associated protective stress granules during CVB3 infection. PLOS ONE *8*, e79546.

Fütterer, J., Kiss-László, Z., and Hohn, T. (1993). Nonlinear ribosome migration on cauliflower mosaic virus 35S RNA. Cell *73*, 789–802.

Fütterer, J., Rothnie, H.M., Hohn, T., and Potrykus, I. (1997). Rice tungro bacilliform virus open reading frames II and III are translated from polycistronic pregenomic RNA by leaky scanning. J. Virol. *71*, 7984–7989.

Gaccioli, F., Huang, C.C., Wang, C., Bevilacqua, E., Franchi-Gazzola, R., Gazzola, G.C., Bussolati, O., Snider, M.D., and Hatzoglou, M. (2006). Amino acid starvation induces the SNAT2 neutral amino acid transporter by a mechanism that involves eukaryotic initiation factor 2alpha phosphorylation and cap-independent translation. J. Biol. Chem. *281*, 17929–17940.

Gamarnik, A.V., and Andino, R. (1997). Two functional complexes formed by KH domain containing proteins with the 5'noncoding region of poliovirus RNA. RNA *3*, 882.

Gietz, D., St Jean, A., Woods, R.A., and Schiestl, R.H. (1992). Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. *20*, 1425.

Gingras, A.-C., Raught, B., and Sonenberg, N. (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. *68*, 913–963.

Gingras, A.C., Raught, B., Gygi, S.P., Niedzwiecka, A., Miron, M., Burley, S.K., Polakiewicz, R.D., Wyslouch-Cieszynska, A., Aebersold, R., and Sonenberg, N. (2001). Hierarchical phosphorylation of the translation inhibitor 4E-BP1. Genes Dev. *15*, 2852–2864.

Graauw, M. de, Tijdens, I., Smeets, M.B., Hensbergen, P.J., Deelder, A.M., and Water, B. van de (2008). Annexin A2 phosphorylation mediates cell scattering and branching morphogenesis via cofilin activation. Mol. Cell. Biol. *28*, 1029–1040.

Graber, T.E., Baird, S.D., Kao, P.N., Mathews, M.B., and Holcik, M. (2010). NF45 functions as an IRES trans-acting factor that is required for translation of cIAP1 during the unfolded protein response. Cell Death Differ. *17*, 719–729.

Gu, L., Zhu, N., Zhang, H., Durden, D.L., Feng, Y., and Zhou, M. (2009). Regulation of XIAP translation and induction by MDM2 following irradiation. Cancer Cell *15*, 363–375.

Guo, J., Hui, D.J., Merrick, W.C., and Sen, G.C. (2000). A new pathway of translational regulation mediated by eukaryotic initiation factor 3. EMBO J. *19*, 6891–6899.

Guo, W., Xu, H., London, B., and Nerbonne, J.M. (1999). Molecular basis of transient outward K+ current diversity in mouse ventricular myocytes. J. Physiol. *521*, 587–599.

Halaby, M.-J., Harris, B.R.E., Miskimins, W.K., Cleary, M.P., and Yang, D.-Q. (2015). Deregulation of internal ribosome entry site-mediated p53 translation in cancer cells with defective p53 response to DNA damage. Mol. Cell. Biol. *35*, 4006–4017.

Haller, A.A., and Semler, B.L. (1992). Linker scanning mutagenesis of the internal ribosome entry site of poliovirus RNA. J. Virol. *66*, 5075–5086.

Hayashi, N., Sasaki, S., Takahashi, H., Yamashita, Y., Naito, S., and Onouchi, H. (2017). Identification of Arabidopsis thaliana upstream open reading frames encoding peptide sequences that cause ribosomal arrest. Nucleic Acids Res. *45*, 8844–8858.

Hellen, C.U., and Sarnow, P. (2001). Internal ribosome entry sites in eukaryotic mRNA molecules. Genes Dev. *15*, 1593–1612.

Hellen, C.U., Witherell, G.W., Schmid, M., Shin, S.H., Pestova, T.V., Gil, A., and Wimmer, E. (1993). A cytoplasmic 57-kDa protein that is required for translation of picornavirus RNA by internal ribosomal entry is identical to the nuclear pyrimidine tract-binding protein. Proc. Natl. Acad. Sci. U. S. A. *90*, 7642–7646.

Hershey, J.W.B., Sonenberg, N., and Mathews, M.B. (2012). Principles of Translational Control: An Overview. Cold Spring Harb. Perspect. Biol. *4*.

Herzog, E., Guilley, H., and Fritsch, C. (1995). Translation of the second gene of peanut clump virus RNA 2 occurs by leaky scanning in vitro. Virology *208*, 215–225.

Holcik, M., and Korneluk, R.G. (2000). Functional characterization of the X-linked inhibitor of apoptosis (XIAP) internal ribosome entry site element: role of La autoantigen in XIAP translation. Mol. Cell. Biol. *20*, 4648–4657.

Holcík, M., Gordon, B.W., and Korneluk, R.G. (2003). The internal ribosome entry site-mediated translation of antiapoptotic protein XIAP is modulated by the heterogeneous nuclear ribonucleoproteins C1 and C2. Mol. Cell. Biol. *23*, 280–288.

Hollås, H., Aukrust, I., Grimmer, S., Strand, E., Flatmark, T., and Vedeler, A. (2006). Annexin A2 recognises a specific region in the 3′-UTR of its cognate messenger RNA. Biochim. Biophys. Acta BBA - Mol. Cell Res. *1763*, 1325–1334.

Holmes, B., Lee, J., Landon, K.A., Benavides-Serrato, A., Bashir, T., Jung, M.E., Lichtenstein, A., and Gera, J. (2016). Mechanistic Target of Rapamycin (mTOR) inhibition synergizes with reduced Internal Ribosome Entry Site (IRES)-mediated translation of Cyclin D1 and c-MYC mRNAs to treat glioblastoma. J. Biol. Chem. *291*, 14146–14159.

Hronová, V., Mohammad, M.P., Wagner, S., Pánek, J., Gunišová, S., Zeman, J., Poncová, K., and Valášek, L.S. (2017). Does eIF3 promote reinitiation after translation of short upstream ORFs also in mammalian cells? RNA Biol. *14*, 1660–1667.

Huang, H., Yoon, H., Hannig, E.M., and Donahue, T.F. (1997). GTP hydrolysis controls stringent selection of the AUG start codon during translation initiation in Saccharomyces cerevisiae. Genes Dev. *11*, 2396–2413.

Hunt, S.L., and Jackson, R.J. (1999). Polypyrimidine-tract binding protein (PTB) is necessary, but not sufficient, for efficient internal initiation of translation of human rhinovirus-2 RNA. RNA *5*, 344–359.

Ingolia, N.T., Lareau, L.F., and Weissman, J.S. (2011). Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. Cell *147*, 789–802.

Jackson, R.J. (2005). Alternative mechanisms of initiating translation of mammalian mRNAs. Biochem. Soc. Trans. *33*, 1231–1241.

Jackson, R.J. (2013). The current status of vertebrate cellular mRNA IRESs. Cold Spring Harb. Perspect. Biol. *5*, a011569.

Jackson, R.J., and Kaminski, A. (1995). Internal initiation of translation in eukaryotes: the picornavirus paradigm and beyond. RNA N. Y. N *1*, 985–1000.

Jackson, R.J., and Standart, N. (1990). Do the poly(A) tail and 3' untranslated region control mRNA translation? Cell *62*, 15–24.

Jackson, R.J., Howell, M.T., and Kaminski, A. (1990). The novel mechanism of initiation of picornavirus RNA translation. Trends Biochem. Sci. *15*, 477–483.

Jang, S.K., and Wimmer, E. (1990). Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA-binding protein. Genes Dev. *4*, 1560–1572.

Jang, G.M., Leong, L.E.-C., Hoang, L.T., Wang, P.H., Gutman, G.A., and Semler, B.L. (2004). Structurally distinct elements mediate internal ribosome entry within the 5'-noncoding region of a voltage-gated potassium channel mRNA. J. Biol. Chem. *279*, 47419–47430.

Jang, S.K., Kräusslich, H.G., Nicklin, M.J., Duke, G.M., Palmenberg, A.C., and Wimmer, E. (1988). A segment of the 5' nontranslated region of Encephalomyocarditis Virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. *62*, 2636–2643.

Janich, P., Arpat, A.B., Castelo-Szekely, V., Lopes, M., and Gatfield, D. (2015). Ribosome profiling reveals the rhythmic liver translatome and circadian clock regulation by upstream open reading frames. Genome Res. *25*, 1848–1859.

Jo, O.D., Martin, J., Bernath, A., Masri, J., Lichtenstein, A., and Gera, J. (2008). Heterogeneous nuclear ribonucleoprotein A1 regulates cyclin D1 and c-myc internal ribosome entry site function through Akt signaling. J. Biol. Chem. *283*, 23274–23287.

Jopling, C.L., and Willis, A.E. (2001). N-myc translation is initiated via an internal ribosome entry segment that displays enhanced activity in neuronal cells. Oncogene *20*, 2664–2670.

Justine, M., and Franck, M. (2018). Viral internal ribosomal entry sites: four classes for one goal. Wiley Interdiscip. Rev. RNA *9*, e1458.

Kahvejian, A., Svitkin, Y.V., Sukarieh, R., M'Boutchou, M.-N., and Sonenberg, N. (2005). Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. Genes Dev. *19*, 104–113.

Kanamori, Y., and Nakashima, N. (2001). A tertiary structure model of the internal ribosome entry site (IRES) for methionine-independent initiation of translation. RNA N. Y. N *7*, 266–274.

Kaspar, R.L., Rychlik, W., White, M.W., Rhoads, R.E., and Morris, D.R. (1990). Simultaneous cytoplasmic redistribution of ribosomal protein L32 mRNA and phosphorylation of eukaryotic initiation factor 4E after mitogenic stimulation of Swiss 3T3 cells. J. Biol. Chem. *265*, 3619–3622.

Khong, A., Matheny, T., Jain, S., Mitchell, S.F., Wheeler, J.R., and Parker, R. (2017). The stress granule transcriptome reveals principles of mRNA accumulation in stress granules. Mol. Cell *68*, 808–820.e5.

Koh, Y.Y., and Wickens, M. (2014). Determining the RNA specificity and targets of RNA-binding proteins using a three-hybrid system. Methods Enzymol. *539*, 163–181.

Koh, D.C., Edelman, G.M., and Mauro, V.P. (2013). Physical evidence supporting a ribosomal shunting mechanism of translation initiation for BACE1 mRNA. Transl. Austin Tex *1*, e24400.

Komar, A.A., and Hatzoglou, M. (2011). Cellular IRES-mediated translation. Cell Cycle *10*, 229–240.

Kondrashov, N., Pusic, A., Stumpf, C.R., Shimizu, K., Hsieh, A.C., Ishijima, J., Shiroishi, T., and Barna, M. (2011). Ribosome-mediated specificity in Hox mRNA translation and vertebrate tissue patterning. Cell *145*, 383–397.

Korostelev, A.A. (2011). Structural aspects of translation termination on the ribosome. RNA *17*, 1409–1421.

Kozak, M. (1979). Migration of 40 S ribosomal subunits on messenger RNA when initiation is perturbed by lowering magnesium or adding drugs. J. Biol. Chem. *254*, 4731–4738.

Kozak, M. (1984). Point mutations close to the AUG initiator codon affect the efficiency of translation of rat preproinsulin in vivo. Nature *308*, 241–246.

Kozak, M. (1987). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. *15*, 8125–8148.

Kozak, M. (1989). The scanning model for translation: an update. J. Cell Biol. *108*, 229–241.

Kozak, M. (2005). A second look at cellular mRNA sequences said to function as internal ribosome entry sites. Nucleic Acids Res. *33*, 6593–6602.

Krishnamoorthy, T., Pavitt, G.D., Zhang, F., Dever, T.E., and Hinnebusch, A.G. (2001). Tight binding of the phosphorylated alpha subunit of initiation factor 2 (eIF2alpha) to the regulatory subunits of guanine nucleotide exchange factor eIF2B is required for inhibition of translation initiation. Mol. Cell. Biol. *21*, 5018–5030.

Kudla, G., Murray, A.W., Tollervey, D., and Plotkin, J.B. (2009). Coding-sequence determinants of gene expression in Escherichia coli. Science *324*, 255–258.

Kuleshov, M.V., Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S.L., Jagodnik, K.M., Lachmann, A., et al. (2016). Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. *44*, W90-97.

Lacerda, R., Menezes, J., and Romão, L. (2017). More than just scanning: the importance of cap-independent mRNA translation initiation for cellular stress response and cancer. Cell. Mol. Life Sci. *74*, 1659–1680.

Lai, M.-C., Lee, Y.-H.W., and Tarn, W.-Y. (2008). The DEAD-box RNA helicase DDX3 associates with export messenger ribonucleoproteins as well as tip-associated protein and participates in translational control. Mol. Biol. Cell *19*, 3847–3858.

Landry, D.M., Hertz, M.I., and Thompson, S.R. (2009). RPS25 is essential for translation initiation by the Dicistroviridae and hepatitis C viral IRESs. Genes Dev. *23*, 2753–2764.

Lebaron, S., Froment, C., Fromont-Racine, M., Rain, J.-C., Monsarrat, B., Caizergues-Ferrer, M., and Henry, Y. (2005). The splicing ATPase prp43p is a component of multiple preribosomal particles. Mol. Cell. Biol. *25*, 9269–9282.

Lee, K.-M., Chen, C.-J., and Shih, S.-R. (2017). Regulation mechanisms of viral IRES-driven translation. Trends Microbiol. *25*, 546–561.

Li, M.Z., and Elledge, S.J. (2007). Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat. Methods *4*, 251–256.

Liberman, N., Gandin, V., Svitkin, Y.V., David, M., Virgili, G., Jaramillo, M., Holcik, M., Nagar, B., Kimchi, A., and Sonenberg, N. (2015). DAP5 associates with eIF2β and eIF4AI to promote Internal Ribosome Entry Site driven translation. Nucleic Acids Res. *43*, 3764–3775.

Lomakin, I.B., Hellen, C.U., and Pestova, T.V. (2000). Physical association of eukaryotic initiation factor 4G (eIF4G) with eIF4A strongly enhances binding of eIF4G to the internal ribosomal entry site of encephalomyocarditis virus and is required for internal initiation of translation. Mol. Cell. Biol. *20*, 6019–6029.

Long, S.B., Campbell, E.B., and Mackinnon, R. (2005). Crystal structure of a mammalian voltage-dependent Shaker family K+ channel. Science *309*, 897–903.

López-Lastra, M., Rivas, A., and Barría, M.I. (2005). Protein synthesis in eukaryotes: the growing biological relevance of cap-independent translation initiation. Biol. Res. *38*, 121–146.

Lu, H., Lu, N., Weng, L., Yuan, B., Liu, Y., and Zhang, Z. (2014). DHX15 senses double-stranded RNA in myeloid dendritic cells. J. Immunol. *193*, 1364–1372.

Macejak, D.G., and Sarnow, P. (1991). Internal initiation of translation mediated by the 5' leader of a cellular mRNA. Nature *353*, 90–94.

Magnuson, B., Ekim, B., and Fingar, D.C. (2012). Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. Biochem. J. *441*, 1–21.

Matsubara, H., Suzuki, J., and Inada, M. (1993). Shaker-related potassium channel, Kv1.4, mRNA regulation in cultured rat heart myocytes and differential expression of Kv1.4 and Kv1.5 genes in myocardial development and hypertrophy. J. Clin. Invest. *92*, 1659–1666.

Matunis, M.J., Michael, W.M., and Dreyfuss, G. (1992). Characterization and primary structure of the poly(C)-binding heterogeneous nuclear ribonucleoprotein complex K protein. Mol. Cell. Biol. *12*, 164–171.

McGivan, J.D., and Pastor-Anglada, M. (1994). Regulatory and molecular aspects of mammalian amino acid transport. Biochem. J. *299*, 321–334.

Mi, H., Huang, X., Muruganujan, A., Tang, H., Mills, C., Kang, D., and Thomas, P.D. (2017). PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. Nucleic Acids Res. *45*, D183–D189.

Michelotti, E.F., Michelotti, G.A., Aronsohn, A.I., and Levens, D. (1996). Heterogeneous nuclear ribonucleoprotein K is a transcription factor. Mol. Cell. Biol. *16*, 2350–2360.

Miller, W.A., Wang, Z., and Treder, K. (2007). The amazing diversity of cap-independent translation elements in the 3'-untranslated regions of plant viral RNAs. Biochem. Soc. Trans. *35*, 1629–1633.

Mitchell, S.A., Brown, E.C., Coldwell, M.J., Jackson, R.J., and Willis, A.E. (2001). Protein factor requirements of the Apaf-1 internal ribosome entry segment: roles of polypyrimidine tract binding protein and upstream of N-ras. Mol. Cell. Biol. *21*, 3364–3374.

Mitchell, S.A., Spriggs, K.A., Coldwell, M.J., Jackson, R.J., and Willis, A.E. (2003). The Apaf-1 internal ribosome entry segment attains the correct structural conformation for function via interactions with PTB and unr. Mol. Cell *11*, 757–771.

Morfoisse, F., Kuchnio, A., Frainay, C., Gomez-Brouchet, A., Delisle, M.-B., Marzi, S., Helfer, A.-C., Hantelys, F., Pujol, F., Guillermet-Guibert, J., et al. (2013). Hypoxia induces VEGF-C expression in metastatic tumor cells via a HIF-1α-independent translation-mediated mechanism. Cell Rep.

Musco, G., Stier, G., Joseph, C., Morelli, M.A.C., Nilges, M., Gibson, T.J., and Pastore, A. (1996). Three-dimensional structure and stability of the KH domain: molecular insights into the Fragile X Syndrome. Cell *85*, 237–245.

Naarmann, I.S., Harnisch, C., Müller-Newen, G., Urlaub, H., Ostareck-Lederer, A., and Ostareck, D.H. (2010). DDX6 recruits translational silenced human reticulocyte 15-lipoxygenase mRNA to RNP granules. RNA *16*, 2189–2204.

Negulescu, D., Leong, L.E., Chandy, K.G., Semler, B.L., and Gutman, G.A. (1998). Translation initiation of a cardiac voltage-gated potassium channel by internal ribosome entry. J. Biol. Chem. *273*, 20109–20113.

Nilius, B., Gerke, V., Prenen, J., Szücs, G., Heinke, S., Weber, K., and Droogmans, G. (1996). Annexin II modulates volume-activated chloride currents in vascular endothelial cells. J. Biol. Chem. *271*, 30631–30636.

Notari, M., Neviani, P., Santhanam, R., Blaser, B.W., Chang, J.-S., Galietta, A., Willis, A.E., Roy, D.C., Caligiuri, M.A., Marcucci, G., et al. (2006). A MAPK/HNRPK pathway controls BCR/ABL oncogenic potential by regulating MYC mRNA translation. Blood *107*, 2507–2516.

Novoa, E.M., and Ribas de Pouplana, L. (2012). Speeding with control: codon usage, tRNAs, and ribosomes. Trends Genet. TIG *28*, 574–581.

Okuse, K., Malik-Hall, M., Baker, M.D., Poon, W.-Y.L., Kong, H., Chao, M.V., and Wood, J.N. (2002). Annexin II light chain regulates sensory neuron-specific sodium channel expression. Nature *417*, 653–656.

Ostareck, D.H., Ostareck-Lederer, A., Wilm, M., Thiele, B.J., Mann, M., and Hentze, M.W. (1997). mRNA silencing in erythroid differentiation: hnRNP K and hnRNP E1 regulate 15-lipoxygenase translation from the 3' end. Cell *89*, 597–606.

Ostareck-lederer, A., Ostareck, D.H., and Hentze, M.W. (1998). Cytoplasmic regulatory functions of the KH-domain proteins hnRNPs K and E1/E2. Trends Biochem. Sci. *23*, 409–411.

Pacheco, A., Reigadas, S., and Martínez-Salas, E. (2008). Riboproteomic analysis of polypeptides interacting with the internal ribosome-entry site element of foot-and-mouth disease viral RNA. Proteomics *8*, 4782–4790.

Pain, V.M. (1996). Initiation of protein synthesis in eukaryotic cells. Eur. J. Biochem. *236*, 747–771.

Park, S., Park, J.M., Kim, S., Kim, J.-A., Shepherd, J.D., Smith-Hicks, C.L., Chowdhury, S., Kaufmann, W., Kuhl, D., Ryazanov, A.G., et al. (2008). Elongation factor 2 and fragile X mental retardation protein control the dynamic translation of Arc/Arg3.1 essential for mGluR-LTD. Neuron *59*, 70–83.

Parsley, T.B., Towner, J.S., Blyn, L.B., Ehrenfeld, E., and Semler, B.L. (1997). Poly (rC) binding protein 2 forms a ternary complex with the 5'-terminal sequences of poliovirus RNA and the viral 3CD proteinase. Rna *3*, 1124.

Paziewska, A., Wyrwicz, L.S., Bujnicki, J.M., Bomsztyk, K., and Ostrowski, J. (2004). Cooperative binding of the hnRNP K three KH domains to mRNA targets. FEBS Lett. *577*, 134–140.

Pelletier, J., and Sonenberg, N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature *334*, 320.

Pestova, T.V., Hellen, C.U., and Shatsky, I.N. (1996). Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. Mol. Cell. Biol. *16*, 6859–6869.

Pestova, T.V., Borukhov, S.I., and Hellen, C.U. (1998). Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. Nature *394*, 854–859.

Pestova, T.V., Kolupaeva, V.G., Lomakin, I.B., Pilipenko, E.V., Shatsky, I.N., Agol, V.I., and Hellen, C.U. (2001). Molecular mechanisms of translation initiation in eukaryotes. Proc. Natl. Acad. Sci. U. S. A. *98*, 7029–7036.

Pilipenko, E.V., Pestova, T.V., Kolupaeva, V.G., Khitrina, E.V., Poperechnaya, A.N., Agol, V.I., and Hellen, C.U. (2000). A cell cycle-dependent protein serves as a template-specific translation initiation factor. Genes Dev. *14*, 2028–2045.

Pisarev, A.V., Skabkin, M.A., Pisareva, V.P., Skabkina, O.V., Rakotondrafara, A.M., Hentze, M.W., Hellen, C.U.T., and Pestova, T.V. (2010). The role of ABCE1 in eukaryotic post-termination ribosomal recycling. Mol. Cell *37*, 196–210.

Pisareva, V.P., Pisarev, A.V., Komar, A.A., Hellen, C.U.T., and Pestova, T.V. (2008). Translation initiation on mammalian mRNAs with structured 5'UTRs requires DExH-box protein DHX29. Cell *135*, 1237–1250.

Poenisch, M., Metz, P., Blankenburg, H., Ruggieri, A., Lee, J.-Y., Rupp, D., Rebhan, I., Diederich, K., Kaderali, L., Domingues, F.S., et al. (2015). Identification of HNRNPK as regulator of Hepatitis C Virus particle production. PLOS Pathog. *11*, e1004573.

Pollard, H.B., and Rojas, E. (1988). Ca2+-activated synexin forms highly selective, voltage-gated Ca2+ channels in phosphatidylserine bilayer membranes. Proc. Natl. Acad. Sci. U. S. A. *85*, 2974–2978.

Poulin, F., Gingras, A.C., Olsen, H., Chevalier, S., and Sonenberg, N. (1998). 4E-BP3, a new member of the eukaryotic initiation factor 4E-binding protein family. J. Biol. Chem. *273*, 14002–14007.

Price, N.T., Mellor, H., Craddock, B.L., Flowers, K.M., Kimball, S.R., Wilmer, T., Jefferson, L.S., and Proud, C.G. (1996). eIF2B, the guanine nucleotide-exchange factor for eukaryotic initiation factor 2. Sequence conservation between the alpha, beta and delta subunits of eIF2B from mammals and yeast. Biochem. J. *318*, 637–643.

Pyronnet, S., Imataka, H., Gingras, A.C., Fukunaga, R., Hunter, T., and Sonenberg, N. (1999). Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E. EMBO J. *18*, 270–279.

Reuter, J.S., and Mathews, D.H. (2010). RNAstructure: software for RNA secondary structure prediction and analysis. BMC Bioinformatics *11*, 129.

Rhoads, R.E. (1999). Signal transduction pathways that regulate eukaryotic protein synthesis. J. Biol. Chem. *274*, 30337–30340.

Robertson, M.E., Seamons, R.A., and Belsham, G.J. (1999). A selection system for functional internal ribosome entry site (IRES) elements: analysis of the requirement for a conserved GNRA tetraloop in the encephalomyocarditis virus IRES. RNA N. Y. N *5*, 1167–1179.

Rogers, G.W., Edelman, G.M., and Mauro, V.P. (2004). Differential utilization of upstream AUGs in the beta-secretase mRNA suggests that a shunting mechanism regulates translation. Proc. Natl. Acad. Sci. U. S. A. *101*, 2794–2799.

Ruppersberg, J.P., Frank, R., Pongs, O., and Stocker, M. (1991). Cloned neuronal IK(A) channels reopen during recovery from inactivation. Nature *353*, 657–660.

Sasaki, J., and Nakashima, N. (2000). Methionine-independent initiation of translation in the capsid protein of an insect RNA virus. Proc. Natl. Acad. Sci. U. S. A. *97*, 1512–1515.

Scheper, G.C., van Kollenburg, B., Hu, J., Luo, Y., Goss, D.J., and Proud, C.G. (2002). Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. J. Biol. Chem. *277*, 3303–3309.

Semler, B.L., and Waterman, M.L. (2008). IRES-mediated pathways to polysomes: nuclear versus cytoplasmic routes. Trends Microbiol. *16*, 1–5.

SenGupta, D.J., Zhang, B., Kraemer, B., Pochart, P., Fields, S., and Wickens, M. (1996). A three-hybrid system to detect RNA-protein interactions in vivo. Proc. Natl. Acad. Sci. *93*, 8496.

Sharathchandra, A., Lal, R., Khan, D., and Das, S. (2012). Annexin A2 and PSF proteins interact with p53 IRES and regulate translation of p53 mRNA. RNA Biol. *9*, 1429–1439.

Singh, L.P., Arorr, A.R., and Wahba, A.J. (1994). Phosphorylation of the guanine nucleotide exchange factor and eukaryotic initiation factor 2 by casein kinase II regulates guanine nucleotide binding and GDP/GTP exchange. Biochemistry (Mosc.) *33*, 9152–9157.

Siomi, H., Choi, M., Siomi, M.C., Nussbaum, R.L., and Dreyfuss, G. (1994). Essential role for KH domains in RNA binding: Impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. Cell *77*, 33–39.

Sivan, G., Aviner, R., and Elroy-Stein, O. (2011). Mitotic modulation of translation elongation factor 1 leads to hindered tRNA delivery to ribosomes. J. Biol. Chem. *286*, 27927–27935.

Somarajan, S.R., Al-Asadi, F., Ramasamy, K., Pandranki, L., Baseman, J.B., and Kannan, T.R. (2014). Annexin A2 mediates Mycoplasma pneumoniae community-acquired respiratory distress syndrome toxin binding to eukaryotic cells. mBio *5*.

Soto-Rifo, R., Rubilar, P.S., Limousin, T., de Breyne, S., Décimo, D., and Ohlmann, T. (2012). DEAD-box protein DDX3 associates with eIF4F to promote translation of selected mRNAs. EMBO J. *31*, 3745–3756.

Spriggs, K.A., Bushell, M., Mitchell, S.A., and Willis, A.E. (2005). Internal ribosome entry segment-mediated translation during apoptosis: the role of IRES-trans-acting factors. Cell Death Differ. *12*, 585–591.

Spriggs, K.A., Cobbold, L.C., Jopling, C.L., Cooper, R.E., Wilson, L.A., Stoneley, M., Coldwell, M.J., Poncet, D., Shen, Y.-C., Morley, S.J., et al. (2009). Canonical initiation factor requirements of the Myc family of internal ribosome entry segments. Mol. Cell. Biol. *29*, 1565–1574.

Spriggs, K.A., Bushell, M., and Willis, A.E. (2010). Translational regulation of gene expression during conditions of cell stress. Mol. Cell *40*, 228–237.

Stuhmer, W., Ruppersberg, J.P., Schroter, K.H., Sakmann, B., Stocker, M., Giese, K.P., Perschke, A., Baumann, A., and Pongs, O. (1989). Molecular basis of functional diversity of voltage-gated potassium channels in mammalian brain. EMBO J. *8*, 3235–3244.

Sturgill, T.W., and Wu, J. (1991). Recent progress in characterization of protein kinase cascades for phosphorylation of ribosomal protein S6. Biochim. Biophys. Acta *1092*, 350–357.

Su, X., Lin, Z., and Lin, H. (2013). The biosynthesis and biological function of diphthamide. Crit. Rev. Biochem. Mol. Biol. *48*, 515–521.

Sutandy, F.X.R., Hsiao, F.S.-H., and Chen, C.-S. (2016). High throughput platform to explore RNA-protein interactomes. Crit. Rev. Biotechnol. *36*, 11–19.

Suthar, M.K., Purva, M., Maherchandani, S., and Kashyap, S.K. (2016). Identification and in silico analysis of cattle DExH/D box RNA helicases. SpringerPlus *5*.

Swanwick, R.S., Pristerá, A., and Okuse, K. (2010). The trafficking of NaV1.8. Neurosci. Lett. *486*, 78–83.

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., Simonovic, M., Roth, A., Santos, A., Tsafou, K.P., et al. (2015). STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res. *43*, D447-452.

Tacheny, A., Dieu, M., Arnould, T., and Renard, P. (2013). Mass spectrometry-based identification of proteins interacting with nucleic acids. J. Proteomics *94*, 89–109.

Takagi, M., Absalon, M.J., McLure, K.G., and Kastan, M.B. (2005). Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. Cell *123*, 49–63.

Tamkun, M.M., Knoth, K.M., Walbridge, J.A., Kroemer, H., Roden, D.M., and Glover, D.M. (1991). Molecular cloning and characterization of two voltage-gated K+ channel cDNAs from human ventricle. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *5*, 331–337.

Tarun, S.Z., Wells, S.E., Deardorff, J.A., and Sachs, A.B. (1997). Translation initiation factor eIF4G mediates in vitro poly(A) tail-dependent translation. Proc. Natl. Acad. Sci. U. S. A. *94*, 9046–9051.

Terenin, I.M., Smirnova, V.V., Andreev, D.E., Dmitriev, S.E., and Shatsky, I.N. (2017). A researcher's guide to the galaxy of IRESs. Cell. Mol. Life Sci. *74*, 1431–1455.

Timmer, R.T., Benkowski, L.A., Schodin, D., Lax, S.R., Metz, A.M., Ravel, J.M., and Browning, K.S. (1993). The 5' and 3' untranslated regions of satellite tobacco necrosis virus RNA affect

translational efficiency and dependence on a 5' cap structure. J. Biol. Chem. *268*, 9504–9510.

Tinton, S.A., Schepens, B., Bruynooghe, Y., Beyaert, R., and Cornelis, S. (2005). Regulation of the cell-cycle-dependent internal ribosome entry site of the PITSLRE protein kinase: roles of Unr (upstream of N-ras) protein and phosphorylated translation initiation factor eIF-2α. Biochem. J. *385*, 155–163.

Topisirovic, I., and Sonenberg, N. (2011). mRNA translation and energy metabolism in cancer: the role of the MAPK and mTORC1 pathways. Cold Spring Harb. Symp. Quant. Biol. *76*, 355–367.

Tsai, B.P., Wang, X., Huang, L., and Waterman, M.L. (2011). Quantitative profiling of in vivo-assembled RNA-protein complexes using a novel integrated proteomic approach. Mol. Cell. Proteomics MCP *10*, M110.007385.

Tsai, C.-J., Sauna, Z.E., Kimchi-Sarfaty, C., Ambudkar, S.V., Gottesman, M.M., and Nussinov, R. (2008). Synonymous mutations and ribosome stalling can lead to altered folding pathways and distinct minima. J. Mol. Biol. *383*, 281–291.

Umate, P., Tuteja, N., and Tuteja, R. (2011). Genome-wide comprehensive analysis of human helicases. Commun. Integr. Biol. *4*, 118–137.

Vagner, S., Galy, B., and Pyronnet, S. (2001). Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. EMBO Rep. *2*, 893–898.

Van Eden, M.E., Byrd, M.P., Sherrill, K.W., and Lloyd, R.E. (2004). Demonstrating internal ribosome entry sites in eukaryotic mRNAs using stringent RNA test procedures. RNA N. Y. N *10*, 720–730.

Waisman, D.M. (1995). Annexin II tetramer: structure and function. Mol. Cell. Biochem. *149–150*, 301–322.

Walden, W.E., and Thach, R.E. (1986). Translational control of gene expression in a normal fibroblast. Characterization of a subclass of mRNAs with unusual kinetic properties. Biochemistry (Mosc.) *25*, 2033–2041.

Walter, B.L., Parsley, T.B., Ehrenfeld, E., and Semler, B.L. (2002). Distinct Poly(rC) Binding Protein KH domain determinants for poliovirus translation initiation and viral RNA replication. J. Virol. *76*, 12008–12022.

Wang, Z., and Sachs, M.S. (1997). Ribosome stalling is responsible for arginine-specific translational attenuation in Neurospora crassa. Mol. Cell. Biol. *17*, 4904–4913.

Wang, P., Zhu, S., Yang, L., Cui, S., Pan, W., Jackson, R., Zheng, Y., Rongvaux, A., Sun, Q., Yang, G., et al. (2015). Nlrp6 regulates intestinal antiviral innate immunity. Science *350*, 826–830.

Wang, X., Paulin, F.E., Campbell, L.E., Gomez, E., O'Brien, K., Morrice, N., and Proud, C.G. (2001). Eukaryotic initiation factor 2B: identification of multiple phosphorylation sites in the epsilon-subunit and their functions in vivo. EMBO J. *20*, 4349–4359.

Wang, Z., Gaba, A., and Sachs, M.S. (1999). A highly conserved mechanism of regulated ribosome stalling mediated by fungal arginine attenuator peptides that appears independent of the charging status of arginyl-tRNAs. J. Biol. Chem. *274*, 37565–37574.

Waskiewicz, A.J., Johnson, J.C., Penn, B., Mahalingam, M., Kimball, S.R., and Cooper, J.A. (1999). Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo. Mol. Cell. Biol. *19*, 1871–1880.

Webb, T.R., Cross, S.H., McKie, L., Edgar, R., Vizor, L., Harrison, J., Peters, J., and Jackson, I.J. (2008). Diphthamide modification of eEF2 requires a J-domain protein and is essential for normal development. J. Cell Sci. *121*, 3140–3145.

Wells, S.E., Hillner, P.E., Vale, R.D., and Sachs, A.B. (1998). Circularization of mRNA by eukaryotic translation initiation factors. Mol. Cell *2*, 135–140.

Welsh, G.I., Miller, C.M., Loughlin, A.J., Price, N.T., and Proud, C.G. (1998). Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. FEBS Lett. *421*, 125–130.

Willimott, S., and Wagner, S.D. (2010). Post-transcriptional and post-translational regulation of Bcl2. Biochem. Soc. Trans. *38*, 1571–1575.

Wilson, J.E., Pestova, T.V., Hellen, C.U., and Sarnow, P. (2000). Initiation of protein synthesis from the A site of the ribosome. Cell *102*, 511–520.

Wymore, R.S., Korenberg, J.R., Kinoshita, K.D., Aiyar, J., Coyne, C., Chen, X.N., Hustad, C.M., Copeland, N.G., Gutman, G.A., and Jenkins, N.A. (1994). Genomic organization, nucleotide sequence, biophysical properties, and localization of the voltage-gated K+ channel gene KCNA4/Kv1.4 to mouse chromosome 2/human 11p14 and mapping of KCNC1/Kv3.1 to mouse 7/human 11p14.3-p15.2 and KCNA1/Kv1.1 to human 12p13. Genomics *20*, 191–202.

Wymore, R.S., Negulescu, D., Kinoshita, K., Kalman, K., Aiyar, J., Gutman, G.A., and Chandy, K.G. (1996). Characterization of the transcription unit of mouse Kv1.4, a voltage-gated potassium channel gene. J. Biol. Chem. *271*, 15629–15634.

Xu, H., Dixon, J.E., Barry, D.M., Trimmer, J.S., Merlie, J.P., McKinnon, D., and Nerbonne, J.M. (1996). Developmental analysis reveals mismatches in the expression of K+ channel alpha subunits and voltage-gated K+ channel currents in rat ventricular myocytes. J. Gen. Physiol. *108*, 405–419.

Xu, H., Guo, W., and Nerbonne, J.M. (1999). Four kinetically distinct depolarization-activated K+ currents in adult mouse ventricular myocytes. J. Gen. Physiol. *113*, 661–678.

Xue, S., Tian, S., Fujii, K., Kladwang, W., Das, R., and Barna, M. (2015). RNA regulons in Hox 5' UTRs confer ribosome specificity to gene regulation. Nature *517*, 33–38.

Yoon, A., Peng, G., Brandenburg, Y., Zollo, O., Xu, W., Rego, E., and Ruggero, D. (2006). Impaired Control of IRES-Mediated Translation in X-Linked Dyskeratosis Congenita. Science *312*, 902–906.

Yueh, A., and Schneider, R.J. (2000). Translation by ribosome shunting on adenovirus and hsp70 mRNAs facilitated by complementarity to 18S rRNA. Genes Dev. *14*, 414–421.

Zhang, Y., You, J., Wang, X., and Weber, J. (2015). The DHX33 RNA Helicase Promotes mRNA Translation Initiation. Mol. Cell. Biol. *35*, 2918–2931.

Zhouravleva, G., Frolova, L., Le Goff, X., Le Guellec, R., Inge-Vechtomov, S., Kisselev, L., and Philippe, M. (1995). Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. EMBO J. *14*, 4065–4072.

Zielinski, J., Kilk, K., Peritz, T., Kannanayakal, T., Miyashiro, K.Y., Eiríksdóttir, E., Jochems, J., Langel, Û., and Eberwine, J. (2006). In vivo identification of ribonucleoprotein-RNA interactions. Proc. Natl. Acad. Sci. U. S. A. *103*, 1557–1562.

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Appendix A: *in vitro* translation assays

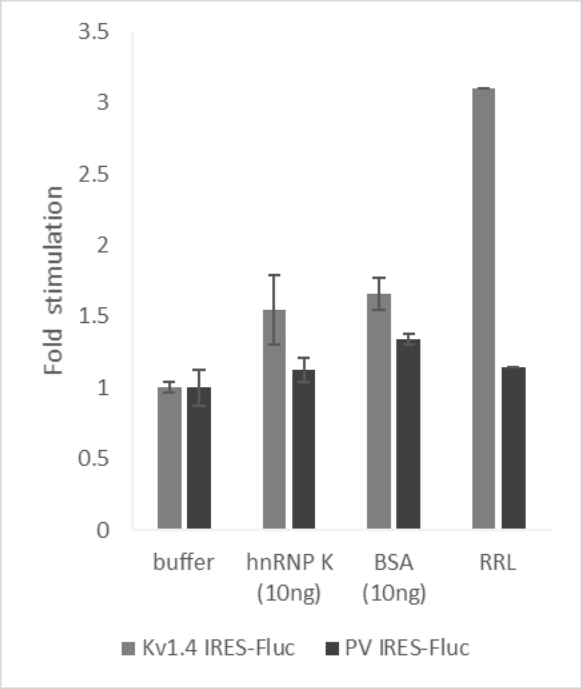

Figure A.1. *in vitro* translation reactions are insensitive to addition of recombinant hnRNP K. HeLa S10 cell-free extract for the translation of Kv1.4 reporter RNA or poliovirus reporter RNA were supplemented with recombinant hnRNP K, bovine serum albumin, or crude rabbit reticulocyte lysate. Firefly luciferase signal was measured after 4 hrs and normalized to buffer only condition. Addition of RRL greatly stimulated translation of Kv1.4 reporter, while not affecting poliovirus reporter translation.

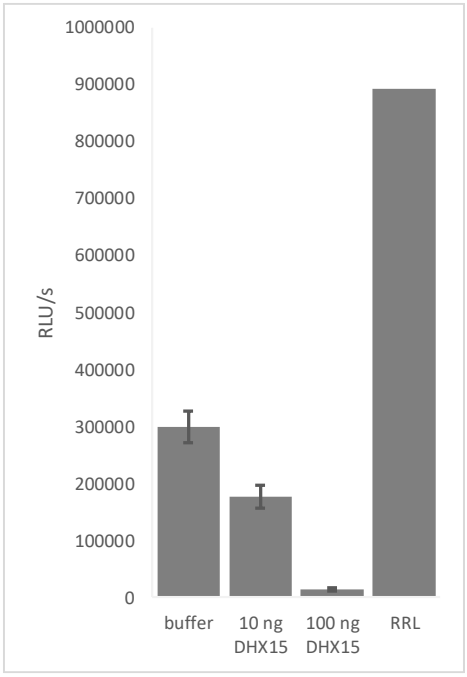

Figure A.2. DHX15 addition to *in vitro* translation reactions inhibits Kv1.4 IRES. HeLa S10 cell-free extract for the translation of Kv1.4 reporter RNA was supplemented with recombinant DHX15 or RRL. RRL addition greatly stimulated IRES-mediated translation whereas addition of small amounts of DHX15 suppressed translation.

Appendix B: Protein interactions detected by yeast 3-hybrid screen

ENTREZ Gene ID	Gene Name	GENE SYMBOL	Summary
26061	2-hydroxyacyl-CoA lyase 1	HACL1	cleaves a 2-hydroxy-3-methylacyl-CoA into formyl-CoA and a 2-methyl-branched fatty aldehyde
214	activated leukocyte cell adhesion molecule	ALCAM	Cell adhesion molecule that binds to CD6. Involved in neurite extension by neurons via heterophilic and homophilic interactions. May play a role in the binding of T- and B-cells to activated leukocytes, as well as in interactions between cells of the nervous system
204	adenylate kinase 2	AK2	Catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP. This small ubiquitous enzyme involved in energy metabolism and nucleotide synthesis that is essential for maintenance and cell growth. Plays a key role in hematopoiesis
8227	A-kinase anchoring protein 17A	AKAP17A	Splice factor regulating alternative splice site selection for certain mRNA precursors
224	aldehyde dehydrogenase 3 family member A2	ALDH3A2	Catalyzes the oxidation of long-chain aliphatic aldehydes to fatty acids. Active on a variety of saturated and unsaturated aliphatic aldehydes between 6 and 24 carbons in length
248	alkaline phosphatase, intestinal	ALPI	Catalyzes the hydrolysis of phosphate monoesters,
302	annexin A2	ANXA2	Calcium-regulated membrane-binding protein whose affinity for calcium is greatly enhanced by anionic phospholipids. It binds two calcium ions with high affinity. May be involved in heat-stress response
50807	ArfGAP with SH3 domain, ankyrin repeat and PH domain 1	ASAP1	Possesses phosphatidylinositol 4,5-biphosphate-dependent GTPase-activating protein activity for ARF1 (ADP ribosylation factor 1) and ARF5 and a lesser activity towards ARF6. May coordinate membrane trafficking with cell growth or actin cytoskeleton remodeling by binding to both SRC and PIP2. May function as a signal transduction protein involved in the differentiation of fibroblasts into adipocytes and possibly other cell types (By similarity)
65117	arginine and serine rich coiled-coil 2	RSRC2	
64860	armadillo repeat containing, X-linked 5	ARMCX5	
261	arthrogryposis multiplex congenita, neurogenic	AMCN	
405	aryl hydrocarbon receptor nuclear translocator	ARNT	Required for activity of the Ah (dioxin) receptor. This protein is required for the ligand-binding subunit to translocate from the cytosol to the nucleus after ligand binding. The complex then initiates transcription of genes involved in the activation of PAH procarcinogens. The heterodimer with HIF1A or EPAS1/HIF2A functions as a transcriptional regulator of the adaptive response to hypoxia

51382	ATPase H+ transporting V1 subunit D	ATP6V1D	Subunit of the peripheral V1 complex of vacuolar ATPase. Vacuolar ATPase is responsible for acidifying a variety of intracellular compartments in eukaryotic cells, thus providing most of the energy required for transport processes in the vacuolar system (By similarity)
483	ATPase Na+/K+ transporting subunit beta 3	ATP1B3	This is the non-catalytic component of the active enzyme, which catalyzes the hydrolysis of ATP coupled with the exchange of Na(+) and K(+) ions across the plasma membrane. The exact function of the beta-3 subunit is not known
546	ATRX, chromatin remodeler	ATRX	Could be a global transcriptional regulator. Modifies gene expression by affecting chromatin. May be involved in brain development and facial morphogenesis
55626	autophagy and beclin 1 regulator 1	AMBRA1	Regulates autophagy and development of the nervous system. Involved in autophagy in controlling protein turnover during neuronal development, and in regulating normal cell survival and proliferation
91408	basic transcription factor 3 like 4	BTF3L4	
10282	Bet1 golgi vesicular membrane trafficking protein	BET1	Required for vesicular transport from the ER to the Golgi complex. Functions as a SNARE involved in the docking process of ER-derived vesicles with the cis-Golgi membrane
23246	block of proliferation 1	BOP1	Component of the PeBoW complex, which is required for maturation of 28S and 5.8S ribosomal RNAs and formation of the 60S ribosome
9790	BMS1, ribosome biogenesis factor	BMS1	May act as a molecular switch during maturation of the 40S ribosomal subunit in the nucleolus
657	bone morphogenetic protein receptor type 1A	BMPR1A	On ligand binding, forms a receptor complex consisting of two type II and two type I transmembrane serine/threonine kinases. Type II receptors phosphorylate and activate type I receptors which autophosphorylate, then bind and activate SMAD transcriptional regulators. Receptor for BMP-2 and BMP-4
673	B-Raf proto-oncogene, serine/threonine kinase	BRAF	Involved in the transduction of mitogenic signals from the cell membrane to the nucleus. May play a role in the postsynaptic responses of hippocampal neuron
10286	breast carcinoma amplified sequence 2	BCAS2	Involved in mRNA splicing (By similarity)
9044	B-TFIID TATA-box binding protein associated factor 1	BTAF1	Regulates transcription in association with TATA binding protein (TBP). Removes TBP from the TATA box in an ATP-dependent manner
10950	BTG anti-proliferation factor 3	BTG3	The protein encoded by this gene is a member of the BTG/Tob family. This family has structurally related proteins that appear to have antiproliferative properties. This encoded protein might play a role in neurogenesis in the central nervous system
23473	calpain 7	CAPN7	Calcium-regulated non-lysosomal thiol-protease (By similarity)
22794	cancer susceptibility candidate 3	CASC3	Component of the dendritic ribonucleoprotein particles (RNPs) in hippocampal neurons. May play a role in mRNA transport

203413	cancer/testis antigen 83	CT83	Specifically expressed in testis. Expressed by cancer cell lines
832	capping actin protein of muscle Z-line beta subunit	CAPZB	F-actin-capping proteins bind in a Ca(2+)-independent manner to the fast growing ends of actin filaments (barbed end) thereby blocking the exchange of subunits at these ends. Unlike other capping proteins (such as gelsolin and severin), these proteins do not sever actin filaments
960	CD44 molecule	CD44	Receptor for hyaluronic acid (HA). Mediates cell-cell and cell-matrix interactions through its affinity for HA, and possibly also through its affinity for other ligands such as osteopontin, collagens, and matrix metalloproteinases (MMPs). Adhesion with HA plays an important role in cell migration, tumor growth and progression. Also involved in lymphocyte activation, recirculation and homing, and in hematopoiesis. Altered expression or dysfunction causes numerous pathogenic phenotypes. Great protein heterogeneity due to numerous alternative splicing and post-translational modification events
1063	centromere protein F	CENPF	Required for kinetochore function and chromosome segregation in mitosis. Required for kinetochore localization of dynein, LIS1, NDE1 and NDEL1. Regulates recycling of the plasma membrane by acting as a link between recycling vesicles and the microtubule network though its association with STX4 and SNAP25. Acts as a potential inhibitor of pocket protein-mediated cellular processes during development by regulating the activity of RB proteins during cell division and proliferation. May play a regulatory or permissive role in the normal embryonic cardiomyocyte cell cycle and in promoting continued mitosis in transformed, abnormally dividing neonatal cardiomyocytes. Interaction with RB directs embryonic stem cells toward a cardiac lineage. Involved in the regulation of DNA synthesis and hence cell cycle progression, via its C-terminus. Has a potential role regulating skeletal myogenesis and in cell differentiation in embryogenesis. Involved in dendritic cell regulation of T-cell immunity against chlamydia
55839	centromere protein N	CENPN	Component of the CENPA-NAC (nucleosome-associated) complex, a complex that plays a central role in assembly of kinetochore proteins, mitotic progression and chromosome segregation. The CENPA-NAC complex recruits the CENPA-CAD (nucleosome distal) complex and may be involved in incorporation of newly synthesized CENPA into centromeres
908	chaperonin containing TCP1 subunit 6A	CCT6A	Molecular chaperone; assists the folding of proteins upon ATP hydrolysis. Known to play a role, in vitro, in the folding of actin and tubulin
205428	chromosome 3 open reading frame 58	C3orf58	
81037	CLPTM1 like	CLPTM1L	Enhances cisplatin-mediated apoptosis, when overexpressed

1314	coatomer protein complex subunit alpha	COPA	The coatomer is a cytosolic protein complex that binds to dilysine motifs and reversibly associates with Golgi non-clathrin-coated vesicles, which further mediate biosynthetic protein transport from the ER, via the Golgi up to the trans Golgi network. Coatomer complex is required for budding from Golgi membranes, and is essential for the retrograde Golgi-to-ER transport of dilysine-tagged proteins. In mammals, the coatomer can only be recruited by membranes associated to ADP-ribosylation factors (ARFs), which are small GTP-binding proteins; the complex also influences the Golgi structural integrity, as well as the processing, activity, and endocytic recycling of LDL receptors (By similarity)Xenin stimulates exocrine pancreatic secretion. It inhibits pentagastrin-stimulated secretion of acid, to induce exocrine pancreatic secretion and to affect small and large intestinal motility. In the gut, xenin interacts with the neurotensin receptor
55704	coiled-coil domain containing 88A	CCDC88A	Enhances phosphoinositide 3-kinase (PI3K)-dependent phosphorylation and kinase activity of AKT1/PKB, but does not possess kinase activity itself. Phosphorylation of AKT1/PKB thereby induces the phosphorylation of downstream effectors GSK3 and FOXO1/FKHR, and regulates DNA replication and cell proliferation (By similarity). Essential for the integrity of the actin cytoskeleton and for cell migration. Required for formation of actin stress fibers and lamellipodia. May be involved in membrane sorting in the early endosome
29097	cornichon family AMPA receptor auxiliary protein 4	CNIH4	
9946	crystallin zeta like 1	CRYZL1	This gene encodes a protein that has sequence similarity to zeta crystallin, also known as quinone oxidoreductase. This zeta crystallin-like protein also contains an NAD(P)H binding site
1429	crystallin zeta	CRYZ	This gene encodes a taxon-specific crystallin protein which has NADPH-dependent quinone reductase activity distinct from other known quinone reductases. It lacks alcohol dehydrogenase activity although by similarity it is considered a member of the zinc-containing alcohol dehydrogenase family. Unlike other mammalian species, in humans, lens expression is low
983	cyclin dependent kinase 1	CDK1	Plays a key role in the control of the eukaryotic cell cycle. It is required in higher cells for entry into S-phase and mitosis. p34 is a component of the kinase complex that phosphorylates the repetitive C-terminus of RNA polymerase II
65992	DDRGK domain containing 1	DDRGK1	Interacts with components of the ubiquitin fold modifier 1 conjugation pathway and helps prevent apoptosis in ER-stressed secretory tissues. In addition, the encoded protein regulates nuclear factor-kB activity
8886	DEAD-box helicase 18	DDX18	DEAD box proteins, characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD), are putative RNA helicases
9416	DEAD-box helicase 23	DDX23	DEAD box protein. A component of the U5 snRNP complex; it may facilitate conformational changes in the spliceosome during nuclear pre-mRNA splicing
9879	DEAD-box helicase 46	DDX46	DEAD box protein. A component of the 17S U2 snRNP complex; plays an essential role in splicing, either prior to, or during splicing A complex formation

1665	DEAH-box helicase 15	DHX15	DEAH box helicase. Pre-mRNA processing factor involved in disassembly of spliceosomes after the release of mature mRNA (By similarity)
54165	defective in cullin neddylation 1 domain containing 1	DCUN1D1	May contribute to neddylation of cullin components of SCF-type E3 ubiquitin ligase complexes. Neddylation of cullins play an essential role in the regulation of SCF-type complexes activity
1603	defender against cell death 1	DAD1	Component of the N-oligosaccharyl transferase enzyme which catalyzes the transfer of a high mannose oligosaccharide from a lipid-linked oligosaccharide donor to an asparagine residue within an Asn-X-Ser/Thr consensus motif in nascent polypeptide chains. N-glycosylation occurs cotranslationally and the complex associates with the Sec61 complex at the channel-forming translocon complex that mediates protein translocation across the endoplasmic reticulum (ER). Loss of the DAD1 protein triggers apoptosis (By similarity)
92737	delta/notch like EGF repeat containing	DNER	Activator of the NOTCH1 pathway. May mediate neuron-glia interaction during astrocytogenesis
1854	deoxyuridine triphosphatase	DUT	This enzyme is involved in nucleotide metabolism: it produces dUMP, the immediate precursor of thymidine nucleotides and it decreases the intracellular concentration of dUTP so that uracil cannot be incorporated into DNA
4733	developmentally regulated GTP binding protein 1	DRG1	May play a role in cell proliferation, differentiation and death
1730	diaphanous related formin 2	DIAPH2	Could be involved in oogenesis. Involved in the regulation of endosome dynamics. Implicated in a novel signal transduction pathway, in which isoform 3 and CSK are sequentially activated by RHOD to regulate the motility of early endosomes through interactions with the actin cytoskeleton
1622	diazepam binding inhibitor, acyl-CoA binding protein	DBI	Binds medium- and long-chain acyl-CoA esters with very high affinity and may function as an intracellular carrier of acyl-CoA esters. It is also able to displace diazepam from the benzodiazepine (BZD) recognition site located on the GABA type A receptor. It is therefore possible that this protein also acts as a neuropeptide to modulate the action of the GABA receptor
22894	DIS3 homolog, exosome endoribonuclease and 3'-5' exoribonuclease	DIS3	Component of the exosome 3'->5' exoribonuclease complex. Required for the 3'-processing of the 7S pre-RNA to the mature nuclear complex. Also associated with the GTPase Ran. Has a 3'-5' exonuclease activity
3337	DnaJ heat shock protein family (Hsp40) member B1	DNAJB1	Member of the DnaJ or Hsp40 (heat shock protein 40 kD) family of proteins. Interacts with HSP70 and can stimulate its ATPase activity. Stimulates the association between HSC70 and HIP.,
84661	dpy-30, histone methyltransferase complex regulatory subunit	DPY30	Integral core subunit of the SET1/MLL family of H3K4 methyltransferases. Directly controls cell cycle regulators and plays an important role in the proliferation and differentiation of human hematopoietic progenitor cells.
29102	drosha ribonuclease III	DROSHA	Executes the initial step of microRNA (miRNA) processing in the nucleus, that is cleavage of pri-miRNA to release pre-miRNA. Involved in pre-rRNA processing. Cleaves double-strand RNA and does not cleave single-strand RNA

1871	E2F transcription factor 3	E2F3	Transcription activator that binds DNA cooperatively with DP proteins through the E2 recognition site, 5'-TTTC[CG]CGC-3' found in the promoter region of a number of genes whose products are involved in cell cycle regulation or in DNA replication. The DRTF1/E2F complex functions in the control of cell-cycle progression from G1 to S phase. E2F-3 binds specifically to RB1 protein, in a cell-cycle dependent manner
54206	ERBB receptor feedback inhibitor 1	ERRFI1	A cytoplasmic protein whose expression is upregulated with cell growth. Shares significant homology with the protein product of rat gene-33, which is induced during cell stress and mediates cell signaling
8894	eukaryotic translation initiation factor 2 subunit beta	EIF2S2	eIF-2 functions in the early steps of protein synthesis by forming a ternary complex with GTP and initiator tRNA. This complex binds to a 40S ribosomal subunit, followed by mRNA binding to form a 43S preinitiation complex. Junction of the 60S ribosomal subunit to form the 80S initiation complex is preceded by hydrolysis of the GTP bound to eIF-2 and release of an eIF-2-GDP binary complex. In order for eIF-2 to recycle and catalyze another round of initiation, the GDP bound to eIF-2 must exchange with GTP by way of a reaction catalyzed by eIF-2B
8666	eukaryotic translation initiation factor 3 subunit G	EIF3G	Component of the eukaryotic translation initiation factor 3 (eIF-3) complex, which is required for several steps in the initiation of protein synthesis. The eIF-3 complex associates with the 40S ribosome and facilitates the recruitment of eIF-1, eIF-1A, eIF-2:GTP:methionyl-tRNAi and eIF-5 to form the 43S preinitiation complex (43S PIC). The eIF-3 complex stimulates mRNA recruitment to the 43S PIC and scanning of the mRNA for AUG recognition. The eIF-3 complex is also required for disassembly and recycling of posttermination ribosomal complexes and subsequently prevents premature joining of the 40S and 60S ribosomal subunits prior to initiation. This subunit can bind 18S rRNA
8667	eukaryotic translation initiation factor 3 subunit H	EIF3H	Component of the eukaryotic translation initiation factor 3 (eIF-3) complex, which is required for several steps in the initiation of protein synthesis. The eIF-3 complex associates with the 40S ribosome and facilitates the recruitment of eIF-1, eIF-1A, eIF-2:GTP:methionyl-tRNAi and eIF-5 to form the 43S preinitiation complex (43S PIC). The eIF-3 complex stimulates mRNA recruitment to the 43S PIC and scanning of the mRNA for AUG recognition. The eIF-3 complex is also required for disassembly and recycling of posttermination ribosomal complexes and subsequently prevents premature joining of the 40S and 60S ribosomal subunits prior to initiation
27335	eukaryotic translation initiation factor 3 subunit K	EIF3K	Component of the eukaryotic translation initiation factor 3 (eIF-3) complex, which is required for several steps in the initiation of protein synthesis. The eIF-3 complex associates with the 40S ribosome and facilitates the recruitment of eIF-1, eIF-1A, eIF-2:GTP:methionyl-tRNAi and eIF-5 to form the 43S preinitiation complex (43S PIC). The eIF-3 complex stimulates mRNA recruitment to the 43S PIC and scanning of the mRNA for AUG recognition. The eIF-3 complex is also required for disassembly and recycling of posttermination ribosomal complexes and subsequently prevents premature joining of the 40S and 60S ribosomal subunits prior to initiation
1982	eukaryotic translation initiation factor 4 gamma 2	EIF4G2	Appears to play a role in the switch from cap-dependent to IRES-mediated translation during mitosis, apoptosis and viral infection. Cleaved by some caspases and viral

			proteases
10827	family with sequence similarity 114 member A2	FAM114A2	
23201	family with sequence similarity 168 member A	FAM168A	
55215	Fanconi anemia complementation group I	FANCI	Required for maintenance of chromosomal stability. Involved in the repair of DNA double-strand breaks by homologous recombination and in the repair of DNA cross-links. Participates in S phase and G2 phase checkpoint activation upon DNA damage. Promotes FANCD2 ubiquitination and recruitment to DNA repair sites
100505641	FGD5 antisense RNA 1	FGD5-AS1	
123811	FGFR1OP N-terminal like	FOPNL	
10211	flotillin 1	FLOT1	May act as a scaffolding protein within caveolar membranes, functionally participating in formation of caveolae or caveolae-like vesicles
8790	fucose-1-phosphate guanylyltransferase	FPGT	Catalyzes the formation of GDP-L-fucose from GTP and L-fucose-1-phosphate. Functions as a salvage pathway to reutilize L- fucose arising from the turnover of glycoproteins and glycolipids
29889	G protein nucleolar 2	GNL2	GTPase that associates with pre-60S ribosomal subunits in the nucleolus and is required for their nuclear export and maturation
9052	G protein-coupled receptor class C group 5 member A	GPRC5A	Unknown. This G-protein coupled receptor could be involved in modulating differentiation and maintaining homeostasis of epithelial cells. The comparable expression level in fetal lung and kidney with adult tissues suggests a possible role in embryonic development and maturation of these organs. This retinoic acid-inducible GPCR provide evidence for a possible interaction between retinoid and G-protein signaling pathways
57798	GATA zinc finger domain containing 1	GATAD1	Contains a zinc finger at the N-terminus, and is thought to bind to a histone modification site that regulates gene expression. Mutations in this gene have been associated with autosomal recessive dilated cardiomyopathy
2958	general transcription factor IIA subunit 2	GTF2A2	TFIIA is a component of the transcription machinery of RNA polymerase II and plays an important role in transcriptional activation. TFIIA in a complex with TBP mediates transcriptional activity
51659	GINS complex subunit 2	GINS2	The GINS complex plays an essential role in the initiation of DNA replication, and progression of DNA replication forks. GINS complex seems to bind preferentially to single-stranded DNA
2932	glycogen synthase kinase 3 beta	GSK3B	Participates in the Wnt signaling pathway. Implicated in the hormonal control of several regulatory proteins including glycogen synthase, MYB and the transcription factor JUN. Phosphorylates JUN at sites proximal to its DNA-binding domain, thereby reducing its affinity for DNA. Phosphorylates MUC1 in breast cancer cells, and decreases the interaction of MUC1 with CTNNB1/beta-catenin

27069	growth hormone inducible transmembrane protein	GHITM	
57801	hes family bHLH transcription factor 4	HES4	Transcriptional repressor. Binds DNA on N-box motifs: 5'-CACNAG-3' (By similarity)
10949	heterogeneous nuclear ribonucleoprotein A0	HNRNPA0	Belongs to the A/B subfamily of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs)
3190	heterogeneous nuclear ribonucleoprotein K	HNRNPK	One of the major pre-mRNA-binding proteins. Binds tenaciously to poly(C) sequences. Likely to play a role in the nuclear metabolism of hnRNAs, particularly for pre-mRNAs that contain cytidine-rich sequences. Can also bind poly(C) single- stranded DNA
3149	high mobility group box 3	HMGB3	Binds preferentially single-stranded DNA and unwinds double stranded DNA (By similarity)
79663	HSPB1 associated protein 1	HSPBAP1	May play a role in cellular stress response (By similarity)
3476	immunoglobulin (CD79A) binding protein 1	IGBP1	Associated to surface IgM-receptor; may be involved in signal transduction. May be involved in regulation of the catalytic activity of type 2A-related serine/threonine phosphatases
3399	inhibitor of DNA binding 3, HLH protein	ID3	ID (inhibitor of DNA binding) HLH proteins lack a basic DNA-binding domain but are able to form heterodimers with other HLH proteins, thereby inhibiting DNA binding. ID-3 inhibits the binding of E2A-containing protein complexes to muscle creatine kinase E-box enhancer
54928	inositol monophosphatase domain containing 1	IMPAD1	Encodes a member of the inositol monophosphatase family. The encoded protein is localized to the Golgi apparatus and catalyzes the hydrolysis of phosphoadenosine phosphate (PAP) to adenosine monophosphate (AMP)
10643	insulin like growth factor 2 mRNA binding protein 3	IGF2BP3	RNA-binding protein that act as a regulator of mRNA translation and stability. Binds to the 5'-UTR of the insulin-like growth factor 2 (IGF2) mRNAs. Binds to sequences in the 3'-UTR of CD44 mRNA
25896	integrator complex subunit 7	INTS7	Component of the Integrator complex, a complex involved in the small nuclear RNAs (snRNA) U1 and U2 transcription and in their 3'-box-dependent processing. The Integrator complex is associated with the C-terminal domain (CTD) of RNA polymerase II largest subunit (POLR2A) and is recruited to the U1 and U2 snRNAs genes
3688	integrin subunit beta 1	ITGB1	Integrins are heterodimeric proteins made up of alpha and beta subunits. At least 18 alpha and 8 beta subunits have been described in mammals. Integrin family members are membrane receptors involved in cell adhesion and recognition in a variety of processes including embryogenesis, hemostasis, tissue repair, immune response and metastatic diffusion of tumor cells. This gene encodes a beta subunit. Multiple alternatively spliced transcript variants which encode different protein isoforms have been found for this gene. [provided by RefSeq, Jul 2008],

23421	integrin subunit beta 3 binding protein	ITGB3BP	Transcription coregulator that can have both coactivator and corepressor functions. Isoform 1, but not other isoforms, is involved in the coactivation of nuclear receptors for retinoid X (RXRs) and thyroid hormone (TRs) in a ligand-dependent fashion. In contrast, it does not coactivate nuclear receptors for retinoic acid, vitamin D, progesterone receptor, nor glucocorticoid. Acts as a coactivator for estrogen receptor alpha. Acts as a transcriptional corepressor via its interaction with the NFKB1 NF-kappa-B subunit, possibly by interfering with the transactivation domain of NFKB1. Induces apoptosis in breast cancer cells, but not in other cancer cells, via a caspase-2 mediated pathway that involves mitochondrial membrane permeabilization but does not require other caspases. May also act as an inhibitor of cyclin A-associated kinase. Also acts a component of the CENPA-CAD (nucleosome distal) complex, a complex recruited to centromeres which is involved in assembly of kinetochore proteins, mitotic progression and chromosome segregation. May be involved in incorporation of newly synthesized CENPA into centromeres via its interaction with the CENPA-NAC complex.,
64135	interferon induced with helicase C domain 1	IFIH1	RNA helicase that, through its ATP-dependent unwinding of RNA, may function to promote message degradation by specific RNases. Seems to have growth suppressive properties. Involved in innate immune defense against viruses. Upon interaction with intracellular dsRNA produced during viral replication, triggers a transduction cascade involving MAVS/IPS1, which results in the activation of NF-kappa-B, IRF3 and IRF7 and the induction of the expression of antiviral cytokines such as IFN-beta and RANTES (CCL5). ATPase activity is specifically induced by dsRNA. Essential for the production of interferons in response to picornaviruses
55699	isoleucyl-tRNA synthetase 2, mitochondrial	IARS2	Aminoacyl-tRNA synthetases catalyze the aminoacylation of tRNA by their cognate amino acid. This gene encodes the mitochondrial isoleucine-tRNA synthetase which belongs to the class-I aminoacyl-tRNA synthetase family
9768	KIAA0101	KIAA0101	May be involved in protection of cells from UV-induced cell death
3801	kinesin family member C3	KIFC3	Minus-end microtubule-dependent motor protein. Involved in apically targeted transport (By similarity)
7071	Kruppel like factor 10	KLF10	Transcriptional repressor involved in the regulation of cell growth. Inhibits cell growth. Binds to the consensus sequence 5'-GGTGTG-3'
688	Kruppel like factor 5	KLF5	Transcription factor that binds to GC box promoter elements. Activates the transcription of these genes
51574	La ribonucleoprotein domain family member 7	LARP7	Negative transcriptional regulator of polymerase II genes, acting by means of the 7SK RNP system. Within the 7SK RNP complex, the positive transcription elongation factor b (P-TEFb) is sequestered in an inactive form, preventing RNA polymerase II phosphorylation and subsequent transcriptional elongation
3945	lactate dehydrogenase B	LDHB	Catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD+ in a post-glycolysis process
81887	LAS1 like, ribosome biogenesis	LAS1L	

	factor		
26020	LDL receptor related protein 10	LRP10	Probable receptor, which is involved in the internalization of lipophilic molecules and/or signal transduction. May be involved in the uptake of lipoprotein APOE in liver (By similarity)
55631	leucine rich repeat containing 40	LRRC40	
84328	leucine zipper and CTNNBIP1 domain containing	LZIC	
4048	leukotriene A4 hydrolase	LTA4H	Hydrolyzes an epoxide moiety of leukotriene A4 (LTA-4) to form leukotriene B4 (LTB-4). The enzyme also has some peptidase activity
64327	limb development membrane protein 1	LMBR1	Putative membrane receptor
57128	LYR motif containing 4	LYRM4	May be involved in mitochondrial iron-sulfur protein biosynthesis
51111	lysine methyltransferase 5B	KMT5B	Histone methyltransferase that specifically trimethylates 'Lys-20' of histone H4. H4 'Lys-20' trimethylation represents a specific tag for epigenetic transcriptional repression. Mainly functions in pericentric heterochromatin regions, thereby playing a central role in the establishment of constitutive heterochromatin in these regions. SUV420H1 is targeted to histone H3 via its interaction with RB1 family proteins (RB1, RBL1 and RBL2)
3920	lysosomal associated membrane protein 2	LAMP2	Implicated in tumor cell metastasis. May function in protection of the lysosomal membrane from autodigestion, maintenance of the acidic environment of the lysosome, adhesion when expressed on the cell surface (plasma membrane), and inter- and intracellular signal transduction
3107	major histocompatibility complex, class I, C	HLA-C	Involved in the presentation of foreign antigens to the immune system (By similarity)Involved in the presentation of foreign antigens to the immune system
9443	mediator complex subunit 7	MED7	Component of the Mediator complex, a coactivator involved in the regulated transcription of nearly all RNA polymerase II-dependent genes. Mediator functions as a bridge to convey information from gene-specific regulatory proteins to the basal RNA polymerase II transcription machinery. Mediator is recruited to promoters by direct interactions with regulatory proteins and serves as a scaffold for the assembly of a functional preinitiation complex with RNA polymerase II and the general transcription factors
112950	mediator complex subunit 8	MED8	Component of the Mediator complex, a coactivator involved in the regulated transcription of nearly all RNA polymerase II-dependent genes. Mediator functions as a bridge to convey information from gene-specific regulatory proteins to the basal RNA polymerase II transcription machinery. Mediator is recruited to promoters by direct interactions with regulatory proteins and serves as a scaffold for the assembly of a functional preinitiation complex with RNA polymerase II and the general transcription factors. May play a role as a target recruitment subunit in E3 ubiquitin-protein ligase complexes and thus in ubiquitination and subsequent proteasomal degradation of target proteins

27249	methylmalonic aciduria and homocystinuria, cblD type	MMADHC	Involved in cobalamin metabolism.,tissue specificity:Widely expressed at high levels
79066	methyltransferase like 16	METTL16	
22924	microtubule associated protein RP/EB family member 3	MAPRE3	May be involved in microtubule polymerization, and spindle function by stabilizing microtubules and anchoring them at centrosomes. May play a role in cell migration
100505738	MIR4458 host gene	MIR4458HG	
359739	mitochondrial ribosomal protein L3 pseudogene 1	MRPL3P1	
51073	mitochondrial ribosomal protein L4	MRPL4	Mitochondrial ribosomal protein component of the large 39S subunit
5601	mitogen-activated protein kinase 9	MAPK9	Responds to activation by environmental stress and pro-inflammatory cytokines by phosphorylating a number of transcription factors, primarily components of AP-1 such as c-Jun and ATF2 and thus regulates AP-1 transcriptional activity. In T-cells, JNK1 and JNK2 are required for polarized differentiation of T-helper cells into Th1 cells
5608	mitogen-activated protein kinase kinase 6	MAP2K6	Catalyzes the concomitant phosphorylation of a threonine and a tyrosine residue in MAP kinase p38 exclusively
84451	mixed lineage kinase 4	MLK4	Activates the JUN N-terminal pathway (By similarity)
25843	MOB family member 4, phocein	MOB4	may be regulated during oocyte maturation and preimplantation following zygotic gene activation (By Simalarity)
114803	Myb like, SWIRM and MPN domains 1	MYSM1	Metalloprotease that specifically deubiquitinates monoubiquitinated histone H2A, a specific tag for epigenetic transcriptional repression, thereby acting as a coactivator. Preferentially deubiquitinates monoubiquitinated H2A in hyperacetylated nucleosomes. Deubiquitination of histone H2A leads to facilitate the phosphorylation and dissociation of histone H1 from the nucleosome. Acts as a coactivator by participating in the initiation and elongation steps of androgen receptor (AR)-induced gene activation
103910	myosin light chain 12B	MYL12B	Myosin regulatory subunit that plays an important role in regulation of both smooth muscle and nonmuscle cell contractile activity via its phosphorylation. Implicated in cytokinesis, receptor capping, and cell locomotion
4637	myosin light chain 6	MYL6	Regulatory light chain of myosin. Does not bind calcium
54893	myotubularin related protein 10	MTMR10	Probable pseudophosphatase. Contains a Glu residue instead of a conserved Cys residue in the dsPTPase catalytic loop which renders it catalytically inactive as a phosphatase (Potential)
653308	N-acylsphingosine amidohydrolase 2B	ASAH2B	Hydrolyzes the sphingolipid ceramide into sphingosine and free fatty acid at an optimal pH of 6.5-8.5. Acts as a key regulator of sphingolipid signaling metabolites by generating sphingosine at the cell surface. Acts as a repressor of apoptosis both by reducing C16-ceramide, thereby preventing ceramide-induced apoptosis, and generating sphingosine, a precursor of the antiapoptotic factor sphingosine 1-phosphate. Probably involved in the digestion of dietary sphingolipids in intestine by acting as a key enzyme for the catabolism of dietary sphingolipids and

			regulating the levels of bioactive sphingolipid metabolites in the intestinal tract
54602	Nedd4 family interacting protein 2	NDFIP2	May be involved in endocytosis. Regulates ENaC surface expression (By similarity). May be involved in NF-kappa-B and MAPK signaling pathways
25983	neuroguidin	NGDN	Involved in the translational repression of cytoplasmic polyadenylation element (CPE)-containing mRNAs (By similarity)
55968	NSFL1 cofactor	NSFL1C	Reduces the ATPase activity of VCP. Necessary for the fragmentation of Golgi stacks during mitosis and for VCP-mediated reassembly of Golgi stacks after mitosis. May play a role in VCP- mediated formation of transitional endoplasmic reticulum (tER) (By similarity). Inhibits the activity of CTSL (in vitro)
25936	NSL1, MIS12 kinetochore complex component	NSL1	Part of the MIS12 complex which is required for normal chromosome alignment and segregation and kinetochore formation during mitosis
4781	nuclear factor I B	NFIB	Recognizes and binds the palindromic sequence 5'-TTGGCNNNNNGCCAA-3' present in viral and cellular promoters and in the origin of replication of adenovirus type 2. These proteins are individually capable of activating transcription and replication (By similarity)Recognizes and binds the palindromic sequence 5'- TTGGCNNNNNGCCAA-3' present in viral and cellular promoters and in the origin of replication of adenovirus type 2. These proteins are individually capable of activating transcription and replication
8650	NUMB, endocytic adaptor protein	NUMB	Implicated in the control of cell fate decisions during development
51526	oxidative stress responsive serine rich 1	OSER1	
55872	PDZ binding kinase	PBK	Phosphorylates MAP kinase p38. Seems to be active only in mitosis. May also play a role in the activation of lymphoid cells. When phosphorylated, forms a complex with TP53, leading to TP53 destabilization and attenuation of G2/M checkpoint during doxorubicin-induced DNA damage
5066	peptidylglycine alpha-amidating monooxygenase	PAM	Bifunctional enzyme that catalyzes 2 sequential steps in C-terminal alpha-amidation of peptides. The monooxygenase part produces an unstable peptidyl(2-hydroxyglycine) intermediate that is dismutated to glyoxylate and the corresponding desglycine peptide amide by the lyase part. C-terminal amidation of peptides such as neuropeptides is essential for full biological activity
25824	peroxiredoxin 5	PRDX5	Reduces hydrogen peroxide and alkyl hydroperoxides with reducing equivalents provided through the thioredoxin system. Involved in intracellular redox signaling
51105	PHD finger protein 20-like 1	PHF20L1	

10056	phenylalanyl-tRNA synthetase beta subunit	FARSB	Encodes a highly conserved enzyme that belongs to the aminoacyl-tRNA synthetase class IIc subfamily. This enzyme comprises the regulatory beta subunits that form a tetramer with two catalytic alpha subunits. In the presence of ATP, this tetramer is responsible for attaching L-phenylalanine to the terminal adenosine of the appropriate tRNA
5144	phosphodiesterase 4D	PDE4D	Regulates the levels of cAMP in the cell
8622	phosphodiesterase 8B	PDE8B	Plays a role in signal transduction by regulating the intracellular concentration of cyclic nucleotides. This phosphodiesterase has high affinity for cAMP and may be involved in specific signaling in the thyroid gland
9373	phospholipase A2 activating protein	PLAA	Plays an important role in the regulation of specific inflammatory disease processes (By similarity)
5198	phosphoribosylformylglycinamidine synthase	PFAS	The enzyme encoded by this gene catalyzes the fourth step of inosine monophosphate (IMP) biosynthesis in the de novo pathway of purine biosynthesis
5411	pinin, desmosome associated protein	PNN	Transcriptional activator binding to the E-box 1 core sequence of the E-cadherin promoter gene; the core-binding sequence is 5'CAGGTG-3'. Capable of reversing CTBP1-mediated transcription repression. Also participates in the regulation of alternative pre-mRNA splicing. Associates to spliced mRNA within 60 nt upstream of the 5'-splice sites. Involved in the establishment and maintenance of epithelia cell-cell adhesion. Potential tumor suppressor for renal cell carcinoma
5361	plexin A1	PLXNA1	Coreceptor for SEMA3A, SEMA3C, SEMA3F and SEMA6D. Necessary for signaling by class 3 semaphorins and subsequent remodeling of the cytoskeleton. Plays a role in axon guidance, invasive growth and cell migration. Class 3 semaphorins bind to a complex composed of a neuropilin and a plexin. The plexin modulates the affinity of the complex for specific semaphorins, and its cytoplasmic domain is required for the activation of down- stream signaling events in the cytoplasm (By similarity)
5420	podocalyxin like	PODXL	Functions as an antiadhesin that maintains an open filtration pathway between neighboring foot processes in the podocyte by charge repulsion
57645	pogo transposable element with KRAB domain	POGK	Unknown. Contains a KRAB domain (which is involved in protein-protein interactions) at the N-terminus, and a transposase domain at the C-terminus, suggesting that it may belong to the family of DNA-mediated transposons in human
51247	poly binding protein interacting protein 2	PAIP2	Acts as a repressor in the regulation of translation initiation of poly(A)-containing mRNAs. Its inhibitory activity on translation is mediated via its action on PABPC1. Displaces the interaction of PABPC1 with poly(A) RNA and competes with PAIP1 for binding to PABPC1. Its association with PABPC1 results in disruption of the cytoplasmic poly(A) RNP structure organization
255738	proprotein convertase subtilisin/kexin type 9	PCSK9	May be implicated in the differentiation of cortical neurons and may play a role in cholesterol homeostasis
5743	prostaglandin-endoperoxide synthase 2	PTGS2	May have a role as a major mediator of inflammation and/or a role for prostanoid signaling in activity-dependent plasticity

5683	proteasome subunit alpha 2	PSMA2	The proteasome is a multicatalytic proteinase complex which is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The proteasome has an ATP-dependent proteolytic activity. This gene encodes a member of the peptidase T1A family, that is a 20S core alpha subunit
5685	proteasome subunit alpha 4	PSMA4	The proteasome is a multicatalytic proteinase complex which is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The proteasome has an ATP-dependent proteolytic activity. This gene encodes a core alpha subunit of the 20S proteosome
10954	protein disulfide isomerase family A member 5	PDIA5	Catalyzes the rearrangement of -S-S- bonds in proteins.,
9063	protein inhibitor of activated STAT 2	PIAS2	Functions as an E3-type small ubiquitin-like modifier (SUMO) ligase, stabilizing the interaction between UBE2I and the substrate, and as a SUMO-tethering factor. Plays a crucial role as a transcriptional coregulator in various cellular pathways, including the STAT pathway, the p53 pathway and the steroid hormone signaling pathway. The effects of this transcriptional coregulation, transactivation or silencing may vary depending upon the biological context and the PIAS2 isoform studied. However, it seems to be mostly involved in gene silencing. Binds to sumoylated ELK1 and enhances its transcriptional activity by preventing recruitment of HDAC2 by ELK1, thus reversing SUMO-mediated repression of ELK1 transactivation activity. Isoform PIAS2-beta, but not isoform PIAS2-alpha, promotes MDM2 sumoylation. Isoform PIAS2-alpha promotes PARK7 sumoylation. Isoform PIAS2-beta promotes NCOA2 sumoylation more efficiently than isoform PIAS2- alpha
8073	protein tyrosine phosphatase type IVA, member 2	PTP4A2	Protein tyrosine phosphatase which stimulates progression from G1 into S phase during mitosis. Promotes tumors. Inhibits geranylgeranyl transferase type II activity by blocking the association between RABGGTA and RABGGTB
5782	protein tyrosine phosphatase, non-receptor type 12	PTPN12	Member of the protein tyrosine phosphatase (PTP) family. This PTP contains a C-terminal PEST motif, which serves as a protein-protein interaction domain, and may regulate protein intracellular half-life. This PTP was found to bind and dephosphorylate the product of the oncogene c-ABL and thus may play a role in oncogenesis. This PTP was also shown to interact with, and dephosphorylate, various products related to cytoskeletal structure and cell adhesion, such as p130 (Cas), CAKbeta/PTK2B, PSTPIP1, and paxillin. This suggests it has a regulatory role in controlling cell shape and mobility
23369	pumilio RNA binding family member 2	PUM2	Sequence-specific RNA-binding protein that regulates translation and mRNA stability by binding the 3'-UTR of mRNA targets. Its interactions and tissue specificity suggest that it may be required to support proliferation and self-renewal of stem cells by regulating the translation of key transcripts
27068	pyrophosphatase (inorganic) 2	PPA2	The protein encoded by this gene is localized to the mitochondrion, is highly similar to members of the inorganic pyrophosphatase (PPase) family, and contains the signature sequence essential for the catalytic activity of PPase. PPases catalyze the hydrolysis of pyrophosphate to inorganic phosphate, which is important for the phosphate

			metabolism of cells
51552	RAB14, member RAS oncogene family	RAB14	May be involved in vesicular trafficking and neurotransmitter release
5861	RAB1A, member RAS oncogene family	RAB1A	Probably required for transit of protein from the ER through Golgi compartment. Binds GTP and GDP and possesses intrinsic GTPase activity
10048	RAN binding protein 9	RANBP9	May act as an adapter protein to couple membrane receptors to intracellular signaling pathways. May be involved in signaling of ITGB2/LFA-1 and other integrins. Enhances HGF-MET signaling by recruiting Sos and activating the Ras pathway. Involved in activation of androgen and glucocorticoid receptor in the presence of their cognate hormones. Stabilizes TP73 isoform Alpha, probably by inhibiting its ubiquitination, and increases its proapoptotic activity. Inhibits the kinase activity of DYRK1A and DYRK1B. Inhibits FMR1 binding to RNA
5910	Rap1 GTPase-GDP dissociation stimulator 1	RAP1GDS1	Stimulates GDP/GTP exchange reaction of a group of small GTP-binding proteins (G proteins) including Rap1a/Rap1b, RhoA, RhoB and KRas, by stimulating the dissociation of GDP from and the subsequent binding of GTP to each small G protein
387	ras homolog family member A	RHOA	Regulates a signal transduction pathway linking plasma membrane receptors to the assembly of focal adhesions and actin stress fibers. Serves as a target for the yopT cysteine peptidase from Yersinia pestis, vector of the plague, and Yersinia pseudotuberculosis, which causes gastrointestinal disorders. May be an activator of PLCE1. Activated by ARHGEF2, which promotes the exchange of GDP for GTP
58480	ras homolog family member U	RHOU	Acts upstream of PAK1 to regulate the actin cytoskeleton, adhesion turnover and increase cell migration. Stimulates quiescent cells to reenter the cell cycle. Has no detectable GTPase activity but its high intrinsic guanine nucleotide exchange activity suggests it is constitutively GTP- bound
57786	RB associated KRAB zinc finger	RBAK	May repress E2F-dependent transcription. May promote AR- dependent transcription
8767	receptor interacting serine/threonine kinase 2	RIPK2	Activates pro-caspase-1 and pro-caspase-8. Potentiates CASP8-mediated apoptosis. Activates NF-kappa-B
5983	replication factor C subunit 3	RFC3	The elongation of primed DNA templates by DNA polymerase delta and epsilon requires the action of the accessory proteins proliferating cell nuclear antigen (PCNA) and activator 1
6117	replication protein A1	RPA1	It participates in a very early step in initiation. RP-A is a single-stranded DNA-binding protein. Absolutely required for simian virus 40 DNA replication in vitro
5955	reticulocalbin 2	RCN2	Calcium-binding protein located in the lumen of the ER. The protein contains six conserved regions with similarity to a high affinity Ca(+2)-binding motif, the EF-hand

6253	reticulon 2	RTN2	This gene belongs to the family of reticulon encoding genes. Reticulons are necessary for proper generation of tubular endoplasmic reticulum and likely play a role in intracellular vesicular transport
57142	reticulon 4	RTN4	Potent neurite growth inhibitor in vitro and plays a role both in the restriction of axonal regeneration after injury and in structural plasticity in the CNS. Isoform 2 reduces the anti-apoptotic activity of Bcl-xl and Bcl-2. This is likely consecutive to their change in subcellular location, from the mitochondria to the endoplasmic reticulum, after binding and sequestration. Isoform 2 and isoform 3 inhibit BACE1 activity and amyloid precursor protein processing
84986	Rho GTPase activating protein 19	ARHGAP19	GTPase activator for the Rho-type GTPases by converting them to an inactive GDP-bound state
9639	Rho guanine nucleotide exchange factor 10	ARHGEF10	May play a role in developmental myelination of peripheral nerves
11102	ribonuclease P/MRP subunit p14	RPP14	Part of ribonuclease P, a protein complex that generates mature tRNA molecules by cleaving their 5'-ends
6155	ribosomal protein L27	RPL27	Encodes a ribosomal protein component of the 60S subunit. The protein belongs to the L27E family of ribosomal proteins
6173	ribosomal protein L36a	RPL36A	This protein is a component of the heterogeneous nuclear ribonucleoprotein (hnRNP) complexes which provide the substrate for the processing events that pre-mRNAs undergo before becoming functional, translatable mRNAs in the cytoplasm. Binds poly(RG)
6133	ribosomal protein L9	RPL9	Encodes a ribosomal protein component of the 60S subunit. The protein belongs to the L6P family of ribosomal proteins
6176	ribosomal protein lateral stalk subunit P1	RPLP1	Plays an important role in the elongation step of protein synthesis
6181	ribosomal protein lateral stalk subunit P2	RPLP2	Plays an important role in the elongation step of protein synthesis
84154	ribosome production factor 2 homolog	RPF2	
284996	ring finger protein 149	RNF149	The RING-type zinc finger domain mediates binding to an E2 ubiquitin-conjugating enzyme
51132	ring finger protein, LIM domain interacting	RLIM	Acts as a negative coregulator for LIM homeodomain transcription factors. Via the recruitment of the SIN3a/histone deacetylase corepressor complex
201965	RWD domain containing 4	RWDD4	
84324	SAP domain containing ribonucleoprotein	SARNP	May have nucleic acid binding capability that may participate in important transcriptional or translational control of cell growth, metabolism and carcinogenesis
23256	sec1 family domain containing 1	SCFD1	Involved in vesicular transport between the endoplasmic reticulum and the Golgi (By similarity)
11231	SEC63 homolog, protein translocation regulator	SEC63	Required for integral membrane and secreted preprotein translocation across the endoplasmic reticulum membrane

56681	secretion associated Ras related GTPase 1A	SAR1A	Involved in transport from the endoplasmic reticulum to the Golgi apparatus (By similarity). Required to maintain SEC16A localization at discrete locations on the ER membrane perhaps by preventing its dissociation. SAR1A-GTP-dependent assembly of SEC16A on the ER membrane forms an organized scaffold defining endoplasmic reticulum exit sites (ERES)
151011	septin 10	43353	Involved in cytokinesis (Potential). Can bind to GTP and exert GTPase activity
6428	serine and arginine rich splicing factor 3	SRSF3	May be involved in RNA processing in relation with cellular proliferation and/or maturation
9263	serine/threonine kinase 17a	STK17A	Acts as a positive regulator of apoptosis
11171	serine/threonine kinase receptor associated protein	STRAP	The SMN complex plays an essential role in spliceosomal snRNP assembly in the cytoplasm and is required for pre-mRNA splicing in the nucleus. STRAP may play a role in the cellular distribution of the SMN complex
6419	SET domain and mariner transposase fusion gene	SETMAR	Histone methyltransferase that methylates 'Lys-4' and 'Lys-36' of histone H3, 2 specific tags for epigenetic transcriptional activation. Specifically mediates dimethylation of H3 'Lys-36'. Binds DNA. May play a role in non-homologous end- joining repair
55209	SET domain containing 5	SETD5	This function of this gene has yet to be determined but mutations in this gene have been associated with autosomal dominant mental retardation-23
6418	SET nuclear proto-oncogene	SET	The protein encoded by this gene inhibits acetylation of nucleosomes, especially histone H4, by histone acetylases (HAT). This inhibition is most likely accomplished by masking histone lysines from being acetylated, and the consequence is to silence HAT-dependent transcription. The encoded protein is part of a complex localized to the endoplasmic reticulum but is found in the nucleus and inhibits apoptosis following attack by cytotoxic T lymphocytes. This protein can also enhance DNA replication of the adenovirus genome. Several transcript variants encoding different isoforms have been found for this gene. [provided by RefSeq, Oct 2011],
6772	signal transducer and activator of transcription 1	STAT1	Signal transducer and activator of transcription that mediates signaling by interferons (IFNs). Following type I IFN (IFN-alpha and IFN-beta) binding to cell surface receptors, Jak kinases (TYK2 and JAK1) are activated, leading to tyrosine phosphorylation of STAT1 and STAT2. The phosphorylated STATs dimerize, associate with ISGF3G/IRF-9 to form a complex termed ISGF3 transcription factor, that enters the nucleus. ISGF3 binds to the IFN stimulated response element (ISRE) to activate the transcription of interferon stimulated genes, which drive the cell in an antiviral state. In response to type II IFN (IFN-gamma), STAT1 is tyrosine- and serine-phosphorylated. It then forms a homodimer termed IFN-gamma-activated factor (GAF), migrates into the nucleus and binds to the IFN gamma activated sequence (GAS) to drive the expression of the target genes, inducing a cellular antiviral state
6742	single stranded DNA binding protein 1	SSBP1	This protein binds preferentially and cooperatively to ss-DNA. Probably involved in mitochondrial DNA replication
23517	Ski2 like RNA helicase 2	SKIV2L2	May be involved in pre-mRNA splicing

7341	small ubiquitin-like modifier 1	SUMO1	Ubiquitin-like protein which can be covalently attached to target lysines as a monomer. Does not seem to be involved in protein degradation and may function as an antagonist of ubiquitin in the degradation process. Plays a role in a number of cellular processes such as nuclear transport, DNA replication and repair, mitosis and signal transduction. Involved in targeting RANGAP1 to the nuclear pore complex protein RANBP2. Covalent attachment to its substrates requires prior activation by the E1 complex SAE1- SAE2 and linkage to the E2 enzyme UBE2I, and can be promoted by an E3 ligase such as PIAS1-4, RANBP2 or CBX4
23049	SMG1, nonsense mediated mRNA decay associated PI3K related kinase	SMG1	Serine/threonine protein kinase involved in both mRNA surveillance and genotoxic stress response pathways. Recognizes the substrate consensus sequence [ST]-Q. Plays a central role in nonsense-mediated decay (NMD) of mRNAs containing premature stop codons by phosphorylating UPF1/RENT1. Acts as part of the SMG1C complex, a mRNA surveillance complex that recognizes and degrades mRNAs containing premature translation termination codons (PTCs). The complex probably acts by associating with ribosomes during tranlation termination on mRNPs. If an exon junction complex (EJC) is located 50-55 or more nucleotides downstream from the termination codon, it phosphorylates UPF1/RENT1, triggering nonsense-mediated decay (NMD). Also acts as a genotoxic stress- activated protein kinase that displays some functional overlap with ATM. Can phosphorylate TP53/p53 and is required for optimal TP53/p53 activation after cellular exposure to genotoxic stress. Its depletion leads to spontaneous DNA damage and increased sensitivity to ionizing radiation (IR). May activate PRKCI but not PRKCZ
4891	solute carrier family 11 member 2	SLC11A2	Macrophage-specific membrane transport. Important in metal transport, in particular iron
399512	solute carrier family 25 member 35	SLC25A35	Belongs to the SLC25 family of mitochondrial carrier proteins
54407	solute carrier family 38 member 2	SLC38A2	Functions as a sodium-dependent amino acid transporter. Mediates the saturable, pH-sensitive and electrogenic cotransport of neutral amino acids and sodium ions with a stoichiometry of 1:1. May function in the transport of amino acids at the blood- brain barrier and in the supply of maternal nutrients to the fetus through the placenta
64116	solute carrier family 39 member 8	SLC39A8	Acts as a zinc-influx transporter
8140	solute carrier family 7 member 5	SLC7A5	Sodium-independent, high-affinity transport of large neutral amino acids such as phenylalanine, tyrosine, leucine, arginine and tryptophan, when associated with SLC3A2/4F2hc. Involved in cellular amino acid uptake. Acts as an amino acid exchanger. Involved in the transport of L-DOPA across the blood- brain barrier, and that of thyroid hormones triiodothyronine (T3) and thyroxine (T4) across the cell membrane in tissues such as placenta. Plays a role in neuronal cell proliferation (neurogenesis) in brain. Involved in the uptake of methylmercury (MeHg) when administered as the L-cysteine or D,L-homocysteine complexes, and hence plays a role in metal ion homeostasis and toxicity. Involved in the cellular activity of small molecular weight nitrosothiols, via the stereoselective transport of L- nitrosocysteine (L-CNSO) across the transmembrane. May play an important role in high-grade gliomas. Mediates blood-to-retina L- leucine transport across the inner blood-retinal barrier which in turn may play a key role in maintaining large neutral amino acids as

			well as neurotransmitters in the neural retina. Acts as the major transporter of tyrosine in fibroblasts
29887	sorting nexin 10	SNX10	May be involved in several stages of intracellular trafficking (By similarity)
57231	sorting nexin 14	SNX14	May be involved in several stages of intracellular trafficking (By similarity)
6667	Sp1 transcription factor	SP1	Binds to GC box promoters elements and selectively activates mRNA synthesis from genes that contain functional recognition sites. Can interact with G/C-rich motifs from serotonin receptor promoter
100506436	SPAG5 antisense RNA 1	SPAG5-AS1	
10924	sphingomyelin phosphodiesterase acid like 3A	SMPDL3A	
51188	SS18 like 2	SS18L2	Synovial sarcomas occur most frequently in the extremities around large joints. More than 90% of cases have a recurrent and specific chromosomal translocation, t(X;18)(p11.2;q11.2), in which the 5-prime end of the SS18 gene (MIM 600192) is fused in-frame to the 3-prime end of the SSX1 (MIM 312820), SSX2 (MIM 300192), or SSX4 (MIM 300326) gene. The SS18L2 gene is homologous to SS18.[supplied by OMIM, Jul 2002],
6780	staufen double-stranded RNA binding protein 1	STAU1	Binds double-stranded RNA (regardless of the sequence) and tubulin. May play a role in specific positioning of mRNAs at given sites in the cell by cross-linking cytoskeletal and RNA components, and in stimulating their translation at the site
2040	stomatin	STOM	Thought to regulate cation conductance. May regulate ACCN1 and ACCN3 gating (By similarity)
90196	SYS1, golgi trafficking protein	SYS1	Involved in protein trafficking. May serve as a receptor for ARFRP1
79101	TATA-box binding protein associated factor, RNA polymerase I subunit D	TAF1D	Component of the transcription factor SL1/TIFIB complex involved in the assembly of the PIC (preinitiation complex)
83940	TatD DNase domain containing 1	TATDN1	
25976	TCDD inducible poly(ADP-ribose) polymerase	TIPARP	This gene encodes a member of the poly(ADP-ribose) polymerase superfamily. Studies of the mouse ortholog have shown that the encoded protein catalyzes histone poly(ADP-ribosyl)ation and may be involved in T-cell

			function
64852	terminal uridylyl transferase 1, U6 snRNA-specific	TUT1	Highly specific terminal uridylyltransferase that exclusively accepts U6 snRNA as substrate. U6 snRNA is unique in that nucleotides are both added to and removed from its 3'-end. U6-TUTase is responsible for a controlled elongation reaction that results in the restoration of the four 3'-terminal UMP-residues found in newly transcribed U6 snRNA
93081	testis expressed 30	TEX30	
54902	tetratricopeptide repeat domain 19	TTC19	This gene encodes a protein with a tetratricopeptide repeat (TPR) domain containing several TPRs of about 34 aa each. These repeats are found in a variety of organisms including bacteria, fungi and plants, and are involved in a variety of functions including protein-protein interactions. This protein is embedded in the inner mitochondrial membrane and is involved in the formation of the mitochondrial respiratory chain III. It has also been suggested that this protein plays a role in cytokinesis. Mutations in this gene cause mitochondrial complex III deficiency
57215	THAP domain containing 11	THAP11	Transcriptional repressor that plays a central role for embryogenesis and the pluripotency of embryonic stem (ES) cells. Sequence-specific DNA-binding factor that represses gene expression in pluripotent ES cells by directly binding to key genetic loci and recruiting epigenetic modifiers (By similarity)
5612	THAP domain containing 12	THAP12	Upstream regulator of interferon-induced serine/threonine protein kinase R (PKR). May block the PKR- inhibitory function of P58IPK, resulting in restoration of kinase activity and suppression of cell growth
25828	thioredoxin 2	TXN2	Has an anti-apoptotic function and plays an important role in the regulation of mitochondrial membrane potential. Could be involved in the resistance to anti-tumor agents. Possesses a dithiol-reducing activity
9325	thyroid hormone receptor interactor 4	TRIP4	Transcription coactivator of nuclear receptors which functions in conjunction with CBP-p300 and SRC-1 and may play an important role in establishing distinct coactivator complexes under different cellular conditions. Plays a pivotal role in the transactivation of NF-kappa-B, SRF and AP1. Acts as a mediator of transrepression between nuclear receptor and either AP1 or NF- kappa-B. Plays a role in androgen receptor transactivation and in testicular function (By similarity)
7082	tight junction protein 1	TJP1	The N-terminal may be involved in transducing a signal required for tight junction assembly, while the C-terminal may have specific properties of tight junctions. The alpha domain might be involved in stabilizing junctions

10318	TNFAIP3 interacting protein 1	TNIP1	Interacts with zinc finger protein A20/TNFAIP3 and inhibits TNF-induced NF-kappa-B-dependent gene expression by interfering with an RIP- or TRAF2-mediated transactivation signal (By similarity). Increases cell surface CD4(T4) antigen expression. Interacts with HIV-1 matrix protein and is packaged into virions and overexpression can inhibit viral replication. May regulate matrix nuclear localization, both nuclear import of PIC (Preintegration complex) and export of GAG polyprotein and viral genomic RNA during virion production., subcellular location:Shuttles between the nucleus and cytoplasm in a CRM1-dependent manner., subunit:Interacts with TNFAIP3 (By similarity). Interacts with HIV-1 matrix protein., tissue specificity:Ubiquitous. Strongly expressed in peripheral blood lymphocytes, spleen and skeletal muscle, and is weakly expressed in the brain
117143	transcriptional adaptor 1	TADA1	Probably involved in transcriptional regulation
9238	transforming growth factor beta regulator 4	TBRG4	May play a role in cell cycle progression
7175	translocated promoter region, nuclear basket protein	TPR	Component of the cytoplasmic fibrils of the nuclear pore complex implicated in nuclear protein import. Its N-terminus is involved in activation of oncogenic kinases. Plays a role in the mitotic spindle checkpoint
23471	translocation associated membrane protein 1	TRAM1	Stimulatory or required for the translocation of secretory proteins across the ER membrane
10972	transmembrane p24 trafficking protein 10	TMED10	Involved in vesicular protein trafficking
153339	transmembrane protein 167A	TMEM167A	
55161	transmembrane protein 33	TMEM33	
55076	transmembrane protein 45A	TMEM45A	
54765	tripartite motif containing 44	TRIM44	
286144	triple QxxK/R motif containing	TRIQK	
81627	tRNA methyltransferase 1 like	TRMT1L	May play a role in motor coordination and exploratory behavior (By similarity)
57570	tRNA methyltransferase 5	TRMT5	Specifically methylates guanosine-37 in various tRNAs. Not dependent on the nature of the nucleoside 5' of the target nucleoside
5756	twinfilin actin binding protein 1	TWF1	Actin-binding protein involved in motile and morphological processes. Inhibits actin polymerization, likely by sequestering G-actin. By capping the barbed ends of filaments, it also regulates motility. Seems to play an important role in clathrin-mediated endocytosis and distribution of endocytic organelles
7323	ubiquitin conjugating enzyme E2 D3	UBE2D3	atalyzes the covalent attachment of ubiquitin to other proteins. Mediates the selective degradation of short-lived and abnormal proteins. Functions in the E6/E6-AP-induced ubiquitination of p53/TP53

10477	ubiquitin conjugating enzyme E2 E3	UBE2E3	Catalyzes the covalent attachment of ubiquitin to other proteins. Participates in the regulation of transepithelial sodium transport in renal cells. May be involved in cell growth arrest., pathway:Protein modification; protein ubiquitination
51377	ubiquitin C-terminal hydrolase L5	UCHL5	Deubiquitinating enzyme associated with the proteasome
92181	ubiquitin domain containing 2	UBTD2	
10054	ubiquitin like modifier activating enzyme 2	UBA2	The dimeric enzyme acts as a E1 ligase for SUMO1, SUMO2, SUMO3, and probably SUMO4. It mediates ATP-dependent activation of SUMO proteins and formation of a thioester with a conserved cysteine residue on SAE2
9736	ubiquitin specific peptidase 34	USP34	Recognizes and hydrolyzes the peptide bond at the C-terminal Gly of ubiquitin. Involved in the processing of poly-ubiquitin precursors as well as that of ubiquinated proteins
84747	unc-119 lipid binding chaperone B	UNC119B	
26019	UPF2 regulator of nonsense transcripts homolog	UPF2	Part of a post-splicing multiprotein complex involved in both mRNA nuclear export and mRNA surveillance. Involved in nonsense-mediated decay (NMD) of mRNAs containing premature stop codons. Binds spliced mRNA
10090	uronyl 2-sulfotransferase	UST	Sulfotransferase that catalyzes the transfer of sulfate to the position 2 of uronyl residues. Has mainly activity toward iduronyl residues in dermatan sulfate, and weaker activity toward glucuronyl residues of chondroitin sulfate. Has no activity toward desulfated N-resulfated heparin
6845	vesicle associated membrane protein 7	VAMP7	Involved in the targeting and/or fusion of transport vesicles to their target membrane during transport of proteins from the early endosome to the lysosome. Required for heterotypic fusion of late endosomes with lysosomes and homotypic lysosomal fusion. Required for calcium regulated lysosomal exocytosis. Involved in the export of chylomicrons from the endoplasmic reticulum to the cis Golgi. Required for exocytosis of mediators during eosinophil and neutrophil degranulation, and target cell killing by natural killer cells. Required for focal exocytosis of late endocytic vesicles during phagosome formation
7417	voltage dependent anion channel 2	VDAC2	Forms a channel through the mitochondrial outer membrane that allows diffusion of small hydrophilic molecules. The channel adopts an open conformation at low or zero membrane potential and a closed conformation at potentials above 30-40 mV. The open state has a weak anion selectivity whereas the closed state is cation-selective
151525	WD repeat, sterile alpha motif and U-box domain containing 1	WDSUB1	
7494	X-box binding protein 1	XBP1	Transcription factor essential for hepatocyte growth, the differentiation of plasma cells, the immunoglobulin secretion, and the unfolded protein response (UPR). Acts during endoplasmic reticulum stress (ER) by activating unfolded protein response (UPR) target genes via direct binding to the UPR element (UPRE). Binds DNA preferably to the CRE-like element 5'-GATGACGTG[TG]N(3)[AT]T-3', and also to some TPA response elements (TRE). Binds to the HLA DR-alpha promoter. Binds to the Tax-responsive element (TRE) of HTLV-I

2547	X-ray repair cross complementing 6	XRCC6	The p70/p80 autoantigen is a nuclear complex consisting of two subunits with molecular masses of approximately 70 and 80 kDa. The complex functions as a single-stranded DNA-dependent ATP-dependent helicase. The complex may be involved in the repair of nonhomologous DNA ends such as that required for double-strand break repair, transposition, and V(D)J recombination
64397	zinc finger protein 106	ZNF106	
22847	zinc finger protein 507	ZNF507	May be involved in transcriptional regulation
162655	zinc finger protein 519	ZNF519	May be involved in transcriptional regulation
27332	zinc finger protein 638	ZNF638	
84146	zinc finger protein 644	ZNF644	May be involved in transcriptional regulation
144348	zinc finger protein 664	ZNF664	May be involved in transcriptional regulation