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RNA G-quadruplexes and their potential regulatory roles in translation

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ABSTRACT

DNA guanine (G)-rich 4-stranded helical nucleic acid structures called G-quadruplexes (G4), have been extensively studied during the last decades. However, emerging evidence reveals that 5'- and 3'-untranslated regions (5'- and 3'-UTRs) as well as open reading frames (ORFs) contain putative RNA G-quadruplexes. These stable secondary structures play key roles in telomere homeostasis and RNA metabolism including pre-mRNA splicing, polyadenylation, mRNA targeting and translation. Interestingly, multiple RNA binding proteins such as nucleolin, FMRP, DHX36, and Aven were identified to bind RNA G-quadruplexes. Moreover, accumulating reports suggest that RNA G-quadruplexes regulate translation in cap-dependent and -independent manner. Herein, we discuss potential roles of RNA G-quadruplexes and associated *trans*-acting factors in the regulation of mRNA translation.

ARTICLE HISTORY

Received 16 August 2016
Revised 26 September 2016
Accepted 28 September 2016

KEYWORDS

Aven; DHX36; FMRP;
G-quadruplexes; RNA
binding proteins; translation

Structure of G-quadruplexes

In 1910, Bang demonstrated that guanylic acid forms gels at high concentration, providing the first evidence that guanine (G)-rich sequences may form higher-order structures.¹ Fifty years later, Gellert et al. (1962) reported that guanylic acid has the ability to form tetrameric structures by self-association.² These non-canonical structures were identified in conserved DNA sequences of telomeres and shown to form so-called G-quadruplexes (G4) structures *in vitro*.^{3,4} Since then, increasing evidence shows that both DNA and RNA containing spaced guanine repeats form G4 structures.^{5,6} G-quadruplexes are folded in G-quartets that are square planar arrangements formed via Hoogsteen pairing of adjacent guanines.⁷⁻¹² The G-quartets lay on the top of each other to form 4-stranded structures which are stabilized by a cation positioned in the middle of the tetrads with preference for potassium (Fig. 1). The ability of potential G4 sequences to form G-quadruplexes is therefore influenced by the nature of the central cation, the number of stacking G-quartets, the length of the sequences connecting the strands (at least in DNA G-quadruplexes), the direction of the strands, and the presence of an alternative Watson-Crick pair-based stable structure.^{13,14}

DNA G-quadruplexes

Bioinformatic search using a script that identifies “regular” G-quadruplexes (i.e. $G_xN_{1-7}G_xN_{1-7}G_xN_{1-7}G_x$, where G stands for guanine, N can be any nucleotide (A, G, C, U) and $x \geq 3$) has shown that the human genome harbors ~376,000 potential G-quadruplex sequences (pG4).^{15,16} With the development of high resolution sequencing-based methods > 716,000 G4 structures were identified, where ~451,000 were not predicted by computational methods.^{17,18} These sequences are highly conserved in mammals, with limited conservation in evolutionarily lower organisms.^{19,20} Genome-wide searches using G-quadruplex-specific probes and structure-based pull-down strategies have revealed that the pG4 sequences are not randomly distributed, being enriched at telomeres, promoter regions and replication origins.^{9,21} The highest abundance of pG4 is in human telomeres, where the G4 formation protects the end of chromosomes by inhibiting telomerase activity.^{16,22,23} Abnormal telomerase overexpression has been observed in > 85% of cancers. Thus, extensive investigations were performed to obtain anti-cancer therapies using small molecules to stabilize telomeric G-quadruplexes as a means to

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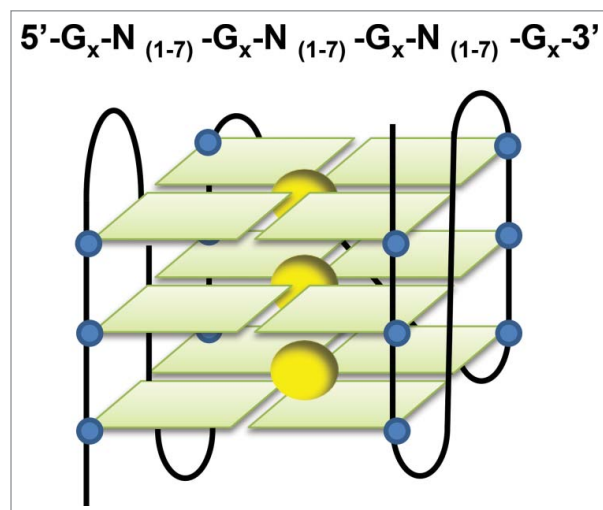


Figure 1. Structure of G-quadruplexes. Guanine-rich sequences fold into G-quadruplex structures (G4 structures), composed of planar G-quartets. This representation is a G-quadruplex parallel structure that could be observed in RNA as well as DNA molecules. The guanines are represented as blue, while cations are yellow.

inhibit telomerase.²⁴⁻²⁷ Moye et al. (2015) characterized the stable human telomeric G-quadruplexes and demonstrated that these G4s are able to extend into parallel, intermolecular conformations, aligning with the intrinsic RNA moiety of the human telomerase RNA (hTR). They also showed that telomerase colocalizes with a subset of telomeric G4 structures *in vivo*.²⁷ Additionally, structural and computational analysis revealed that pG4 sequences are found in ~40% of gene promoter regions, mostly acting as transcriptional repressors.^{23,28,29} It has been shown that pG4 sequences occur with high frequency in the promoter regions of genes encoding oncogenes such as *c-Myc*, *c-Kit*, *KRAS*, *PDGF-A* (platelet derived growth factor-A), and *hTERT* (human telomerase reverse transcriptase), while tumor suppressor genes correlate with low pG4 abundance in their promoter regions.^{12,30,31} Recently, Onel et al. (2016) showed that *Bcl-2* forms G-quadruplexes in its promoter by nuclear magnetic resonance (NMR) spectroscopy and dimethylsulfate (DMS) footprinting assays, and these G4s were shown to inhibit transcription by promoter-driven luciferase assay.³² Another study also demonstrated that the human tyrosine hydroxylase (*hTH*) gene harbors a G4 structure in the 3' proximal promoter region, acting as a necessary element for transcriptional regulation. Since hTH is linked to several neurological and psychiatric disorders such as Parkinson disease and Schizophrenia,³³ these

findings suggest that promoter G4 sequences may be linked with these diseases. Furthermore, emerging evidence suggests that ~90% of DNA replication origins contain pG4 sequences.^{7,34-36} Recently, it was discovered by performing nascent strand sequencing that G4 sequences may also position nucleosomes at a subset of human replication origins.³⁷ Moreover, it was reported that Potential DNA:RNA Hybrid G-Quadruplex Sequences (PHQS) are present in > 97% of human genes, and these PHQS may modulate transcription.³⁸ Additional studies show that helicases, which are the molecular motors to unwind DNA and RNA, are involved in the active resolution of G4s. The best-characterized DNA G4 helicase are Pif1, RecQ, FANCI, DDX11, BLM (Bloom syndrome protein) and WRN (Werner syndrome protein).⁸ Known dual RNA-DNA G4 helicases are DHX9 and RHAU (DHX36).^{39,40}

RNA G-quadruplexes

G-quadruplexes also form in RNA and are more stable than their DNA counterparts.¹¹ RNA G-quadruplexes almost exclusively adopt a parallel conformation in which the 4 strands all have the same directionality. The 2'-hydroxyl group of the ribose locks the RNA in an anti-conformation, which favors the parallel topology. Consequently, RNA G-quadruplexes have less topological diversity than DNA G-quadruplexes.

In eukaryotes, RNA G4 structures are enriched at telomeres and within specific protein encoding transcripts i.e., mRNAs. In telomeres, the G-rich TERRA (telomeric repeat-containing RNAs, or TelRNA) RNA which is transcribed from the human C-rich telomeric DNA, was shown to form G4 structures *in vitro*^{41,42} and *in cellulo*.⁴³ The high resolution of the G4 structure in TERRA revealed that the 2'-hydroxyl group provides intramolecular hydrogen bonding within the parallel-stranded structures, and this is important for ligand targeting and higher-order arrangement.⁴⁴ The human telomerase RNA (hTR) forms G4 structures at its 5' end in the presence of potassium, as visualized using gel electrophoresis and UV, CD, and NMR spectroscopy.^{45,46}

RNA G-quadruplexes are frequently present in mRNAs. Kumari et al. (2007) demonstrated that among all the genes in the human transcriptome available at that time, ~3,000 of the 5'-UTRs were identified to possess at least one pG4 sequence.⁴⁷ This was revised, when Beaudoin and Perreault (2010) identified

9,979 5'-UTRs to contain at least one pG4 sequence, among the 124,315 transcripts from the human UTR-full dataset based on a “regular” definition of G-quadruplex.⁴⁸ Subsequently, it was discovered that 1,453 human pG4 sequences possess 2 short distal loops of 1 nucleotide in length and a long central loop of up to 70 nucleotides long in the 5'-UTRs, which significantly expands the number of pG4s in the transcriptome.⁴⁹ A bioinformatic search for “regular” G-quadruplex in 3'-UTRs of the human transcriptome, detected 8,903 pG4 sequences showing that the enrichment for G-quadruplex is not limited to the 5'-UTRs.⁵⁰ Additionally, other bioinformatic analyses, in-line probing and luciferase reporter assays revealed the existence of pG4 sequences in both the 5'- and 3'-UTR of transcripts, indicating G4 structures function in the post-transcription regulation *in cellulo*, and neighboring C-rich sequences that affect G4 folding.^{14,49,51} Moreover, a bioinformatics search for pG4 sequences in mRNA-coding regions was performed, which revealed that ~1600 pG4s are present in human ORFs.⁵² Recently, the existence of G-quadruplex formation in RNA was further confirmed in human cells by using stabilizing ligands that specifically trap RNA G-quadruplexes.⁵³ The authors were able to visualize G4 structures in the cytoplasm of human cells using G-quadruplex-specific antibodies.

RNA G-quadruplexes in TERRA and hTR RNAs regulate telomerase function, whereas G4 formation in mRNAs has been implicated in several gene expression steps, including pre-mRNA processing, mRNA turnover and targeting, and translation.¹² While recent reviews have broadly covered the G4 structures in DNA and RNA molecules as well as their functions in telomere maintenance and RNA metabolism in physiology and pathology conditions,^{9,10,12} we will discuss in detail the emerging areas of RNA G4 structures in mRNA translation and known *trans*-acting factors that bind these structures.

G-quadruplexes in the control of mRNA translation

mRNA translation is one of the most fundamental processes in RNA metabolism, and its regulation is tightly controlled. Protein synthesis is composed of 4 main steps: translation initiation, elongation, and termination as well as ribosome recycling.⁵⁴ For most eukaryotic mRNAs, translation initiation involves the association of the 7-methylguanosine cap with the cap-binding complex called eukaryotic initiation

factor 4F (eIF4F, Fig. 2A).^{55,56} eIF4F complex contains the cap-binding protein eIF4E, the scaffold protein eIF4G that bridges interaction between eIF4F and multifactor complex (MFC), thus allowing recruitment of the mRNA to the ribosome, and the ATP-dependent DEAD box RNA helicase eIF4A required to unwind secondary structures in the 5'-UTRs.^{57,58} The association between the mRNA and eIF4F is the first step of translation, followed by the recruitment of 43S initiation complex, composed of the 40S ribosomal subunit, the eukaryotic initiation factors eIF3, eIF1, eIF1A and eIF5, as well as the ternary complex containing methionine-loaded tRNA, eIF2 and GTP (Fig. 2A).^{56,59} The 43S complex recognizes the initiation codon, where it is joined by a 60S ribosomal subunit to form the 80S ribosome.^{57,58} Excessive secondary structures in 5'-UTRs impede mRNA translation in a cap-dependent manner in eukaryotes.^{60,61} The 3'-UTRs also participate in translational regulation, where the added poly (A) tail is bound by the poly(A)-binding protein (PABP) and eIF4G, resulting in the circularization of mRNAs and enhanced overall initiation rate.⁵⁷ An alternative mode of translation is driven by IRES (Internal Ribosome Entry Sites), and it occurs in a cap-independent mode.⁶² Independent of the presence or integrity of several canonical initiation factors (especially eIF4E), IRES directly recruits ribosomes, bypassing the requirement for the 5' cap and eIF4E. Efficient IRES-driven translation is facilitated by the IRES *trans*-acting factors (ITAFs, Fig. 2B).^{59,63} Collectively, secondary structure in 5'-UTR is thought to have a major impact on translation efficiency.⁵⁵

G-quadruplexes in 5'-UTR and translational control

Kumari et al. (2007) reported that pG4 sequences in the human *NRAS* mRNA are conserved in different organisms.⁴⁷ They documented the formation of G4 structures *in vitro* by circular dichroism (CD) spectroscopy and UV-melting experiments, while luciferase reporter assays revealed that the RNA G4 in 5'-UTR of *NRAS* inhibits translation by ~80% in rabbit reticulocyte lysates.⁴⁷ Moreover, it was established that the human *ZIC-1* mRNA forms a 27 nucleotide G4 structure within its 5'-UTR and represses protein production by ~80% in HeLa cells using the dual-luciferase plasmid based assay.⁶⁴ The presence of G4 structures in 5'-UTR of various human mRNAs and multiple strategies such as bioinformatic analyses, mutagenesis

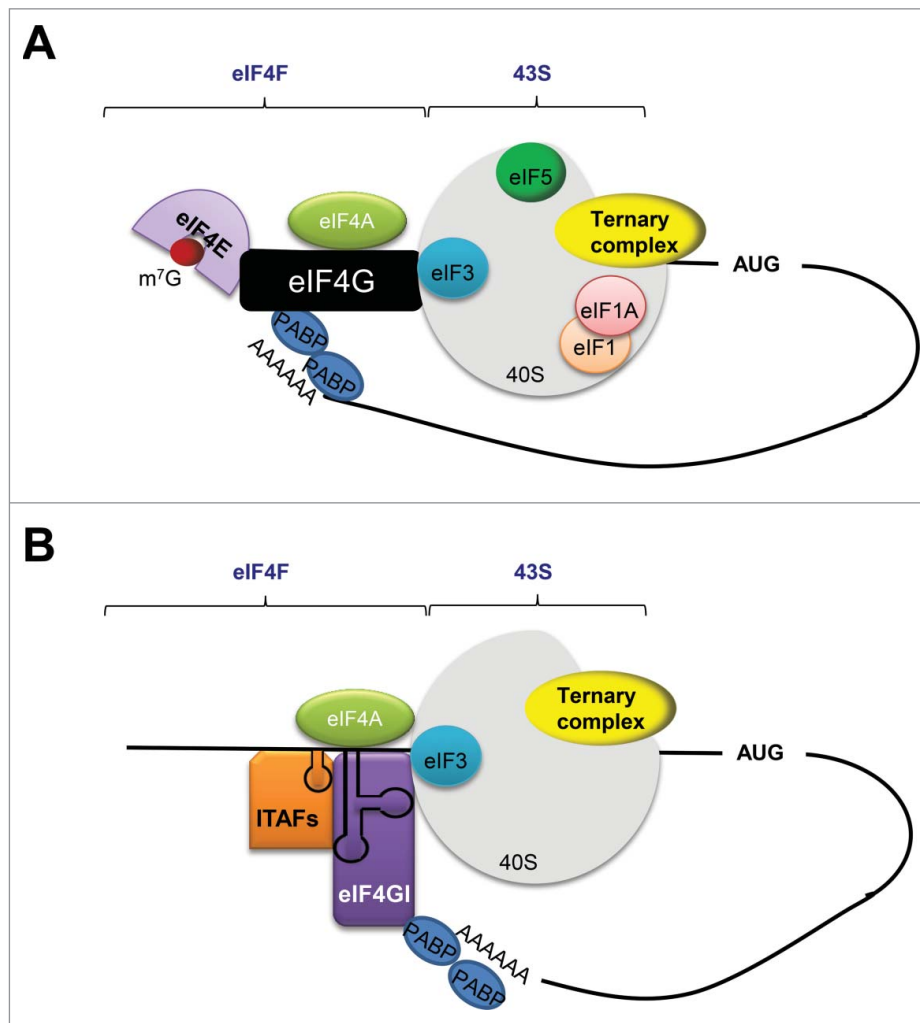


Figure 2. Cap-dependent and Cap-independent translation initiation. (A) In cap-dependent translation, eIF4E binds to the 5'-m⁷GpppN of the mRNA (m⁷G). The capped 5'-end is associated with 43S complex by a bridging protein called eIF4G. eIF4G is also bound to eIF4A, the RNA helicase that unwinds 5' secondary structures. PABP binds the poly (A) tail and brings the 5'-end and 3'-end of the mRNA together through the interaction with eIF4G. eIF3, eIF5, eIF1/eIF1A and ternary complex are shown as represented. (B) ITAFs and eIF4GI (also known as p97/DAP5/NAT1, purple) facilitates IRES cap-independent translation.⁵⁹

and reporter gene-based expression assays showed that G4s in 5'-UTRs correlate with translational repression of various mRNAs including *MT3-MMP*,⁶⁵ *ERS1*,⁶⁶ *BCL-2*,⁶⁷ *TRF2*,⁶⁸ *ADAM10*^{69,70} and *TGFβ2*⁷¹ (Fig. 3). Moreover, in-depth analysis using CD spectroscopy and in-line probing, identified several 5'-UTRs that harbor pG4 sequences including *EBAG9*, *AASDHPPT*, *FZD2*, *BARHL1*, *NCAM2*, and *THRA*⁴⁸ (Fig. 3). Most of these genes are involved in transcriptional regulation, protein modification, G-protein-mediated signaling, cation transport and developmental processes. The C-to-A substitution, known to destabilize G4 formation, was able to rescue the repressed translation of all but one gene.⁴⁸ Recently, G4 structures with longer central loops (> 7 nucleotides) in the *HIRA*, *TOM112*

and *APC* 5'-UTR were also shown to have the ability to repress translation when tested by luciferase reporter assays (Fig. 3).⁴⁹ Similar conclusions were reached in the study where the “irregular” G4 structures were discovered in the *H2AFY* and *AKIRIN* 5'-UTR (Fig. 3).⁷² It was also shown that antisense oligonucleotides can be used to inhibit or promote the formation of RNA G4 structures.⁷² Additionally, by using ribosome footprinting on a transcriptome-wide scale, Wolfe et al. (2014) reported that the 12-nucleotide guanine quartet motifs that can form G4 structures in 5'-UTRs rendering mRNAs exceptionally sensitive to eIF4A. As a key factor in cap-dependent translation initiation, eIF4A plays a role in scanning the 5'-UTR of the mRNAs for start codons.⁷³ Notably, eIF4A inhibitors including

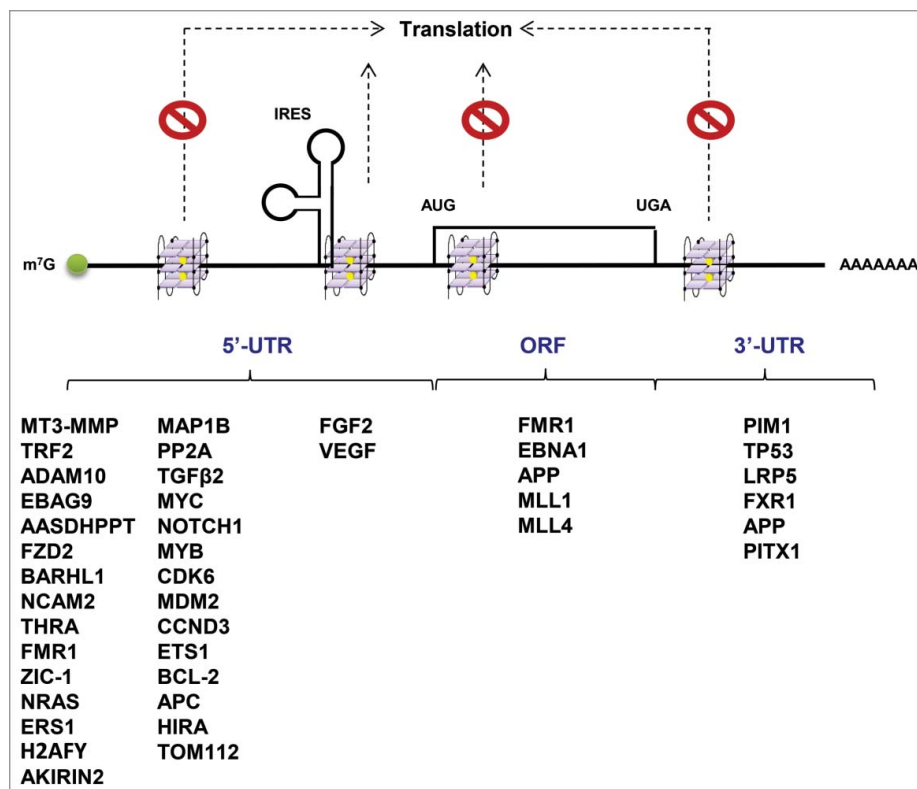


Figure 3. Possible roles of G-quadruplexes in mRNA translation and mRNAs that harbor G4 structures. G-quadruplexes in 5'-UTRs, ORF and 3'-UTRs mainly repress cap-dependent translation, whereas G-quadruplexes in 5'-UTR near IRESs likely enhance the IRES-mediated translation. The genes harboring G4 structures in 5'-UTRs, ORF and 3'-UTRs are listed below.

silvestrol, hippuristanol and pateamine A exhibit potent anticancer activity.^{74,75} By using silvestrol in murine T-cell acute lymphoblastic leukemia (T-ALL) models and primary human T-ALL samples, Wolf et al. observed that eIF4A promotes the T-ALL development and maintenance by unwinding the G4 structures and stimulating translation of mRNAs encoding oncogenes, superenhancers-associated transcription factors and epigenetic regulators including *MYC*, *NOTCH1*, *MYB*, *CDK6*, *MDM2*, *CCND3*, *ETS1*, and *BCL-2* (Figs. 3, 4B).⁷⁶ It was, however, suggested that motifs other than 5'-UTR G4 structures may be required to render mRNA translation sensitive to eIF4A.⁷⁷ To this end, mRNAs with long, but not short 5'-UTRs, appear to exhibit eIF4A-sensitivity, thereby suggesting that the length of 5'-UTR may also determine eIF4A requirement.^{78,79}

G4 structures in 5'-UTRs also influence cap-independent, IRES-driven-translation. The IRES within the 5'-UTR of the *FGF2* mRNA forms a G4 structure affecting cap-independent translation.⁸⁰ Deletion analysis in human liver adenocarcinoma cells showed that the pG4 sequences are sufficient to facilitate IRES

activity.⁸¹ Another example was shown by Morris et al. (2010) who reported that the *hVEGF* (human vascular endothelial growth factor) mRNA forms a G4 structure essential for IRES-mediated translation.⁸² Interestingly, it was also shown that the stabilization of the G4 structure leads to inhibition of IRES-mediated translation of VEGF-A.⁸³ These findings show that G4 structures may influence IRES-mediated cap-independent translation, although the mechanism on how this is achieved is unclear.⁸⁴

G-quadruplexes in open reading frames and translational control

The role of G4 structures in translational control has been focused mainly on G4 sequences in the 5'-UTRs. However, ORFs also contain G4 sequences and these sequences frequently encode low complexity amino acid sequences, amino acid repeats or short motifs.⁵² While G-quadruplexes in 5'-UTRs decreases protein expression by inhibiting ribosome scanning processes, G4 structures in ORFs contribute to other translation-related processes, such as elongation,⁸⁵ ribosomal

frameshift,⁸⁶ no-go mRNA decay^{87,88} and translational folding of newly synthesized proteins.⁸⁹⁻⁹¹ Originally, it was demonstrated that the Herpes virus thymidine kinase ORF contains pG4 sequences, leading to the expression of full-length thymidine kinase.⁹² Subsequently, the ORFs containing G-quadruplexes were found in *FMR1* and *APP* (amyloid precursor protein) mRNAs.^{93,94} Murat et al. (2014) revealed that the mRNA encoding Epstein-Barr virus-encoded nuclear antigen 1 (EBNA1) forms a G4 in its ORF and using antisense oligonucleotides to G4 sequences to destabilize G4 formation, they observed that the structure impairs translation elongation (Fig. 3).⁹⁵ Recently, a bioinformatics analysis identified 2 mixed lineage leukemia (MLL) proto-oncogenes *KMT2A* (lysine methyltransferase 2A or mixed lineage leukemia, *MLL1*) and *KMT2B* (*MLL4*) to harbor pG4 sequences in their ORFs ~200 nucleotides downstream of the start codon (Fig. 3).⁵² By performing *in-line* probing analysis with G-A mutagenesis and the dual luciferase reporter assay, it was confirmed that *MLL1* and *MLL4* mRNAs form *bona-fide* G4 structures that block translation by >75%.⁵² Endoh & Sugimoto (2016) observed that the positioning of the G4 in ORFs, but not in 5'-UTR has a dramatic impact on translational efficiency.⁹⁶ These results suggest that by acting as 'roadblocks', G-quadruplexes in ORFs may play a significant role in protein synthesis by inhibiting ribosomal progression during elongation.

G-quadruplexes in 3'-UTR and translational control

3' end processing is an essential process in post-transcriptional regulation through which the mRNA is endonucleolytically cleaved and a poly (A) tail is added downstream of a canonical polyadenylation signal (AAUAAA).^{97,98} G-quadruplexes are located in the 3'-UTR of some mRNAs and their presence affects translational output. The 3'-UTR of the proto-oncogene *PIM1* harbors a conserved pG4 sequence (Fig. 3) and Arora & Sues (2011) showed using reporter assays that this pG4 sequence inhibits translation.⁹⁹ A pG4 structure in *TP53* 3'-UTR maintains the 3'-end processing under DNA damage and the G4 formation is critical for p53 protein expression contributing to p53-induced apoptosis.¹⁰⁰ More recently, a study combining *in silico*, *in vitro* and *in cellulo* approach, demonstrated that *LRP5* and *FXR1* mRNAs form G4 structures in their 3'-UTR, affecting the ratio of short/

long isoforms produced (Fig. 3).⁵⁰ Moreover, via a bioinformatic approach and CD spectrophotometry, Crenshaw et al. (2015) identified a candidate G-quadruplex in the 3'-UTR of *APP* mRNA, whose overexpression leads to Alzheimer disease. The authors also showed that this G-quadruplex inhibits APP protein expression by dual luciferase reporter assay (Fig. 3).¹⁰¹

In addition to translational control, G-quadruplexes in the 3'-UTR also play a role in alternative splicing, polyadenylation and mRNA targeting. For instance, G4 structures in the 3'-UTR of *TP53* and *hTERT* mRNAs regulate alternative splicing.^{102,103} G-quadruplex in the intron 6 of *hTERT* mRNA appears to act as a splicing silencer, inasmuch as a potent G-quadruplex-stabilizing agent impaired the splicing of *hTERT*.¹⁰² Similarly, Marcel et al. (2011) reported that the G-quadruplex in intron 3 of *TP53* mRNA modulates the splicing of intron 2.¹⁰³ Additionally, G4 structures within the *LRP5* and *FXR1* 3'-UTRs increase the efficiency of alternative polyadenylation site selection, leading to the expression of shorter transcripts.⁵⁰ Finally, G4 structures in 3'-UTR of 2 dendritic mRNAs *PSD95* and *CaMKIIa* facilitate their localization in neurites.¹⁰⁴

G-quadruplexes and trans-acting proteins

Many cellular RNAs associate with RNA-binding proteins to form ribonucleoprotein (RNP) complexes.^{105,106} A large number of RNA binding proteins contain RGG/RG motif¹⁰⁷ or the related RGG/YGG motif.¹⁰⁸ The RGG/RG motifs of nucleolin and fragile X mental retardation protein (FMRP) have been shown to associate with RNA G4 forming sequences.^{109,110} Some RNA G-quadruplex binding proteins are required for the unwinding of G4 structures during translation progression.¹¹¹ The *trans*-acting factors that bind G4 sequences and their links with diseases are discussed below.

Nucleolin

Nucleolin is a 100kDa nucleolar phosphoprotein that contains an RGG/RG motif.¹⁰⁹ Nucleolin interacts with both DNA and RNA G-rich sequences and plays a role in translational repression of specific mRNAs.^{107,112,113} *C9orf72*, a disease-related gene, harbors the expansion of a (GGGGCC)_n(GGCCCC)_n repeats within the first intron, leading to amyotrophic lateral sclerosis and frontotemporal dementia.^{114,115} It

has been reported that the DNA/RNA hexanucleotide repeat expansion (HRE), $(GGGGCC)_n$, forms extremely stable G-quadruplex structures and this may play a role in *C9orf72* gene activity, protein binding as well as translation into pathologic dipeptides.^{116,117} It was also elucidated that the DNA of *C9orf72* HRE forms antiparallel- and parallel-G-quadruplexes, while the HRE RNA sequence only adopts the parallel-stranded G4s. Nucleolin preferentially binds the HRE G-quadruplexes, which is mislocalized in patient cells carrying the *C9orf72* mutation.¹¹⁷ These studies show DNA/RNA G4 sequences bound by nucleolin as a determinant of repeat-associated neurodegenerative diseases.

FMRP

Fragile X syndrome (FXS) is caused by expansion of $(CGG)_n$ repeats in the *FMR1* gene, and subsequent hypermethylation of the *FMR1* gene promoter, leading to the loss of FMRP expression.^{118,119} FXS is recognized to be the most-frequent heritable syndrome of mental insufficiency.¹²⁰ This implicates the role of RNA G-quadruplexes in neuronal function.¹²⁰ In addition to binding to the G4s, the RGG/RG motif of FMRP also modulates its association with polysomes,¹²¹ being consistent with its role in translational regulation.^{120,122} Originally, it was demonstrated that 432 mRNAs were co-immunoprecipitated with the FMRP RNP complex from mouse brain, nearly 70% of which contain a G4 sequence. Dramatic changes in polysome-association of these mRNAs were observed in the absence of FMRP, indicating that translational dysregulation of these mRNAs may underpin FXS.¹²³ Recently, this was redefined by large-scale CLIP (crosslinking immunoprecipitation) studies, where 34,218 consensus FMRP binding sites in 3,703 genes were identified.¹²⁴ It was reported that a G4 sequence in the *FMR1* mRNA provides the docking site for FMRP. Luciferase reporter assays demonstrated that the binding of FMRP to a G4 sequence in 5'-UTR of a reporter gene strongly represses translation initiation *in vitro*.⁹³ Strikingly, *FMR1* G4 structures are bound by several FMRP isoforms, thereby suggesting the presence of a feedback mechanism regulating different FMRP isoform production.¹²⁵ Additionally, *MAP1B*, *PP2A* and *Shank1* genes that are essential for neural development have been shown to harbor one or more

G-quadruplexes in their 5'-UTRs and/or 3'-UTRs, whereby the absence of FMRP increases production of the corresponding proteins, indicating that FMRP plays pivotal roles in neonatal brain development (Fig. 3).¹²⁶⁻¹²⁸ Furthermore, to explore the functions of FMRP in translational control in neurons, Napoli et al. (2008) found that FMRP inhibits cap-dependent translation by recruiting and/or stabilizing CYFIP1 in synaptoneuroosomes. As a novel eIF4E binding protein, CYFIP1 binds eIF4E independently of other factors to impede cap-dependent translation of many mRNAs (Fig. 4A). FMRP is dephosphorylated in response to synaptic activation, resulting in the release from mRNAs encoding synaptic proteins and their derepression.^{129,130} In contrast, Bechara et al. (2009) reported a novel role of FMRP in translation activation. They observed that FMRP binds *SOD1* mRNA through a motif called SoSLIP (*SOD1* mRNA Stem Loops Interacting with FMRP), which competes with FMRP binding to G4 structures favoring translation.¹³¹ The solution structure of FMRP in complex with the *in vitro* selected G-rich RNA sequence, sc1 RNA, revealed that arginines within the RGG/RG motif of FMRP are positioned in the major groove of the G4 structure.¹³² Recently, X-ray crystallography analysis demonstrated that an RGG peptide of human FMRP was able to bind to the selected G-rich RNA *in vitro*.¹³³ The RGG peptide was shown to stabilize G-quartets and facilitate G4 formation. It was also revealed that the specific binding of these RNAs with FMRP likely also involves the hydrogen binding with RNA duplexes, shown by mutagenesis and footprinting.¹³³ High throughput sequencing of RNAs collected by HITS-CLIP (high-throughput sequencing-crosslinking immunoprecipitation) identified that FMRP also interacts with ORFs and stalls ribosomes on mRNA encoding pre-synaptic and postsynaptic proteins implicated in autism spectrum disorders (ASD) (Fig. 4A).¹³⁴ Moreover, FMRP was shown to repress translation by direct binding to the L5 protein on the 80S ribosome,¹³⁵ and this activity is depended on the integrity of the RGG/RG motif.¹³⁶

RHAU (DHX36)

RHAU, the RNA helicase associated with AU-rich elements, also known as DHX36, is a member of ATP-dependent DEAH-box RNA helicase family.^{137,138}

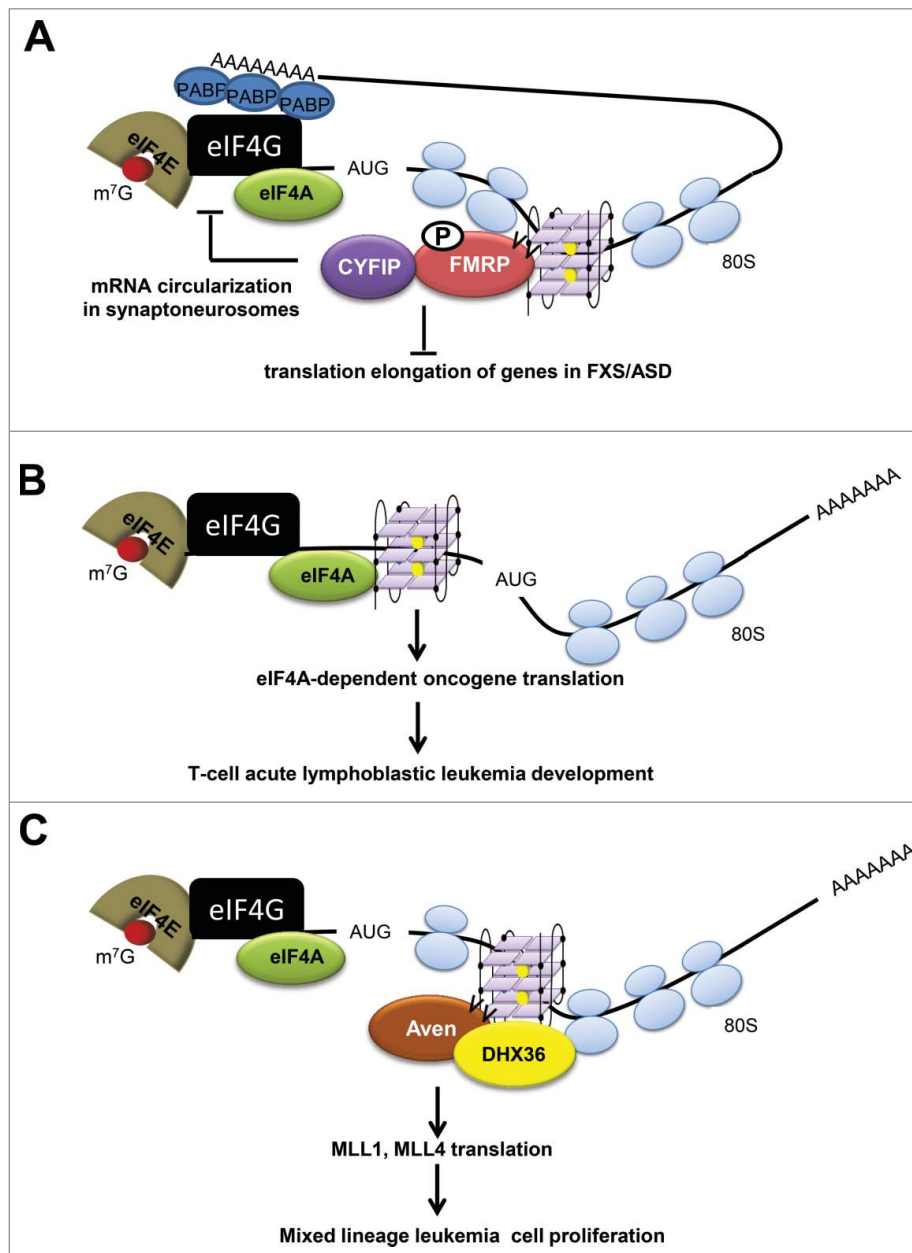


Figure 4. Schematic illustration of the functions of RNA binding proteins that bind RNA G4 structures in mRNAs. (A) Phosphorylated FMRP binds ORFs of mRNAs and inhibits translation. It stalls ribosomes in the elongation stage, resulting in the repressed translation of transcripts related to FXS/ASD. It recruits the co-factor CYFIP in synaptoneuroosomes. By interacting with CYFIP, FMRP prevents the complex assembly between eIF4E, eIF4G and PABP, thereby inhibiting translation initiation. RGG/RG motif is denoted as **vv**. (B) eIF4A unwinds the G4 structures in 5'-UTR of many key transcription factors and oncogenes, thereby contributing to the T cell-acute lymphoblastic leukemia development. (C) Methylated Aven binds G4 structures in the ORFs of MLL1 and MLL4 mRNAs in an RGG/RG motif (denoted as **vv**) dependent manner. Aven also recruits DHX36 onto the polysomes that may facilitate unwinding of G4 structures. Thus Aven favors the translation of oncogenic proteins to increase leukemic cell proliferation.

DHX36 has been shown to be the predominant G-quadruplex resolving helicase *in cellulo*, with high activity in unwinding RNA G4 structures.¹³⁹⁻¹⁴¹ The solution structure of DHX36 recognizing a G-quadruplex was resolved and identified a 3-anchor-point electrostatic interaction.¹⁴² It was also elucidated that DHX36 uses a local, non-processive mechanism to

unwind G4 structures, mimicking the DEAD-box RNA helicase eIF4A.³⁹ By applying genome-wide analysis, DHX36 was shown to bind ~106 RNAs, the majority harboring pG4 sequences including the human telomerase RNA.^{143,144} Sexton et al. (2011) also discovered the association between DHX36 and *TERC* (*hTR*) pG4 sequences in the HEK293 cells.¹⁴⁵

Another study confirmed that the interaction between DHX36 and the *TERC* G-quadruplex disrupts the formation of P1 helix, a structure which defines template boundary for reverse transcription. DHX36 was sufficient to unwind the quadruplexes and promote the formation of a stable P1 helix, and DHX36 depletion led to a reduction in average telomere length.^{138,141} Besides the function on telomere maintenance, DHX36 regulates translation, although the precise mechanism has not yet been elucidated. By performing a RNA-co-immunoprecipitation screen, Booy et al. (2014) discovered that DHX36 interacts with G-quadruplexes in 3'-UTR of *PITX1* mRNA *in cellulo*, and depletion of DHX36 results in increased *PITX1* expression, a transcription factor with roles in development and cancer.¹⁴⁶ Finally, it was also demonstrated that the DHX36-mediated regulation of *PITX1* involves miRNA regulatory components.¹⁴⁶ Moreover, the impact of *Aven* on mRNA translation regulation is dependent on DHX36 (see below).

Aven

Aven is an anti-apoptotic protein first shown to interact and stabilize the pro-survival factor Bcl-xl, while inhibiting Apaf-1, thereby stimulating survival in particular when the cells are exposed to stress.¹⁴⁷ *Aven* is overexpressed in acute myeloid and acute lymphoblastic leukemia where its expression has been associated with poor prognosis.^{148,149} Recently, *Aven* was shown to bind RNAs with G4 structures via its RGG/RG motif.⁵² The RGG/RG motif of *Aven* is arginine methylated by PRMT1 and this promotes association with the methylarginine interactors SMN and TDRD3. The arginine methylation and binding to SMN and TDRD3 is required for association of *Aven* with polysomes. *In vitro* binding and photocrosslinking immunoprecipitation assays revealed that *Aven* associates with *MLL1* and *MLL4* mRNAs. Interestingly, the G4 sequences bound by *Aven* were in the coding regions regulating their mRNA translation (Fig. 4C). Mechanistically, *Aven* recruits DHX36 onto the polysomes likely to facilitate unwinding of the G4 structures during translation. Depletion of *Aven*/DHX36 inhibits *KMT2A/KMT2B* translation, whereas luciferase assays with G-to-A mutagenesis revealed that both *Aven* and DHX36 are required to rescue translation in the presence of G4 motifs.⁵² Thus, *Aven* represents a G4 interacting protein that recruits DHX36 to unwind G4

structures containing mRNAs during translational elongation. These findings foster our understanding that RNA G4 binding proteins can play a key role in modulating mRNA translation.

Conclusions and perspectives

Increasing evidence shows that G-quadruplexes play essential roles in RNA metabolism. Advances in computational analyses and genome-wide sequencing have facilitated the characterization of RNA quadruplexes and their implication in translational control. Further advancements are required to confirm their occurrence *in vivo*. Moreover, the *trans*-acting factors that bind G4 structures are the key to understanding the role of G-quadruplexes in the regulation of mRNA fate. Nucleolin, FMRP, DHX36 and *Aven* represent the tip of the iceberg of *trans*-acting factors that recognize G4 RNA structures. Large scale analyses including HITS-CLIP-seq, SHAPE-seq, and HITS-RAP (high-throughput RNA affinity profiling) hold great promise for knowledge advancement as to the role of G4 structures and interacting proteins *in vivo*.¹⁵⁰⁻¹⁵⁴ It is possible that multiple RNA binding proteins recognize one G-quadruplex, and there could be functional interplay. The binding of different RNA binding proteins are likely to: 1) stabilize the G4 structures, 2) unwind the G4s by recruiting helicases, 3) act as chaperones to transport G4 containing mRNAs, and 4) serve as scaffold proteins to recruit other proteins or RNAs.

A growing number of disease-related genes are regulated by G4 structures and their RNA binding proteins. This includes mRNAs encoding the tumor suppressor *TP53*,¹⁰⁰ oncogene *NRAS*,^{47,53} oncogenes *KMT2A/KMT2B*,⁵² anti-apoptotic *Bcl-2*,^{67,76} *FMR1*,^{125,155} and the telomerase *hTERT*.^{102,156} This indicates that by affecting mRNA translation, splicing, and polyadenylation, G4 structures may be implicated in numerous human diseases, especially cancer. Thus, targeting G4 structures with synthesized small molecules is attractive to modify oncogene expression. Extensive studies have evaluated some “drug-like” molecules including the pyridostatins (PDS),¹⁵⁷ cationic porphyrins and derivatives¹⁵⁸⁻¹⁶⁰ and bisquinolinium compounds.⁶⁸ Short antisense oligonucleotides affect RNA G4 folding and translation regulation of specific mRNAs⁷² and may eventually be of therapeutic potential. A new polyaromatic molecule, RGB-1, was recently shown to

specifically stabilize RNA G-quadruplexes, but not DNA G-quadruplexes or other RNA structures. RGB-1 inhibits translation in mammalian cells and decreases NRAS expression, providing a new tool to understand G4 structures and therapeutic applications.¹⁶¹ Thus, a better understanding of the various contributions of G4 structures in the human transcriptome may provide important insights into strategies targeting G4s in molecular medicine.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This manuscript was funded by a grant from the Canadian Institute of Health Canada (CIHR) MOP-67070 to S.R.

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