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# CGG Repeat RNAs Regulate Granule Translation

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# CGG Repeat RNAs Regulate Granule Translation

René N Rovozzo, PhD

University of Connecticut, 2017

The fragile X family of disorders is associated with CGG repeat expansions in the 5'UTR of the FMR1 gene on the X chromosome. The full mutation (~200 CGG repeats) causes hypermethylation of the DNA that results in silencing of the FMR1 transcript and Fragile X Syndrome (FXS), a protein loss of function mechanism that results in early onset mental retardation. The premutation (~55-200 CGG repeats) is associated with Fragile X Tremor Ataxia Syndrome (FXTAS), a late onset neurodegenerative disorder, and Fragile X primary ovarian insufficiency (FXPOI), causing early menopause from reduced estrogen levels. The purpose of this thesis is to determine if FXTAS and FXPOI are protein loss of function disorders or RNA gain of function disorders.

RNAs containing an hnRNP A2 response element (A2RE) are localized and transported in discrete ribonucleoprotein particles called hnRNP A2 granules that are organized by a series of multivalent interactions. We show that RNAs containing CGG repeat sequences are also localized in hnRNP A2 granules. Additionally, exogenous premutation CGG repeat RNA microinjected in mouse hippocampal neurons or mouse oocytes inhibits granule RNA translation. Endogenous premutation CGG repeats in the FMR1 gene in human fibroblast cells from females at risk for FXPOI also inhibit granule RNA translation. We suggest that CGG repeat RNAs encoding calcium regulatory proteins may be in RNA granules and show that CGG repeat RNAs in premutation human fibroblast cells affect calcium homeostasis which may contribute to FXTAS and FXPOI pathogenesis. Additionally, premutation CGG repeat RNA containing the 5'UTR, a small part of the ORF and part of intron 1 from FMR1 in the transgenic TG296 mouse does not inhibit granule RNA translation. We interpret these results to show that FXTAS and FXPOI are granule based phenomena and RNA gain of function disorders. We

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suggest two mechanisms for RNA gain of function mechanisms, sequestration of translational machinery, supported by ribosomal profile data and activation of the double stranded binding protein, protein kinase R (PKR). Differences in the magnitude of translational inhibition by CGG repeat RNA in neurons, fibroblasts and oocytes suggests that activation of PKR is most likely the pathogenic mechanism for FXTAS and FXPOI.

# **CGG Repeat RNAs Regulate Granule Translation**

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B.S., Cedar Crest College, 2010

A Dissertation

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Requirements for the Degree of Doctor of Philosophy

at the

University of Connecticut

2017

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# APPROVAL PAGE

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**Abbreviations used in text:**

RNA	ribonucleic acid
MBP	myelin basic protein
CAMK2A	calcium, calmodulin-dependent protein kinase 2A
NRGN	neurogranin
ARC	activity regulated cytoskeletal associated protein
A2RE	hnRNP A2 response element
hnRNP A2	heterogeneous nuclear ribonucleoprotein A2
TOG	tumor overexpressed gene
FCS	fluorescence correlation spectroscopy
SPR	surface plasmon resonance
DNA	deoxyribonucleic acid
LTP	long term potentiation
AMPA	$\alpha$ -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
mGluR	metabotropic glutamate receptor
AMPA	AMPA receptor
LTD	long term depression
P granules	polar granules
P bodies	processing bodies
RNP	ribonucleoprotein particle
EIF	eukaryotic initiation factor
5'-UTR	5' untranslated region
ORF	open reading frame
GTP	guanosine-5'-triphosphate
mRNA	messenger RNA
EIF2AK2	eukaryotic initiation factor 2 alpha kinase 2
PKR	protein kinase R
FXTAS	fragile X tremor ataxia syndrome
RAN	repeat associated non-ATG
SCA8	spinocerebellar ataxia type 8

DM1	myotonic dystrophy type 1
FMR1	fragile X mental retardation 1
ALS	amyotrophic lateral sclerosis
FTD	frontotemporal dementia
FXPOI	fragile X primary ovarian insufficiency
FXS	fragile X syndrome
POI	primary ovarian insufficiency
FSH	follicle stimulating hormone
LH	luteinizing hormone
KH domain	K homology domain
FMRP	fragile X mental retardation protein
TMPyP4	Tetra(4-N-methyl-pyridyl)porphyrin
CGG KI	CGG repeat knock-in
NSD	non-stop decay
DIC	differential interference contrast
dsRBD	double stranded RNA binding domain
PACT	protein activator of the interferon induced protein kinase
TRBP	transactivation responsive RNA binding protein
dsRNA	double stranded RNA
IFN	interferon
CACNA1A	voltage dependent calcium channel
KCNMA1	calcium activated potassium channel



**Chapter1:**  
**Introduction**

## RNA localization

RNA localization is a specialized method for obtaining distinct gene expression patterns in polarized cells such as neurons and oligodendrocytes (1). *In situ* hybridization of RNA localization in *Drosophila* during early development reveals that more than 70% of transcripts exhibit characteristic localization patterns, suggesting that most RNAs are actually localized and that RNA localization is necessary for local protein expression and function (2). In fact, localization of mRNAs has been observed in multiple cell types providing functional advantages. For example, *bicoid* RNA is localized to the anterior pole in developing *Drosophila* oocytes and is necessary for patterning of the head and thorax (3). *Oskar* RNA is localized to the posterior pole of the *Drosophila* oocyte during formation of the germ plasm. *Oskar* protein localization directs localization of a second RNA, *nanos*, which is necessary for proper abdominal patterning (4). Mammalian cells exhibit RNA localization as well. *Actin* RNA is concentrated at the lamellopodia of skeletal myoblasts and fibroblasts from chick embryos and correlates with localized actin protein expression (5). Also, *myelin basic protein* (MBP) RNA is localized to the peripheral processes in cultured mouse oligodendrocytes and in vivo and correlates with myelin formation (6, 7). Understanding the mechanism for RNA localization is critical to understanding localized translation and localized protein function.

RNA localization occurs via trans-acting protein-RNA interactions driven by RNA localization elements. One particular localization element, an eleven nucleotide sequence known as the hnRNP A2 Response Element (A2RE) (GCCAAGGAGCC), first identified as a localization signal in MBP RNA (7), drives dendritic localization of several different RNAs in neurons and oligodendrocytes. Similar sequences have been identified in RNAs encoding proteins involved in synaptic plasticity such as calcium, calmodulin-dependent protein kinase 2A (CAMK2A), neurogranin (NRGN) and activity regulated cytoskeletal associated (ARC) protein, (8). While the

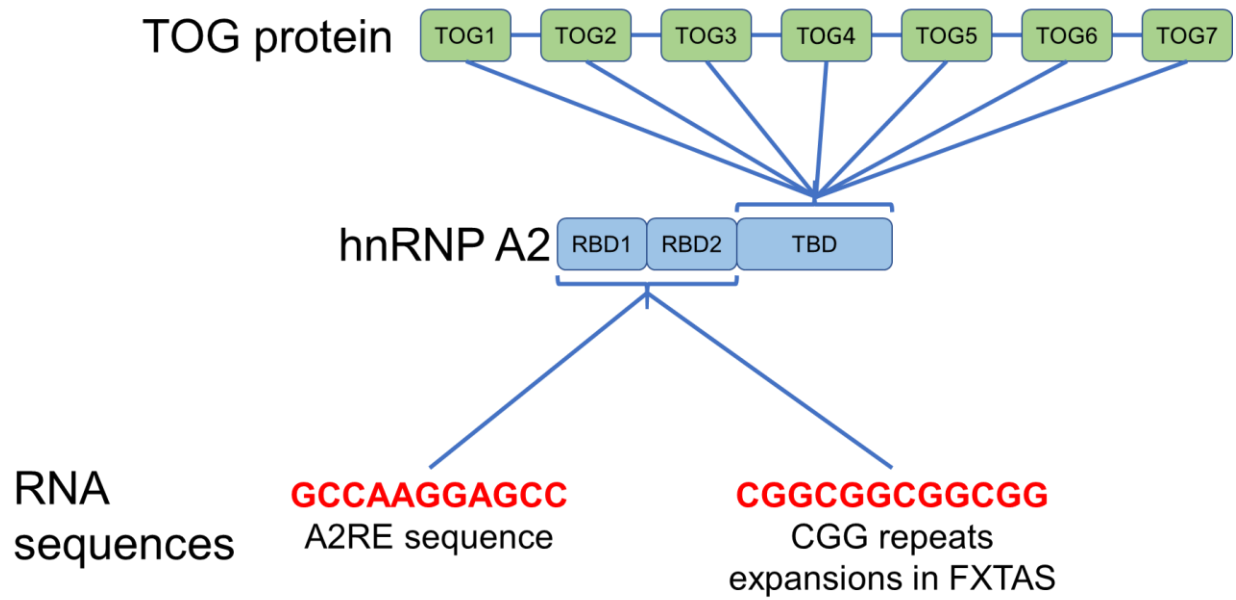
A2RE and signals like it are necessary for localization of specific RNAs, other interactions are also required for RNA localization.

### **hnRNP A2 RNA granules**

Localization signals are not sufficient for local translation. In addition, a series of multivalent molecular interactions are necessary to organize the RNA molecules with trans-acting proteins into units called RNA granules (9), which move via molecular motors to sites of local translation (10). Affinity purification of the binding partners for the A2RE, followed by protein sequencing and mass spectrometry identified heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2) as the major protein that binds the A2RE (11). In turn, yeast-two-hybrid screening identified a binding partner for hnRNP A2 known as tumor overexpressed gene (TOG) (12). Fluorescence correlation spectroscopy (FCS) and surface plasmon resonance (SPR) indicated that TOG protein contains seven  $\alpha$ -helical hnRNP A2 binding domains, suggesting that TOG may serve as a multivalent scaffold for hnRNP A2 binding during granule assembly (13) (Figure 1).

hnRNP A2, found in abundance in both the nucleus and cytoplasm, is a member of the hnRNP family of proteins that are found in the RNA splicing complex, and are believed to be involved in selection of the RNA splice site depending on the abundance of the protein (14). hnRNP A2 is also believed to function as an adapter between telomeric DNA and telomerase, thus protecting DNA from degradation (15). TOG is a microtubule binding protein required for proper spindle pole formation during mitosis (16). Additionally, TOG is necessary for organization of microtubule minus ends at spindle poles, centrosome integrity and bipolarity of spindle poles (17).

TOG is colocalized with A2RE RNA and hnRNP A2 in RNA granules (12). This suggests that multivalent interactions of TOG characterized *in vitro* mediate RNA granule assembly *in vivo*. In



**Figure 1: hnRNP A2 RNA granules consist of multivalent interactions.** TOG protein can act as a scaffold for hnRNP A2 binding each of its seven binding domains. hnRNP A2 also has two RNA binding domains. The A2RE sequence and CGG repeats can bind to these domains. These are the interactions that form hnRNP A2 RNA granules.

addition to the key components, A2RE RNA, hnRNP A2 and TOG, hnRNP A2 RNA granules also contain many translational components, including Arginyl-tRNA synthetase, elongation factor 1a and ribosomal RNA (18). Single molecule imaging indicates that hnRNP A2 RNA granules serve as sites for translation in dendrites and at synapses (19).

As previously mentioned, A2RE RNAs that are associated with synaptic plasticity are found in hnRNP A2 RNA granules. In particular, I will focus on the immediate early gene ARC, which is involved in regulation of actin dynamics, promoting long term potentiation (LTP) consolidation (20) and also binds to dynamin and endophilin to mediate endocytosis of AMPA receptors during LTD (21). Interaction of ARC with the cytoskeleton makes it a good reporter for local translation because newly synthesized ARC is anchored to the cytoskeleton in the vicinity of the site of translation. ARC is also involved in metabotropic glutamate receptor (mGluR) dependent Long Term Depression (LTD) by interacting with components of the endocytic machinery, dynamin and endophilin 2/3, resulting in internalization of AMPA receptors (AMPA receptors) (20).

However, A2RE RNAs are not the only RNAs that bind hnRNP A2 and are therefore localized to hnRNP A2 RNA granules. CGG repeat containing RNAs also bind to hnRNP A2 (22, 23) (Figure 1). Less than a dozen different A2RE RNAs have been identified, but there are more than 200 different CGG repeat containing RNAs in the human exome (24), which suggests that CGG repeat RNAs may be the predominant RNAs in hnRNP A2 RNA granules. In chapter 2, we will show that some granules have up to ten fold more CGG RNA than ARC RNA, a reporter for A2RE RNAs in hnRNP A2 RNA granules.

### **Other types of RNA granules**

hnRNP A2 RNA granules are one of many categories of RNA granules. Understanding the mechanisms and functions of hnRNP A2 RNA granules aids in understanding other RNA transport granules and perhaps even other classes of granules. As previously mentioned, 70%

of RNAs in *Drosophila* embryos exhibit characteristic granule-like localization patterns (2). Approximately 35 discrete RNA localization patterns have been identified, which may correspond to types of RNA granules. Each type of RNA granule presumably contains RNAs with characteristic cis-acting localization sequences, which bind to cognate RNA binding proteins, which in turn associate with cognate multivalent scaffold proteins to form the RNA granule (9). Different types of RNA granules may have different functions. In addition to RNA transport granules, three other major types of RNA granules have been identified in living cells, polar granules (P granules), processing bodies (P bodies) and stress granules. Investigating granule assembly, localization and function of A2 granules may be relevant to understanding assembly, localization and function of other granule types.

P granules are ribonucleoprotein particles (RNPs) that contain maternal RNAs that specify germ cell differentiation (25). The function of P granules is to regulate timing of translation of maternal RNAs to control the development of germ cells and establish the germ line (25). P granules have been identified in germ cells from *Myastor metraloas*, *Xenopus laevis*, *Drosophila melanogaster* and *Caenorhabditis elegans* (25).

P bodies, sometimes confused with P granules, are somatic cell granules containing components of the 5'→3' mRNA decay machinery, the nonsense-mediated decay pathway, and the RNA-induced silencing complex (25). Assembly of P bodies is increased in response to cellular stress, and is mediated by the colocalization of translationally silent RNAs with decapping machinery (26). Although the function of P bodies is debatable, the local concentration of translationally silent RNAs is increased in P bodies, which may facilitate their function (26).

Stress granules are functional RNPs that decrease translation of housekeeping genes but increase translation of molecular chaperones and enzymes involved in repair in response to

environmental stress (25). The key component of stress granule assembly is eukaryotic initiation factor 2 $\alpha$  (EIF2 $\alpha$ ) which is phosphorylated during environmental stress (25). This prevents formation of the EIF2–GTP–tRNA<sup>Met</sup> ternary complex, inhibiting AUG initiated translation and promoting polysome disassembly (25). It is believed that some RNAs shuttle between stress granules and P bodies suggesting that these are functionally distinct types of granules (26).

### **Eukaryotic translation**

One of the most important cellular processes that occurs in hnRNP A2 RNA granules is translation. Translation is the process of producing peptides or proteins from an mRNA sequence. It is believed that this process initiates with ribosome scanning in which the 40S subunit of a ribosome carrying a Met-tRNA<sup>Met</sup> and initiation factors binds to the 5' end of an mRNA and scans in a 5'-3' direction until encountering the first AUG (start codon) in an appropriate context (27). The scanning complex is known as the 43S preinitiation complex, and consists of EIF2, which binds to the Met-tRNA<sup>Met</sup> and GTP, EIF1A, which is involved in transfer of EIF2 to the 40S subunit, EIF5, which activates the GTPase activity of EIF2, EIF1, which is involved in recognition of the start codon and EIF3 which prevents elongation (28). In the nucleus, mRNAs are capped with a m<sup>7</sup>GpppN structure (29) that is believed to recruit the 40S ribosomal subunit (28). EIF4E binds to the 5' cap (30). EIF4G then forms a bridge between EIF4E, EIF3 on the 40S ribosomal subunit (31) and EIF4A (32) which likely has helicase activity to unwind secondary structure in the 5'-untranslated region (5'-UTR) during scanning (33). Upon recruitment of the 43S preinitiation complex, the 48S initiation complex forms and scans the mRNA until finding the first AUG, followed by the processes of initiation, elongation and termination.

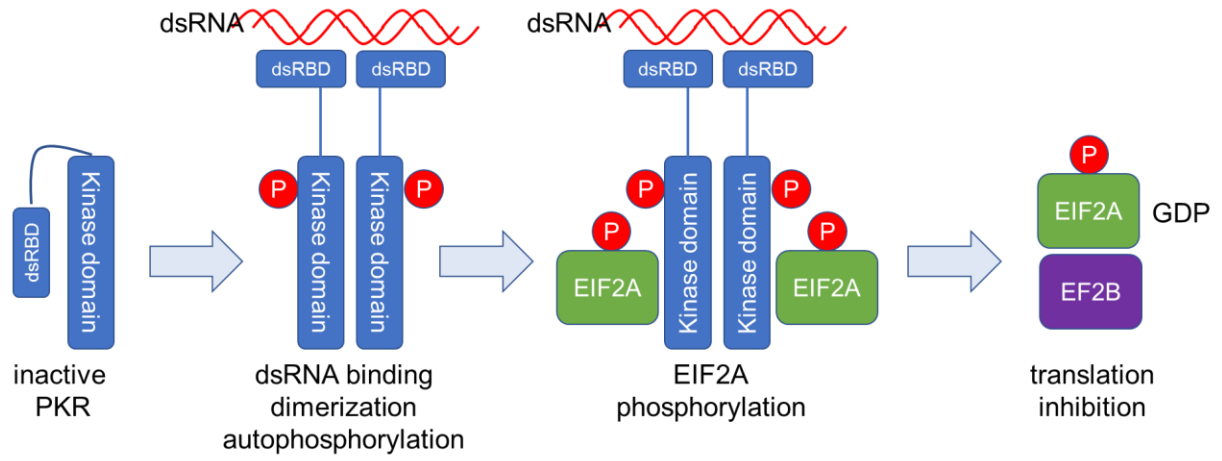


## RNA secondary structure and translation

During translation, translocation of the ribosome along the mRNA is dependent on the mRNA helicase activity of the ribosome, which is believed to unwind secondary structures in the mRNA (34). As a result of this process, the ribosome undergoes a series of translocation-pause-translocation steps that are dependent on the RNA hairpin structure (35, 36). Highly structured mRNA molecules have decreased translation rates (35, 36). Additionally, G-quadruplex structures in either the 5'-UTR or the open reading frame (ORF) of mRNA in human cells decreases cellular translation of transfected mRNA (37). Trinucleotide repeat sequences, which are found in certain RNAs in hnRNP A2 RNA granules can form secondary structures and quadruplex structures, suggesting that they may regulate granule RNA translation.

hnRNP A2 RNA granule RNA translation may also be regulated by translation factors encoded by granule RNAs. These include EIF2 $\alpha$  RNA containing an A2RE sequence and multiple other translation factor RNAs containing CGG repeats. Eukaryotic translation initiation factor 2 alpha kinase 2, EIF2AK2, also known as protein kinase R (PKR), contains CGG repeats in the 5'-UTR which bind to hnRNP A2 and mediate localization in A2 RNA granules (21). PKR is a serine/threonine kinase that autophosphorylates when bound to double stranded RNA (38). CGG repeat sequences in hnRNP A2 granule RNAs can form double stranded secondary structures that can activate PKR. Activated PKR can phosphorylate EIF2 $\alpha$ , which may regulate conventional translation in hnRNP A2 RNA granules (39) (Figure 2).

In CGG repeat expansion disorders, such as fragile X tremor ataxia syndrome (FXTAS), the CGG repeat expansions may form stable CGG repeat secondary structures, which may increase PKR activation. In chapter 2, we will show that introducing one or more CGG repeat RNAs into an hnRNP A2 RNA granule inhibits translation of all other RNAs in that granule in



**Figure 2: PKR activation by double stranded RNA prevents translation.** Inactive PKR is folded in such a way that the double stranded RNA binding domains block autophosphorylation (40). Upon binding to double stranded RNA, autophosphorylation activates PKR and allows it to facilitate phosphorylation of EIF2 $\alpha$  (38). Phosphorylated EIF2 $\alpha$  forms an inactive complex with EIF2B which prevents translation (39).

trans, demonstrating that CGG repeat RNA regulates granule RNA translation. Whether translation in hnRNP A2 RNA granules is regulated directly by CGG repeats or indirectly by activation of a translational regulator, CGG repeat secondary structure may serve as a regulatory element in mRNA translation.

### **Nucleotide repeat expansions**

Trinucleotide repeat regions in RNA molecules can be highly structured. Multiple different neurological disorders, including Huntington disease, spinal and bulbar atrophy and many ataxias are associated with trinucleotide repeats in different genes. A unique characteristic of each of these disorders is that individuals of successive generations have decreased age of onset and increased severity, which is known as “genetic anticipation” (41). Genetic anticipation is associated with increasing expansions of trinucleotide repeats in DNA (41). Approximately 30 human disorders have been identified that are associated with trinucleotide, tetranucleotide, pentanucleotide and dodecanucleotide repeat expansions (41). In most cases increasing repeat number above a threshold of ~100-150 repeats results in destabilization of the repeats, and further expansions become more likely (41).

Two causes for repeat instability in successive generations have been identified, replication-dependent and repair-dependent (42). Replication-dependent instability occurs via DNA polymerase slippage when the nucleotide repeat sequence misaligns, forming an extrahelical loop that bypasses repair (42). On the daughter strand, this results in an expansion, while on the template strand, it results in a deletion (42). Repair-dependent instability can occur in two different ways. First, MutS $\beta$ , a DNA mismatch repair protein, recognizes and binds to slipped DNA, protecting it from repair and thus enhancing replication-dependent instability (43). Second, during base excision repair to remove oxidized guanines, a single stranded break is formed followed by gap filling synthesis by the polymerase (44). This displaces the single strand of the DNA being repaired (44). If the repair occurs in a region with nucleotide repeats, the single

strand may fold back on itself in a hairpin structure. When ligation subsequently occurs this can result in an expansion (44). These mechanisms suggest that any repeat RNA has the potential to expand, although thus far expansions have only been identified in ~ 30 different genes.

The majority of trinucleotide repeats can form secondary structures, with stability based on GC content (45). Trinucleotide repeat motifs can be separated into four structural classes: unstructured, semi-stable hairpin, stable hairpin and quadruplex. For example, CGG and CCG repeats form stable hairpin structures (45) which may impede translocation of the ribosome or interact with translation factors. In chapter 2 we will show that ribosomes accumulate in the vicinity of CGG repeats in FMR1 RNA. The human exome contains 878 trinucleotide repeat containing genes (24), suggesting that secondary structure regulation of translation is a common occurrence in the human exome.

### **RAN translation**

In addition to conventional translation, repeat containing RNAs can undergo an unconventional nonAUG form of translation called repeat associated non-ATG (RAN) translation (46). RAN translation in the absence of an AUG start codon occurs at lower rates than conventional AUG initiated translation and can occur in multiple and bidirectional reading frames (46). RAN translation of CAG repeats in spinocerebellar ataxia type 8 (SCA8) and myotonic dystrophy type 1 (DM1) results in synthesis of polyglutamine, polyalanine and polyserine peptides (48). RAN translation of CGG repeats in fragile X mental retardation 1 (FMR1) RNA in FXTAS, results in polyglycine and polyalanine tracts (47). Potentially, CGG repeats in FMR1 may produce polyarginine tracts as well. RAN translation in C9orf72 RNA in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), results in synthesis of six RAN proteins (antisense: Pro-Arg, Pro-Ala, Gly-Pro; and sense: Gly-Ala, Gly-Arg, Gly-Pro) (48). RAN translation products may contribute to pathogenesis of repeat disorders (46-48).

## **FXTAS, FXPOI and FXS**

CGG repeat expansions in the 5'-UTR of the FMR1 gene are associated with a family of disorders known as Fragile X disorders. In unaffected individuals, the number of CGG repeats in the FMR1 gene is usually less than 55. However, when the repeats are expanded to a premutation level between 55 and 200, individuals are often affected by FXTAS or Fragile X Primary Ovarian Insufficiency (FXPOI). Expansions above 200 usually result in Fragile X Syndrome (FXS) (49).

FXTAS is a late onset neurodegenerative disorder affecting primarily males as the FMR1 gene is located on the X chromosome. The average age of onset for FXTAS is  $61.6 \pm 7.9$  years (50). The disorder is characterized by progressive cerebellar ataxia, intention tremors, Parkinsonism, cognitive decline and sometimes psychiatric disturbances and autonomic and peripheral neuropathy (50). Approximately 1 in 260 females and 1 in 813 males are carriers. Penetrance is 40% in males over the age of 50 while only 8% in females over the age of 40 (50). The disorder is also characterized by cerebellar and cerebral white matter loss that affects males more than females (50).

Primary ovarian insufficiency is a disorder affecting ~1% of the female population under the age of 40 resulting in reproductive problems and early menopause (51). Premutation Fragile X alleles occur in ~7% of sporadic POI and ~21% of familial POI (51). FXPOI individuals have decreased ovarian function that is dependent on the number of CGG repeats (51). The resultant symptoms include decreased numbers of growing follicles in ovaries and altered serum hormone levels, including Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and  $17\beta$ -estradiol (52). Additionally, LH-induced ovulation-related gene expression is altered (52). In chapter 3, we show that translation of Venus-ARC RNA is inhibited in mouse oocytes coinjected with CGG99 RNA, suggesting that translation inhibition in oocytes may contribute to FXPOI pathogenesis.

Fragile X Syndrome is the most common cause of inherited intellectual disability and autism in children (53). It has a frequency of 1:4000 males and 1:6000 females (53). Lack of FMRP, a translation regulator, is believed to stimulate protein expression following mGluR activation resulting in increased AMPAR internalization and LTD (54).

Both FXTAS and FXPOI are considered toxic RNA gain-of-function disorders because the levels of FMR1 transcript are increased while the levels of fragile X mental retardation protein (FMRP) protein are decreased (50, 51). Fragile X Syndrome is considered a protein loss-of-function disorder since hypermethylation of the DNA causes silencing of the transcript and thus absence of the resulting protein (54).

### **FMRP function and targets**

FMRP is a protein that is most commonly associated with Fragile X disorders as described previously, however, it also serves an important regulatory function in the cell. Two K homology domains (KH domains) that recognize and bind RNA were identified in a region of FMRP that is not involved in alternative splicing (55). These KH domains bind stoichiometrically to ~4% of human fetal brain messages (55). Importantly, FMRP also binds to its own message (55). Understanding the function of FMRP is essential since FMRP plays such an integral role in causing Fragile X related disorders. Multiple potential FMRP binding partners have been identified via high throughput microarray analysis (56, 57).

Approximately 70% of FMRP-binding transcripts identified by microarray analysis were found to contain G-quartet structures, suggesting that this may be a target for FMRP binding (57). In addition to two KH domains, FMRP contains a C-terminal RGG box thought to enhance sequence specific binding at the KH domains (58, 59). The RGG box was found to bind specifically to G-quartet motifs (58, 59).

The function of FMRP in relation to its binding to RNAs is unknown. However, FMRP suppresses translation of brain poly(A) RNA in rabbit reticulocyte lysate in a dose dependent manner (60). Specifically, translation of RNAs that bind FMRP is suppressed, while translation of RNAs that do not bind FMRP is not suppressed (60). Additionally, the phosphorylation state of FMRP is important to its function because phosphorylated FMRP tends to associate with stalled polyribosomes while non-phosphorylated FMRP tends to associate with actively translating RNAs (61). Since FMRP phosphorylation state is altered in conjunction with synaptic activity, this may represent a mechanism for translation regulation in RNA granules in response to synaptic activity (61).

### **TMPPyP4**

Tetra(4-N-methyl-pyridyl)porphyrin (TMPPyP4) is a non-toxic, membrane-permeant porphyrin ring compound. TMPPyP4 alters secondary structure of CGG99 RNA and rescues translation of CGG repeat RNA in vitro and in Human Embryonic Kidney 293 cells (62). TMPPyP4 alters the secondary structure in the 5'-UTR of MT3-MMP mRNA and rescues in vitro translation of the RNA (63). These results suggest that TMPPyP4 can be used for investigating the effect of CGG repeat secondary structure on translation. In chapter 2, we show that TMPPyP4 reverses the effects of CGG repeat RNA on translation of ARC RNA in hnRNP A2 RNA granules in rat neurons and in human fibroblast cells.

### **Calcium homeostasis**

Several RNAs encoding proteins associated with calcium homeostasis contain CGG repeats or A2REs and thus are likely found in hnRNP A2 RNA granules (8,24). Since translation of these RNAs may be regulated by CGG repeats in the same granules, it is important to analyze calcium homeostasis as a marker for cellular homeostasis in premutation cells. Cortical astrocytes from CGG KI mice exhibit spontaneous asynchronous calcium oscillations (65). Furthermore, iPSC-derived fragile X premutation neurons, exhibit calcium transients with



increased amplitude and frequency (66). This suggests that calcium signaling and calcium homeostasis may be affected by granule RNA translation in premutation cells. We show in chapter 2 that premutation human fibroblast cells exhibit secondary calcium transients in response to stimulation with Bradykinin.

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## **Chapter2:**

### **CGG repeats in the 5'UTR of FMR1 RNA regulate translation of other**

### **RNAs localized in the same RNA granules.**

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## Translation regulation in RNA granules

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### Abstract

CGG repeats in the 5'UTR of Fragile X Mental Retardation 1 (FMR1) RNA mediate RNA localization and translation in granules. Large expansions of CGG repeats (> 200 repeats) in FMR1, referred to as full mutations, are associated with fragile X syndrome (FXS). Smaller expansions (55-200 repeats), referred to as premutations, are associated with fragile X tremor ataxia syndrome (FXTAS) and fragile X primary ovarian insufficiency (FXPOI). TMPyP4 is a porphyrin ring compound that destabilizes CGG repeat RNA secondary structure. Here we show that exogenous CGG repeat RNA by itself, lacking the FMRP ORF, microinjected into hippocampal neurons is localized in RNA granules and inhibits translation of ARC RNA, which is localized in the same granules. TMPyP4 rescues translation of ARC RNA in granules. We also show that in human premutation fibroblasts with endogenous CGG repeat expansions in the FMR1 gene, translation of ARC RNA is inhibited and calcium homeostasis is disrupted and both phenotypes are rescued by TMPyP4. Inhibition of granule RNA translation by expanded CGG repeats and rescue of granule RNA translation by TMPyP4, represent potential pathogenic mechanism and therapeutic strategy, respectively, for FXTAS and FXPOI.

## Introduction

CGG repeat sequences have been identified in >200 different RNAs in the human exome (1). In some cases expansion of CGG repeats is associated with neurological or neuromuscular disorders (2). For example, the Fragile X Mental Retardation 1 (FMR1) gene, encoding fragile X mental retardation protein (FMRP), normally contains 5-55 CGG repeats in the 5'UTR. Large expansions of CGG repeats (> 200 repeats) in the FMR1 gene, referred to as full mutations, cause DNA methylation and transcriptional silencing, resulting in fragile X syndrome (FXS), a neurodevelopmental disorder characterized by intellectual disability and autism (3). Smaller expansions of CGG repeats (55-200 repeats) in the same gene, referred to as premutations, are associated with fragile X tremor ataxia syndrome (FXTAS), a late onset neurodegenerative disorder characterized by tremor, ataxia and cognitive decline (4-6) and fragile X primary ovarian insufficiency (FXPOI), characterized by infertility and early menopause (7).

Most eukaryotic RNAs undergo conventional translation, which initiates at an AUG start codon at the beginning of the open reading frame (ORF) and terminates at a stop codon at the end of the ORF, resulting in synthesis of the protein encoded by the ORF. RNAs such as FMR1, which contain trinucleotide repeats in the 5'UTR, can also undergo an unconventional type of translation, called repeat associated non-AUG (RAN) translation, which initiates at non-AUG sites in the vicinity of the repeats in the 5'UTR, resulting in synthesis of poly-amino acid RAN translation products encoded by the repeat sequences (8-11). Pathogenesis of FXTAS is believed to reflect toxicity of either the CGG repeat expansion RNA itself or of RAN translation products encoded by CGG repeat expansion RNA, although the mechanism(s) of toxicity are unclear.

CGG repeats can form secondary structures (hairpins, duplexes) by a combination of canonical C(anti)::G(anti) and non-canonical G(syn)::G(anti) base pairing (12), which may cause

ribosomes to stall in the CGG repeat region of the 5'UTR. Expanded CGG repeats in FMR1 RNA are associated with reduced translation of the downstream FMRP ORF (13,14), suggesting that expanded CGG repeats might increase stalling of ribosomes in the 5'UTR, decreasing translation of the downstream ORF. TMPyP4 (tetra-(N-methyl-4-pyridyl) porphyrin) is a membrane-permeant porphyrin ring compound that binds to CGG repeat RNA and destabilizes RNA secondary structure. TMPyP4 reverses the effect of expanded CGG repeats on FMRP translation (13,14), possibly by preventing ribosome stalling in the CGG repeat region. Heterogeneous nuclear ribonucleoprotein (hnRNP) A2 binds to CGG repeats, which are found in multiple different RNAs, including FMR1 RNA (15-17), and also to A2 response elements (A2RE), which are also found in multiple RNAs, including activity regulated cytoskeletal associated protein (ARC) RNA (18). Single molecule imaging reveals that both FMR1 RNA and ARC RNA are localized and translated in granules and that newly-synthesized FMRP and ARC protein molecules both accumulate in the vicinity of the granule where they are synthesized (19).

Since FMR1 RNA, and ARC RNA, are both localized and translated in the same RNA granules (19), and since expanded CGG repeats in FMR1 RNA inhibit translation of FMRP, possibly by causing ribosomes to stall in the 5'UTR, the presence of expanded CGG repeat RNA in granules might affect conventional translation of ARC RNA localized in the same granules and TMPyP4 might block this effect. In this regard, expression of CGG repeat expansion RNA in transgenic mice and flies does affect translation of other RNAs (20).

Several RNAs containing CGG repeats or A2RE sequences and therefore may be localized in the same granules as FMR1 RNA, encode proteins that regulate calcium homeostasis in the cell (1,18). Previous work has shown that calcium transients are increased in astrocytes from CGG KI mice (21) and in iPSC-derived neurons from individuals with FXTAS (22), both of which

contain CGG repeat expansions. If CGG repeat expansions in FMR1 RNA affect translation of these RNAs this could potentially affect calcium homeostasis in the cell.

Here we show that exogenous CGG repeat RNA, microinjected into neurons and endogenous CGG repeat expansions in FMR1 RNA expressed in premutation fibroblasts both inhibit translation of ARC RNA, which is localized in the same granules as FMR1 RNA and serves as a reporter for translation in granules, and that calcium homeostasis is also affected in premutation fibroblasts. Furthermore, TMPyP4 rescues translation of ARC RNA in neurons and in premutation fibroblasts and also rescues calcium homeostasis in premutation fibroblasts.

## **Materials/Methods**

### ***Ethics statement***

Use of animals as described in this study followed the guidelines of UConn Health and those of the National Institutes of Health for the Care and Use of Laboratory Animals, and was approved under Protocol # 100686-0416 by the Institutional Animal Care and use Committee (IACUC) of UConn Health. UConn Health complies with all applicable provisions of the Animal Welfare Act and other Federal statutes and regulations relating to animals. UConn Health Animal Welfare Assurance number is A3471-01.

Skin biopsies were obtained from selected patients at the Waisman Center at the University of Wisconsin - Madison following recruitment, consent and sample collection procedures approved by the University of Wisconsin-Madison Health Sciences Institutional Research Board of the Human Research Protection Program (HRPP). Prior to consent and sample collection potential subjects were screened in person to determine study eligibility. Subjects found to be eligible underwent approved consent procedures, including written informed consent. Skin biopsies were obtained by a physician at the Waisman Center and fibroblasts were isolated from skin biopsies.

The University of Connecticut Health Institutional Review Board has specifically exempted the use of human cell lines (fibroblasts produced at the Waisman Center at the University of Wisconsin-Madison and fibroblasts obtained from the Coriell Institute for Medical Research) in this study from IRB approval because the cell lines are de-identified.

### ***CGG repeat profiles***

Profiles of sequential CGG repeats in target sequences were calculated using a sliding sequence algorithm that compares a query sequence consisting of four sequential CGG repeats to sequential consecutive overlapping 12 nt sequences in the target RNA sequence and calculates homology scores at each position. Perfect homology between the query sequence and the target sequence yields a homology score of 1 at that position. Homology scores < 1 indicate less than perfect homology. Multiple sequential CGG repeats in the target sequence produce a profile of peaks with homology scores of 1 at positions where the CGG repeats in the query and target sequences are aligned, separated by intervening lower scores where the CGG repeats in the query and target sequences are misaligned.

### ***Ribosome profiles***

Ribosome profiling is a technique that reveals the probability of ribosomes localized at each position on an RNA molecule (23). In most RNAs, ribosome probability is high in the ORF of the RNA, reflecting active translation in this region, and low in the 5'UTR and 3'UTR, indicating less active translation in these regions. In RNAs with trinucleotide repeats or upstream ORFs in the 5'UTR, ribosome probability in the 5'UTR may be increased if translation is increased in these regions. However, ribosome probability in regions of CGG repeats may be underestimated by this technique because sequencing through long regions of CGG repeats is inefficient (24). Ribosome profiles for FMR1 RNA, representing global aggregate data from multiple published studies with multiple different cell types under multiple different conditions (25-35), were obtained from the GWIPS-viz database (<http://gwips.ucc.ie>).

### ***In vitro transcription***

ARC RNA and Venus-ARC RNA were prepared by in vitro transcription of linearized plasmid DNA, as previously described (18,19). CGG 0, 30, 62 and 99 RNAs were prepared by in vitro transcription from plasmid DNA containing FMR1 cDNA with 0, 30, 62 or 99 CGG repeats, respectively, obtained from Dr. Fry (13). Plasmid DNAs were linearized at the Xho 1 site immediately downstream of the CGG repeat region. Labeled RNAs were prepared by in vitro transcription in the presence of Cy3- or Cy5-conjugated UTP (all UTPs from GE Healthcare Biosciences, Pittsburgh, PA) using T7 mScript Standard mRNA Production System, according to manufacturer's protocol (CellScript, Madison, WI) or Amplicap-Max T3 High Yield Message Maker Kit (CellScript, Madison, WI) followed by capping and polyadenylation using reagents supplied in the T7 mScript Standard mRNA Production System (CellScript, Madison, WI) or Amplicap-Max T3 High Yield Message Maker Kit (CellScript, Madison, WI). Intactness and purity of in vitro transcribed RNAs were analyzed by gel electrophoresis and fluorescence correlation spectroscopy (FCS).

### ***Surface plasmon resonance analysis***

Biotin labeled CGG 0, 30, 62 and 99 RNA ligands prepared by in vitro transcription in the presence of biotin-UTP, were immobilized on a streptavidin Biacore chip, and binding to serial dilutions of TMPyP4 analyte was measured using a Biacore T100 instrument. Sensorgrams were fitted to a heterogeneous ligand model to determine binding parameters for specific and non-specific binding of TMPyP4 to each RNA.

### ***Cell culture, microinjection and confocal imaging***

Hippocampal neurons obtained from C57Bl/6 E19 mouse embryos were grown in culture as described previously (18). Human fibroblasts were obtained from unaffected normal controls with normal numbers of CGG repeats in the FMR1 gene and from apparently unaffected female premutation carriers with expanded numbers of CGG repeats in the FMR1 gene (see “*Human*

*fibroblasts*” below). Fibroblasts were grown in culture as recommended by the Coriell Institute cell repository with the addition of  $\alpha$ -tocopherol (10  $\mu$ g/ml) (Sigma Cat# T3251-5G), a lipophilic radical scavenger used to reduce cellular autofluorescence in cultured human fibroblast cells (36).

Primary mouse hippocampal neurons (14 to 21 days in culture) or human fibroblasts, grown in the presence or absence of 1  $\mu$ M TMPyP4 (Calbiochem, Darmstadt, Germany) were microinjected using an electronic microinjection system (Eppendorf, Hamburg, Germany). Injected cells were identified by fluorescence microscopy. Three channel confocal images of injected cells were collected by recording simultaneously in the 488 $\lambda$ , 560 $\lambda$  and 633 $\lambda$  channels using a Zeiss LSM 510 confocal laser scanning microscope with a 63X 1.4 numerical aperture oil immersion objective lens (Carl Zeiss, Thornwood, NY). Images were collected with maximum dynamic range and minimum background in each channel, while avoiding image saturation (pixels >255) or image offset below background (pixels <0). Individual RNA granules were masked and fluorescent intensity for each granule was integrated in each channel after subtracting background fluorescence using Image J. Specific translational activities were calculated by dividing the fluorescence intensity of newly-synthesized Venus-ARC protein, measured in the 488 $\lambda$  channel, by the fluorescence intensity of Venus-ARC RNA, measured in the 633 $\lambda$  channel.

### ***Fluorescence Correlation Spectroscopy (FCS) photobleaching***

The numbers of fluorescent RNA molecules in individual granules were determined by FCS (37,38). Counts per molecule for Cy5-conjugated-UTP-labelled CGG 99 RNA and Alexafluor 488-conjugated-UTP-labelled ARC RNA were first determined by FCS in solution. Both RNAs were co-injected into primary mouse hippocampal neurons in culture. Dual channel confocal images of injected cells were collected to visualize individual granules containing both fluorophores. The FCS observation volume was positioned to encompass a single RNA granule

and FCS counts were recorded for 10 s, during which the count rate decayed due to photobleaching of fluorescent RNA molecules in the granule, which is immobile. The numbers of fluorescent RNA molecules of each type in each granule were determined by subtracting counts remaining after 10 s from counts at time 0 and dividing by counts per molecule.

### ***Calcium transients***

Fibroblasts in culture were loaded with Fluo-4 calcium indicator, according to the manufacturer's protocol (Life Technologies). Bradykinin was bath applied to the cells to induce calcium transients. Fluo-4 fluorescence was recorded by time lapse confocal microscopy after bradykinin addition. Fluo-4 fluorescence in individual cells was quantified by masking each cell and integrating total fluorescence in the cell at each time step. Calcium transients were expressed as  $\Delta F/F_0$ .

### ***Human fibroblasts***

Fibroblasts were isolated from skin biopsies obtained from selected patients at the Waisman Center at the University of Wisconsin-Madison per Institutional Review Board–approved human subject protocols. The following fibroblast lines were analyzed: FX08-01 (full mutation), FX08-02 (premutation), FX11-01 (full mutation), FX11-02 (premutation), FX13-01 (full mutation), FX13-02 (premutation), WC26 (double premutation), C0603 (control). In addition GM00497 (control) and GM00498 (control) fibroblasts from apparently unaffected individuals were purchased from the Coriell Cell Repository.

In the case of FX08-02, FX11-02, FX13-02 and C0603 cell lines the numbers of FMR1 CGG repeats were determined using a PCR-based protocol previously described (39). The protocol combines gene-specific primers that flank the CGG repeat region of the FMR1 gene with gender-specific primers, a polymerase mixture, and a reaction buffer optimized for amplification of GC rich DNA. PCR was performed on an ABI Veriti thermal cycler (Applied Biosystems,



Grand Island, NY). CGG repeat numbers were determined by agarose gel electrophoresis of PCR products and confirmed by capillary electrophoresis on an ABI 3730xl DNA Analyzer with POP-7 195 polymer using a 50-cm array (40), which is capable of defining exact CGG repeat number on samples with 200 CGG repeats or less.

In the case of WC26 and C0603 a different, previously described assay was used to determine the FMR1 CGG repeats (41). Amplification of CGG repeats and flanking sequences was performed with AccuPrime Pfx DNA Polymerase (Thermo Fisher Scientific) in 15 ul reactions containing 0.15 ul of Pfx polymerase, 1x Reaction Mix, 2.5 M Betaine (Sigma), 7.5% DMSO (Sigma), 50 ng of genomic DNA, and 0.3 uM of each primer CGG-F (TCAGGCGCTCAGCTCCGTTTCGGTTTCA) and CGG-R (AAGCGCCATTGGAGCCCCGCACTTCC). Samples were amplified with an initial denaturation step of 98°C for 5 min; 35 cycles of 98°C for 35 s, 64°C for 35 s, and 68°C for 2 min, and a final extension step at 68°C for 5 min. PCR products were resolved on agarose gel electrophoresis. The CGG repeat number was estimated by comparing to DNA sizing ladder visualized on agarose gel electrophoresis.

### ***Western blotting***

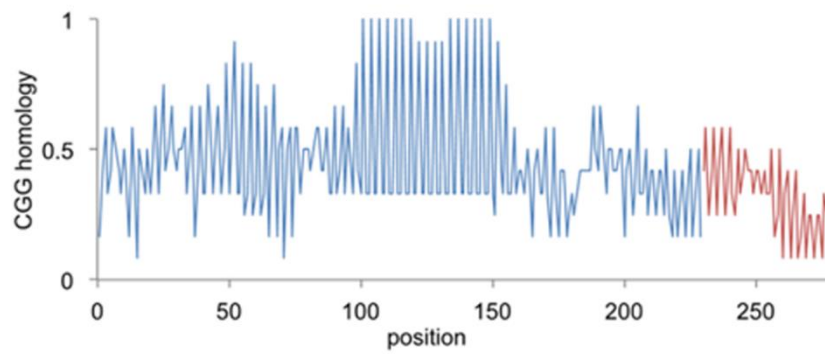
Expression of FMRP in fibroblast cell lines was analyzed by Western blotting with antibody to FMRP as described previously (42,43).

## **Results**

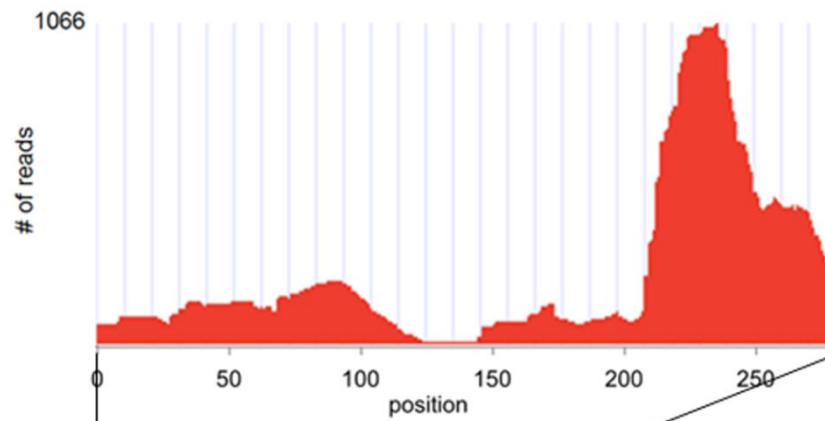
### ***CGG repeat and ribosome profiles for FMR1 RNA***

Figure 1 shows CGG repeat profiles and ribosome profiles for exon 1 of FMR1 RNA, which includes the 5'UTR and initial portion of the ORF. The CGG profile reveals a region of CGG repeats between positions 100-150 in the 5'UTR of FMR1 RNA. The ribosome profile reveals regions of increased ribosomal density between position 200-250, presumably reflecting

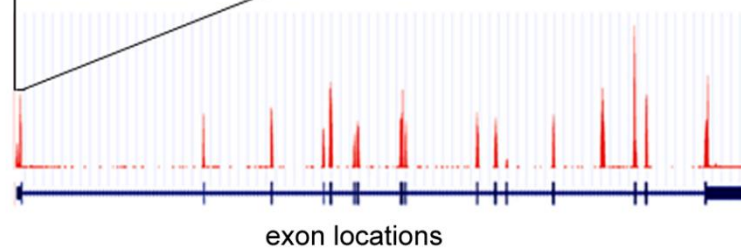
**A** FMR1 RNA exon 1 CGG repeat profile



**B** FMR1 RNA exon 1 ribosome profile



**C** FMR1 gene ribosome profile

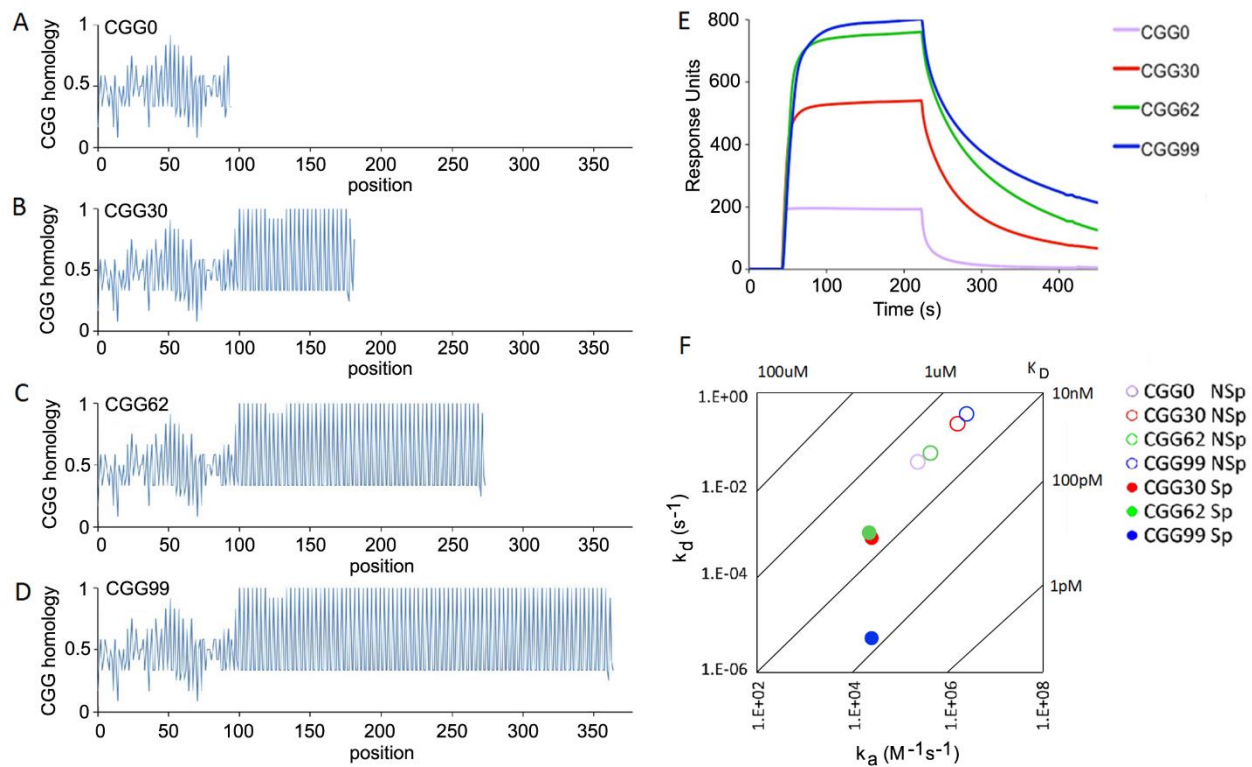


**Figure 1: CGG repeat profile and ribosome profile for FMR1 RNA.** Panel A shows the CGG profile for exon 1 of FMR1 RNA, including the 5'UTR (blue) and the initial portion the ORF (red), calculated as described in Materials and Methods. There is a region of CGG repeats between positions 100-150, interrupted by a single AGG at position 131. Panel B shows the corresponding ribosome profile for exon 1 of FMR1 RNA. The number of sequence reads at each position reflects the probability of ribosomes located at that position. Increased ribosome probability downstream of position 200 presumably reflects ribosomes engaged in conventional translation of the FMRP ORF beginning at the AUG at position 230. Increased ribosome probability in upstream regions (1-100 and 150-200) may reflect ribosomes initiating translation at non-canonical AUG-like sites upstream of the CGG repeats or ribosomes engaged in RAN translation of 5'UTR CGG repeats, consistent with reports that translation of CGG repeats in FMR1 RNA may require non-canonical AUG-like sequences upstream of the CGG repeats (11). The paucity of reads in the region between 100-150, which corresponds to the CGG repeat region, may reflect inefficient sequencing through long regions of CGG repeats (24). Panel C shows the ribosome profile for the entire FMR1 gene (red) and the locations of individual exons (blue), with 5' and 3'UTR regions indicated by thinner lines and ORF regions indicated by thicker lines. The exon 1 region shown in panels A and B is indicated.

ribosomes engaged in conventional translation of the FMR1 ORF and between positions 0-125, which may reflect ribosomes stalled in the CGG repeat region in the 5'UTR of FMR1. The number of sequence reads in the CGG repeat region may underestimate the actual ribosome density in this region because sequencing through long sequences of CGG repeats is inefficient (24). For comparison, the ribosome profile and exon locations for the entire FMR1 gene are shown in Fig. 1C. Ribosome densities for most exons are comparable, indicating that translational activity is relatively uniform across the ORF. Ribosome density in the 5'UTR is slightly lower compared to ribosome density in the ORF, indicating that RAN translation activity in the CGG repeat region is slightly lower than conventional translational activity in the ORF.

Expansion of CGG repeats in the 5'UTR of FMR1 RNA in premutation cells could potentially further increase ribosome density in this region. This has not been tested directly because ribosome profiling has not been performed with premutation cells.

TMPyP4, is a membrane-permeant tetraporphyrin ring compound that binds to, and disrupts, CGG repeat RNA secondary structure (13,14). TMPyP4 binding to CGG repeat RNA was characterized by surface plasmon resonance (SPR) analysis with CGG repeat RNA as ligand and TMPyP4 as analyte, as shown in Fig. 2. CGG repeat profiles for RNA transcripts derived from FMR1 RNAs with 0, 30, 62 and 99 CGG repeats are shown in panels A-D. In each transcript the 5' flanking region from position 1-101 in FMR1 RNA, corresponding to the region with increased ribosome density in Fig. 1, is identical, followed by different numbers of CGG repeats (0, 30, 62, 99) for each transcript. To compare the TMPyP4 binding parameters for nonCGG repeat regions and for CGG repeat regions, SPR sensorgrams for TMPyP4 binding to RNAs with 0, 30, 62 and 99 repeats (shown in panel E) were globally fitted to a heterogeneous ligand binding model to determine non-specific and specific binding parameters for each transcript (shown in panel F). One set of binding parameters is similar for all transcripts ( $K_D \sim$



**Figure 2: CGG repeat profiles and TMPyP4 binding for FMR1, CGG0, CGG30, CGG62 and CGG99 RNAs.** Panels A-D - CGG repeat profiles for RNAs derived from the 5'UTR of FMR1 RNAs containing CGG 0, 30, 62, 99 were calculated using a sliding sequence algorithm (described in Materials Methods). Values of 1 in the profile indicate four consecutive CGG repeats in a row. Panel E – Binding of each CGG repeat RNA to serial dilutions of TMPyP4 was analyzed by SPR. Panel E shows representative SPR sensorgrams for binding of TMPyP4 to CGG 0, 30, 62 and 99 RNAs. Panel F – On-rates and off-rates determined by fitting SPR sensorgrams for each RNA to a heterogeneous ligand binding model. Apparent  $K_D$  values were calculated by dividing off rates by on rates. NSp indicates non-specific binding, Sp indicates CGG-specific binding for each RNA.

0.5  $\mu\text{M}$ ), presumably corresponding to non-specific binding of TMPyP4 to common nonCGG repeat regions present in each transcript. In addition, CGG 30, 62 and 99 RNAs, exhibit a second set of binding parameters, presumably corresponding to specific binding of TMPyP4 to different number of CGG repeats in each transcript. Apparent on-rates for CGG-specific binding are similar for CGG 30, 62 and 99 RNAs ( $k_a \sim 2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ) but apparent off-rates are slower for CGG99 RNA ( $k_d \sim 2 \times 10^{-6} \text{ s}^{-1}$ ) compared to CGG30 and CGG62 RNAs ( $k_d \sim 2 \times 10^{-4} \text{ s}^{-1}$ ). This may reflect tighter binding of TMPyP4 to CGG 99 RNA compared to CGG 30 and CGG 62 RNAs, possibly because of increased numbers of CGG repeats, or alternatively, may reflect rebinding of TMPyP4 to CGG99 RNA during the dissociation phase. In any case, these results indicate that TMPyP4 binds non-specifically to nonCGG repeat sequences and specifically to CGG repeat sequences. Overall the apparent  $K_D$  for TMPyP4 binding to all RNAs is  $< 1 \mu\text{M}$ . Accordingly, in subsequent experiments, cells were incubated with TMPyP4 at a final concentration of  $1 \mu\text{M}$  to ensure saturation binding to all CGG repeat RNA molecules in the cell.

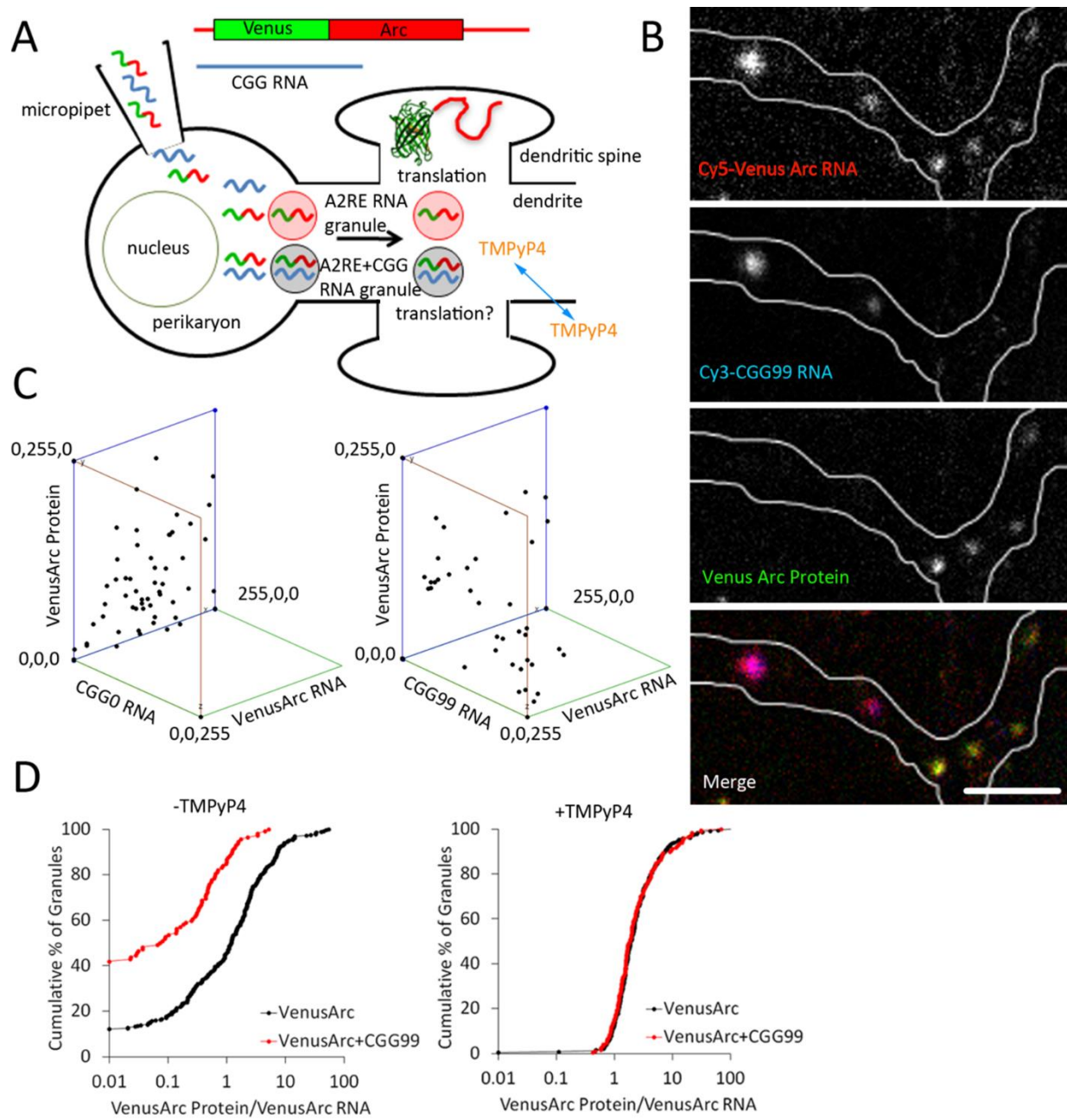
***CGG repeat RNA molecules and Venus-ARC RNA molecules are co-localized in granules and CGG repeat RNA inhibits translation of Venus-ARC RNA in the same granule.***

FMR1 RNA containing CGG repeats is localized and translated in RNA granules (19). To determine if CGG repeats by themselves are sufficient for RNA localization in granules and if CGG repeat RNA affects translation of other RNAs localized in the same granules, CGG0 and CGG99 RNAs were microinjected into hippocampal neurons, along with Venus-ARC RNA, as a marker for granules and as a reporter for granule RNA translation. CGG0 and CGG99 RNAs were labelled by in vitro transcription with Cy3-UTP and Venus-ARC RNA was differentially labeled by in vitro transcription with Cy5-UTP. Co-injected neurons were incubated for 2 hours to allow time for localization and translation of the injected RNAs in individual granules. Fluorescent Venus-ARC RNA, fluorescent CGG repeat RNA and fluorescent newly synthesized Venus-ARC protein were imaged simultaneously by three channel confocal microscopy. The

relative amounts of each component associated with each granule were quantified by integrating fluorescent intensities in each channel for each granule. In some experiments neurons were incubated with TMPyP4, prior to microinjection, to disrupt secondary structure in CGG repeat RNA. The experimental protocol is outlined schematically in Fig. 3A.

The experimental protocol outlined Fig. 3A is based on several considerations. Hippocampal neurons were used to analyze localization and translation in granules because individual granules are well resolved in neuronal dendrites. Venus-ARC RNA was used as a marker for granules because it contains an A2RE sequence that binds to hnRNP A2 and mediates localization in granules (18). It also serves as a reporter for translation in granules because Venus-ARC RNA is translated in granules and newly-synthesized Venus-ARC protein molecules remain in the vicinity of the granule where they are synthesized (19). Truncated CGG repeat RNAs derived from the 5'UTR of FMR1 RNA but lacking the FMRP ORF, were injected to test the effect of CGG repeat RNA itself, without potential confounding effects of FMRP protein. CGG0 and CGG99 RNAs were used because they do not represent substrates for conventional translation, since they lack initiation and termination codons, although the region upstream of the CGG repeats in FMR1 does contain several non-canonical AUG-like sequences that could potentially mediate translation initiation at low efficiency (11). Because CGG99 contains CGG repeats it is localized in granules and may represent a substrate for RAN translation. CGG repeats can also form secondary structures, which may cause ribosomes to stall in the vicinity of the CGG repeats. Fluorescent RNAs were injected directly into the cytoplasm rather than into the nucleus in order to assay effects of CGG repeat RNA on cytoplasmic translation, while avoiding effects of CGG repeats on nuclear processes, such as nuclear inclusion formation (44), nuclear processing or nuclear transport that could potentially affect subsequent translation or decay in cytoplasm. There are several reasons to believe that CGG repeat RNA is not degraded during the course of the experiment. Eukaryotic cells have





**Figure 3: CGG repeat RNA and ARC RNA are co-localized in granules and CGG repeat RNA inhibits translation of ARC RNA in hippocampal neurons.** Panel A shows a schematic outline of the experimental protocol. Differentially labeled CGG repeat RNA and Venus-ARC RNA were co-injected into the cytoplasm of hippocampal neurons where they are localized in granules in dendrites. Venus-ARC RNA is translated in granules near dendritic spines. In some cases hippocampal neurons were incubated with membrane-permeant TMPyP4 before injecting RNA. Panel B shows the distribution of Venus-ARC RNA (red), CGG99 RNA (blue) and newly-synthesized Venus-ARC protein (green) in discrete granules in a three channel confocal images of a dendritic segment. Seven granules are visible in this dendritic segment, all of which contain Venus-ARC RNA, two of which also contain CGG99 RNA, and five of which lack detectable CGG99 RNA. Newly synthesized Venus-ARC protein is detected in the five granules lacking CGG99 RNA but not in the two granules containing CGG99 RNA. Scale bar indicates 1  $\mu\text{m}$ . Panel C shows 3-dimensional graphs of fluorescence intensities for Venus-ARC RNA, Venus-ARC protein and CGG99 (right panel) or CGG0 (left panel) RNAs in a population of individual granules in hippocampal neurons. Panel D shows Kolmogorov-Smirnov cumulative frequency plots of specific translational activities (Venus-ARC protein/Venus-ARC RNA) for granules containing detectable CGG99 RNA (red), defined as integrated fluorescence intensity > 10 arbitrary units, or in granules where CGG99 RNA was undetectable (black), defined as integrated fluorescence intensity < 10 arbitrary units. Specific translational activities were measured for granules in untreated (left panel) or in TMPyP4-treated (right panel) hippocampal neurons.

multiple different decay pathways for degrading dysfunctional RNAs including: nonsense mediated decay (NMD), triggered by premature nonsense codons in the RNA (45), non-stop decay (NSD), triggered by lack of a termination codon preceding the polyA tail on the RNA (46), and no-go decay (NGD), triggered by stalled elongation on the RNA (47). CGG repeat RNAs do not represent substrates for NMD, because they lack termination codons or for NSD, because they lack a polyA tail. CGG repeat RNAs could potentially represent substrates for NGD, if translation elongation is stalled by CGG repeat secondary structure in the injected RNA. However injection of fluorescent RNA into the cytoplasm provides an internal control for RNA degradation because fluorescent fragments produced by RNA decay in the cytoplasm are translocated to the nucleus (18). In this experiment none of the injected cells showed detectable nuclear fluorescence, indicating that RNA injected into the cytoplasm was not degraded during the time of the experiment. RNA localization and translation in granules were measured 2 hours after microinjection in order to analyze primary effects of CGG repeat RNA on granule RNA translation, while minimizing potential secondary effects due to RNA toxicity, decay or changes in cellular physiology caused by long term presence of exogenous CGG repeat RNA in the cell.

Fig. 3B shows several individual granules in a representative dendritic segment from a microinjected neuron and Fig. 3C shows total integrated fluorescence intensities for microinjected Venus-ARC RNA, CGG99 RNA (right panel) or CGG0 RNA (left panel) and newly-synthesized Venus-ARC protein in multiple individual granules. Venus-ARC RNA is detected in all RNA granules, CGG99 RNA is detected in a subset of Venus-ARC RNA granules and Venus-ARC protein is detected in granules that do not contain detectable CGG99 RNA but not in granules that contain detectable CGG99 RNA, indicating that CGG repeat RNA inhibits translation of Venus-ARC RNA localized in the same granule. Microinjected CGG0 RNA is not detected in granules and does not affect synthesis of Venus-ARC protein in granules. These

results indicate that CGG repeat RNA is localized in granules and inhibits translation of Venus-ARC RNA in the same granules.

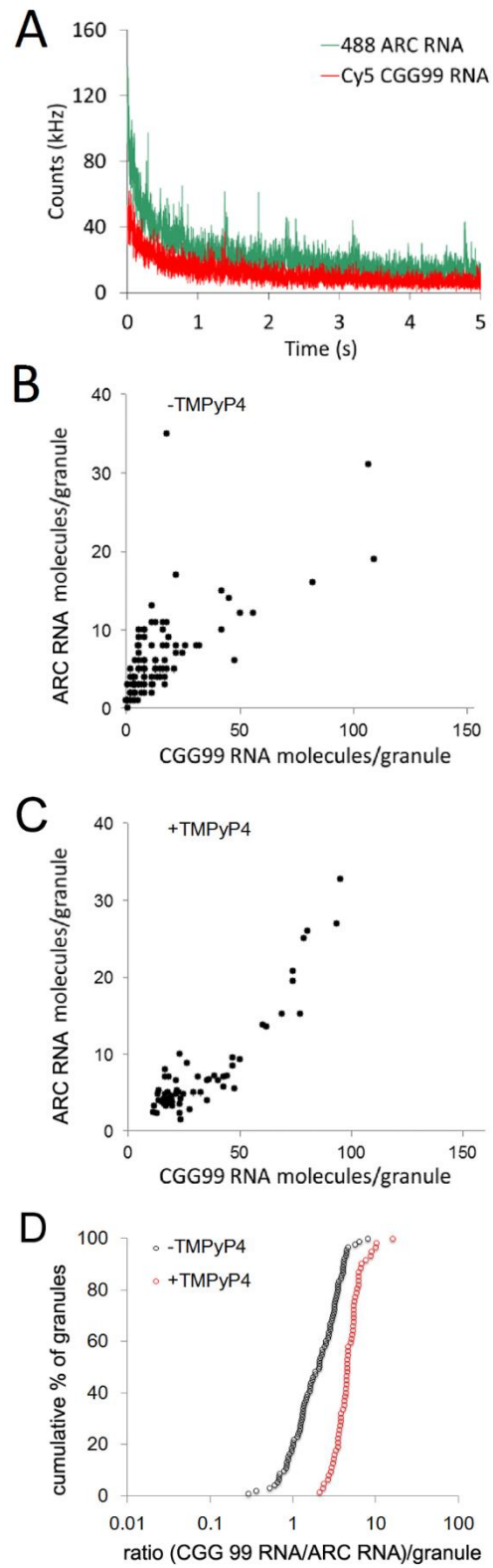
To quantify specific translational activities in individual granules, Venus-ARC RNA and newly-synthesized Venus ARC protein were measured in multiple granules, with and without detectable CGG99 RNA (Fig. 3, panels C and D). Specific translational activities were calculated by dividing the amount of newly-synthesized Venus-ARC protein by the amount of Venus-ARC RNA in each granule. Individual granules exhibit variable translational activities, which is consistent with previous single molecule imaging observations of translational output from individual granules (19). In order to compare translational activities for a large population of granules the results are presented in the form of a Kolmogorov-Smirnov plot showing cumulative frequencies for granules with different specific translational activities. Granules without detectable CGG99 RNA (black symbols) exhibit a range of translational activities (0.01 - 50), with a small proportion (~10%) of translationally inactive granules. Variation in translational activity among granules without exogenous CGG99 RNA may be due to variable amounts of endogenous CGG repeat RNAs in different granules. Granules with detectable CGG99 RNA (red symbols) exhibit reduced translational activities (0.01 - 5) with a larger proportion (~40%) of translationally inactive granules. This indicates that CGG99 RNA localized in granules inhibits translational activity of Venus-ARC RNA localized in the same granules.

TMPyP4, binds to CGG repeat RNA and disrupts CGG repeat secondary structure. To determine if TMPyP4 rescues granule RNA translation, neurons were treated with TMPyP4 prior to injection with Cy5-labeled Venus-ARC RNA, by itself or with CGG99 RNA (Fig. 3D). In TMPyP4-treated cells, the ranges of translational activities in individual granules, with detectable CGG99 RNA (red symbols) or without detectable CGG99 RNA (black symbols), are similar (the cumulative frequency plots are superimposable) and translationally inactive granules are not detected in either case. Since TMPyP4 binds specifically to CGG repeats and since

Venus-ARC RNA does not contain CGG repeats, and since TMPyP4 differentially affects translation of granules containing CGG repeat RNA, the effect of TMPyP4 on translation of Venus-ARC RNA may be mediated by its binding to CGG repeat RNA molecules in the same granules. These results indicate that TMPyP4 rescues translation of Venus-ARC RNA in granules containing CGG99 RNA.

If CGG repeat RNA inhibits translation of other RNAs in the same granule, the magnitude of inhibition may reflect the number of CGG repeat RNA molecules relative to other RNA molecules in the same granule. The numbers of CGG repeat RNA and ARC RNA molecules in individual granules were determined by fluorescence correlation spectroscopy (FCS) photobleaching (Fig. 4) (37,38). Alexafluor 488-labeled ARC RNA and Cy5-labeled CGG99 RNA were first analyzed by FCS in solution to determine fluorescence counts per molecule for each RNA. For these experiments ARC RNA was used instead of Venus-ARC RNA in order to avoid fluorescence from newly-synthesized Venus-ARC protein. The numbers of Alexafluor 488-ARC RNA and Cy5-CGG99 RNA molecules in individual granules in live cells were determined by positioning the FCS volume to encompass a single immobile RNA granule and recording decay of counts over time in each channel due to photobleaching of fluorescent RNA molecules in the immobile granule during the FCS measurement. Dividing the total loss of counts per granule by counts per individual RNA molecule provides a measure of the number of RNA molecules of each type in the granule encompassed by the FCS volume (Fig. 4A). Endogenous CGG repeat or A2RE RNA molecules in individual granules are not detected by FCS photobleaching because they are not fluorescent.

In the absence of TMPyP4 (Fig. 4B), the relative numbers of ARC RNA molecules and CGG99 RNA molecules per granule are correlated over the population of granules but the absolute number of CG99 RNA molecules is greater than the number of ARC RNA molecules in most granules. This suggests that CGG99 RNA molecules and ARC RNA molecules are assembled



**Figure 4: FCS photobleaching of ARC RNA and CGG 99 RNA molecules in individual granules in hippocampal neurons.** Panel A – the FCS observation volume was positioned to encompass a single individual immobile granule containing differentially labeled fluorescent ARC RNA and CGG99 RNA molecules in a hippocampal neuron. Continuous illumination during the FCS measurement results in photobleaching of fluorescent RNA molecules of each type in the granule, which is recorded as count rate decay in each FCS channel. The numbers of fluorescent RNA molecules of each type in the granule are determined by dividing the total decay in counts during photobleaching by the counts per molecule for each RNA determined by FCS in solution. Panel B shows a scatter plot for numbers of ARC RNA and CGG99 RNA molecules in individual granules in hippocampal neurons in the absence of TMPyP4. Panel C shows a scatter plot for numbers of ARC RNA and CGG99 RNA molecules in individual granules in hippocampal neurons in the presence of TMPyP4. Panel D shows Kolmogorov-Smirnov plots of the ratios of CGG99 RNA molecules to ARC RNA molecules in individual granules in the absence (black symbols) and presence (red symbols) of TMPyP4.

into granules by similar mechanisms but CGG 99 RNA is assembled more efficiently than ARC RNA. In the presence of TMPyP4 (Fig. 4C) the number of CGG99 RNA molecules compared to ARC RNA molecules is slightly increased. Fig. 4D shows the ratios of CGG99 RNA molecules to ARC RNA molecules for granules in the absence and presence of TMPyP4. In the absence of TMPyP4 the ratio varies from 1-10, indicating that CGG99 RNA molecules are assembled into granules more efficiently than ARC RNA molecules. CGG99 RNA contains multiple CGG repeat sequences that can potentially bind to multiple hnRNP A2 molecules, which may mediate more efficient assembly into granules, while ARC RNA contains a single A2RE sequence that binds to a single hnRNP A2 molecule, which may mediate less efficient assembly into granules. In the presence of TMPyP4 the ratios are shifted slightly to higher values indicating that assembly of CGG99 RNA molecules into granules is slightly enhanced. Disruption of secondary structure by TMPyP4 may increase binding of hnRNP A2 molecules to CGG repeat RNA molecules, which could facilitate more efficient assembly into RNA granules. This could result in increased hnRNP A2 concentration in CGG99 RNA granules in the presence of TMPyP4 but this was not tested experimentally.

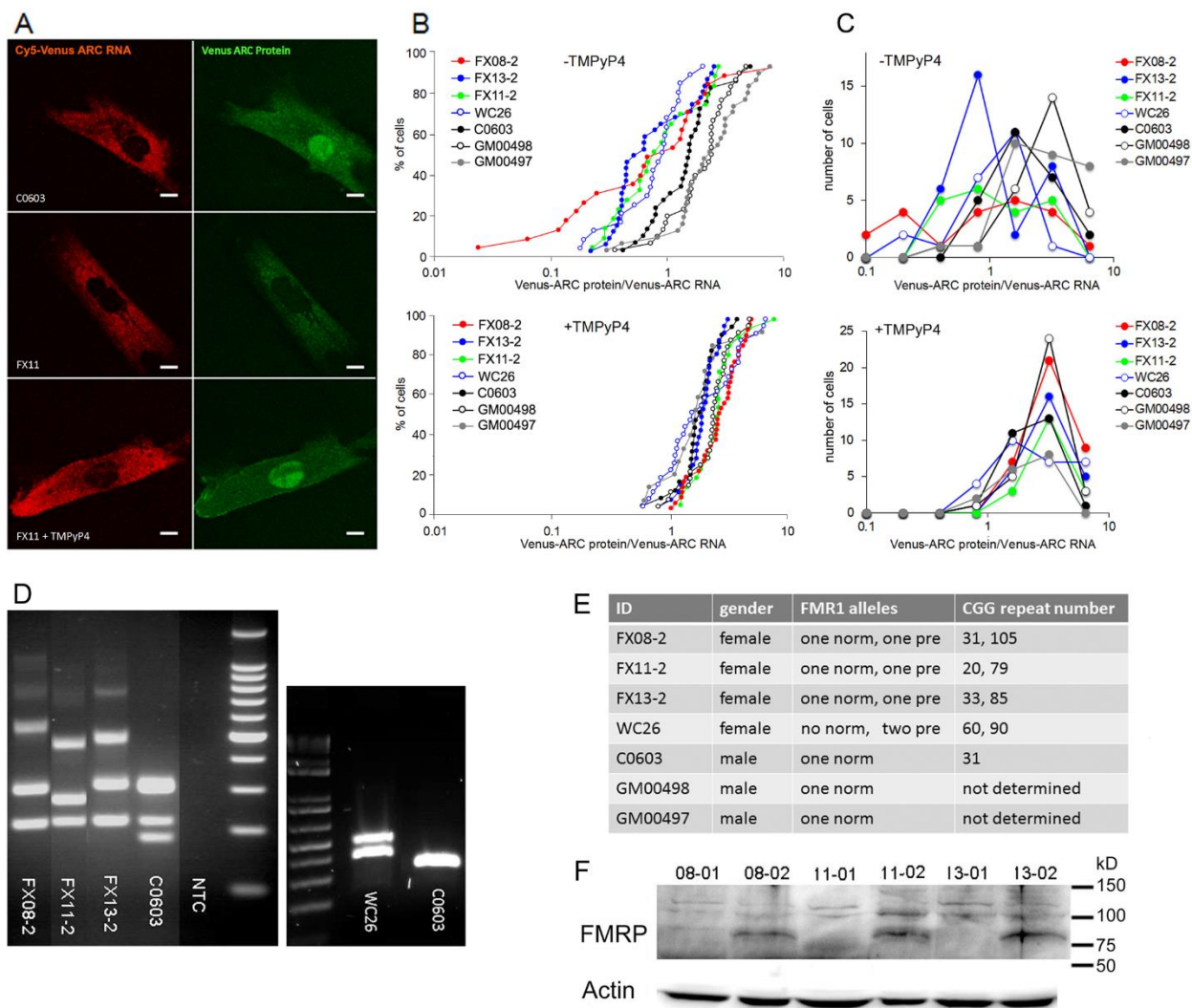
In the absence of TMPyP4 the range of values for the ratio of CGG99 RNA to ARC RNA in individual granules (1-10) is narrower than the range of values for translational activities in CGG99 containing granules (compare Fig. 4B to Fig. 3D). This indicates that inhibition of granule RNA translation by CGG repeat RNA is not linearly proportional to the ratio of CGG99 RNA and ARC RNA in individual granules. This non-linearity could be due to CGG repeat RNA affecting translation of other endogenous RNAs that encode proteins known to regulate translation [1]. If CGG99 RNA inhibits translation of such endogenous RNAs this could amplify the effect of CGG99 RNA on translation of ARC RNA in granules.



### **CGG repeat expansions in endogenous FMR1 RNA inhibit translation of Venus-ARC RNA in premutation human fibroblasts.**

The effect of CGG repeat expansions in endogenous FMR1 RNA on translation of Venus-ARC RNA was analyzed by microinjecting Venus-ARC RNA labeled with Cy5-UTP into human fibroblasts from premutation carrier individuals with different numbers of CGG repeats in the endogenous FMR1 gene (Fig. 5). Microinjected Venus-ARC RNA and newly-synthesized Venus-ARC protein were imaged in multiple cells by dual channel confocal microscopy two hours after injection to allow time for injected RNA to be translated. Fluorescent Venus-ARC RNA was restricted to the cytoplasm and appeared to have a granular distribution, suggesting that the injected RNA was localized in granules and was not degraded (Fig. 5A). However in most cells discrete individual granules were not sufficiently well resolved from each other to quantify RNA in individual granules. Newly-synthesized Venus-ARC protein accumulates in both cytoplasm and nucleus of fibroblasts, as reported previously (48).

Total fluorescent RNA and total fluorescent protein were integrated over the entire cell as a measure of the total amount of injected Venus-RNA and the total amount of newly-synthesized Venus-ARC protein, respectively, in each cell. Specific translational activities were calculated by dividing the amount of Venus-ARC protein by the amount of Venus-ARC RNA in each cell. The results for multiple individual cells from 3 control and 4 premutation fibroblast cell lines are shown in cumulative Kolmogorov-Smirnov plots (Fig. 5B). In control fibroblasts (C0603, GM00497, GM00498) from individuals with normal numbers of CGG repeats in the FMR1 gene, most cells exhibit translational activities between 1-10. Intrinsic variation in control cells may be due to cell-to-cell variations in expression of other endogenous CGG repeat RNAs that affect translation in these cells. In premutation fibroblasts (FX08-2, FX11-2, FX13-2) from individuals with CGG repeat expansions in one copy of the FMR1 gene, some cells exhibit translational activities comparable to control cells (1-10) while other cells exhibit reduced translational



**Figure 5: CGG repeat expansions in the FMR1 gene inhibit translation of Venus-ARC RNA in human fibroblasts.** Panel A - Human fibroblasts from control individuals C0603 (control male, 31 repeats), GM00497 (control male, unknown repeat number), GM00498 (control male, unknown repeat number) and FMR1 premutation carriers FX08-2 (female, 31, 105 repeats), FX11-2 (female, 20, 79 repeats), FX13-2 (female, 33, 85 repeats), and WC26 (female, two premutation alleles, 60, 90 repeats) were microinjected with Venus-ARC RNA and after 2 hours Venus-ARC RNA and newly-synthesized Venus-ARC protein were imaged by dual channel confocal microscopy. Representative images are shown for untreated control and premutation cells and for premutation cells treated with TMPyP4. Scale bars indicate 5  $\mu$ m. Panel B – Cumulative Kolmogorov Smirnov plots for specific translational activities (newly-synthesized Venus-ARC protein/microinjected Venus-ARC RNA) in individual cells for 3 control and 4 premutation fibroblast cell lines as described for Panel A, untreated (top) and treated with TMPyP4 (bottom). Panel C – Frequency distribution plots for specific translational activities (newly-synthesized Venus-ARC protein/microinjected Venus-ARC RNA) in cells for 3 control and 4 premutation fibroblast cell lines as described for Panel B, untreated (top) and treated with TMPyP4 (bottom). Panel D – PCR analysis of CGG repeat numbers in FX08-02, FX11-02, FX13-02 and C0603 fibroblasts with a 100 bp DNA ladder (left panel) and in WC26 and C0603 fibroblasts with a 1 Kb ladder (right panel). In the panel on the left, in the FX08-2, FX11-2, and FX13-2 lanes, the band at the bottom of the gel represents female gender specific PCR product, the bands immediately above the gender specific band represent PCR products from CGG repeat alleles in the normal range and the bands above represent PCR products from expanded CGG repeat alleles. The fainter products near the top of the gel are of unknown origin. In the C0603 lane, the two bands near the bottom of the gel represent male and female gender specific PCR products, and the band the gender specific bands represents PCR product from the normal CGG repeat allele. In the panel on right, the two bands in the WC26 lane both represent PCR products from expanded CGG repeat alleles and the single band in the C0603 lane represents PCR product from the CGG repeat allele in the normal range. Panel E – Table showing ID, gender, FMR1 alleles and CGG repeat numbers (based on panel D) for each cell line. Panel F – Western blotting of FMRP expression in full mutation (FX08-1, FX11-01, FX13-01) and premutation (FX08-2, FX11-02 and FX13-02) cell lines with actin loading controls.

activities (0.02-1). The translational activities in the second sub-population exhibit the same rank order as the number of CGG repeats in the expanded FMR1 allele in the different cell lines (FX08>FX13>FX11), suggesting that inhibition of translational activity is proportional to CGG repeat number in different cell lines. In cells from one individual with CGG repeat expansions in both FMR1 alleles (WC26), specific translational activities are reduced in all cells.

To determine if TMPyP4 rescues translation in premutation fibroblasts, cells were treated with TMPyP4 for 24 hours prior to injection with Venus-ARC RNA. After TMPyP4 treatment, control and premutation fibroblasts exhibit comparable specific translational activities, which are similar to untreated control cells. This indicates that TMPyP4 rescues translation in premutation fibroblasts from individuals with CGG repeat expansions in the endogenous FMR1 gene, which is consistent with the effect of TMPyP4 on granule RNA translation in neurons microinjected with exogenous CGG repeat RNA. These results indicate that translation of Venus-ARC in human fibroblasts is inhibited by CGG repeat expansions in FMR1 RNA and rescued by TMPyP4.

The existence of two subpopulations of cells with different translational activities could reflect random X inactivation of different FMR1 alleles in different sub-populations of fibroblasts. In cells with reduced translational activities, the FMR1 allele with expanded CGG repeats may be active, while in cells with normal translational activities, the FMR1 allele with normal CGG repeat numbers may be active. In WC26 cells, with reduced translational activities in all cells, either FMR1 allele may be active because both alleles contain CGG repeat expansions. To test this possibility, translational activities in different cell lines were plotted as a frequency distribution plot (Fig. 5C). Control cells (C0603, GM00497, GM00498) with a single FMR1 allele with normal numbers of CGG repeats, exhibit a unimodal distribution with normal translational activities. Premutation cells (FX08-2, FX11-2, FX13-2) with one normal and one FMR1 allele with expanded CGG repeats exhibit a bimodal distribution of translational activities (one normal

and one reduced) and the double premutation cells (WC26) with expanded CGG repeats in both FMR1 alleles, exhibit a unimodal distribution with reduced translational activities in all cells. In cells treated with TMPyP4 all cell lines exhibit unimodal distributions with normal translational activities. These results indicate that in premutation carrier cells with one normal FMR1 allele and one premutation FMR1 allele with expanded CGG repeats, translational activity is determined by the active FMR1 allele in each cell.

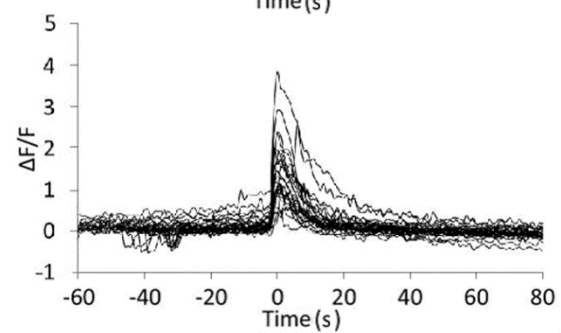
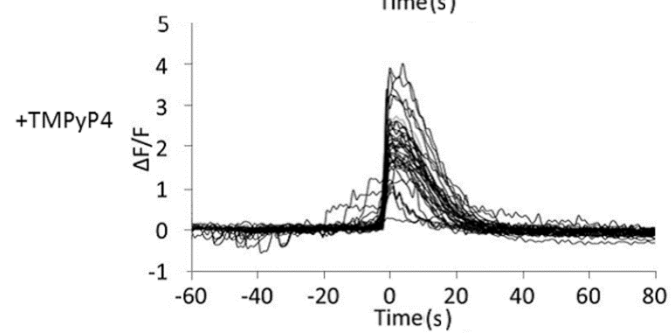
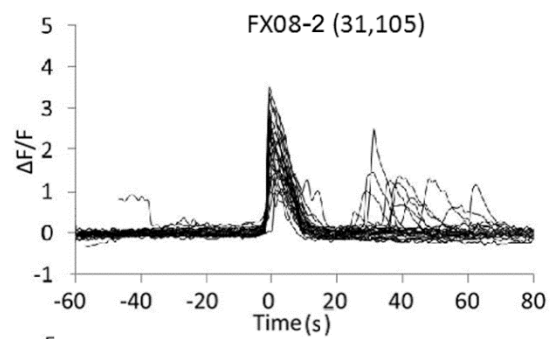
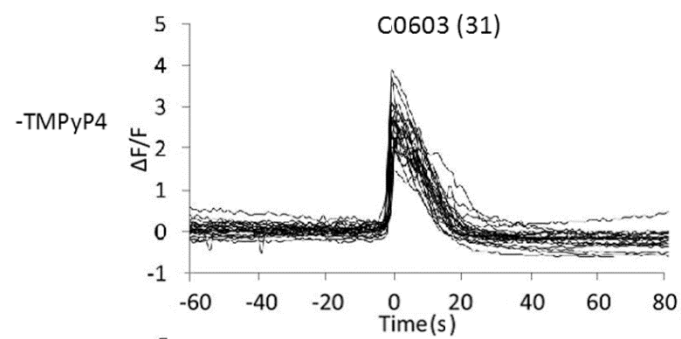
FMRP is a translational repressor that inhibits ARC translation. Levels of FMRP expression are generally not affected much in premutation cells (42,43). However, variation in Venus-ARC translation in different premutation fibroblast cell lines could conceivably reflect variation in endogenous FMRP expression in these specific lines. Western blotting showed comparable levels of FMRP expression in FX08-2, FX11-2 and FX13-2 cell lines (Fig. 5F). This analysis would not necessarily detect variations of FMRP expression due to X-inactivation of different FMR1 alleles in different subpopulations of cells. Since expanded CGG repeats in FMR1 RNA may reduce expression of FMRP from the downstream ORF, subpopulations of cells where the FMR1 allele with expanded CGG repeats is active might have reduced FMRP expression, which would be expected to result in increased ARC translation. However the results show decreased ARC translation in these cells. Moreover, TMPyP4 increases FMRP expression from FMR1 RNA with expanded CGG repeats, which might decrease ARC translation. However the results show increased ARC translation in the presence of TMPyP4. For these reasons it is unlikely that variation in Venus-ARC translation in different cell lines is primarily due to variations in FMRP expression.

### **CGG repeat expansions in the FMR1 gene cause increased calcium transients in human fibroblasts.**

Expanded CGG repeats in endogenous FMR1 RNA inhibit translation of Venus-ARC RNA, which is a reporter for translation of RNAs localized in the same granules as FMR1 RNA.

Translation of endogenous RNAs localized in the same granules may also be inhibited, which may result in additional cellular phenotypes. In this regard, previous studies have shown that calcium transients are increased in astrocytes from CGG KI mice, which contain a CGG repeat transgene (21), and in iPSC-derived neurons from individuals with expanded CGG repeats in the endogenous FMR1 gene (22). These observations suggest that expanded CGG repeat RNA inhibits translation of endogenous RNAs encoding proteins that mediate calcium homeostasis. To determine if calcium transients are affected in fibroblasts from individuals with CGG repeat expansions in the endogenous FMR1 gene, control and premutation human fibroblasts were loaded with Fluo-4 as a calcium indicator and bradykinin was added to induce calcium transients (Fig. 6). Fluo-4 fluorescence in individual cells was imaged by time lapse confocal microscopy. Calcium transients were detected by integrating Fluo-4 fluorescence over each cell at each time point. In control fibroblasts (C0603), bradykinin induced a single primary calcium transient, which returned to baseline after ~ 20 sec in all cells. In premutation fibroblasts with expanded CGG repeats in the endogenous FMR1 gene (FX08-2), the primary calcium transient was followed by secondary calcium transients in some cells. The time interval between primary and secondary calcium transients was variable (20-60 sec) among different cells. These results indicate that CGG repeat expansions in the endogenous FMR1 gene disrupt calcium homeostasis in premutation fibroblasts. Premutation cells that did not exhibit secondary calcium transients may represent cells in which the normal FMR1 allele is active and the allele with expanded CGG repeats is inactivated.

To determine if TMPyP4 rescues calcium homeostasis in premutation fibroblasts, cells were treated with TMPyP4 for 24 hours before analyzing bradykinin-induced calcium transients. In TMPyP4-treated control and premutation fibroblasts, bradykinin induced a single primary calcium transient with no secondary transients. This indicates that TMPyP4 rescues calcium



**Figure 6: CGG repeat expansions in the FMR1 gene disrupt regulation of calcium transients in human fibroblasts.** Control (C0603) and premutation (FX08, 31,105 repeats) fibroblasts were loaded with fluorescent calcium indicator Fluo-4 and incubated with bradykinin to induce calcium transients. Cells were imaged by time-lapse confocal microscopy to visualize changes in intracellular calcium concentrations over time. Total fluorescence intensity over the entire cell was integrated in each time frame for 20 control and 20 premutation cells, in the absence or presence of TMPyP4.  $F/F_0$  values for each cell are plotted, with initiation of primary calcium transients aligned at time = 0 s.



homeostasis in premutation fibroblasts and that CGG repeat expansions in the endogenous FMR1 gene are associated with a phenotype that is manifested in fibroblasts.

The control fibroblasts used in these experiments were obtained from apparently normal male individuals with normal numbers of CGG repeats in the FMR1 gene, while the premutation fibroblasts were obtained from apparently normal female individuals carrying premutation CGG expansions in the FMR1 gene. This raises the possibility that the observed differences in granule RNA translation and calcium homeostasis between control and premutation fibroblasts could reflect gender differences rather than differences in CGG repeat expansions in the FMR1 gene. We believe this possibility is unlikely for several reasons. First, in cells with one normal FMR1 allele and one premutation allele, some cells exhibit normal granule RNA translation and calcium homeostasis, while others exhibit reduced granule RNA translation and disrupted calcium homeostasis, while in cells where both FMR1 alleles contain CGG repeat expansions, all cells exhibit reduced granule RNA translation. Since the FMR1 gene is located on the X chromosome, which is subject to random X inactivation in different cells, this suggests that granule RNA translation and calcium homeostasis are regulated by the number of CGG repeats in the active FMR1 allele in each cell, rather than by the gender of the donor. Second, the extent of inhibition of granule RNA translation in premutation cells is proportional to the number of CGG repeats in the premutation allele of the FMR1 gene, suggesting that granule RNA translation is regulated by the number of CGG repeats in the FMR1 gene rather than by gender differences. Third, both translation and calcium homeostasis in premutation cells are rescued by TMPyP4, which binds to CGG repeat RNA and destabilizes CGG repeat secondary structure, suggesting that both translation and calcium homeostasis are regulated by CGG repeats rather than by gender differences. For these reasons we believe the differences in granule RNA translation and calcium transients between control and premutation human

fibroblasts observed in this study are likely due to differences in CGG repeat number in the endogenous FMR1 gene rather than to gender differences.

## Discussion

CGG repeat RNAs, such as FMR1 RNA, and A2RE RNAs, such as Venus-ARC RNA, are localized and translated in the same RNA granules. CGG repeat RNA inhibits translation of ARC RNA in the same granule and TMPyP4 rescues ARC RNA translation in granules containing CGG repeat RNA. These observations can be explained by multiple potential mechanisms involving component(s) in granules that: 1) regulate translation; 2) are affected by CGG repeats; and 3) whose effects are reversed by TMPyP4. Here we will discuss three potential mechanisms involving components that meet these criteria: hnRNP A2, ribosomes, and EIF2AK2.

The first potential mechanism involves hnRNP A2, which binds to CGG repeat RNA (15-17) and is known to enhance translation of A2RE RNAs in granules (49). If RNA molecules with expanded CGG repeats bind increased numbers of hnRNP A2 molecules this could potentially reduce the number of hnRNP A2 molecules available to bind to ARC RNA molecules, which could potentially reduce translation of ARC RNA in granules. Several considerations argue against this possibility. First, the concentration of hnRNP A2 in cytoplasm is quite high (6  $\mu$ M), which suggests that this component may not be rate limiting for translation of ARC RNA. Second, since hnRNP A2 is required for assembly of ARC RNA into granules, reduced binding of hnRNP A2 to ARC RNA might result in reduced assembly of ARC RNA into granules, which was not observed (Fig. 4). Third, TMPyP4 rescues translation but is not known to interact with hnRNP A2. For these reasons it seems unlikely that intragranule competition between CGG repeat RNA and A2RE RNA for hnRNP A2 is responsible for inhibition of ARC RNA translation by CGG repeat RNA in the same granule.

The second mechanism involves ribosomes, which are required for translation of ARC RNA and also accumulate in the vicinity of CGG repeats. Ribosome profiling reveals increased ribosome density in the vicinity of CGG repeats in the 5'UTR of FMR1. If the number of CGG repeats is expanded, the ribosome density in this region could be further increased, although this has not been tested experimentally. Increased ribosome density in the CGG repeat region might reduce the number of ribosomes available for translation of ARC RNA in the same granule. The magnitude of such an effect would depend on the relative number of ribosomes associated with expanded CGG repeats in the 5'UTR of premutation FMR1 RNA molecules compared to the total number of ribosomes in each granule. In this regard, single molecule imaging reveals that granules with increased numbers of RNA molecules do not have increased numbers of translational events per granule (19), which implies that the number of ribosomes per granule may be rate limiting for translation. Furthermore, if secondary structure in CGG repeat RNA causes ribosomes to accumulate in the 5'UTR of FMR1 RNA, TMPyP4 might reduce ribosome density by disrupting CGG repeat secondary structure in this region, which might increase availability of ribosomes for translation of ARC RNA in the same granules, reversing the effects of CGG repeat expansion. These considerations are consistent with intragranule competition between CGG repeat RNA and ARC RNA for ribosomes as a potential mechanism for inhibition of ARC RNA translation by CGG repeat RNA in the same granule.

The third mechanism involves EIF2AK2, eukaryotic translation initiation factor 2 alpha kinase 2, a serine/threonine protein kinase activated by dsRNA that inhibits conventional translation by phosphorylating translation initiation factor EIF2a (50). EIF2AK2 RNA itself contains CGG repeats in the 5'UTR (1), which may mediate localization and translation of EIF2AK2 RNA in the same granules as FMR1 RNA and ARC RNA. CGG repeats in FMR1 RNA can form dsRNA secondary structure, which may activate EIF2AK2, thereby inhibiting ARC RNA translation in the same granule. Expansion of CGG repeats in FMR1 RNA may increase stability of dsRNA

secondary structure, thereby increasing activation of EIF2AK2 and reducing translation of ARC RNA. TMPyP4 may destabilize CGG repeat dsRNA secondary structure in FMR1 RNA, thereby reversing activation of EIF2AK2 and rescuing translation of ARC RNA. These considerations are consistent with activation of EIF2AK2 by CGG repeat RNA as a potential mechanism for inhibition of ARC RNA translation in the same granule.

The above, and other, potential mechanisms could be distinguished by knocking out or inhibiting specific components that are: localized in the same granules as FMR1 RNA, regulate translation of other RNAs in the same granule, are potentially affected by CGG repeat expansions in FMR1 RNA, and whose effects are potentially reversed by TMPyP4. However, without such experimental validation it is not possible to identify specific components that account for the observations reported here.

The human exome contains multiple different CGG repeat RNAs besides FMR1 RNA and multiple different A2RE RNAs besides ARC RNA, some of which may be localized in the same RNA granules as FMR1 RNA because CGG repeat sequences and A2RE sequences both bind to hnRNP A2, which mediates granule assembly. This may create an intra-granule translational regulatory network, where expansion of CGG repeats in one RNA increases the overall CGG repeat burden in the granule, thereby decreasing translation of other RNAs in the same granule. Since CGG repeat RNAs and A2RE RNAs encode multiple different proteins that mediate multiple different cellular functions, regulating translation of these RNAs in granules could have pleiotropic effects on the cell. In this regard, expression of a CGG repeat transgene is known to affect translation of multiple different RNAs in mice and flies (20), which could explain why expansions of CGG repeats in various genes are associated with pleiotropic pathological manifestations such as: late onset, tremors, ataxia, dysphagia (difficulty swallowing), dysarthria (difficulty speaking).

Calcium homeostasis is a complex cellular process mediated by multiple different proteins, some of which are encoded by CGG repeat and/or A2RE RNAs. For example: CGG repeat RNAs such as: CACNA1A (voltage dependent calcium channel) and KCNMA1 (calcium activated potassium channel), could potentially affect calcium homeostasis and A2RE RNAs such as CAMK2A (calcium, calmodulin-dependent protein kinase 2A) and NRGN (neurogranin/calmodulin binding protein) could also potentially affect calcium homeostasis. If calcium homeostasis is mediated by proteins encoded by CGG repeat and/or A2RE RNAs that are localized in granules and translationally regulated by CGG repeat RNA this could explain why calcium homeostasis is disrupted in premutation fibroblasts from individuals with CGG repeat expansions in the endogenous FMR1 gene. However it is difficult to identify one specific gene or group of genes that mediates the calcium phenotype in premutation fibroblasts to because multiple different CGG repeat and/or A2RE RNAs encode proteins could potentially affect calcium homeostasis and TMPyP4 can potentially affect multiple different pathways in the cell.

TMPyP4 binds to CGG repeat RNA and rescues granule RNA translation and calcium homeostasis. The effect of TMPyP4 on calcium homeostasis may be secondary to the effect on granule RNA translation if translation of RNAs that mediate calcium homeostasis is inhibited by CGG repeat RNA and rescued by TMPyP4. However, it is also possible that TMPyP4 affects calcium homeostasis directly. Previous studies have shown that TMPyP4 can induce calcium release by direct interaction with the Ca<sup>2+</sup> release protein from sarcoplasmic reticulum (51). Furthermore, studies in yeast suggest that TMPyP4 can induce a cellular oxidative stress response that could affect calcium homeostasis (52). TMPyP4 rescue of translation and calcium homeostasis in premutation fibroblasts could theoretically involve similar mechanisms. The observation that granule RNA translation and calcium homeostasis are dysregulated in premutation fibroblasts indicates that pathogenic effects of CGG repeat expansions in FMR1

(and possibly other CGG repeat genes) are manifested in non-neuronal cell types. In this study premutation fibroblasts that exhibit dysregulated granule RNA translation and calcium homeostasis were obtained from apparently healthy young female premutation carriers with no apparent neurological or neuromuscular symptoms. This indicates that disrupted granule RNA translation and calcium homeostasis in human fibroblasts may provide early preclinical biomarkers for identifying individuals at increased risk to develop late onset neurological or neuromuscular disorders such as FXTAS, FXPOI or other CGG repeat disorders. Chronic, sustained disruption of granule RNA translation and/or calcium homeostasis may be deleterious to the cell over the long term and may contribute to late onset development of neurological or neuromuscular pathology in these individuals. Restoring normal granule RNA translation and/or calcium homeostasis with TMPyP4 could potentially reduce the risk of subsequent onset of these disorders in premutation carriers. Inhibition of translation in granules by CGG repeat expansions in FMR1 RNA represents a potential pathogenic mechanism for CGG repeat expansion disorders such as FXTAS and FXPOI. Rescue of translation in granules by TMPyP4 could potentially ameliorate pathogenic effects in CGG repeat expansion disorders.

### **My contributions to this work**

George Korza and I performed the in vitro transcription of all RNAs in this project. I also performed SPR experiments with the help of George Korza. I performed all microinjection experiments and the confocal imaging to follow. I maintained neurons and fibroblasts in culture, but the initial plating was done by Dr. Elisa Barbarese. I am responsible for the FCS experiments and analyses. I also performed and analyzed the calcium transient experiments. I was involved in conceptualization and interpretation of all experimental approaches and results as well as writing and editing of the publication.

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### **Chapter3:**

**CGG repeat RNA inhibits translation of granule RNA in oocytes.**

## **Abstract**

An expanded CGG repeat tract in the 5'UTR of the FMR1 gene is associated with the fragile X family of disorders, including smaller expansions (~55-200), referred to as premutation expansions, associated with fragile X tremor ataxia syndrome (FXTAS) and fragile X primary ovarian insufficiency (FXPOI) and larger expansions (>200), referred to as full mutation expansions, associated with fragile X syndrome (FXS). One current controversy in the field centers on whether the premutation disorders are RNA gain of function disorders or protein/peptide gain of function disorders. Here we show that exogenous CGG repeat RNA (not in the context of FMR1) microinjected into mouse oocytes inhibits translation of VenusArc, a reporter hnRNP A2 granule RNA, but a transgenic construct containing 90 CGG repeats, a small part of the FMR1 open reading frame and part of intron 1, does not inhibit VenusArc RNA microinjected into transgenic TG296 mouse oocytes. By comparison to experiments in chapter 2 with Venus Arc coinjected with synthetic CGG repeat RNA and Venus Arc microinjected into human fibroblast cells from carriers for the premutation, we show that translational inhibition is likely an RNA gain of function mechanism. Furthermore, we show that PKR activation may be the mechanism for translational inhibition.

## **Introduction**

The fragile X family of disorders is associated with the expansion of a CGG repeat tract in the 5'UTR of the FMR1 gene located on the X chromosome. In unaffected individuals, the number of CGG repeats is less than ~55 repeats. When the number of CGG repeats expands to between 55 and 200 CGG repeats, this is referred to as the premutation. Premutation expansions are often associated with fragile X tremor ataxia syndrome (FXTAS), a late onset neurodegenerative disorder, or fragile X primary ovarian insufficiency (FXPOI). Expansion to more than ~200 CGG repeats, called the full mutation, is associated with fragile X syndrome (FXS), an early onset mental retardation (1). Experiments in chapter 2 show that exogenous

CGG repeat expansion RNA inhibits translation in RNA granules in neurons, which may represent a mechanism for FXTAS, which affects neuronal function. Experiments in chapter 2 also show that CGG repeat expansions in the endogenous FMR1 gene inhibit translation in RNA granules in fibroblasts from females at risk for FXPOI (2), which may represent a mechanism for FXPOI. In chapter 3, we will investigate whether expanded CGG repeats affect translation in RNA granules in mouse oocytes and whether this may represent a potential mechanism for FXPOI.

Primary ovarian insufficiency (POI) is a disorder characterized by infertility, irregular menses and early menopause (3). The corresponding reduced estrogen levels result in a number of deleterious side effects, including increased rates of twinning, hot flashes, vaginal dryness, insomnia, decreased bone mineral density and reduced overall cardiovascular health (4, 5). Even more serious is the increased risk for disorders that accompany early menopause, including increased risk of coronary heart disease and osteoporosis (6). FXPOI comprises 4-6% of all cases of POI (6). In females, up to 24% of premutation carriers present with FXPOI (7, 8).

In FXS, the full mutation expansion results in hypermethylation of the DNA resulting in silencing of the transcript (9). As a result, this is considered a protein loss of function disorder. However, the FMR1 gene is not silenced in the premutation, so FXTAS and FXPOI are not considered protein loss of function disorders. The current controversy in the field concerns whether these disorders result from protein gain of function or RNA gain of function (10). Chapter 3 will provide some insight into which of these mechanisms causes FXTAS and FXPOI.

The ribosome profile for FMR1 RNA (shown in Figure 1 Chapter 2) reveals increased ribosome density in the ORF, presumably representing ribosomes engaged in conventional translation of the ORF. Additionally, ribosome density is also increased in the 5'UTR suggesting that

ribosomes are stalled in the CGG repeat region (2). This may be indicative of an unconventional type of translation called repeat associated nonAUG translation (RAN translation). RAN translation products may be toxic to the cell, which would represent a protein gain of function mechanism. Alternatively, increased ribosome density in the 5'UTR of FMR1 RNA may be due to ribosomal stalling at CGG repeat secondary structure in this region (as suggested in chapter 2), which would represent an RNA gain of function mechanism for FXTAS and FXPOI.

In chapter 2 exogenous CGG repeat RNA, not in the context of FMR1 RNA, was microinjected into mouse hippocampal neurons. 99 CGG repeats were sufficient to co-localize CGG repeat RNA in the same granules as VenusArc RNA, a reporter A2RE RNA (2). This verifies that CGG repeats are a localization signal, similar to an A2RE, and since A2RE RNAs localize to hnRNP A2 RNA granules (11), we can assume CGG repeat RNA localizes to hnRNP A2 RNA granules as well. In chapter 3, we will show that hnRNP A2 appears to be granular in oocytes, suggesting that hnRNP A2 RNA granules are present in oocytes, which would allow the mechanism for RNA toxicity seen in mouse neurons and human fibroblast cells to be recapitulated in mouse oocytes.

Also in chapter 2 we show that the microinjected exogenous CGG99 RNA inhibits translation of VenusArc RNA in granules, but granule RNA translation is rescued by TMPyP4, which binds to CGG repeat RNA (2) and destabilizes secondary structure (12). This suggests that CGG repeat secondary structure is necessary for translational inhibition. In chapter 3 three different potential mechanisms are discussed to explain CGG repeat RNA inhibition of translation in RNA granules. The first potential mechanism, competition of expanded CGG repeat RNA with other granule RNAs for hnRNP A2, is not likely because the concentration of hnRNP A2 in the cytoplasm is believed to be in excess (2). The second potential mechanism, sequestration of translational machinery by CGG repeat RNA, and the third potential mechanism, activation of PKR by CGG repeat RNA, both represent RNA gain of function mechanisms. In chapter 3, we

will show that microinjected CGG99 RNA inhibits translation of VenusArc RNA in oocytes, and we will discuss the different possible RNA gain of function mechanisms for this affect.

In chapter 2, we showed that CGG repeat expansions in endogenous FMR1 RNA inhibit translation in RNA granules in human fibroblast cells (2). This suggests that the CGG repeat microinjection experiment is a good model for FXTAS and FXPOI. In this chapter we explore translational inhibition in oocytes as it relates to FXPOI. However, it is difficult to obtain oocytes from human subjects, especially in a disease model. Therefore, we investigated the effects of exogenous CGG repeat RNA on granule RNA translation in control mouse oocytes and the effects of transgenic CGG repeat RNA in transgenic mouse oocytes. The transgenic mouse available to us contains a construct with 90 CGG repeats in the 5'UTR of FMR1, part of the open reading frame and part of intron 1, making it slightly different from a model with CGG repeat expansions in the context of a complete FMR1 RNA ORF (13). In chapter 3, we show that endogenous transgenic CGG repeat RNA does not inhibit translation of VenusArc RNA in transgenic oocytes. Possible reasons for the different results in FXPOI human fibroblasts and transgenic mouse oocytes will be discussed.

In addition to potential RNA gain of function mechanisms, it is possible that RAN translation products of the FMR1 gene with premutation CGG repeats are somehow toxic to the cell causing FXTAS or FXPOI by a protein gain of function mechanism (14). In chapter 3, we will demonstrate that this is likely not the case.

## **Materials/Methods**

### ***CGG repeat profiles***

A sliding sequence algorithm was used to generate CGG repeat homology profiles for the various constructs as described in chapter 2 (2).



### ***Western blotting***

For Western blots, oocytes were boiled in 1X sample buffer (Laemmli et al) and proteins were separated on 4-20% gradient gels (BioRad) and electrophoretically transferred to nitrocellulose membranes.

### ***FMRP***

Membranes were blocked for at least 30 minutes in blocking buffer (TBST + 1% nonfat milk), then incubated overnight in primary antibody. FMRP antibody, obtained from Abcam (cat #ab17722), was diluted 1:2500 in blocking buffer (TBST+2% milk). Blots were washed in TBST, and incubated for at least 60 minutes in secondary antibody. Secondary antibody obtained from Santa Cruz Biotechnology (cat #sc-2004) was diluted 1:5,000 in blocking buffer. Blots were developed using ECLPrime (Life Technologies).

### ***PKR***

Membranes were blocked for 45min in blocking buffer (4% Bovine Serum Albumin in Tris/NaCl, 0.2% Tween, pH7.7 (Blotto buffer)) then incubated overnight in primary antibody. PKR antibody, obtained from Abcam, was diluted 1:2000 in Blotto buffer. Blots were washed in 8 to 10mL Blotto buffer at room temperature for a total of three times and incubated in secondary antibody for 1hr at room temperature. Secondary antibody (goat anti-rabbit HRP) obtained from Millipore was diluted 1:10,000 in Blotto buffer. Membranes were washed with 8 to 10 mL Blotto buffer for 10min at room temperature a total of three times, then rinsed with approx. 10ml water five times. After the final rinse blots were incubated with 15 mL Tris/NaCL 0.1%Tween, pH7.6 for 15 minutes. The buffer was discarded and 8 mL ECL reagent (Pierce Super Signal West Pico Chemiluminescent Substrate; #34078) was added for 10 minutes.

## ***Mice***

Female NSA(CF1) mice were obtained from Harlan Laboratories. Transgenic mice carrying a yeast artificial chromosome with the human FMR1 5'UTR containing 90 CGG repeats (13) were obtained from Peng Jin (Emory University). These mice were genotyped from ear punches to determine that they were positive for the transgene using the REDEExtract-N-AmpTissue PCR Kit from Sigma and primer sets 5'-TCAAATTGCGAAGGAGCAG-3' (forward) and 5'-AACGAATATGTGTTGAGGACCC-3' (reverse). Males testing positive for the transgene were mated with wild type females of the same strain (FVB) to generate transgenic and wild type mice. In some cases, female FVB/J mice were obtained from Charles River. FMR1 KO mice were obtained from Elisa Barbarese (UConn Health). All experimental protocols involving mice were performed with prior approval of the Animal Care and Use Committee at UConn Health.

## ***Media and Reagents***

Except where noted, all reagents were from Sigma Aldrich (St. Louis, MO). The medium for oocyte collection was MEM $\alpha$  (Life Technologies, Carlsbad, CA) supplemented with 20mM HEPES (Life Technologies), 75  $\mu$ g/mL penicillin G, 50  $\mu$ g/mL streptomycin, 0.1% polyvinyl alcohol (PVA), and 10 $\mu$ M milrinone to inhibit spontaneous meiotic resumption. For overnight culture, oocytes were placed in bicarbonate-buffered MEM $\alpha$ , in which the HEPES was replaced with 25mM sodium bicarbonate.

## ***Collection and culture of oocytes***

Fully grown germinal vesicle (GV)-stage oocytes were obtained from the ovaries of >6 wk-old mice primed 42-46 hrs previously with 5 IU pregnant mare serum gonadotropin (PMSG). For experiments utilizing TG296 mice, oocytes were obtained from ovaries of 8-9 wk-old mice that were not primed with PMSG. Cumulus cells were removed by repeated pipetting through a small-bore pipet. Oocytes were cultured in 200  $\mu$ L drops of medium under light mineral oil on a

warming tray set to 37°C (when in HEPES-buffered medium) or in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>, 95% air (when in bicarbonate-buffered medium).

### ***RNA preparation***

Venus DNA was a gift from Dr. Ji Yu (UConn Health, Farmington, CT). Venus-ARC construct was derived from ARC cDNA clone (pBS II (SK+) r ARC obtained from Dr. Paul Worley (Johns Hopkins University, Baltimore, MD). The ARC open reading frame (ORF) was inserted in-frame at the C-terminus of the Venus ORF in a Venus vector. The *NotI-NdeI* fragment from this clone, including the Venus ORF and the first 16 bases from ARC ORF, was excised and religated with the corresponding *NotI-NdeI* fragment of the original ARC cDNA construct. The final construct carries the complete ARC ORF and 3'-UTR but not the 5'-UTR. Venus-Arc RNA was prepared by in vitro transcription of linearized plasmid DNA as previously described (15, 16). CGG99 RNA was prepared from plasmid DNA containing FMR1 cDNA with 99 CGG repeats obtained from Dr. Fry (12). Plasmid DNA was linearized with XhoI (New England Biolabs) immediately downstream of the CGG repeat region followed by use of a MinElute reaction cleanup kit (Qiagen) according to the manufacturer's protocol. In vitro transcription was performed in the presence of Cy5-labelled UTP. All UTPs were obtained from GE Healthcare Biosciences, Pittsburgh, PA. In vitro transcription of CGG99 RNA was performed using T7 mScript Standard Standard mRNA Production System (CellScript, Madison, WI) according to manufacturer's protocol. In vitro transcription of VenusArc RNA and Venus RNA was performed using Amplicap-Max T3 High Yield Message Maker Kit (CellScript, Madison, WI) followed by capping and polyadenylation using reagents from the T7 mScript Standard mRNA Production System. RNA quality and purity was analyzed by gel electrophoresis and FCS.

### ***Microinjection***

Quantitative microinjection was performed as described previously (17), using a direct pressure system in which a small volume of mercury in a micropipette permits controlled injection of

picoliter quantities. Concentrations of injected substances were calculated based on an oocyte volume of 200 pL. GFP vector with a CMV promoter was obtained from Clontech. Oocytes that were injected with Venus-Arc  $\pm$  CGG99 RNA were cultured overnight to allow protein expression and then were fixed for 1 hr in 2% formaldehyde in 100mM HEPES, 50mM EGTA, 10mM MgSO<sub>4</sub>, and 0.2% Triton X-100 at 37°C 23-24 hrs following injection, washed into PBS containing 0.1% PVA, and stored at 4°C until imaging.

### ***Confocal microscopy***

Fluorescent images were acquired using a Zeiss LSM 510 inverted confocal-laser scanning microscope with a 40X 1.2 numerical aperture water immersion objective lens at zoom 2 with a line average of 8. Multi-channel acquisition was used to scan Venus and Cy5 in channel 1 by excitation with a 488nm argon laser and a 633nm HeNe laser followed by scanning of Cy3 in channel 2 by excitation with a 561 nm laser. Images without Cy3 were acquired using one channel. GFP was imaged with the same properties as Venus in one channel. The emitted light was collected in a bandwidth of 500-550nm for Venus and GFP, 650-710nm for Cy5 and 575-615nm for Cy3. Differential interference contrast (DIC) images were also collected in channel 1. The excitation laser intensity was adjusted so that control signals were minimized near the detection limit, but sample signals were maximized near the saturation limit.

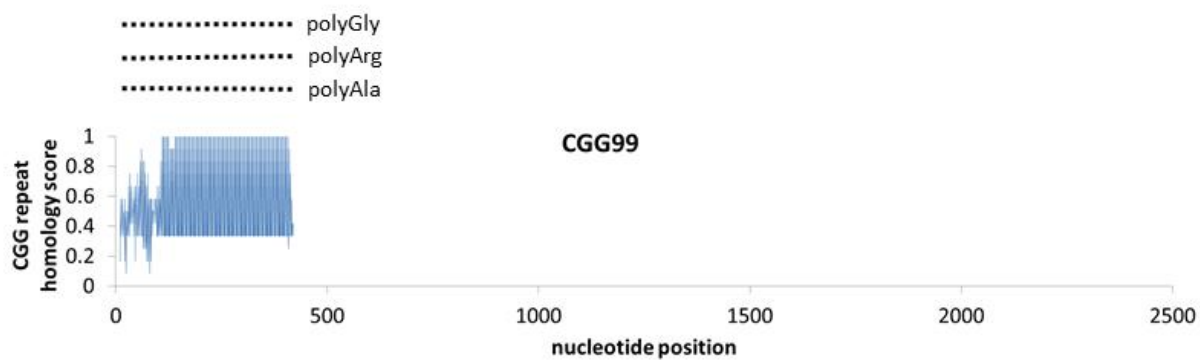
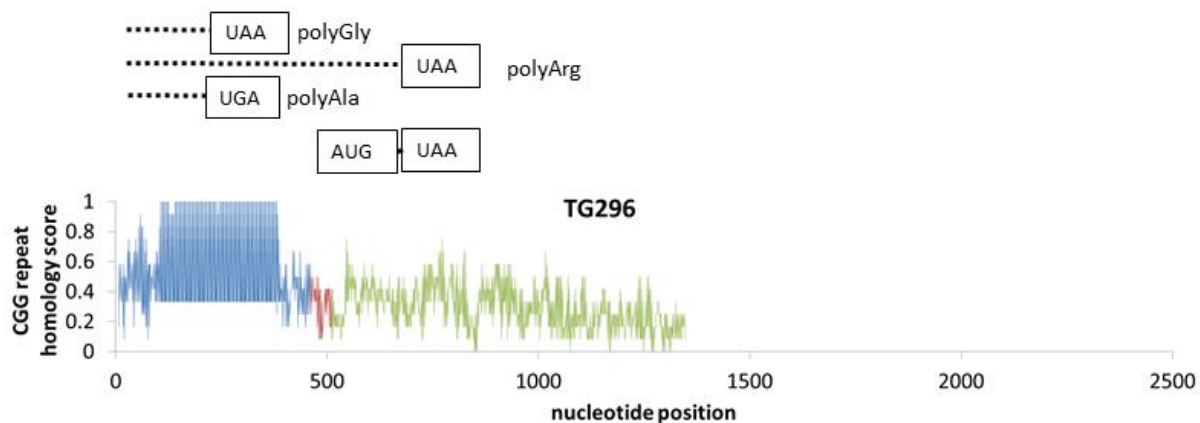
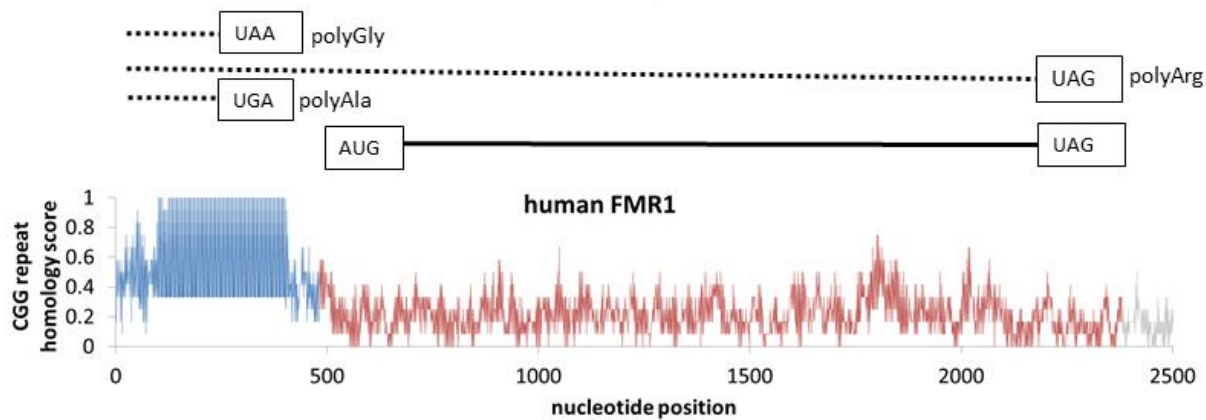
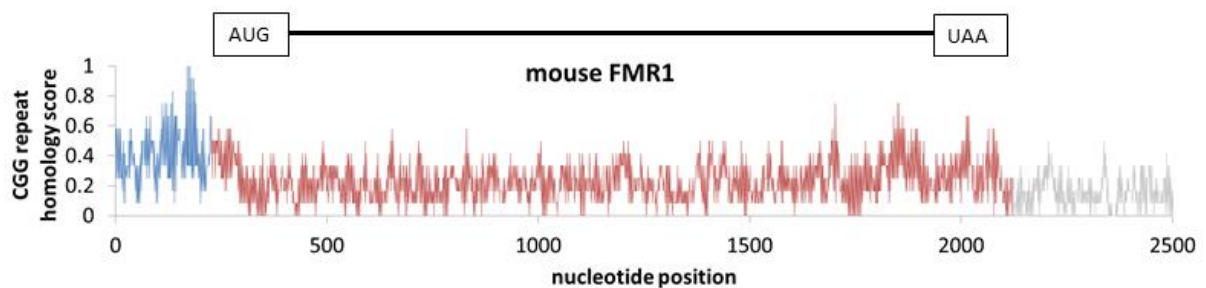
### ***Immunofluorescence***

Oocytes were fixed for 1 hr in 2% formaldehyde in 100mM HEPES, 50mM EGTA, 10mM MgSO<sub>4</sub>, and 0.2% Triton X-100 at 37°C. After fixation, oocytes were incubated in blocking buffer (PBS containing 0.01% Triton X-100, 0.1% PVA, and 3% BSA), then in primary antibody, anti-hnRNP A2 mouse clone EF-67 from Santa Cruz (cat #sc-53531) overnight, diluted 1:200 in blocking buffer. Oocytes were washed in PBS containing 0.1% PVA. The secondary antibody, anti-mouse FITC obtained from Santa Cruz (cat #sc-2010), was diluted 1:200 in blocking buffer

and incubated for 90min. GFP RNA-injected oocytes were incubated overnight and were fixed and imaged as above.

## Results

Figure 1 shows CGG repeat homology profiles for mouse FMR1 RNA, human FMR1 RNA, the construct used to generate TG296 transgenic mice and the microinjected CGG99 RNA construct. Mouse and human FMR1 RNAs contain the 5'UTR containing CGG repeats, the ORF and the RNA sequence downstream of the ORF. Notably, there are fewer CGG repeats in mouse FMR1 than in human FMR1 RNA. Consequently, it is unclear whether RAN translation of mouse FMR1 RNA is as efficient as human FMR1 RNA. Both mouse and human FMR1 RNA are substrates for conventional translation. Human FMR1 RNA with premutation CGG repeats may be a substrate for three potential RAN translation products. RAN translation in the GCG reading frame can potentially produce a peptide consisting of a poly-alanine tract terminated by an in-frame stop codon in the 5'UTR. RAN translation in the CGG reading frame can potentially produce a peptide beginning with a poly-arginine tract and continuing in frame through the conventional FMRP translation product, and ending at the conventional FMRP stop codon. RAN translation in the GGC reading frame can potentially produce a short peptide poly-glutamine tract terminating at a stop codon in the 5'UTR. Each of these RAN translational products could potentially be generated in cells from a FXPOI affected individual with CGG repeat expansions in the endogenous FMR1 gene. In the TG296 construct conventional translation could also initiate at the AUG codon in exon 1 and continue through intron 1 terminating at a stop codon in intron 1. This could potentially produce a partially nonsense translation product. RAN translational in the GCG and GGC reading frames for the TG296 construct would be the same as for endogenous human FMR1 RNA because the sequence is identical in the 5'UTR. RAN translation in the CGG reading frame for the TG296 construct would terminate at the in frame



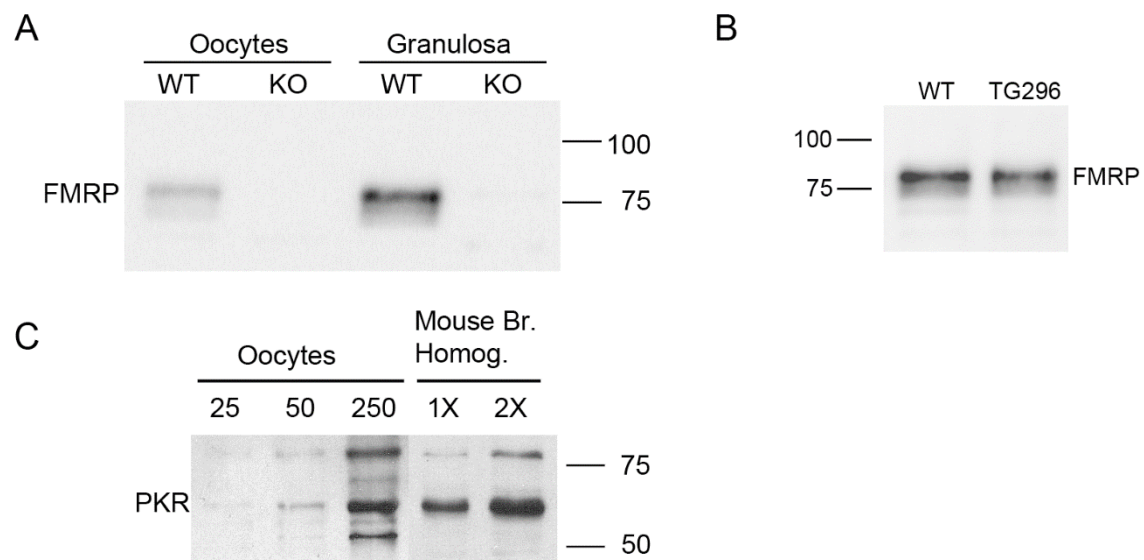
**Figure 1: CGG repeat profiles and potential translation products for CGG repeat RNAs.**

CGG repeat homology profiles are shown for mouse FMR1, human FMR1, the TG296 RNA construct and the microinjected CGG99 RNA, in order. The blue regions represent the 5'UTRs. The red regions represent the ORFs. The gray region in FMR1 is the RNA sequence just downstream of the ORF. The green region in the TG296 RNA construct represents intron 1 from FMR1. Start codons are located to the left of the box labelled start. Stop codons are located to the right of the box labelled stop. Shown above the homology profiles are the potential translational products. Conventional translation products are represented by a solid line while potential RAN translation products are represented by a dotted line. Start and stop codons are indicated where applicable, and the amino acid repeat tract encoded by the repeat region in the 5'UTR is shown for each RAN translation product.

stop codon in intron 1 producing a partially nonsense translation product. The microinjected CGG99 RNA contains no start or stop codons in any frame. Therefore, it would not be subject to conventional translation. RAN translation in all three reading frames could potentially produce poly-alanine, poly-arginine or poly-glutamine. However, with no stop codon, the fates of these peptides and the translational machinery associated with them is not clear. Normally, this type of RNA would be subject to non-stop decay (NSD), a mechanism for releasing the translational machinery from the RNA when there is no stop codon present. However, NSD requires a polyA tail (18), and the CGG99 RNA is not polyadenylated. The potential RAN translation products for the transgene and for endogenous human FMR1 gene are more similar to each other than the potential RAN translation products for microinjected CGG repeat RNA. If granule RNA translation is inhibited by RAN translation products by a protein gain of function mechanism then translation inhibition in the transgenic mouse oocytes might be similar to translation inhibition in human premutation fibroblast cells, as reported in chapter 2. However if granule RNA translation is inhibited by CGG repeat RNA by an RNA gain of function mechanism then translation inhibition may be similar in transgenic mouse oocytes expressing CGG repeat RNA and in oocytes injected with exogenous CGG repeat RNA unless CGG repeat RNA is not exported to the cytoplasm or localized in granules in the transgenic oocytes .

There is conflicting data as to where and when FMRP is expressed in the ovary. Two reports show expression of FMRP in oocytes and granulosa cells (4, 19) and one shows FMRP protein in granulosa cells but not in the oocyte (20). To resolve this issue, we performed western blots on oocytes and granulosa cells for FMRP. We found that oocytes and granulosa cells both express FMRP (Figure 2A). Levels of FMRP were significantly higher in granulosa cells compared to oocytes confirming previous reports (19). To determine if endogenous FMRP expression is affected in TG296 mice (Figure 2B) we analyzed FMRP in granulosa cells. FMRP





**Figure 2: FMRP and PKR expression in oocytes and granulosa cells.** Panel A – Western blot analysis of FMRP expression in wildtype (WT) and FMR1 knockout mouse (KO) oocytes and granulosa cells. The expected molecular weight for FMRP is 71kDa. Panel B – Western blot analysis of FMRP expression in wildtype (WT) and TG296 transgenic mouse granulosa cells. Panel C – Western blot analysis of PKR expression in 25, 50 or 250 WT oocytes and 1X or 2X mouse brain homogenate. The expected molecular weight for PKR is 58kDa.

expression was comparable in both wild type and transgenic granulosa cells, confirming previous results that showed similar amounts of FMRP in whole ovaries (19).

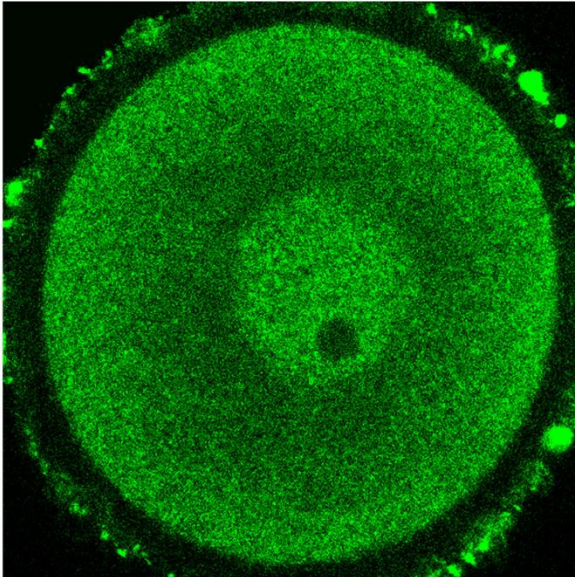
Inhibition of granule RNA translation may be mediated by PKR activation by CGG repeat RNA. To determine if oocytes express endogenous PKR, we performed western blots for PKR in mouse oocytes (Figure 2C). The results show that PKR is expressed in mouse oocytes suggesting that granule RNA translation in oocytes could be inhibited by activation of PKR by CGG repeat RNA.

Oocytes were immunostained for hnRNP A2 to determine if hnRNP A2 is expressed and localized in granules in mouse oocytes. Figure 3 shows that hnRNP A2 in oocytes appears granular compared to GFP which appears diffuse. The size range of the granular structures containing hnRNP A2 in oocytes is consistent with the size of hnRNP A2 RNA granules in neurons (0.5-1 $\mu$ m) (15).

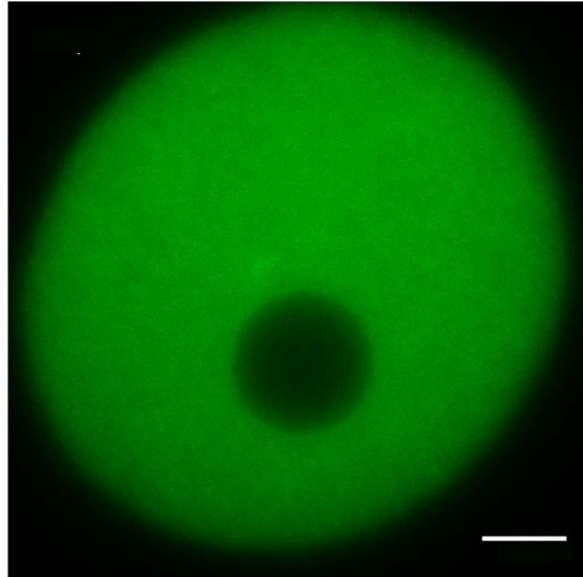
To measure granule RNA translation in oocytes, we microinjected Cy5-labelled RNA encoding a Venus-tagged ARC (VenusArc) and imaged the oocytes with confocal microscopy after overnight incubation. Although individual granules are difficult to resolve due to the large volume of the oocyte and the optical sectioning limitations of the confocal microscope, the injected RNA was dispersed throughout the oocyte in a granular distribution (Figure 4C). Expression of Venus-ARC detected in the 488 nm channel indicated that the injected RNA was translated in oocytes.

To determine if CGG repeat RNA affects translation in oocytes, Cy5-VenusArc RNA with Cy3-labelled RNA was co-injected with RNA containing 99 CGG repeats (CGG99). The CGG99 RNA also exhibited a granular appearance (Figure 4C). When this RNA was co-injected with VenusArc RNA, translation of VenusArc was inhibited. Specific translational activity was calculated by dividing whole cell mean fluorescent intensity of VenusArc protein by whole cell

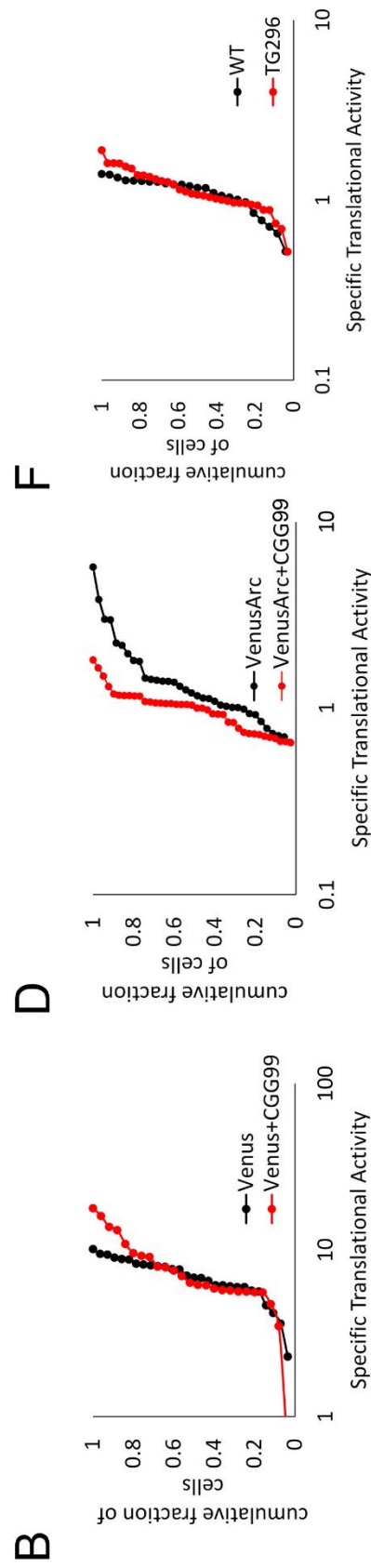
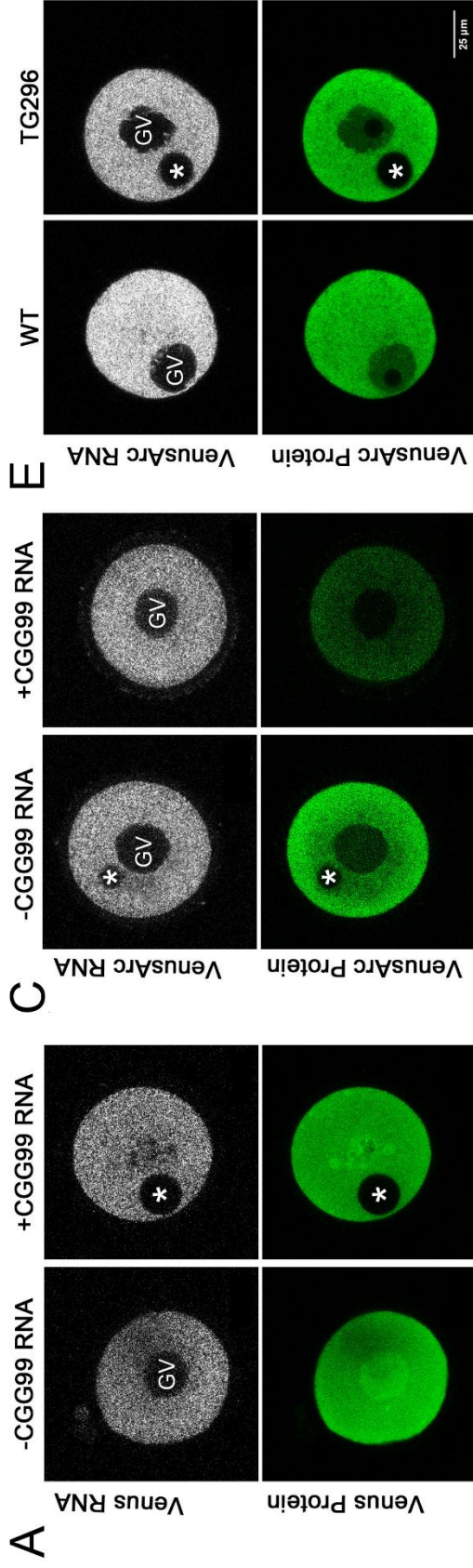
anti-hnRNP A2



GFP



**Figure 3: hnRNP A2 is granular in oocytes.** Immunostaining for hnRNP A2, left panel, shows that hnRNP A2 is localized in granule-type structures when compared to GFP, right panel) microinjected into oocytes. Scale bar is 25µm.



**Figure 4: Translation of VenusArc RNA is inhibited in oocytes microinjected with CGG99 RNA but not in transgenic TG296 oocytes.** Panel A, control – Wildtype oocytes were microinjected with Venus RNA (green) without CGG99 RNA, left, or with CGG99 RNA (red), right. Newly synthesized Venus-ARC protein is shown in white. Panel B – Cumulative Kolmogorov Smirnov plots for specific translational activities (newly synthesized Venus protein/microinjected Venus RNA) for oocytes microinjected with VenusArc RNA  $\pm$  CGG99 RNA. Panel C – Wildtype oocytes were microinjected with VenusArc RNA (green) without CGG99 RNA, left, or with CGG99 RNA (red). Newly synthesized Venus-ARC protein is shown in white. Panel D – Cumulative Kolmogorov Smirnov plot for specific translational activities (newly synthesized VenusArc protein/microinjected VenusArc RNA) for oocytes microinjected with VenusArc RNA  $\pm$  CGG99 RNA. Panel E – Wildtype oocytes, left, or transgenic TG296 oocytes were microinjected with VenusArc RNA (green). Newly synthesized Venus-ARC protein is shown in white. Panel F – Cumulative Kolmogorov Smirnov plot for specific translational activities (newly synthesized VenusArc protein/microinjected VenusArc RNA) for wildtype or TG296 oocytes microinjected with VenusArc RNA. GV is the germinal vesicle and \* is the oil droplet that enters the oocytes during the microinjection process (17). Representative images are shown. Comparable results were obtained with at least 24 oocytes in each experiment.

mean fluorescent intensity of VenusArc RNA. VenusArc alone had a specific translational activity between 1 and 10 while the specific translational activity of VenusArc coinjected with CGG99 was usually ~1 (Figure 4D). In contrast, Venus, which does not contain an A2RE and therefore should not accumulate in hnRNP A2 RNA granules was not inhibited by coinjection of CGG99 RNA (Figure 4A,B).

To determine if endogenous CGG repeat RNA inhibits granule RNA translation in oocytes we microinjected transgenic TG296 and wild type oocytes with VenusArc RNA and compared VenusArc translation in each case. We found that transgenic oocytes expressed VenusArc protein with comparable specific translational activities as wild type oocytes (Figure 4E,F) suggesting that endogenous CGG repeat RNA expressed in TG296 transgenic mouse oocytes does not inhibit granule RNA translation. Possible explanations for the differential effects of exogenous and endogenous CGG repeats on granule RNA translation are discussed in the following section.

## **Discussion**

We have shown that CGG repeat RNA inhibits translation of other RNAs localized in hnRNP A2 RNA granules in neurons and in oocytes injected with an RNA containing 99 CGG repeats, not in the context of FMR1. We have also shown that granule RNA translation is inhibited in human fibroblast cells expressing endogenous FMR1 RNA containing a CGG expansion in the 5'UTR but not in the transgenic TG296 oocytes containing a truncated FMR1 gene containing exon1 and part of intron 1. Several possible RNA gain of function or a protein gain of function mechanisms for inhibition of granule RNA translation have been proposed.

If the mechanism for translational inhibition is protein gain of function, then toxic protein expressed should be the same in the endogenous FMR1 RNA with CGG repeat expansions in human fibroblast cells and in wildtype neurons and oocytes injected with exogenous CGG



repeat RNA because translation of VenusArc RNA was inhibited in each case. However the toxic protein species should be different in transgenic TG296 mice because translation of VenusArc RNA is not inhibited. As described in Figure 1, RAN translation products (which represent the potential toxic protein species) are different for human FMR1, for the TG296 RNA and the synthetic CGG99 RNA. This suggests that the mechanism for translational inhibition is not protein gain of function.

There are two alternative RNA gain of function mechanisms for inhibition of granule RNA translation by CGG repeat RNA. One is competition for translational machinery in the granule. Ribosome profiling in chapter 2 showed that ribosomes tend to accumulate in the 5'UTR of the FMR1 gene in the region of CGG repeats suggesting that CGG repeats may inhibit granule RNA translation by sequestering translational machinery. In this case the CGG repeat region would be the part of the RNA that is responsible for sequestering the translational machinery. The full length endogenous FMR1 RNA, the transgenic TG296 RNA and the exogenous CGG99 RNA all contain CGG repeats in the 5'UTR that should be equally capable of sequestering translational machinery. Yet, we do not see translational inhibition in the TG296 oocytes. One possible explanation may be that transgenic CGG repeat RNA expressed in the TG296 oocytes is not exported to the cytoplasm because it lacks appropriate exon-exon junctions, which are required for proper splicing and nuclear export.

A second possible RNA gain of function mechanism is PKR activation by RNA secondary structures formed by the CGG repeats. According to this hypothesis, all of the constructs that contain CGG repeats should be capable of forming RNA secondary structures that could potentially activate PKR and thereby inhibit granule RNA translation. Still, we do not see translational inhibition in the TG296 oocytes. So, does this rule out an RNA gain of function mechanism? As discussed above, RNAs that are not spliced may become trapped in the nucleus of the cell and not exported to the cytoplasm (21). Since the TG296 RNA construct

contains a truncated intron 1 from FMR1, It is possible that TG296 transcripts are not exported from the nucleus, are not localized to RNA granules, do not activate PKR and therefore do not inhibit granule RNA translation. To determine if this is the case, it would be important to use in situ hybridization for the TG296 RNA transcript to determine if it is localized to the nucleus or the cytoplasm. To distinguish between these two RNA gain of function mechanisms, a PKR knockout mouse has been generated and characterized. The PKR KO mouse is physically normal, fertile, and produces litters of normal size. PKR KO mice exhibit normal induction of Type I IFN by virus and poly(I)poly(C) but a reduced antiviral response by IFN- $\gamma$  and poly(I)poly(C) (22). It would be informative to analyze granule RNA translation in oocytes from a PKR KO mouse injected with VenusArc RNA and CGG99 RNA. If granule RNA translation were not inhibited this would suggest that PKR activation is responsible for translation inhibition. If granule RNA translation is inhibited, this would be consistent with the sequestration of translation machinery mechanism.

One result that supports the PKR hypothesis over the hypothesis for sequestration of translational machinery is that translational inhibition in oocytes as seen in Figure 4 of chapter 3 is less pronounced than translational inhibition in neurons as seen in Figure 3 of chapter 2. If the mechanism for translational inhibition were sequestration of translational machinery, there would be no obvious explanation for this result. CGG repeats in neurons and oocytes should both sequester translational machinery to the same extent. However, if the PKR hypothesis were the mechanism for translational inhibition, this could provide a possible explanation because levels of PKR are much lower in oocytes than in neurons or fibroblasts.

We conclude that CGG repeats inhibit granule RNA translation in the context of FXTAS and FXPOI most likely by RNA gain of function. Furthermore, it is most likely that CGG repeats inhibit granule RNA translation by activating PKR.

**My contributions to this work**

Dr. Lisa Mehlmann was responsible for performing FMRP western blotting, and George Korza was responsible for performing PKR western blotting. Dr. Lisa Mehlmann was also responsible for all mouse work and acquisition and culturing of oocytes. Dr. Lisa Mehlmann injected GFP into oocytes and performed hnRNP A2 immunostaining. I was responsible for all preparation of RNAs by in vitro transcription. For the granule RNA translation experiments, I performed most oocyte injections with some help from Dr. Lisa Mehlmann. I imaged all oocytes on the confocal microscope. I was involved in conceptualization and interpretation of all experimental approaches and results as well as writing and editing of this chapter.

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**Chapter4:**  
**Discussion**

### **The FXTAS and FXPOI translational phenotype is a granule based phenomenon.**

RNAs are localized in cells, and RNA localization is necessary for localized translation and localized protein function (1, 2). An 11 nucleotide sequence known as the A2RE (GCCAAGGAGCC) is necessary for dendritic localization of a subset of RNAs in neurons and oligodendrocytes (3, 4). The main binding protein for the A2RE is hnRNP A2 (5) which in turn binds the multivalent protein TOG (6), which links together multiple RNA molecules in granules. CGG repeat RNA also binds hnRNP A2 (7, 8) and is localized in granules (9). CGG repeats in the 5'UTR of FMR1 are believed to be causal of FXTAS and FXPOI (10).

CGG repeats inhibit translation in-cis and in vitro, in a dose dependent manner (11). We show for the first time that expanded CGG repeat RNA microinjected into mouse hippocampal neurons and expressed in human fibroblast cells (chapter 2) and injected into mouse oocytes (chapter 3) inhibits translation of granule RNA in trans and in vivo. This indicates that regulation of granule RNA translation by CGG repeat RNA occurs in multiple cell types and may represent a widespread physiological regulatory mechanism. Furthermore, since individual granules are well resolved in dendrites of mouse hippocampal neurons, we were able to analyze translation in individual granules. While translational output from individual RNA granules has been explored with single molecule imaging (12), this is the first time that expanded CGG repeat RNA has been shown to inhibit translation in individual granules. It is likely that translation inhibition in human fibroblasts from individuals with endogenous expanded CGG repeats in the FMR1 gene also represents translation inhibition in individual granules (although individual granules are difficult to resolve in fibroblasts). We also show that endogenous CGG repeat expansions in fibroblast cells affects regulation of calcium transients, a phenotypic effect that may reflect translational inhibition of calcium regulatory proteins encoded by granule RNAs in these cells. If inhibition of granule RNA translation by CGG repeat expansions represents a pathogenic mechanism for FXTAS and FXPOI, this suggests that these are granule based disorders and

raises the possibility that translation in granules is normally regulated by CGG repeats in unaffected individuals.

**Translational inhibition in FXTAS and FXPOI is an RNA gain of function mechanism.**

Full mutation expansions (usually >200) of the CGG repeat tract in the 5'UTR of FMR1 gene cause hypermethylation and transcriptional silencing of the gene (13), resulting in FXS, which is considered a protein loss of function disorder. Premutation expansions (typically between 55 and 200) are associated with FXTAS and FXPOI (10). In FXTAS and FXPOI, the premutation expansion is associated with an increase of FMR1 RNA compared to normal cells, but a reduced quantity of FMRP protein (14, 15), suggesting a translational deficit. For this reason, it is believed that FXTAS and FXPOI are not protein loss of function disorders like FXS but may instead represent RNA or protein gain of function disorders.

The current controversy in the field is whether FXTAS and FXPOI are caused by RNA gain of function or protein gain of function (16). In chapters 2 and 3 we show that synthetic CGG repeat RNA, not in the context of FMR1, microinjected into mouse hippocampal neurons and mouse oocytes inhibits granule RNA translation. In chapter 2, we show that endogenous CGG repeat RNA with premutation expansions inhibits granule RNA translation in human fibroblast cells. However, in chapter 3, we show CGG repeat RNA in oocytes from the transgenic TG296 mouse, containing the 5'UTR, part of the ORF and part of intron 1 from FMR1 does not inhibit granule RNA translation. These results are relevant to the question of whether FXTAS and FXPOI represent RNA gain of function or protein gain of function disorders.

The protein gain of function mechanism is based on the idea that repeat associated non-AUG (RAN) translation products of FMR1 RNA with expanded CGG repeats have a toxic effect that leads to FXTAS and FXPOI (17-19). Based on our results it seems unlikely that RAN translation is causing CGG repeat translational inhibition of granule RNAs. We see the same inhibition of



granule RNA translation with exogenous CGG99 RNA in neurons as with endogenous FMR1 RNA with expanded CGG repeats in human fibroblasts. If RAN translation products were responsible for inhibition of granule RNA translation, one would expect the RAN translation products to be the same or similar for both CGG repeat constructs. Endogenous CGG99 RNA can potentially generate three RAN translation products, one in each reading frame. However since the exogenous RNA does not contain stop codons in any reading frame it is not clear how potential RAN translation products from these RNAs could be released from the ribosome in the absence of a termination codon. Endogenous FMR1 RNA in human fibroblast cells can also generate three different potential RAN translation products in different reading frames, but these RAN translation products would be longer than the synthetic CGG99 RNA products and each would terminate at a stop codon. Potential RAN translation products for the transgenic TG296 RNA are similar to potential RAN translation products for endogenous FMR1 RNA from human fibroblast cells except that one would terminate at a stop codon in intron 1 instead of at the end of the endogenous FMR1 ORF (Chapter 3 Figure 1). Since granule RNA translation is inhibited in human fibroblasts with CGG repeat expansions in the endogenous FMR1 gene but not in mouse oocytes with CGG repeat expansion in a transgenic FMR1 construct this argues against the idea that inhibition of granule RNA translation by CGG repeat expansion represents a protein gain of function mechanism.

This leaves the possibility of an RNA gain of function mechanism for inhibition of granule RNA translation by CGG repeat RNA. In chapter 2 we propose two potential RNA gain of function mechanisms. The first potential mechanism involves intra-granule competition between CGG repeat RNA and other RNAs for rate-limiting translation machinery in the granule. We show by ribosome profiling that density of ribosomes is increased in the 5'UTR CGG repeat region of the FMR1 gene, which may reflect RAN translation of CGG repeats or stalled ribosomal scanning in this region. In either case, translational machinery could be sequestered in the CGG repeat

region of FMR1 RNA, reducing availability for other granule RNAs, thereby inhibiting granule RNA translation. The second potential mechanism involves activation of protein kinase R (PKR) by CGG repeat RNA in the granule. RNA encoding PKR contains CGG repeats in the 5'UTR (20), which have been shown to be necessary and sufficient for localization and translation in hnRNP A2 RNA granules (12). CGG repeat RNA can form double stranded secondary structures (21-24) that may bind to and activate PKR in granules. Activated PKR phosphorylates EIF2 $\alpha$  which forms an inactive complex with EIF2b, inhibiting translation (25). The results in chapters 2 and 3 are generally consistent with either mechanism.

The following considerations and observations favor the PKR activation hypothesis. CGG repeat RNA inhibits granule RNA translation in mouse hippocampal neurons and human fibroblasts by more than 10 fold while in chapter 3, CGG repeat RNA inhibits translation in mouse oocytes by about 2 fold. This difference in the magnitude of inhibition may reflect differences in PKR expression levels in the different cell types. PKR is expressed at lower levels in mouse oocytes compared to neurons and fibroblast cells (26-28), which may reduce the magnitude of inhibition of granule RNA translation due to PKR activation in oocytes compared to neurons and fibroblasts. This favors the PKR activation hypothesis. By contrast, if CGG repeat RNA inhibits granule RNA translation by sequestering translational machinery in the granule, one might expect that oocytes should have the same level of inhibition as neurons and fibroblasts.

The PKR activation hypothesis could be tested experimentally in several ways. First, it would be important to determine if PKR is actually localized in hnRNP A2 RNA granules. PKR exhibits a punctate distribution in human lung cancer cell lines (29, 30) suggesting that PKR is localized in granules. Immunostaining for hnRNP A2 and PKR in VenusArc injected neurons would reveal definitively if PKR is localized in A2 RNA granules because in neurons, granules are well resolved in the dendrites, and ARC RNA and hnRNP A2 provide markers for A2 granules.

The following unpublished observations in the Carson lab support the PKR activation hypothesis (personal communication). CGG repeat RNA binds to and activates PKR in vitro. CGG repeat RNA and PKR inhibit translation in vitro but neither component alone affects in vitro translation. In PKR KO cells, EIF2a phosphorylation is reduced. In MSH2 KO cells, which have reduced numbers of CGG repeats, PKR activation and EIF2a phosphorylation are reduced. In lymphocytes with different number of CGG repeats in the FMR1 gene, PKR activation and EIF2a phosphorylation are proportional to the number of CGG repeats in the FMR1 gene. In lymphocytes with increased numbers of CGG repeats in the FMR1 gene PKR activation and EIF2a phosphorylation are decreased by treating the cells with TMPyP4, which binds to CGG repeats. In TMPyP4 treated lymphocytes RAN translation of other CGG repeat RNAs in granules is increased. These observations are consistent with the hypothesis that CGG repeat RNA inhibits translation in granules by activating PKR.

Single molecule imaging studies show that translation in granules is bursty, with extended periods of low translational activity lasting several minutes interrupted by short periods of active translation lasting 1-2 minutes. Cycles of PKR activation/inactivation might provide an explanation for bursty translation in granules. Periods of low translational activity might correspond to periods when granule RNA translation is inhibited because PKR is activated, whereas periods of active translation might correspond to periods when PKR is inactivated. In this regard, experimentally measured on-rates and off rates for CGG repeat RNA binding to PKR in vitro are consistent with characteristic times for bursty granule RNA translation measured in vivo. It is possible that bursty translation in granules reflects the kinetics of PKR activation. This hypothesis could be tested by analyzing bursty translation in neurons from PKR knockout mice (31), and in the presence of various PKR inhibitors (32-35).

### **PKR may be a master regulator for translation in hnRNP A2 RNA granules.**

PKR contains three important domains, essential to its function in regulating translation: a C-terminal serine threonine kinase domain that activates EIF2 $\alpha$ , and two N-terminal double stranded RNA binding domains (dsRBDs) that bind to CGG repeat and other double stranded RNAs to activate the kinase domain (36). In response to cellular stress, protein activator of the interferon induced protein kinase (PACT) protein can activate a protein called transactivation responsive RNA binding protein (TRBP), which inhibits PKR by interacting with the dsRBDs, even in the absence of double stranded RNA (dsRNA) (37), suggesting a role for PKR translational inhibition in response to cellular stress. When either dsRNA or one of these proteins is bound to the two dsRBDs, a conformational change occurs that allows for autophosphorylation of PKR (38). This allows PKR to phosphorylate EIF2 $\alpha$ , a subunit of EIF2 in the translation preinitiation complex, which causes it to form an inactive complex with EIF2B that no longer functions in preinitiation complex formation necessary for translation (25). Thus PKR activation induced by expanded CGG repeats in FMR1 RNA or by stress, may inhibit translation of all other RNA localized in the same granule.

### **CGG repeats may mediate homeostatic translational scaling in A2 RNA granules.**

Neurons have developed a system called homeostatic synaptic scaling to sense their level of activation and respond to correct their strength (41, 42). Long term potentiation (LTP) and long term depression (LTD) are responses by excitatory synapses to strengthen or weaken the synapse based on whether calcium entry into the cell is high (LTP) or low (LTD) (40). If LTP strengthens the synapse in response to presynaptic and postsynaptic firing, the synapse may become too sensitive to excitation, thus creating a positive feedback loop leading to unconstrained LTP. The same would be true in the opposite direction with LTD (39). Homeostatic synaptic scaling provides a mechanism to avoid “maxing out” or “zeroing out” the synapse and maintains synaptic excitability within a range where it can respond appropriately to

changes in LTP and LTD (39). If PKR activation regulates translation in A2 RNA granules, this may provide a mechanism for granule homeostatic translational scaling analogous to homeostatic synaptic scaling, in response to changes that alter the need for translation activation or inhibition. One example is viral infection. If the host cell translates viral RNA, the virus will spread, and the cell will die. It has already been shown that PKR is activated in response to viral infection (43). It is possible that CGG repeat RNA present in hnRNP A2 RNA granules provides a mechanism for regulating PKR activation of translation inhibition and maintaining it at a homeostatic level. CGG repeat RNA may regulate PKR activation in A2 RNA granules so that the cell can quickly respond with changes to translation. Interferons (IFNs) are proteins that are released in response to viral infection. The PKR promoter contains an IFN response element, and PKR expression is induced in response to type 1 IFN (43). For these reasons, PKR in the cell is believed to mediate an antiviral response when PKR becomes activated upon accumulation of viral dsRNAs (44). If PKR activation inhibits granule RNA translation in response to CGG repeat RNA expansions, it may also regulate granule RNA translation under other circumstances, such as viral infection. Thus PKR may play a role in maintaining homeostasis in granule RNA translation. Under circumstances where granule RNA translation is too high PKR activation may decrease granule RNA translation and under circumstances where granule RNA translation is too low, PKR inactivation may increase granule RNA translation.

**PKR may mediate inter-conversion of A2 RNA granules and stress granules.**

PKR plays a key role in the cellular response to stress (37) and viral infection (44). Formation of stress granules may be part of the cellular response to viral infection (45). Stress granules are functional RNPs, containing mostly translationally inhibited RNAs (46, 47). Stress granule formation is poorly understood, but is believed to involve molecular interactions between components of the preinitiation complex (48). Furthermore, the mechanism often involves EIF2 $\alpha$

kinase, one of which is PKR (49). If the mechanism for translational inhibition of granule RNAs is PKR activation, it is possible that when PKR binds to expanded CGG repeat RNA, it converts A2 RNA granules into stress granules as a protective mechanism for the cell. One way to investigate this possibility is to look at markers for stress granules in A2 RNA granules in FXTAS and FXPOI. Possible markers to look at would be phospho-EIF2 $\alpha$ , EIF3, EIF4A1, EIF4B, EIF4G, FXR1, G3BP-1 and PABP-1 (50).

### **Calcium homeostasis is disrupted by CGG repeat expansions in FXTAS and FXPOI.**

Several RNAs that are involved in regulating calcium homeostasis contain either CGG repeats or A2RE sequences (51-53), which mediate localization in A2 RNA granules. Calcium transients are increased in astrocytes from CGG knock-in mice (54) and in iPSC derived neurons from individuals with CGG repeat expansions in the FMR1 gene (55). This suggests that disruption of calcium homeostasis may result from CGG repeat RNA inhibition of granule RNA translation. In chapter 2, we show that premutation human fibroblast cells loaded with the calcium indicator Fluo-4 exhibit secondary calcium transients in response to Bradykinin, which indicates that CGG repeat expansions disrupt calcium homeostasis in premutation fibroblast cells.

Among the RNAs regulating calcium homeostasis are voltage dependent calcium channel (CACNA1A), calcium activated potassium channel (KCNMA1), calcium, calmodulin-dependent protein kinase 2A (CAMK2A) and neurogranin (NRGN) (9). CACNA1A mediates entry of calcium into excitable cells. KCNMA1 is the pore-forming subunit of the MaxiK calcium-sensitive potassium channel. CAMK2A is a serine/threonine protein kinase necessary for LTP and spatial learning (56), and NG is a protein kinase substrate that binds calmodulin in the absence of calcium (57). One experiment to determine if translation of these RNAs in granules affects calcium homeostasis is to systematically knock out each of these genes and to determine if calcium homeostasis is restored in human fibroblast cells. Additionally, it would be important to analyze calcium transients in VenusArc injected mouse oocytes with or without synthetic

CGG99 RNA. This can be done using thimerosal to induce calcium spikes from internal stores in the oocyte (58). If calcium homeostasis is altered in the CGG99 injected oocytes, there may be a change in the patterning of the calcium spikes.

### **Computational modeling of granules containing CGG repeat RNA and PKR.**

Agent based modeling is a form of computational modeling in which a system is modeled as a collection of individual agents, and each agent makes a decision at defined time steps. Agent based models keep track of every binding and unbinding event as well as which agents are involved (59). This makes agent based modeling very computationally expensive. Rule based modeling is a form of computational modeling in which biomolecular reactions are represented as a set of local rules (60). These rules define conditions that are both necessary and sufficient for the set of interactions and transformations that one wishes to model (61). In other words, information that is not pertinent to the system being modeled is left out. For this reason, rule-based modeling allows some reduction in combinatorial complexity (60). The rules are then coupled to rate laws for association and dissociation (61). hnRNP A2 RNA granules lend themselves to these computational modeling approaches because granule components, including TOG, hnRNP A2 and A2RE RNAs are multivalent and bind to one another under very specific conditions. Furthermore, specific and non-specific affinities have been measured for many of these components, as have molecule numbers and valencies, providing reasonable starting parameters and rate laws (62).

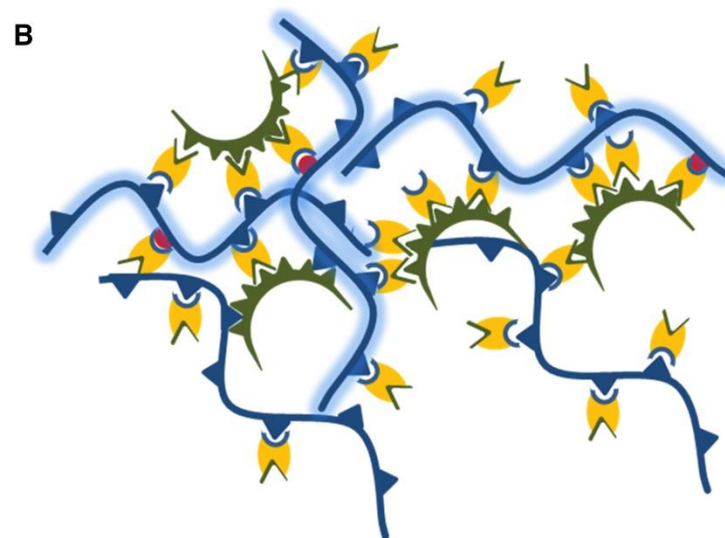
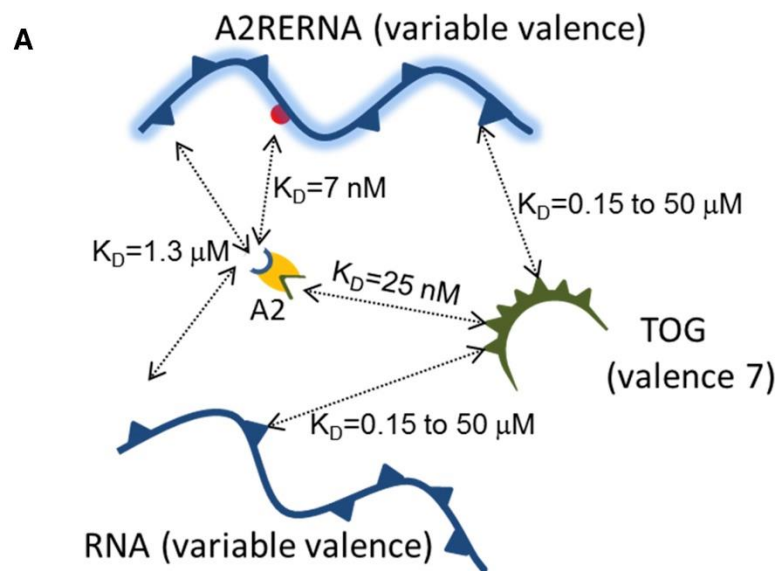
Falkenberg et al. describes a hybrid deterministic stochastic model for hnRNP A2 RNA granule assembly that further reduces combinatorial complexity. The model deconstructs each multivalent molecule into its individual binding sites based on the assumption that binding at each site is independent of the others. Next, the probability of bond formation at each site is calculated deterministically based on all possible interactions at that site. Finally, bound and free

states are assigned stochastically, and the granule is reconstructed by identifying which molecules are directly or indirectly connected.

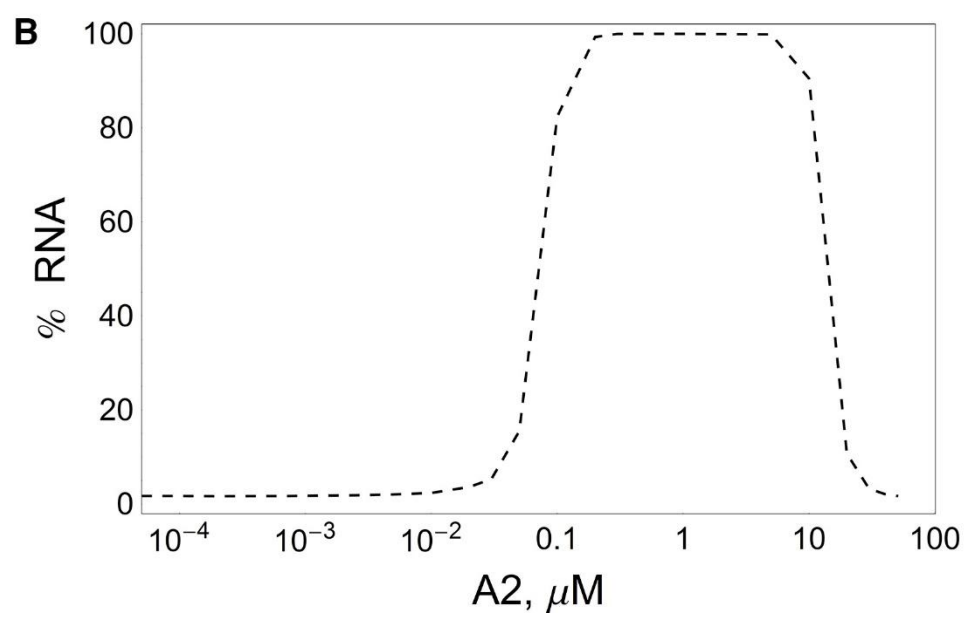
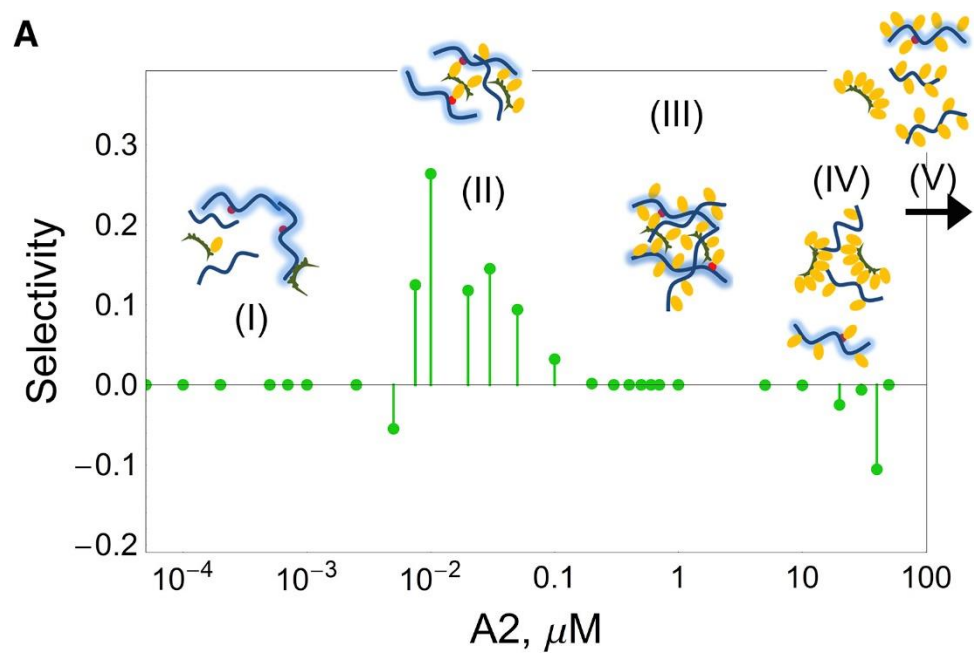
Figure 1A shows the binding sites that were included in the model and the dissociation constants associated with each binding pair. TOG is heptavalent, so TOG binding sites are represented as multiple individual binding sites with seven times the concentration of intact TOG. Each TOG binding site can bind specifically to hnRNP A2 with a 25nM KD or non-specifically to RNA binding sites with KD ranging from 0.15 to 50 $\mu$ M. hnRNP A2 is bivalent, so hnRNP A2 binding sites are represented as individual binding sites with two times the concentration of intact hnRNP A2. hnRNP A2 can bind specifically to the A2RE with a KD of 7nM or non-specifically to RNA with a KD of 1.3 $\mu$ M. Figure 1B is a schematic of some of the interactions within a representative granule after reconstruction. Parameter sensitivity analysis with this model indicates that the key parameters are: the concentration of the multivalent RNA binding proteins and the number of sites available for interaction with other molecules (valency of the RNA).

The key findings from the model are that high affinity binding between hnRNP A2 and TOG and between hnRNP A2 and the A2RE allows hnRNP A2 to either promote or inhibit the interactions between the other multivalent components in a concentration dependent manner. Figure 2A shows that there are five ranges of hnRNP A2 concentration that correspond to simulations in which hnRNP A2 concentration differentially affected granule selectivity for A2RE RNA. First, when hnRNP A2 and TOG concentrations are very low, there is a low probability of RNA interaction driving granule assembly (region I). As the concentration of hnRNP A2 increases, this drives interaction between A2RE RNA and TOG, increasing specificity (region II). The granule loses this specificity when hnRNP A2 concentration is high enough to drive the assembly of all specific and non-specific RNAs into a single large aggregate (region III) as





**Figure1: Schematic of multivalent interactions in granule assembly modeled in Falkenberg et al.** This figure is being reused from Falkenberg et al. (62) with permission from Elsevier. A) Specific and non-specific interactions between granule components and their valency are shown with respective dissociation constants represented by arrows. TOG is heptavalent, hnRNP A2 is bivalent and RNAs are allowed variable valency. B) Schematic representation of some of the interactions within a representative granule after granule reconstruction.



**Figure 2: hnRNP A2 concentration affects granule specificity.** This figure is being reused from Falkenberg et al. (62) with permission from Elsevier. A) Selectivity scores are represented as a function of hnRNP A2 concentration. Five regions of hnRNP A2 concentration reflect scenarios that affect granule specificity. Region V reflects hnRNP A2 concentrations beyond the chart. Region II represents an ideal hnRNP A2 concentration that allows granule assembly and specificity. B) Percentage of available RNA associated with the largest simulated granule is represented as a function of hnRNP A2 concentration. Region III drives assembly into one large aggregate.

shown in Figure 2B. Eventually, there are so many hnRNP A2 molecules that they saturate both RNA and TOG, so hnRNP A2 no longer functions as an intermediate between RNA and TOG and granule size is reduced (region IV). Finally, complete saturation of RNA and TOG with hnRNP A2 prevents granules from forming (region V). This defines a narrow region of hnRNP A2 concentration in which granule specificity occurs. Additionally, low affinity binding between TOG and A2RE RNA prevents the RNA from aggregating into an infinitely large complex. These findings are based on a model containing only A2RE RNA and non-specific RNA molecules and only hnRNP A2 and TOG protein molecules (62). However the model can be extended to include CGG repeat RNA molecules and RNA secondary structure as well as PKR molecules, using the same deconstruction/reconstruction strategy.

CGG repeat RNA is a key component of hnRNP A2 RNA granules. Based on FCS photobleaching in chapter 2, we know the ratio of CGG repeat and A2RE RNAs in individual granules. We can assume that one hnRNP A2 molecule binds four CGG repeats (by analogy with hnRNP A2 binding to A2RE sequences). Therefore, the maximal valency of CGG repeat RNAs for hnRNP A2 can be calculated by dividing the repeat number by 4. It would be of interest to introduce CGG repeat RNAs with different numbers of CGG repeats into the model to see how granule size and composition are affected. It is possible that introduction of different numbers of CGG repeats may affect the ratios of other components in individual granules analogous to the way hnRNP A2 concentration defines granule specificity. The most interesting result of this scenario would be that the optimal window for CGG repeat number would reflect actual CGG repeat numbers in normal individuals. If the model recapitulates granule assembly in vivo this could have important implications for regulating granule function.

Falkenberg et al. suggest that RNA secondary structure in the granule may lower the affinity and valency for RNA binding to TOG or hnRNP A2 in vivo and that these effects could be represented in the model by altering those parameters (62). CGG repeat RNA is capable of

forming secondary structure because CGG repeats can form both hairpins and quadruplex structures in vitro (21, 23). By altering the parameters in the model to account for CGG repeat secondary structure, we could gain insight into the quantity of hairpin, quadruplex and unstructured CGG repeat RNA in the granule. A simple way to approach this would be to add a 4 CGG repeat motif as a binding site to the model. The CGG repeat could bind to other CGG repeats or to hnRNP A2, allowing RNA secondary structure to form or CGG repeat RNA to bind to hnRNP A2. This is a realistic first step because hnRNP A2 binds to single stranded RNA (63). It would not take into consideration what secondary structure would form if two RNAs on the same TOG molecule came into contact with one another, which is something that would eventually need to be addressed. This approach is important because if the PKR hypothesis for CGG repeat inhibition of granule RNA translation is correct, it is dependent on CGG repeats being double stranded. Additionally, two possible outcomes could result from increasing CGG repeat number while also allowing CGG repeat secondary structure to form. First, increasing CGG repeat number could increase RNA secondary structure to a level that would inhibit granule RNA translation by PKR. Second, increasing CGG repeat number could drive granule assembly to one large complex as hnRNP A2 concentration did in the Falkenberg et al. model. It is possible that CGG repeats regulate PKR activation through alterations in secondary structure.

To determine if this is possible, PKR binding should also be added to the model. Unlike hnRNP A2, PKR binds to double stranded CGG repeats (64). Therefore, it would be unnecessary to complicate the model by adding activated PKR as a separate PKR species. Instead, PKR activation could be represented indirectly by secondary structure formation. In other words, if secondary structure is present, it can be assumed that PKR is activated. This approach assumes that nothing is competing with PKR for binding to CGG repeat secondary structure. An extension of this model would be to consider PKR explicitly and model PKR dimer formation

depending on the length of double stranded RNA. Modeling each of these aspects along with CGG repeat RNA secondary structure could reveal how PKR affects granule assembly, but more importantly, how CGG repeat length and secondary structure affects PKR activation and granule RNA translation.

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