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Austin Lai April 12, 2020

Novel role of Cdh1-APC as a regulator of neuronal protein synthesis and stress granule dynamics

by

Austin Lai

Gary Bassell

Adviser

Biology

Gary Bassell

Adviser

Anita Corbett

Committee Member

Jie Jiang

Committee Member

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An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

of Emory University in partial fulfillment

of requirements of the degree of

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Abstract

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Protein homeostasis, the balance between protein synthesis and degradation, is crucial to synaptic function and long-term synaptic modifications. Dysregulated protein homeostasis can therefore lead to defects in learning and memory, as seen in the neurodevelopmental disorder Fragile X Syndrome (FXS)—the most prevalent form of inherited intellectual disability. FXS is caused by the loss of Fragile X Mental Retardation protein (FMRP) and the excess protein synthesis that results. Previously observed dysregulation of the ubiquitin-proteasome system (UPS) in related neurodevelopmental disorders suggests the possible involvement of dysregulated ubiquitination in FXS. Furthermore, the E3 ligase complex Cdh1-APC has been shown to associate with FMRP *in vivo*. However, the interaction of the APC coactivator Cdh1 with FMRP and the consequences of such an interaction remain unclear. We hypothesized that Cdh1-APC downregulates FMRP through interaction with the destruction (D)-box domain and promotes protein synthesis. Here, we show that the interaction of FMRP with Cdh1 affects the protein expression of FMRP. Using FMRP mutants, we demonstrate that mutation in the D-box motif sequence and lysine residues of the *Fmr1* gene results in higher levels of FMRP protein expression, and a lower steady-state protein synthesis.

Additionally, unpublished studies from our lab showed that Cdh1 interacts with multiple stress granule-associated proteins, suggesting that stress granule dynamics may be regulated by the Cdh1-APC-mediated ubiquitination of FMRP. Stress granules are membrane-less organelles comprised of translationally repressed mRNAs and ribonucleoproteins. We hypothesized that Cdh1-APC regulates stress granule dynamics through its regulation of FMRP. We show that ubiquitination by Cdh1-APC reduces the assembly of stress granules in an FMRP-dependent manner. We further show that stimulation of group 1 metabotropic glutamate receptor reduces stress granule formation. Altogether, these results clarify the role of Cdh1-APC as a neuronal translational regulator and a potential target for Fragile X Syndrome.

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Acknowledgements

First, I would like to thank Dr. Bassell for accepting me to his laboratory as a freshman to work with Dr. Rossoll. My first semester in the Bassell Lab showed me what can be done in neurobiology and the questions yet to be answered. My experiences in the Bassell Lab have solidified my interest in pursuing this as my career. Dr. Bassell's patience and willingness to allow me to contribute towards the lab's overarching research goals have motivated me to increase my involvement.

Second, I would like to thank Dr. Arielle Valdez-Sinon. Since February 2017, I have worked with Arielle on the project herein reported. Over the three years, I have had many opportunities to learn to overcome many sorts of hurdles in our project—experiments and beyond. Arielle's patience towards me and people around her, as well as her persistence, were major motivating factors for me to put my maximum effort in not only performing my experiments but thinking critically about the problems at hand. I am very grateful to have worked alongside her, and to be a part of this project in the Bassell Lab.

I would also like to thank Dr. Rossoll for his guidance of during the Fall of 2016, as well as Summer 2018. Through his guidance at Emory and Mayo Clinic, I learned to think about complex research questions, and design troubleshooting experiments, by creatively applying fundamental knowledge of molecular biology.

Finally, I would like to thank the committee members Dr. Corbett and Dr. Jiang, for providing helpful feedback and advising me through the final year on this project. I am so grateful to have the support from all the different mentors throughout my undergraduate career.

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Introduction

Partially adapted from: **Austin Lai**, Arielle Valdez-Sinon, Gary Bassell, Regulation of RNA granules by FMRP and implications for neurological diseases, ***Traffic (Under Review)***.

Fragile X Syndrome (FXS) is the most frequently inherited form of intellectual disability, resulting from the lack of fragile X mental retardation protein (FMRP). Over the past three decades, FXS has been a subject of intense research. Yet, as there continues to be no FDA-approved treatment for FXS, novel therapeutic strategies need to be explored by studying the upstream events regulating FMRP, as well as noncanonical mechanisms of translational regulation by FMRP. Through the sections below, I begin by summarizing the clinical presentation and epidemiology of FXS. Next, I discuss our current understanding of the molecular underpinnings of FXS: the function of FMRP as a translational repressor, the metabotropic glutamate receptor (mGluR) theory of FXS, and how this understanding informs past and future clinical trials. With this primer in FXS pathophysiology, I discuss the roles of ubiquitination and Cdh1-APC in synaptic plasticity and FMRP regulation, the role of stress granules as a mode of translational control, and the involvement of FMRP in stress granule dynamics.

Fragile X Syndrome: etiology and clinical manifestations

Fragile X Syndrome is the most common genetic form of intellectual impairment. Fragile X patients possess a CGG trinucleotide expansion in the 5' untranslated region (UTR) of the Fragile X Mental Retardation 1 (*Fmr1*) gene that leads to hypermethylation and repressed expression of the gene. Consequently, the loss of the protein which it encodes, Fragile X Mental Retardation Protein (FMRP), leads to dysregulated protein synthesis in cells. Several groups have reported varying frequencies of Fragile X Syndrome. Located on the long (q) arm of the X

chromosome at position 27.3 (Xq27.3), mutations in *Fmr1* affect males approximately twice as frequently as females. An estimated 1 in 8000 females suffer from Fragile X syndrome worldwide. However, the reported frequencies are distributed across a considerable range, as estimates for females span from 1 in 4,000 to 1 in 8,000 (Randi J. Hagerman et al. 2017). A study conducted in the U.S. found that of 36,124 male neonates screened, 1 in 5,161 possessed the full mutation (Randi J. Hagerman et al. 2017; Coffee et al. 2009). Another study in Quebec saw a prevalence of 1 in 6,209 males from a sample size of 24,449 neonates (Lévesque et al. 2009). Several other studies concluded higher prevalence of pathogenic mutations in *Fmr*1. However, the robustness of these studies was limited by the sample sizes. Beyond the factor of small size of study, the ethnicity and race of subjects also influence the risk of repeat expansion. Fascinatingly, a case study by Maia and colleagues reported a haplotype that predisposes individuals with both pathologic and normal length of trinucleotide repeat in *Fmr*1 to large contractions of such repeats (Maia et al. 2017). Additionally, the prevalence of pathologic expansion in *Fmr1* has also been seen in populations influenced by the founder effect—a phenomenon in which a small group of individuals results in a more genetically homogeneous descendant population. As seen in Recaurte, a small town in Colombia where the 1 in 19 males and 1 in 36 females had Fragile X Syndrome (Randi J. Hagerman et al. 2017). In 2014, a meta-analysis performed by Hunter and colleagues applied a statistical model that took into account individual characteristics in 50 FXS prevalence studies (Hunter et al. 2014). The study found the prevalence of FXS to be approximately 1 in 7,000 for males, and 1 in 11,000 females. The significantly lower frequency of FXS concluded by Hunter and colleagues is indicative of three broader limitation in the smaller studies. First, studies in which only individuals with intellectual disabilities are screened most likely overestimates the true prevalence of FXS. Furthermore, the noticeable fluctuation in the estimates of FXS frequency is

contributed partly by the inconsistent repeat numbers that were defined as full mutations and pre-mutations. Additionally, different techniques were used to measure the size of alleles (i.e. the length of trinucleotide repeats). Finally, the ethnic backgrounds of study subjects vary across different reports (Randi J. Hagerman et al. 2017).

As FMRP is expressed ubiquitously, the loss-of-function mutation in *Fmr1* results in wide ranging clinical symptoms. The pathogenesis of FXS manifests as clinical symptoms within the first years of a child's life. Newborn FXS patients suffer hypertonia, which results in clear difficulty in the ability to suck, as well as emesis and reflux (R. Hagerman and Hagerman 2002). The neuromuscular dysfunction continues as the child experiences delays in the development of motor system during the crawling years. In the second year, symptoms begin to include delays in language development, increasing hyperactivity, anxiety, and sensory over-reactivity. Psychiatric impairments of FXS patients include seizures, and epileptiform activity. Patients demonstrate impairments in social learning and language learning, as well as stereotyped and restricted behaviors (Bhakar, Dölen, and Bear 2012a). Further, FXS is considered a form of autism spectrum disorder (ASD) and is considered the most-common single-gene cause of ASD. Beyond the neurological defects, the loss of FMRP in other organs results in craniofacial abnormalities and macroorchidism that developed during puberty.

FXS is caused by the lack of transcription of the Fragile X Mental Retardation 1 (*Fmr1*) gene, which locates on the X chromosome. The $(CGG)_n$ repeat sequence in the 5' untranslated region (UTR) of the *Fmr1* gene normally ranges from 20 to 50 repeats in healthy individuals. The expansion of this repeat to over 200 copies generates an excess number of CpG islands that can be methylated. This expansion thus leads to the hypermethylation of *Fmr1* and repression of gene transcription. The lack of FMRP leads to abnormal synaptic function and morphology, which in

turn impairs higher-order brain function. The mutation in *Fmr1* gene for most patients is the aforementioned trinucleotide expansion in the 5' untranslated region (UTR). However, in rare cases, few patients have been found to have normal length of $(CGG)_n$ repeat, but with a substitution mutation in the KH1 domain (and RNA binding domain) of the *Fmr1* sequence, in which the isoleucine 304 (I304) was substituted for an asparagine (I304N) (De Boulle et al. 1993).

FMRP is a translational repressor

Fragile X Mental Retardation Protein (FMRP) is an RNA binding protein encoded by the *Fmr1* gene that regulates protein synthesis in animal models and humans. FMRP consists of three major RNA-binding domains (**Figure 2A**), including two hnRNP K-homology (KH) domains, and an RGG box domain. The two KH domains recognize the kissing-complex tertiary motif in target RNAs (Darnell et al. 2005), while the RGG box recognizes the stem-G-quartet loop (Blackwell, Zhang, and Ceman 2010).

FMRP has been well characterized as a translational repressor enriched in the postsynaptic terminals of glutaminergic synapses (Bassell and Warren 2008). Multiple modes of translational repression have been attributed to FMRP. While several of these mechanisms of translational regulation by FMRP have been reviewed in detail elsewhere (Richter, Bassell, and Klann 2015; Bhakar, Dölen, and Bear 2012a), here we provide a preliminary overview for these major roles of FMRP.

Translation can be evaluated in terms of three steps: initiation, elongation, and termination. FMRP regulates both initiation and elongation stages of translation, by acting alone (Darnell et al. 2011) or through association with factors such as miRNAs, and the RISC complex (Jin et al. 2004). Additionally, FMRP can inhibit cap-dependent translation of mRNAs by forming a complex with

an eIF4E binding protein (4EBP) called cytoplasmic FMR1-interacting protein 1 (CYFIP1) (Schenck et al. 2003). Previous studies have shown that reducing the levels of CYFIP1 in neurons increased the level of proteins encoded by FMRP-targeted mRNAs, including *Map1b*, *aCamk2*, and *App* (Napoli et al. 2008). The binding of FMRP to CYFIP1 inhibits the binding of eIF4G to, and the phosphorylation of, eIF4E. As a result, the formation of FMRP-CYFIP1 complex prevents cap-dependent translation. This regulatory mechanism is linked to extracellular signaling, as BDNF or glutamate signaling can release CYFIP1 and FMRP from eIF4E to promote translation. In *Fmr1*-KO mice, eIF4E-eIF4G binding (Ronesi et al. 2012) and eIF4E phosphorylation (Gkogkas et al. 2014) are both increased. Taken together, these observations suggest that FMRP both regulates initiation through direct and indirect mechanisms.

Additionally, FMRP has previously been shown to stall polyribosomes at the elongation phase (Darnell et al. 2011). *In vivo* high-throughput sequencing of RNAs isolated by CLIP showed that FMRP binds most frequently to coding regions of mRNAs, with fewer binding sites within 5' and 3' UTRs (Darnell et al. 2011; Ascano et al. 2012). High frequency of binding to coding regions suggests that FMRP acts by physically blocking ribosomes transit. Experiments specifically measuring ribosome transit with and without FMRP showed that polypeptide elongation was 40-50% faster in *Fmr1*-KO than WT brain lysates (Darnell et al. 2011), which further implies a role for FMRP in regulation of ribosomal transit.

Previous work has found that FMRP associates directly or indirectly with miRNAs, Argonaut of RISC, Dicer, and miRNA precursors. In *Drosophila* model, FMRP modulates miRNA expression to control neuronal development (Jin et al. 2004; X.-L. Xu et al. 2008). In mice, FMRP associates with RISC and/or miRNAs that cooperate to regulate protein synthesis and regulate dendritic spine morphology (Muddashetty et al. 2011). The stimulation of group 1 mGluR results

in the PP2A-mediated dephosphorylation of FMRP, which subsequently promotes the release of RISC from target mRNAs (Muddashetty et al. 2011). Additionally, this dephosphorylation event also enhances the ubiquitination of FMRP and its degradation by the proteasome (Nalavadi et al. 2012). Together this pathway serves as a switch for regulating translational activity in response of environmental cues. Through the well-established mechanisms outlined above, FMRP maintains the proper physiologic levels of translation for its target mRNAs.

The mGluR theory of Fragile X Syndrome

As discussed above, translational control is crucial to not only synaptic transmission but also synaptic plasticity. Regulation of neuronal protein synthesis has a well-established role in information storage in the nervous system. How synaptic activity regulates local protein synthesis is a question crucial to our understanding of learning, memory, and the molecular pathology of Fragile X Syndrome. Electron microscopy evidence presented by Steward and Levy in 1982 showed the accumulation of polyribosomes in dendrites and led to interest in studying the link between synaptic activity and protein synthesis (Steward and Schuman 2003). In the decades that followed, numerous types of excitatory synaptic receptors, the roles which they play, and then mechanisms by which they regulate protein synthesis were discovered and elucidated.

Excitatory neurons in the central nervous system express two major types of glutamate receptors that have been implicated in the regulation of local protein synthesis: the calcium-permeable N-methyl-d-aspartate ionotropic receptors (NMDARs); and the G_q-coupled (group 1) metabotropic glutamate receptors (mGluR). The NMDARs are widely expressed throughout the brain, and their activation in hippocampus leads to the release of brain-derived neurotrophic factor, which can promote protein synthesis (Scheetz, Nairn, and Constantine-Paton 2000). The group 1

mGluRs consists of two members, mGluR1 and mGluR5, which are differentially expressed across the brain. The forebrain has been found to show the highest level of mGluR5 expression, while mGluR1 expression is most pronounced in the cerebellum (Shigemoto et al. 1993). In 1993, pioneering work by Weiler and Greenough demonstrated that the stimulation of group 1 mGluRs using several glutamate analogues resulted in increased postsynaptic protein synthesis. This was the first work that demonstrated the effect of mGluR activation in upregulating protein synthesis by coupling with the phosphatidylinositol second messenger system. Since then two major pathways downstream of group 1 mGluRs have been found to regulate the translational machinery: the mammalian target of rapamycin (mTOR) pathway and the extracellular signal-regulated kinase (ERK) pathway. Both pathways promote 5' cap-dependent translation by regulating different factors required for translation initiation.

Initiation is one of three phases in translation (the latter two being elongation and termination), during which protein complexes such as the eukaryotic initiation factors (eIF) forms at the 5' cap of an mRNA and recruit ribosomal components to begin translation elongation. The mTOR pathway disinhibits translation initiation by phosphorylating eIF4E binding protein, releasing the 5' cap which it sequesters, and allow eIF4E and other initiation factors to bind (Takei and Nawa 2014). Alternatively, the mTOR pathway can also result in the phosphorylation of p70 ribosomal S6 kinases (S6Ks). Upon phosphorylation, the active S6Ks can phosphorylate ribosomal protein S6 and eIF4B (Bhakar, Dölen, and Bear 2012b). The ERK pathway stimulate initiation in an analogous fashion. On one arm activated ERK can phosphorylate the p90 ribosomal protein S6 kinases (RSKs), which phosphorylates S6 and eIF4B, thereby promoting initiation. On another arm, ERK phosphorylates MAP kinase-interacting protein kinase (MNK), which can subsequently phosphorylate eIF4E, reducing its binding affinity for the 5' cap, and thereby lowering the rate of

translation. Paradoxically, these two arms of ERK signaling antagonize each other. One potential explanation under investigation is that these two arms impinge on different subpopulations of mRNAs, allowing the ERK signaling pathway to stimulate translation of one set of mRNAs, while modulating the translation of another (Costa-Mattioli et al. 2009).

Crucially, translational control downstream of group 1 mGluR activation is not limited to initiation of translation. Stimulation of both mGluR5 and NMDAR can promote calcium/calmodulin-dependent eEF2 kinase activity, and increase the phosphorylation of eEF2, resulting in its selective stalling of a particular set of mRNAs. More important within the context of FXS is the mGluR-activation-dependent dephosphorylation of FMRP. Phosphorylated FMRP binds to coding regions of target mRNAs such as Arc and MAP-1B, stalling the transit of polyribosomes. Dephosphorylation of FMRP reduces its repressive activity, and enhances its susceptibility towards ubiquitination and proteasomal degradation (Nalavadi et al. 2012).

With these cumulative findings by the turn of the 20th century, one of the significant challenges in our understanding Fragile X Syndrome was finding a molecular mechanism that links the different psychiatric and neurological symptoms of FXS. In 2000, the seminal work by Kim Huber and colleagues showed that the increase in protein synthesis resulting from Gp1 activation in the CA1 region of the hippocampus underlies, among other processes, long term depression (LTD) (K. M. Huber, Kayser, and Bear 2000), a persisting downregulation of synaptic transmission mediated by translation of various proteins that ultimately facilitates the internalization of AMPA-type receptors from the post-synaptic membrane (Snyder et al. 2001). A following study by Huber and colleagues observed that *Fmr1*-KO mice showed exaggerated mGluR-dependent LTD (Kimberly M. Huber et al. 2002). The paradigm in which FMRP serves as a translational repressor and maintains protein synthesis at physiologically appropriate levels

and can be removed upon mGluR activation has since been well supported. This paradigm suggests mGluR as a point of convergence for the multitude of protein synthesis symptoms.

Challenges in the development of FXS treatment

Currently, there is no approved treatment targeting the molecular mechanism underlying FXS. The lack of effective treatment stands in contrast with the extent of preclinical data available on potentially viable drug targets. Preclinical studies in search of therapeutic strategies for FXS have mostly converged at two major receptors, the signaling pathways downstream of which regulate protein synthesis and long-term synaptic plasticity: group 1 mGluRs and gamma-Aminobutyric acid (GABA) receptor (Berry-Kravis et al. 2018). In addition to these two receptors, enzymatic intermediates in the downstream signaling pathways have also shown preclinical promise—most of which with no matching clinical success. Below, I summarize currently available options for symptomatic treatment of FXS, notable FXS therapeutic targets that have been studied in clinical trials, and future directions as the field continues the search for an effective cure.

Currently, there are five general classes of pharmacological treatment that are used to ameliorate the symptoms of FXS. Since more than three decades ago, psychostimulants such as methylphenidate (brand names include Daytrana and Quillivant XR) have been validated by clinical trials to reduce hyperactivity, impulsivity, and attention deficit disorder (R. J. Hagerman, Murphy, and Wittenberger 1988). The alpha-2 adrenergic receptors agonist clonidine (brand names include Kapvay and variations of Catapres) was validated in the mid-2000s to reduce over-stimulation from sensory inputs, hyperarousal, hyperactivity, and sleep disturbances (Ingrassia and Turk 2005). Anticonvulsants such as carbamazepine (brand names include Tegretol XR, and

Equetro) and valproic acid (brand name Valproic) have been shown to effectively control seizures and mood instability (Randi J. Hagerman et al. 2009). Other anticonvulsants such as phenytoin, phenobarbital, gabapentin, and levetiracetam have been avoided due to significant secondary effects (Randi J. Hagerman et al. 2009). Selective serotonin reuptake inhibitors (SSRIs), antipsychotics, and antidepressants have been effective in remediating anxiety experienced by FXS patients. Antipsychotics and antidepressants such as the most frequently used Risperidone (brand name Risperdal) or the more recent Aripiprazole have also been prescribed for aggression and sleep disturbance (Randi J. Hagerman et al. 2009; Erickson et al. 2010).

Recognition of the therapeutic potential in antagonizing mGluR1/5 followed the introduction of the mGluR theory in the early 2000s. After the observation of exaggerated mGluR-dependent protein synthesis and LTD in *Fmr1*-KO mice, genetic reduction of mGluR5 expression was shown to rescue a many but not all *Fmr1*-KO phenotypes including ocular dominance plasticity, dendritic spine density, and basal protein synthesis (Dölen et al. 2007). The potential of antagonizing mGluR5 was further supported by several work demonstrating the rescue of disease measures in mice and flies tried with mGluR5 antagonists such as MPEP, CTEP, and mavoglurant. These studies have shown the correction of molecular, synaptic, behavioral, and physiological phenotypes of FXS. However, the behavioral phenotypes have seen the most variability and inconsistency. This consistency was carried forward into the clinical trials. Two mGluR5 antagonists, basimglurant (Lindemann et al. 2015) and mavoglurant (Vranesic et al. 2014), have undergone large clinical trials.

Beyond this arsenal of drugs to combat symptoms of FXS, there is currently no treatment that directly targets the molecular bases of FXS. Recent phase IIb trials of glutamate antagonist mavoglurant (Vranesic et al. 2014) and basimglurant (Lindemann et al. 2015) showed no

significant improvement in behavior regardless of methylation status. Phase III trial of the $GABA_B$ agonist arbaclafen have ended similarly in disappointment. While the continual identification of novel drug targets is essential, it is equally important to expand the phenotypes studied in preclinical studies to focus on higher-order behavioral and cognitive traits (Berry-Kravis et al. 2018).

The neurobiological roles of ubiquitination and Cdh1-APC

Ubiquitination is an evolutionarily conserved post-translational modification, through which a 76-amino acid peptide ubiquitin is conjugated to a lysine residue of a protein substrate (Swatek and Komander 2016). The conjugated ubiquitin can be subsequently subjected to further modifications, leading to a variety of downstream regulatory responses. For instance, initial ubiquitin tag can be removed, phosphorylated, acetylated, SUMOylated, or further ubiquitinated (Swatek and Komander 2016). The wide variety of moieties that result from these sequential modifications on ubiquitin can be recognized be specific binding domains on proteins and trigger numerous signaling cascades. For instance, mono-ubiquitination, the addition of multiple single ubiquitin peptides onto lysine residues of a protein substrate, has been shown to regulate endocytosis, endosomal sorting, and DNA repair (Haglund and Dikic 2005). Polyubiquitination through lysine 63 (K63) is seen to drive in NFκB signaling and regulate high-fidelity DNA polymerases for DNA repair (Mabb and Ehlers 2010). In contrast, polyubiquitination through lysine 48 (K48) of ubiquitin allows the modified substrate to be recognized and degraded by the proteasome (Mabb and Ehlers 2010).

The process of ubiquitination is generally a sequential transfer of ubiquitin or modified ubiquitin moieties through E1, E2, and E3 ligases, ultimately conjugating onto the target protein.

As the substrate specificity depends mostly on the E3 ligases, mammalian genomes encode several hundreds of E3 ligases. There are two families of E3 ligases: HECT (homologous to E6-associated protein C-terminus) domain and RING (Really Interesting New Gene) domain. The HECT domain E3 ligases include UBE3A; and NEDD4. The RING domain is a significantly larger family, with more than 300 members including the anaphase-promoting complex/cyclosome (APC/C).

Until the 1990s, the prevailing understanding is that synaptic plasticity is regulated at the transcriptional and translational level. However, it later became clear that post-translational modification plays an indispensable role in long-term synaptic modifications. One of the first evidence for the role of ubiquitination in synaptic plasticity was that mice with diminished expression of HECT domain E3 ligase UBE3A in the brain were demonstrated deficient learning and abnormal long-term potentiation. Furthermore, mutation in the UBE3A gene has seen been established as a hallmark of Angelman Syndrome (Greer et al. 2010). Duplication in the 15q11-q13 chromosomal region, which contains the *Ube3a* gene, results in the loss of UBE3A function in cells (Vorstman et al. 2006). Its deficiency in neurons in particular leads to defects in hippocampal LTP, increased dendritic spine density, and an overall cognitive impairment in mice (Clayton-Smith and Laan 2003). Mice with maternally inherited loss-of-function mutant form of Ube3a also demonstrated defective experience-dependent maturation of the neocortex and abnormal development of several brain regions including the visual cortex (Sato and Stryker 2010). Under normal physiological context, UBE3A has been observed to ubiquitinate protein Arc/Arg3.1, leading to its proteasomal degradation and inhibiting endocytosis of AMPA receptors (Greer et al. 2010). Altogether, these results suggest the crucial nature of Ube3a and

the ubiquitin proteasome system in mediating synaptic modifications, and its loss of function to underlie Autism-related disorders such as Angelman Syndrome.

Another E3 ligase, the RING E3 APC/C, has been extensively studied for its role in the cell cycle, and more recently in synaptic plasticity. APC/C is a 1.5 MDa E3 complex comprised of 13 subunits (da Fonseca et al. 2011). Like other RING domain E3 ligases, APC/C recognizes its substrate through one of two domains: the destruction (D)-box or the KEN (lysine-glutamate-asparagine)-box domain (da Fonseca et al. 2011). Structural studies by da Fonseca and colleagues found that APC/C recognition of the D-box domain involves the coactivator Cdh1 and subunit APC10 (da Fonseca et al. 2011). It has been well established that the APC/C-mediated ubiquitination of regulators of mitosis such as securin and cyclin B1 (Peters 2006). Additionally, it was observed that mice deficient in the expression of Cdh1 showed abnormal late-phase LTP in the CA1 Schaffer collateral, and impaired contextual fear-conditioning (Li et al. 2008). More recently, Huang and colleagues reported that Cdh1-APC ubiquitinates FMRP, and that this ubiquitination event necessary for the mGluR-dependent LTD in the brain (Huang et al. 2015).

Stress granules as a mechanism for translational regulation

Stress granules are membrane-less ribonucleoprotein assemblies that form during cellular stress (Protter and Parker 2016). mRNAs sequestered into stress granules are translationally stalled at translation initiation; the precise regulation of stress granules assembly and disassembly is therefore a mechanism for fine-tuning of protein synthesis. The structure, composition, and dynamics of stress granules have been subjects of most interest in the recent years.

Stress granules contain mRNAs, RNA-binding proteins, translation initiation factors, and other non-RNA-binding proteins. Some non-RNA-binding proteins that have been observed in

stress granules include enzymes catalyzing post-translational modifications, and those that metabolize nucleic acids. Studies on the morphology of stress granules have identified substructures that are noticeably higher in concentration of proteins and mRNAs under super-resolution fluorescence microscopy (Jain et al. 2016). These substructures are known as the stress granule "core", while the regions surrounding the cores are known as the shell. Stress granule cores can be biochemically purified to study the difference in proteome between the core and the shell. One proteomic study on the core have revealed that more than half of the mammalian stress granule core proteome has RNA-binding ability (226/411 identified proteins) (Jain et al. 2016). In contrast to the core, the shell is in dynamic equilibrium with cytoplasmic mRNAs and proteins, as it has been visualized through FRAP studies that translating mRNAs and rapidly enter and exit stress granules (Moon et al. 2019). This dynamic nature of the shell has implications for the composition of stress granules, the favorability stress granule formation, and even the formation of other RNP granules. Processing bodies (P-bodies), a similar form of membrane-less RNP granule, has been shown to exchange protein and nucleic acid components with stress granules. Through this bidirectional exchange, P-bodies can promote the formation, and remodel the composition, of stress granules.

Until recently, there had been two models for stress granule assembly. In one model, known as the "core first" model, oligomeric ribonucleoproteins (RNPs) assemblies first nucleate the formation of a core. Then, the core continues to accumulate more translationally repressed RNPs, enlarging the growing granule. During the third phase, multiples of these precursor stress granules are transported by the microtubule network to close proximity with each other, and fuse to form a larger mature stress granule (Protter and Parker 2016). A second model, known as the "Liquid-liquid Phase Separation (LLPS) First" model, nearly all translationally repressed RNPs to be in

the eventual stress granule undergo LLPS to form a droplet in which RNPs are held together by weak interactions. In the second phase, more translationally repressed RNPs may enter and enlarge the droplet. In the third and final phase, a core forms by the increased concentration of proteins in certain regions within the droplet, and the formation of amyloid interactions (Protter and Parker 2016). Since the proposal of these two models, the "core-first" model has been more strongly supported by subsequent time-course analyses of granule assembly that saw an initial formation of the core during assembly, and the initial dissolution of the shell during disassembly (Wheeler et al. 2016). In fact, LLPS driven by intrinsically disordered regions (on RNA binding proteins, for example) have been observed to have the opposite effect *in vitro* compared to *in vivo*. In addition to characterizing the proteome of the stress granule core, Jain et al. also observed the in their landmark paper a redundancy in protein-protein interactions facilitating stress granule formation. For instance, the stress granule assembly factor RasGAP SH3 domain binding protein (G3BP) has two well-studied paralogs G3BP1 and G3BP2 that drives stress granule formation by interacting with caprin-1 (Solomon et al. 2007), a conserved phosphor-protein that localizes to RNA granules, or other copies of themselves (Tourrière et al. 2003). However, stress granules can be induced by multiple forms of stimuli, including arsenic sensing, heat shock, and aberrant osmotic pressure. During stress granule formation in response to changes in osmotic pressure, this observed interactions between G3BP-1, G3BP-2, and Caprin-1 are not necessary.

Approximately 50% of the stress granule proteome is RNA-binding proteins (RBPs). Stress granule connectomes show that non-RBPs can be recruited to stress granules through protein-protein interactions. Interestingly, the composition of stress granules varies depending on the type of cellular stress used to induce stress granule formation, cell type, and disease-contexts (Markmiller et al. 2018). In this study by Markmiller et al, nearly 25% of the RBPs under study

was biased towards sodium arsenite or heat shock stress. Just under half of the RBPs tested were found to localize to stress granules in a cell-type dependent manner. Neuronal stress granules are particularly diverse in their composition, which varies depending on whether they are localized in the dendrites, soma, or axon.

Much work has been done on the elucidating how cellular processes influence stress granule dynamics (Snead and Gladfelter 2019). Molecular chaperones play a significant role in the dissolution of stress granules during stress recovery (Cherkasov et al. 2013; Walters et al. 2015; Kroschwald et al. 2015), and can inhibit the aberrant formation of stress granules in amyotrophic lateral sclerosis (ALS) (Mateju et al. 2017). RNA helicases, which regulate many aspects of RNA metabolism, have also been shown to mediate stress granule dissolution. Proteomic analyses of stress granule cores reveal RNA helicases as a conserved component, and abolishing the activities of such RNA helicases reduces stress granule formation (Jain et al. 2016).

Additionally, several post-translational modifications (PTMs) have been found to regulate stress granule dynamics. PTMs can rapidly alter the interactome of target substrates by inducing conformational changes or alteration in charge of the target substrate. Three classes of PTMs have been found to oppose stress granule formation. Glycosylation of α-synuclein and tau have been shown to inhibit their pathologic aggregation into stress granules and other mRNP structures (Marotta et al. 2015; Yuzwa et al. 2012). Methylation of RNA-binding proteins (RBPs), such as the ALS-associated FUS, has been found to inhibit the cation-π interactions—in which a π-bond system stabilizes cationic group—and stress granule assembly(Qamar et al. 2018; Lorton and Shechter 2019). Similarly, lysine acetylation can disrupt cation-π interactions, and is protective against pathologic tau aggregation characteristic of multiple neurodegenerative disorders (Carlomagno et al. 2017).

By far the most well-characterized post-translational modification in regulating stress granule dynamics is phosphorylation. The rapid and reversible conjugation of a charged phosphate moiety serves as an ideal pathway for rapidly altering the RBP interactome in response to environmental cues. Interestingly, phosphorylation can promote or oppose stress granule formation, depending on the kinase involved. Several cases of phosphorylation-mediated inhibition of phase separation have been reported (Wang et al. 2018, 4; Monahan et al. 2017). Liquid-liquid phase separation (LLPS) occurs when a set of molecules form a dense network of weak interactions, which concentrate these molecules into a separate phase. By altering the conformation and charge of a biomolecule, phosphorylation can switch the ability of a molecule to form the necessary interactions that drive LLPS. For instance, the hyperphosphorylation of tau drastically alters the electrostatic characteristic of the molecule in a way that favors phase separation (Ambadipudi et al. 2017).

The Role of FMRP in stress granule dynamics

More recently, phosphorylation of FMRP was observed to result in its phase separation with caprin-1 (Kim et al. 2019), a protein that positively regulates and localizes to stress granule assembly. This suggests a mechanism by which FMRP localizes to and promotes the formation of stress granules upon post-translational modification. Other studies have supported this hypothesis. For instance, FMRP and its autosomal homologs are well described to be present in stress granules (Gareau et al. 2013; Herman Allison B. et al. 2019). Furthermore, the loss of FMRP results in reduced levels of stress granule formation (Didiot et al. 2009). As described above, it is well established that dephosphorylation is a critical node downstream of the stimulation of group 1 metabotropic glutamate receptors (mGluRs), which are critical for learning and memory (Nalavadi

et al. 2012). Therefore, the phase separation of FMRP as a result of phosphorylation points to a potential link between the activation of group 1 mGluRs and stress granule dynamics. A better understanding of the mechanistic relationship between extracellular signaling and the role of FMRP in stress granules may uncover novel therapeutic strategies that target stress granule dynamics through receptors such as mGluRs.

Methods

Publication: Arielle Valdez-Sinon, **Austin Lai**, Liang Shi, Carly L. Lancaster, Avanti Gokhale, Victor Faundez, Gary J. Bassell. Cdh1-APC interactions with FMRP and ribosomal components regulate protein synthesis and stress granule dynamics in neural cells. ***Cell Reports (Under Review)***.

Site-directed Mutagenesis and Generating FMRP constructs.

To investigate the Cdh1-APC-dependant ubiquitination of the FMRP protein, 3 mutations were considered: FMRP 6xKTR, FMRP DBM, and FMRP I304N. All mutants were generated using the QuickChange II XL Site-Directed Mutagenesis Kit (#200521). A GFP-FLAG tagged mouse FMRP (ex17b variant) construct (GFP-FLAG-FMRP) was used as template. All primers were designed by my graduate mentor using the QuikChange II XL online site.

First, the 6xKTR mutant was generated by mutating 6 lysine (K) residues on the FMRP amino acid sequence into arginine residues. Specifically, the mutation sites were K295R, K299R, K302R, K310R, K324R, K424R. These sites were chosen based on: 1) preliminary results from mass spectrometry on FMRP and 2) looking at the tertiary structure of FMRP to determine the lysine most proximal to the D-box. Because Cdh1-APC complex ubiquitinates FMRP by ligating ubiquitin at lysine residues, mutations at all lysine sites on FMRP should generate a ubiquitin-resistant mutant. Because of the structural similarity between lysine and arginine residues, the substitution of lysine residues for arginine residues does not alter the polarity or charge of the FMRP protein.

Next, we generated a mutation in the D-box motif of FMRP. According to the current model of FMRP ubiquitination, Cdh1-APC complex interacts with the D-box motif of FMRP prior

to the ligation of ubiquitin on a FMRP lysine residue. The D-box is a conserved motif across species (i.e. mouse, human, rat, etc.). Therefore, D-box mutation (DBM) should inhibit Cdh1-APC complex binding, consequently inhibiting FMRP ubiquitination. The specific mutations were R276A, and L279A. These site mutations were utilized in the 2015 paper demonstrating Cdh1-FMRP interactions (Huang et al. 2015).

The final mutation that we included was the I304N mutant. This mutation was identified as a rare a point mutation in the *Fmr1* gene that substitutes an isoleucine residue (I) to an asparagine (N). The presence of this gene alone has been shown to lead to FXS phenotype.

These mutations were combined to produce 6 mutants: FMRP 6xKTR, FMRP DBM, FMRP DBM 6xKTR, FMRP DBM I304N, FMRP I304N. All mutants were GFP-FLAG tagged (e.g. GFP-FLAG-FMRP-DBM).

Cell Culture, and Transfection

Neuro2A cells were plated at a density of 150,000 cells per well in a 12-well plate or 300,000 cells per well in a 6-well plate. Cells were transfected with their respective experimental plasmids with Lipofectamine 2000 (Invitrogen) for 24 hours. For the transfection with increasing levels of Cdh1, 8 samples were used, each with increasing amounts of Cdh1 plasmid (0, 0.1, 0.25, 0.75, 1.00, 1.75 μg). 0.25 μg of WT FMRP plasmid were co-transfected. An empty vector plasmid was transfected to bring the total amount of transfected DNA to 2.00 μg. For transfections of mutant FMRP constructs, 0.5 μg of plasmids were used with the same Lipofectamine 2000 protocol. For co-transfections of MEF2 and FMRP, 1 μg of each construct was transfected in addition to 1 μg of the transcription reporter, MRE. For the control samples, 2 μg of MRE and 1 μg of FMRP were transfected into each of the 12 wells.

Puromycin Labelling

24 hours after transfection of Neuro2A cells (described above), puromycin labeling was performed to measure global protein synthesis. Neuro2A cells were treated with Neuro2A media with 50 μg/mL puromycin (Sigma-Aldrich) for 45 minutes. For the negative control, Neuro2a cells were treated with $4x10^{-2}$ mM Aniso. After treatment, the cells were lysed (described below).

Neuro2A Cell Lysis

Prior to assaying protein levels in the N2A cells, the cells were lysed with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM MgCl2, 1% NP40, pH 7.4, supplemented with protease and phosphatase inhibitors), following washes with 1x phosphate buffered saline (PBS) solution. For 6-well and 12-well plates, each well was washed with 1 mL 1x PBS twice. For 6-well plates, each well received 200 μL of lysis buffer; each well of 12-well plates received 100 μL. The cell lysates were then transferred into the microcentrifuge tubes and incubated on ice for 30 minutes. After incubation, the lysates were centrifuged at 20,000 rpm for 20 minutes at 4 degrees Celsius. The supernatants were transferred and kept on ice, while the resulting pellets were discarded. For puromycin labeling, lysates were not centrifuged.

Bicinchonic (BCA) Assay

Prior to performing western blots, protein concentrations of each culture were measured using BCA assay. The BCA assays were performed following the Thermo Scientific Pierce™ BCA Protein Assay Kit (#23227).

Western Blotting

The western blotting procedure was performed following the procedure provided by Thomas and colleague (Thomas et al. 2017). 8 micrograms of protein per sample were resolved using SDS-polyacrylamide gel electrophoresis. The samples were then transferred to a nitrocellulose membrane. Blots were blocked in Odyssey Blocking Buffer (PBS) (LI-COR, #927-40000) and incubated overnight at 4°C in primary antibodies diluted in a 1:1 mix of blocking buffer and PBS-Tween-0.1%. The following primary antibodies were used: rabbit anti-FMRP (Sigma; 1:5000), mouse anti-actin (Sigma; 1:8000), mouse anti-Cdh1 (Abcam; 1:750), rabbit anti-GAPDH (Cell Signaling 1:2000), and mouse anti-puromycin (Millipore 1:1000). Blots were incubated in fluorescent secondary antibodies (LI-COR) diluted in PBS-Tween-0.1% 1 hour at room temperature. Three 10-minute washes were performed in PBS-Tween-0.1% before and after addition of the secondary antibodies.

Primary cortical neuron cultures

For experiments in which wildtype cortical neurons were treated with the Apcin and arsenite, primary cortical neurons were prepared from C57BL/6J mouse embryos (Charles River) of either sex on embryonic day 17. For experiments involving knock-out (KO) and wildtype FMRP postnatal cortical neurons, *Fmr1*-KO and littermate wildtype control mice were obtained as described in work by Buelow et al (Bülow et al. 2019). Heterozygous Fmr1-KO (*Fmr1*^{HET}) with C57BL/6J background were crossed with each other, generating progeny with *Fmr1*-KO, heterozygous *Fmr1*, or wildtype. Cerebral cortices were dissected and cultured from genotyped WT and Fmr1-KO pups on postnatal day 0 to day 3.

Cortices were dissociated using trypsin (Thermo Fischer Scientific), and mechanically dissociated in Minimum Essential Medium (MEM; Fischer) supplemented with 10% Fetal Bovine Serum (Hyclone). Neurons were plated on dishes or coverslips previously coated with poly-l-lysine (Sigma). After allowing two to four hours for adherence to coverslips, the neurons were grown in standard growth medium consisting of glial conditioned medium (Fischer), Glutamax (Gibco), and B27 (Invitrogen). Culture medium was changed once a week until experiments were performed. Cultures were included under 37ºC and 5% CO_2. Animal protocols were approved by the institutional Animal Care and Use Committee at Emory University. All direct handling of mice was completed by Arielle Valdez-Sinon, Ph.D.

Pharmacological treatment of cortical neurons

Mouse cortical neurons were collected from embryonic day 17 mice. At day *in vitro* (DIV) 14, neurons were treated with 2μM of either APC inhibitor Apcin or DMSO as control for 16-18 hours. The APC complex is thought to house the catalytic site for ubiquitination. On DIV 15, neurons were treated with sodium arsenite ($NaAsO_2$), a well-established method of inducing cellular stress or water as control for 45 minutes. Following optimized protocol for immunofluorescence, neurons will be fixed, permeabilized, and incubated overnight with primary antibodies against one of three stress granule proteins (FXR1, Caprin1, or G3BP) and MAP2.

To study the effect of gp1 mGluR stimulation on stress granule formation, mouse cortical neurons were collected from embryonic day 17 mice. At day *in vitro* (DIV) 14, neurons were treated either DHPG or water as control. After DHPG treatment, neurons were treated with $NaAsO_2$ or water as control. Neurons were then fixed, and immunofluorescence protocol was performed

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 15 minutes, washed three times for 10 minutes in PBS. Cells are blocked for 1 hour in blocking solution consisting of 5% normal donkey serum, 0.1% bovine serum albumin, and 0.1% Triton-X 100 in PBS. Cells were incubated overnight in primary antibodies diluted in blocking solution. The next day, cells were washed 3 times for 5 to 10 minutes in PBS. They were incubated in secondary antibodies in blocking solution for one hour at room temperature. Cells were washed 3 times for 5 minutes. Coverslips with cells were lightly rinsed with ultrapure water and then mounted using Prolong Gold Antifade mounting media (Invitrogen). Cells were imaged using Keyence BZ-810 or a Nikon Eclipse TE300 widefield fluorescence microscope with a 60X objective.

Proteomics Analysis

Samples were analyzed for interactome analysis by MS Bioworks. Proteomics samples were separated on a 10% Bis-Tris Novex mini-gel (Invitrogen) using the MES buffer system. The gel was stained with Coomassie blue and excised into ten equally sized segments. Gel segments were processed using a robot (Progest, DigiLab). First, gel segments were washed with 25mM ammonium bicarbonate followed by acetonitrile. They were then reduced with 10mM dithiothreitol at 60°C followed by alkylation with 50mM iodoacetamide at RT. Then, segments were digested with trypsin (Promega) at 37°C for 4 hours. They were then quenched with formic acid and the supernatant was analyzed directly without further processing. The gel digests were analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. Peptides were loaded on a trapping column and eluted over a 75μm

analytical column at 350nL/min; both columns were packed with Luna C18 resin (Phenomenex). The mass spectrometer was operated in data-dependent mode, with MS and MS/MS performed in the Orbitrap at 70,000 FWHM resolution and 17,500 FWHM resolution, respectively. The fifteen most abundant ions were selected for MS/MS. Data were searched using a local copy of Mascot.

Bioinformatic Analysis

Gene ontology analysis was performed with Database Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov). Cytoscape with Enrichment Map plugin for visualizing DAVID outputs was used to represent the Biological Processes enriched within the Cdh1 interactome.

Quantification and statistical analysis

Statistical analyses and graphs were prepared in GraphPad Prism (v.8). All data are expressed as mean +/- SEM. Replicates are reported in the figure legends or directly on the figures. For all experiments, α was set as 0.05. See figure legends for specific statistical analyses.

Quantification of Stress Granules

Coverslips for immunocytochemistry were blinded during imaging and quantification. Imaged neurons were classified as being stress granule positive or negative based on G3BP1 staining. Diffuse G3BP1 staining was classified as stress granule negative whereas cells with punctate G3BP1 staining were classified as stress granule positive. The total number of stress granule positive or negative neurons for each condition were then input into GrapPad Prism and a z-test was ran on the data.

Number of stress granules in the soma of each cell were quantified using Image J plugin TrackMate (Tinevez et al., 2017) with the Laplacian of Gaussian detector (threshold set from 5-15). Total area of the cell body was quantified using Image J plugin Mitomorphology macro(Dagda et al. 2009) with threshold set as 130-180. The number of granules were normalized to the cell body area.

Results

Publication: Arielle Valdez-Sinon, **Austin Lai**, Liang Shi, Carly L. Lancaster, Avanti Gokhale, Victor Faundez, Gary J. Bassell. Cdh1-APC interactions with FMRP and ribosomal components regulate protein synthesis and stress granule dynamics in neural cells. ***Cell Reports (Under Review)***.

FMRP mutants show different expression levels

Previous work by Huang and colleagues demonstrate that FMRP is a substrate of Cdh1-APC (Huang et al. 2015). Our first goal was to determine the physical interactive relationship between FMRP and Cdh1. To investigate this, genetic constructs of different FMRP mutants were transfected into Neuro2A cells with lipofectamine for 24 hours. The cells were lysed, and immunoblotting was done to measure the levels of expression of FMRP. The levels of expression were measured with Image J software by quantifying the density of fluorescence. The signals were normalized to the level of expression of beta actin.

FMRP-D-box mutant showed an increased expression compared to wildtype, suggesting that the mutant is degraded less than wildtype FMRP (**Figure 3**). This observation is consistent with our hypothesis that Cdh1-APC interaction at the D-box promotes the ubiquitination of FMRP. However, the WT 6xKTR showed a decrease in expression, which suggests that it is being ubiquitinated and then degraded more compared to WT FMRP expression. This observation could potentially be explained by the fact that the six lysine residues that were mutated are not the only lysine residues on FMRP. Therefore, by mutating these 6, other lysine residues of FMRP were more likely to be ubiquitinated. Different patterns of ubiquitination on lysine residues can determine other downstream signaling aside from degradation. It is possible that the other lysine

residues of WT 6xKTR FMRP that are now more likely to be ubiquitinated allow for more robust downstream degradation than the 6 lysine residues we have chosen to mutate. Mutating the D-box in addition to the six lysine residues seemed to rescue the 6xKTR phenotype, suggesting that disrupting Cdh1 interaction with FMRP makes FMRP expression more stable. The expression levels for the two I304N mutants trend towards increased stability of FMRP. This suggests that the KH domain of FMRP in which the I304N mutation is located may be a site involved in post-translational modification and stability of FMRP expression.

Cdh1-FMRP interaction promotes global protein synthesis

Given the function of FMRP as a translational repressor, the increased expression of DBM FMRP mutant compared to wildtype led us to consider the effects of DBM FMRP on global protein synthesis. N2A cells were transfected with wildtype FMRP or DBM FMRP for 24 hours. Subsequently, a 45-minute puromycin labeling was performed **(Figure 4A)**. The cells were lysed and immunoblotting against puromycin and GAPDH as control was done to measure the levels of global protein synthesis. The levels of expression were measured with Image J software by quantifying the density of fluorescence. The signals were normalized to the level of expression of GAPDH.

The level of global protein synthesis for samples with DBM FMRP was significantly lower than that with WT FMRP (**Figure 4B, C**). Thus, mutation in the destruction-box binding domain, where Cdh1-APC complex is thought to interact with FMRP during ubiquitination, significantly lowered protein synthesis. This data suggests that the interaction between Cdh1-APC complex and FMRP promotes downstream protein synthesis, which is in agreement with the current paradigm for the post-translational regulation of FMRP downstream of mGluR-induced LTD (**Figure 1**).

Cdh1 associates with stress granule-associated proteins

Cdh1-APC-mediated ubiquitination of FMRP, a translational repressor, suggests a new role for Cdh1-APC in cell biology. Whereas past work on APC and co-activators Cdh1 and Cdc20 have mostly been within the context of chromatid segregation during mitosis, our results point to a function of Cdh1-APC in translational regulation post-mitotic cells. With this result, we became interested in clarifying the broader mechanism by which Cdh1-APC regulates protein synthesis. To this end, we sought to identify other RNA-binding proteins that interact with Cdh1-APC. Neuro2A cells were transfected with FLAG-Cdh1, and subsequently lysed. FLAG-Cdh1 and its interactors were pulled down in an unbiased manner through immunoaffinity chromatography FLAG-peptide elution (Comstra et al. 2017; Gokhale et al. 2012). A portion of the lysate was incubated with FLAG-peptide and underwent silver staining. FLAG-peptide outcompetes FLAG-Cdh1 for proteins that are nonspecifically bound to bead-antibody complexes. The following silver staining of this sample accomplishes two goals: the identification of these nonspecific binding proteins and the determination of sufficient protein pulldown for subsequent mass spectrometry.

Mass spectrometry identified 185 unique proteins as the Cdh1 interactome, including well-established subunits of the thirteen-subunit Cdh1-APC complex such as APC1, APC2, APC7, CDC16, CDC23, and CDC27. The enrichment of these proteins validated the specificity of our immunoaffinity procedure. To identify enriched biological processes among the Cdh1 interactome, we performed a DAVID analysis. As anticipated, we found enrichment in categories related to the ubiquitin-proteasome system such as protein K11-linked ubiquitination (GO: 00709791, $p = 7.57x10^{-6}$). Surprisingly, the analysis also showed the interactome to be highly enriched in categories related to protein synthesis (**Figure 5A, Table 1**). More than a third (36.7%) of the

Cdh1 interactome was categorized into translation (GO: 0006412, $p = 4.09x10^{-73}$). Multiple cellular processes that regulate translation were enriched, including the formation of translation preinitiation complex (GO: 0001731, $p = 8.07 \times 10^{-13}$), regulation of translational initiation (GO:0006446, $p = 2.22x10^{-12}$), positive regulation of translation (GO: 0045727, $p = 2.16x10^{-4}$), negative regulation of translation (GO: 001748, $p = 1.39x10^{-7}$), and ribosomal subunit assembly (GO: 0000027, $p = 7.76x10^{-12}$; GO: 0000028, $p = 2.05x10^{-11}$). Enrichment in these categories of translational regulation strongly supports a novel role of Cdh1-APC in translation regulation—a role that extends beyond the canonical role of Cdh1-APC in ubiquitination-mediated progression during the cell cycle.

Notably, the Cdh1 interactome includes FXR1—one of two FMRP autosomal homologs **(Figure 5B)**. Like FMRP, FXR1 have been observed to form cytoplasmic RNA granules in transformed embryonic primary cell cultures (Mazroui et al. 2007) and rodent nervous system, forming what is termed Fragile X granules (FXG) that are found in neurons (Christie et al. 2009). Additionally, *Drosophila* FMR1 (dFMRP) , which has no paralog, was found to localize in stress granules in *Drosophila*. The formation of both FXGs and stress granules repress translation of mRNAs (Gareau et al. 2013). Cdh1 interaction with FXR1 homolog points to a potential role which Cdh1-APC plays in stress granule regulation.

We cross-referenced our Cdh1 interactome to previously published compendium of stress granule-associated proteins (Jain et al. 2016; Markmiller et al. 2018). Interestingly, nearly a third (28%) are stress granule proteins. Through an additional immunoprecipitation and immunoblotting, we found that stress granule proteins HNRNPU, Caprin1, GB3P1, ELAVL1, and RPS3 interact with Cdh1. This strengthens the potential that Cdh1-APC plays a role in the regulation of stress granule dynamics.

Cdh1-APC activity inhibits stress granule formation

Our finding that DBM FMRP expression lowers global protein synthesis suggests that Cdh1-FMRP interaction and Cdh1-APC activity increases global protein synthesis. This observation, in light of our finding stress granule-associated protein in the Cdh1 interactome, led us to the hypothesis that Cdh1-APC activity may inhibit stress granule formation as a mechanism of promoting protein synthesis.

To evaluate this hypothesis, we sought to observe the effect of pharmacological inhibition of APC function on stress granule dynamics. DIV15 mouse cortical neurons were treated with sodium arsenite ($NaAsO_2$) (0.5mM, 45 minutes) and immunostained for G3BP1. As discussed above, G3BP1 is a well-established stress granule-associated protein identified in previously published stress granule proteomes. Stress granule formation was clearly observed in both neurons treated with dimethyl sulfoxide (DMSO) as a vehicle control and APC inhibitor Apcin upon $NaAsO_2$ treatment (**Figure 6A, B**). Apcin is a competitive inhibitor of the D-box binding site on Cdc20 and Cdh1 (Sackton et al. 2014). To thoroughly assess effect of Apcin treatment on stress granule formation, we compared stress granule formation in both control and Apcin-treated samples using two parameters. First, we compared the percentages of stress granule-positive neurons. Representative samples of stress granule-positive neurons were imaged, and an objective threshold was applied to determine the proportion of image neurons that are considered to have formed stress granules. We observed that while merely 26% of the vehicle-treated neurons were stress-granule positive, more than half (56%) of the Apcin-treated neurons were stress granule-positive after arsenite treatment. The second parameter that we evaluated pointed to a similar difference; we found that Apcin-treated neurons formed a significantly higher number of stress

granules per 100 square microns. These results demonstrate that inhibition of the enzymatic activity of Cdh1-APC promotes stress granule formation and suggests a potential mechanism in which Cdh1-APC activity regulates stress granule dynamics under the physiological context.

Because Apcin acts by inhibiting the association between APC and both coactivator Cdh1 and Cdc20, we sought to replicate our results using pharmacological approach that more specifically targets Cdh1. Subsequent experiments were performed following a similar treatment regimen but substituting Apcin for a modified version of tosyl-L-arginine methyl ester (TAME), termed ProTAME. ProTAME competitively inhibits the physical association between APC and Cdh1. Cortical neurons were treated with 12 μM ProTAME for 4 hours, followed by sodium arsenite stimulation of stress granule formation. Successful stimulation of stress granule formation was observed in neurons treated with either ProTAME or vehicle **(Figure 7A)**. Neurons treated with ProTAME demonstrated significantly higher percentage of stress-granule positive cells **(Figure 7B)**, as well as a higher density of granules (**Figure 7C**), in comparison to vehicle-treated neurons.

Cdh1-APC does not localize to stress granules

Our observation Cdh1 interacts with stress granule-associated proteins such as G3BP1, and that Cdh1-APC activity inhibits stress granule formation led us to the initial hypothesis that the regulation of stress granules of Cdh1-APC may be dependent upon its localization to stress granules. To study the localization of Cdh1-APC in neural cells, Neuro2A (N2A) cells were transfected with the mCherry-Cdh1-myc construct for 24 hours. The cells were then treated with $NaAsO_2$ (0.5mM, 45 minutes) or water as control, and immunostained for G3BP1. In N2A cells treated with sodium arsenite, we saw clear formation of cytoplasmic granules containing G3BP1.

Cdh1-APC was not observed to co-localize with G3BP1 (**Figure 8**), suggesting that Cdh1-APC is not recruited to stress granules.

Our data is in agreement with previously reported stress granule proteomes, which do not include Cdh1-APC. The method we utilized to determine the Cdh1 interactome shows all protein-protein interactions with Cdh1 without distinguishing the spatiotemporal nature of different interactions. Our finding that Cdh1 does not localize to stress granules therefore suggests a potential mechanism in which Cdh1-APC forms transient interactions with stress granule-associated proteins and regulate stress granule dynamics by modifying one or more of these interactors.

Cdh1-FMRP interaction reduces stress granule formation

As mentioned above, FMRP and its paralog FXR1 have been found to localize to stress granules. Furthermore, the lack of FMRP has been seen to result in a diminished level of stress granule, suggesting that FMRP is a positive regulator of stress granule formation. Our findings that Cdh1-FMRP interaction promote global protein synthesis, and that Cdh1-APC inhibits stress granule formation without localizing to stress granules, pointed to a mechanism in which Cdh1-APC regulates stress granule dynamics interacting with and ubiquitinating FMRP.

To study whether the interaction between Cdh1-APC and FMRP affects stress granules dynamics, we investigated the difference in stress granule formation between Neuro2A (N2A) cells expressing FMRP DBM mutant and cells expressing wildtype FMRP (FMRP WT). N2A cells were transfected with either construct for 24 hours, and treated with $NaAsO_2$ (0.5 mM, 45 minutes) or water as control, and immunostained for G3BP1. Stress granule formation was clearly observed in cells treated with $NaAsO_2$ (**Figure 9A, B**). Furthermore, FMRP was observed to co-localized

with G3BP1 (**Figure 9A, B**), supporting previously reported observations of FMRP as a stress-granule associated protein (Jain et al. 2016; Markmiller et al. 2018).

Following a similar quantitative approach that we used for determining Apcin effects in mice cortical neurons, we measured the percentage of stress-granule positive N2A cells and the density of stress granule (given as number of granules per 100 microns) for cells expressing either FMRP WT or FMRP DBM and treated with arsenite. Among cells expressing FMRP DBM, more than half (59%) of the cells formed stress granules. This percentage was significantly higher than the 35% of cells expressing FMRP WT that formed stress granules. Moreover, cells expressing DBM FMRP had on average a significantly higher number of stress granules per 100 square microns.

These data demonstrate that the interaction between Cdh1 and FMRP through the destruction box motif results in lower stress granule formation. Presumably, this interaction allows the Cdh1-APC-mediated ubiquitination of FMRP, and the subsequent proteasomal degradation. As seen in Figure 2, the D-box mutation results in a higher steady-state levels of FMRP DBM. The upregulated levels of stress granule formation in cells expressing FMRP DBM therefore supports the previous reports of FMRP as a positive regulator of stress granule. Additionally, these results are in agreement with our proposed paradigm in which Cdh1-APC regulates stress granule dynamics by interacting with and modifying stress granule-associated proteins.

Cdh1-APC inhibits stress granule formation in an FMRP dependent manner

Based on our observations of FMRP WT- and FMRP DBM-expressing N2A cells, FMRP thus appears to mediate the effect of Cdh1-APC on stress granules. However, the E3 ligase Cdh1-APC has been shown to ubiquitinate multiple other substrates, and it is possible that the regulation

of other substrates influence stress granule dynamics in parallel with the FMRP-mediated effects we have observed. Whether Cdh1-APC regulation of stress granule requires FMRP needs to be specifically addressed.

To investigate FMRP-dependence of Cdh1-APC inhibition of stress granule formation, postnatal neurons from *Fmr1*-KO mice was used. *Fmr1*-KO mice has been commonly studied as a model of Fragile X Syndrome; the findings in this has been found to be have preclinical value. Postnatal neurons were cultured from wildtype littermates. Neurons from both genotypes underwent treatment with Apcin and sodium arsenite to induce stress granule formation. Stress granule formation was observed **(Figure 10)**. Similar to arsenite-inducing experiments on embryonic cortical neurons and N2A, two parameters were measured: percentage of stress granule-positive cells and number of stress granules per unit area. In the wildtype cortical neurons treated with arsenite, we expected Apcin treatment to increase the formation of stress granules, as seen before in embryonic neurons. Our observations was in agreement: 62% of Apcin-treated wildtype cells were stress granule-forming, in contrast to 42% of vehicle-treated cells ($p = 0.0046$) **(Figure 11A)**; the number of granules per 1000 square micron in Apcin-treated wildtype neurons was also statistically significantly higher ($p = 0.0008$) than that in vehicle-treated wildtype neurons **(Figure 11C)**. Fascinatingly, this significant effect of APC inhibition was abolished in the *Fmr1*-KO neurons. We observed no significant difference in either percentage of stress granule formation **(Figure 11B)**, and density of stress granules **(Figure 11C)**. For both Apcin and vehicle *Fmr1*-KO treatment groups, the number of stress granules per unit area was noticeably lower than wildtype-neurons, supporting the previously reported role of FMRP as a positive regulator of stress (Vanderklish and Edelman 2002; Booker et al. 2019)granule formation. The absence of APC-inhibition effects in upregulating stress granule formation in *Fmr1*-KO neurons suggests that

FMRP not only mediates Cdh1-APC antagonism of stress granule formation, but that this effect is dependent on the modification of FMRP.

Stimulation of group 1 mGluR reduces stress granule formation

As discussed above in the context of the mGluR Theory of FXS, stimulation of group 1 mGluR results in upregulated protein synthesis through multiple mechanisms. Furthermore, previous work by Nalavadi and colleagues from the Bassell Laboratory have shown that the stimulation of group 1 mGluR by DHPG results in the dephosphorylation of FMRP, thus enhancing its ubiquitination. The ubiquitination of FMRP allows it to be recognized and degraded by the 26S proteasome. Given that stress granule is a cellular process that negative regulates translation, and that FMRP has previously been shown to be a translational regulator we hypothesized that the stimulation of mGluR1 and mGluR5 by pharmacological treatment with dihydroxyphenylglycine (DHPG) inhibits stress granule formation.

Embryonic cortical neurons were cultured until DIV 14-16, and underwent DHPG treatment (1μM, 15 minutes) or water as control. Immediately following the 15-minute stimulation, neurons were treated with $NaAsO_2$ (0.5mM, 45 minutes) or water as control. In neurons treated with $NaAsO_2$, stress granules were clearly observed (**Figure 12A**). We compared neurons treated with DHPG and arsenite to those treated with water and arsenite. The percentage of stress granule-positive neurons in the stimulated cohort was significantly higher than those without DHPG treatment ($p < 0.0015$) (**Figure 12B**). This robust effect suggests that the activation of mGluR1/5 results inhibits stress granule formation.

The observed effect of DHPG stimulation is in agreement with the current paradigm. Activation of mGluR1/5 has previously been shown to result in the dephosphorylation, Cdh1-

APC-mediated ubiquitination, and proteasomal degradation of FMRP. The activation of group 1 mGluRs is therefore a mechanism to downregulate steady state levels of FMRP—a positive regulator of stress granule formation. Our result further demonstrates that this mGluR-FMRP pathway is sufficient to significant influence the level of stress granules formed.

Discussion

Conclusions

In this study, we elucidate a novel role of E3 ligase Cdh1-APC in regulating protein synthesis in neural cells. We show the interaction between the Cdh1 subunit of the Cdh1-APC complex and FMRP significantly reduces the level of FMRP and upregulates the steady-state level of global protein synthesis. Our observations that Cdh1 interacts with stress granule-associated proteins led us to elucidate a broader mechanism in which Cdh1-APC regulates translation by inhibiting stress granule formation through an FMRP-dependent mechanism.

First, previous studies by Nalavadi et. al. demonstrated that ubiquitination and degradation of FMRP is a part of the molecular pathway leading to upregulation of FMRP target proteins. The E3 ligase complex Cdh1-APC was shown to ubiquitinate FMRP. However, the interaction between Cdh1-APC complex with FMRP remains unclear. To address this, we tested two hypotheses: that Cdh1-APC interacts with FMRP, and that Cdh1 is directly responsible for the ubiquitination of FMRP. Here we show that mutating the D-box motif of the FMRP led to a significant increase in FMRP stability, which suggests that Cdh1 interacts with FMRP at the D-box motif. The disruption of D-box inhibits interaction between Cdh1 and FMRP, thus stabilizing FMRP. This observation supports the hypothesis that Cdh1-APC plays a role in the regulation of FMRP expression.

Excitingly, using DBM and wildtype FMRP, we demonstrate that the ubiquitination of FMRP leads to an increase in global protein synthesis. Previous works studies have identified numerous FMRP targets that play crucial roles in synaptic plasticity. Thus, the increased global protein synthesis by Cdh1-APC ubiquitination of FMRP suggest that specific FMRP targets could potentially be upregulated.

Furthermore, we show that increase in levels of Cdh1 has no significant correlation with FMRP stability. The lack of correlation between increased Cdh1 levels and FMRP levels of expression, together with the D-box mutant results, suggest that Cdh1 may be involved in other non-canonical regulatory molecular events. It is suspected that Cdh1-APC only regulates FMRP degradation following a stimulation paradigm, such as mGluR5 stimulation in neurons.

In our experiments examining the different mutant expressions, we also observe that the I304N FMRP mutant trends towards increased stability. This stands in contrast with the previous hypothesis. The data suggest that I304N FMRP appears to be destabilized through the Cdh1-APC-mediated event. The I304N mutation has been characterized to be a substitution mutation that alters a neutral isoleucine to a charged asparagine residue in the KH2 domain, impairing the RNA-binding capability of the domain. However, the proximity of the KH2 domain to the D-box domain **(Figure 2)** may alter the conformation of the D-box domain to an extent that inhibits recognition of FMRP by Cdh1-APC. The loss of FMRP in patients with I304N mutation in FMRP could be caused by other molecular events.

We then considered the functional relationship between Cdh1-APC, FMRP, and stress granules. We show that Cdh1, without localizing to stress granules, interacts with FMRP, and that the *in vivo* function of Cdh1-APC regulates stress granule in an FMRP-dependent manner. Cdh1-APC has been well-studied in the context of mitotic exit. The anaphase-promoting complex APC/C controls sister chromatid segregation by ubiquitinating cell-cycle regulatory proteins. Evidence for its role in post-mitotic cells, however, has been minimal. Our results demonstrate that Cdh1-APC has two noncanonical roles in regulating translation. For one, the targeted ubiquitination of FMRP and subsequent downregulation of FMRP de-represses the translation of numerous target mRNAs,

many of which have notable roles in regulating synaptic plasticity, which underlies the broader processes of learning and memory.

Recently, a publication by Markmiller et al. reports that poly-ubiquitination does not have a significant effect on stress granule formation. In particular, they observed that the pharmacological inhibition of the ubiquitin activating enzyme (UAE), which is required for the sequential transfer of ubiquitin onto E3 ligases and the protein target, does not significantly affect stress granule formation in HeLa and 293T cells. Importantly, our observations are confined strictly to neural cells. All experiments have been complete in murine embryonic cortical neurons, murine postnatal cortical neurons, and Neuro2A cells—a neuroblastoma cell line. Given the differing results in nonneuronal cell types and the cell-type specific composition of stress granules, we posit that our results suggest a cell-type specific role of Cdh1-APC that is relevant to the proper translational control in neuronal cells.

Future work

Our data demonstrating the increased expression of DBM FMRP and the decreased global protein synthesis for DBM FMRP provide an exciting insight into the role of Cdh1-FMRP interaction in regulation of FMRP expression and global protein synthesis. Using a FLAG-FMRP co-immunoprecipitation experiment, we will test whether the interaction between Cdh1 and FMRP is indeed disrupted by the mutation in the D-box motif of FMRP. In this experiment we will transfect WT FMRP or DBM FMRP with or without the Cdh1 construct. If the mutation in the D-box motif disrupts the Cdh1-FMRP interaction, then no Cdh1 should be co-immunoprecipitated with DBM FMRP. After confirming the physical interaction between Cdh1-FMRP, we can determine whether the D-box mutation results in less ubiquitination of FMRP by comparing the

amount of ubiquitination present on DBM FMRP and WT FMRP. In this experiment, we can co-transfect an HA-tagged ubiquitin construct with either WT FLAG-FMRP or DBM FLAG-FMRP. We can then perform an immunoprecipitation using antibodies against FLAG, followed by a western blot using antibodies to detect HA. This way, we assay for and compare the levels of ubiquitinated WT FMRP and DBM FMRP. This experiment would allow us to draw a clearer mechanistic connection between Cdh1-FMRP interaction and the ubiquitination of FMRP.

Previous work from the Bassell lab has shown that post-translational modification of FMRP regulates the translation of postsynaptic density protein 95 (PSD-95) translation (Muddashetty et al. 2011). This knowledge, in addition to our new understanding of the Cdh1 interaction with FMRP and the effect of this interaction on global protein synthesis, leads to a new question about whether there is a change in expression of specific proteins crucial to synaptic plasticity. To answer this question, we can transfect Neuro2A cells with WT FMRP or DBM FMRP, perform puromycin labeling, and purify the newly synthesized proteins using immunoprecipitation with antibodies against puromycin. Following the IP experiment, we will probe for the expression of target proteins through western blot, using antibodies against specific proteins of interest. We are interested in the changes in the levels of expression of PSD-95, CamKIIa, and Arc, due the importance of these proteins for forms of learning and memory. We are interested in the levels of the PSD-95 downstream of this regulatory event because PSD-95 has been shown to regulate the endocytosis of AMPA receptors (Bhattacharyya et al. 2009), influence synaptic strength by regulating AMPA receptor function (W. Xu et al. 2008), promote LTD (W. Xu et al. 2008), and stabilize dendritic spines (De Roo et al. 2008). We are interested in CamKIIa for its well-established roles in synaptic plasticity (Fink and Meyer 2002). Finally, we're interested

in the effects of FMRP regulation on the Arc, as it has been recognized as the master regulator of synaptic plasticity (Shepherd and Bear 2011).

Furthermore, neurons from postmortem FXS patient samples and *Fmr1* KO mouse models demonstrate increased protein synthesis and elongated dendritic spines. Once the effects of FMRP mutants on protein synthesis is determined, these effects can be connected to abnormalities in dendritic spine morphology (Vanderklish and Edelman 2002; Booker et al. 2019).

Efforts to elucidate the ubiquitination of FMRP as a molecular switch for protein synthesis is crucial to further understand the pathophysiology of FMRP. Furthermore, it has been demonstrated that other neurodevelopmental disorders share this regulatory mechanism (Mabb and Ehlers 2010). For instance, the UBE3A-mediated ubiquitination of arc and other synaptic proteins has been shown to have physiologic significance in the context of Angelman's syndrome. The extensive role of ubiquitination makes the efforts to better understand this mechanism particularly urgent, in the process of finding therapeutic targets for related neurodevelopmental disorders. Our findings of a novel interplay between protein synthesis and stress granules have implications to understand how decreased assembly of RNA granules may contribute to neurodevelopmental disorders including those linked to alterations in E3 ligase expression and function, such as Angelman syndrome. It is unlikely that modulation of in Cdh1-APC function by pharmaceutical intervention can be directly employed for FXS treatment. However, new methods of promoting or disinhibiting RNA granule formation can be studied as potential treatment strategies for FXS and other neurodevelopmental disorders.

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Figures

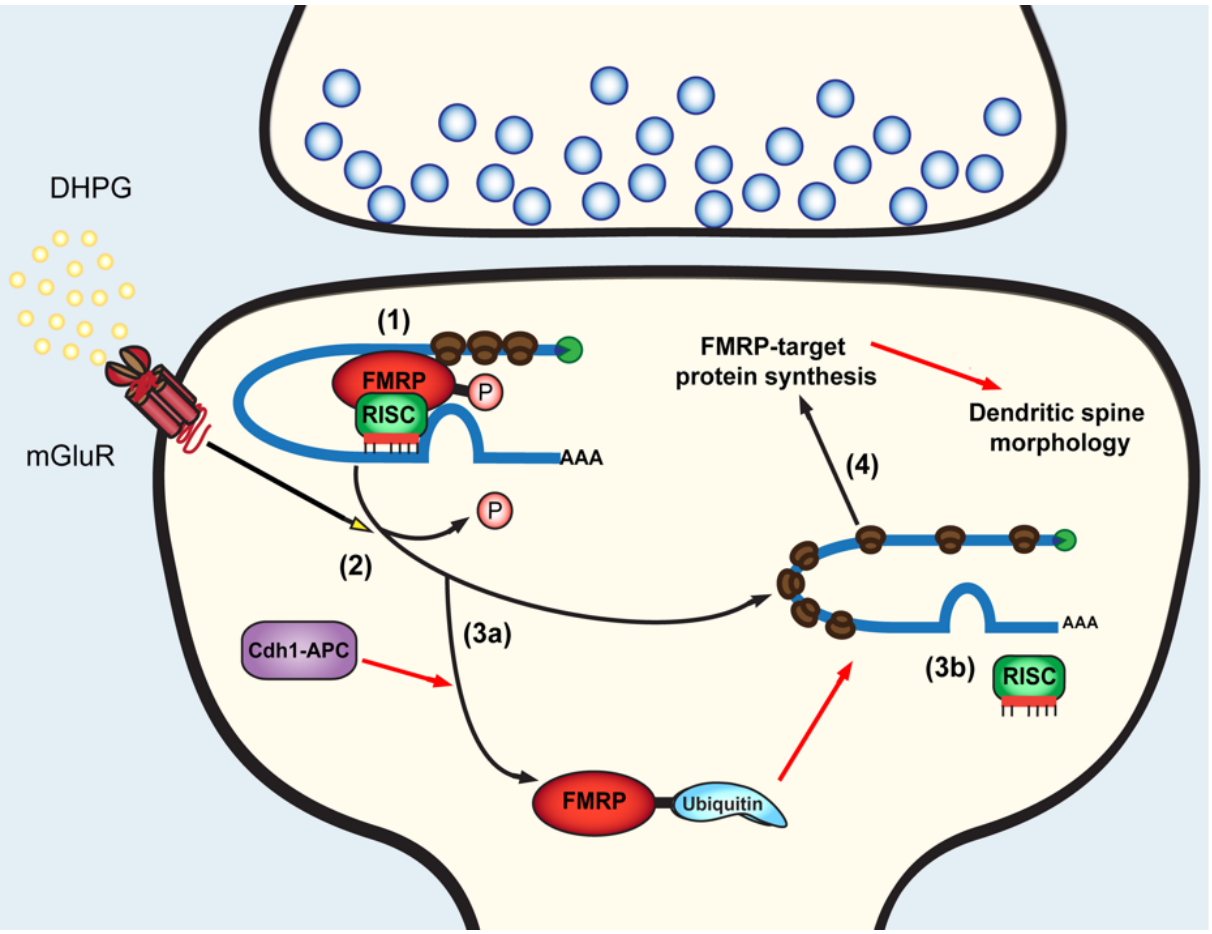

Figure 1. Current model of FMRP ubiquitination. A phosphorylated form of FMRP binds to RISC. (1) FMRP-RISC complex represses the translation of mRNA by binding to the 5' UTR of target transcripts. (2) Signaling events downstream of mGluR stimulation lead to the dephosphorylation of FMRP. (3a) The dephosphorylation event leads to ubiquitination of FMRP. (3b) dephosphorylation also leads to RISC dissociation. (4) Dissociation of RISC leads to increased protein synthesis. Whether FMRP ubiquitination (3a) is necessary for RISC dissociation (3b) to occur is not yet known. Red arrows indicate ongoing research questions.

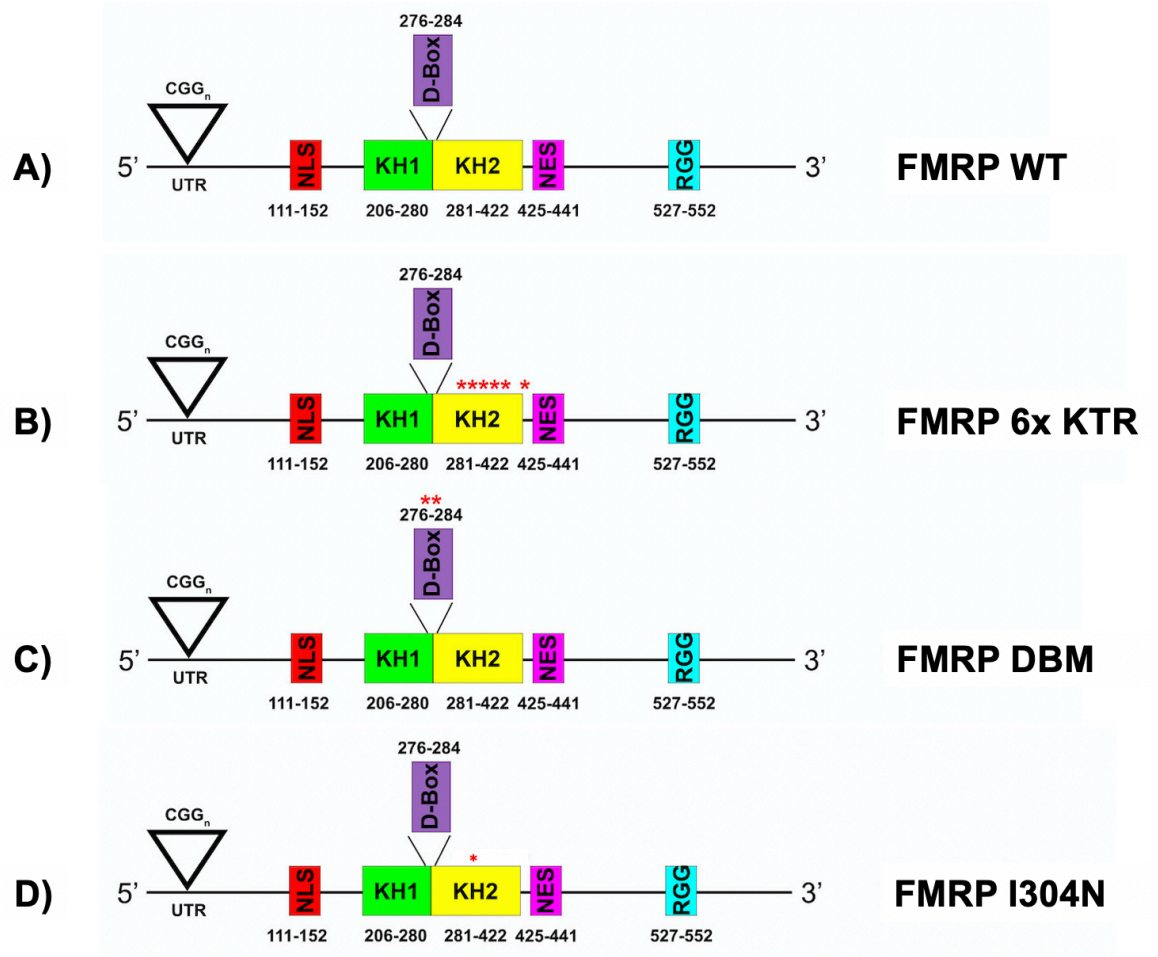

Figure 2. Wildtype and mutant constructs of FMRP. (A) The wildtype FMRP construct of FMRP. The 5' untranslated region (UTR) is annotated at the 5' end. The nuclear localization sequence (NLS) is shown in Red, and nuclear export sequence (NES) is shown in magenta. The RNA-binding domains K homology domains KH1 (green) and KH2 (yellow), as well as the RGG motif (turquoise) are shown. The destruction box motif (D-box) is indicated by the inset in purple. (B) The FMRP 6xKTR mutant contains six point mutations through which 6 lysine (K) residues are substituted with 6 arginine (R) residues. (C) FMRP D-box mutant (DBM) contains two points mutations in the D-box motif. (D) FMRP I304N mutant recapitulates the rare point mutation at the 304th amino acid residue, causing the normal isoleucine (I) residue to be replaced by as asparagine (N) residue.

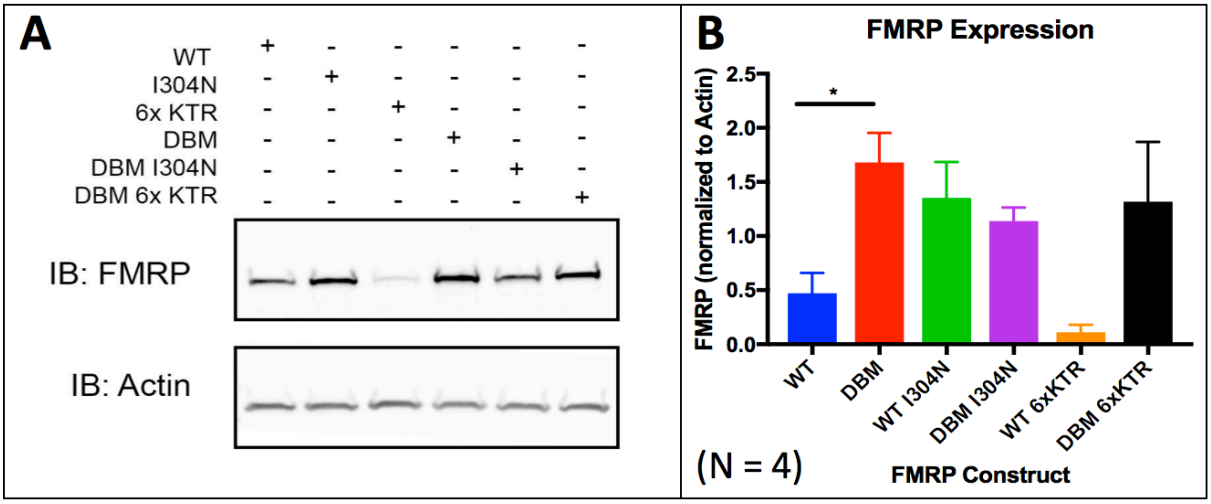

Figure 3. Different FMRP mutants show varying levels of expression. (A) FMRP mutant constructs were transfected to Neuro2A cells with lipofectamine for 24 hours. FMRP was quantified by western blotting with an anti-FMRP antibody. **(B)** Quantification of FMRP levels in Neuro2A cells after 24 hours of transfection**.** The quantified FMRP expression was normalized to that of endogenous beta actin. Performed one-way ANOVA with Sidak's multiple comparisons test ($n = 4$, $p < 0.05$).

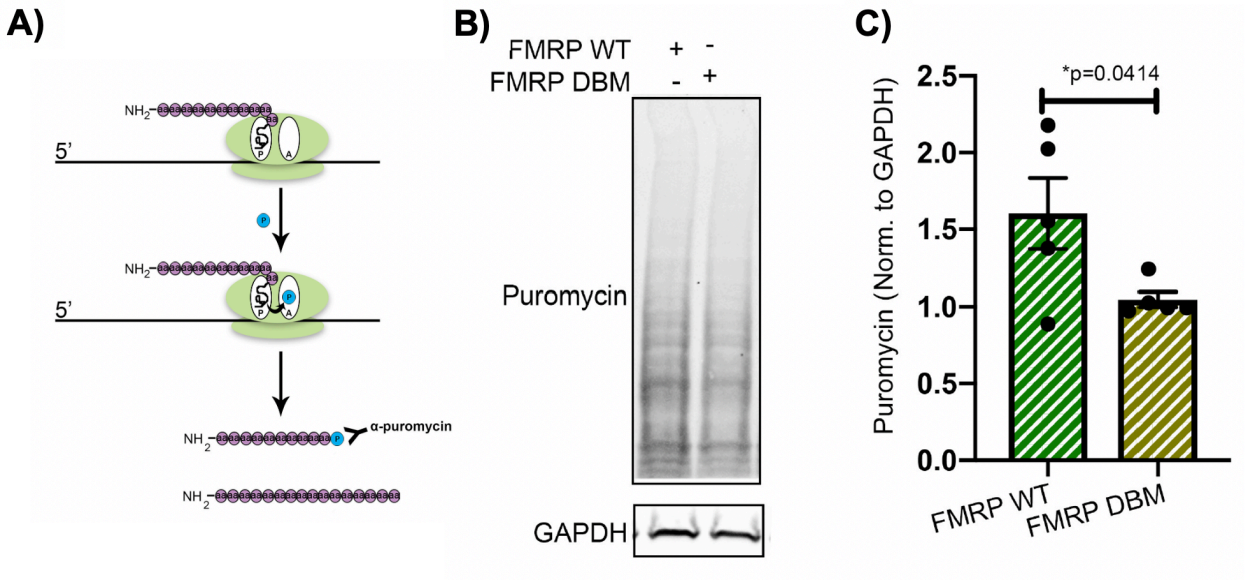

Figure 4. Cells expressing DBM FMRP mutant showed decreased protein synthesis. Neuro2A cells were transfected with either WT FMRP or DBM FMRP with lipofectamine for approximately 24 hours. (**A**) Cells were treated with 50µg puromycin for FMRP was quantified by western blotting. (**B**) Quantification FMRP levels in Neuro2A cells after 24 hours of transfection**.** The quantified FMRP expression was normalized to beta actin expression. Performed one-way ANOVA ($n = 4$, $p < 0.05$). (**C**) Quantification of puromycin levels in Neuro2A cells after 24 hours of transfection**.** The quantified FMRP expression was normalized to that of endogenous beta actin. Performed one-way ANOVA with Sidak's multiple comparisons test ($n = 4$, $p < 0.05$).

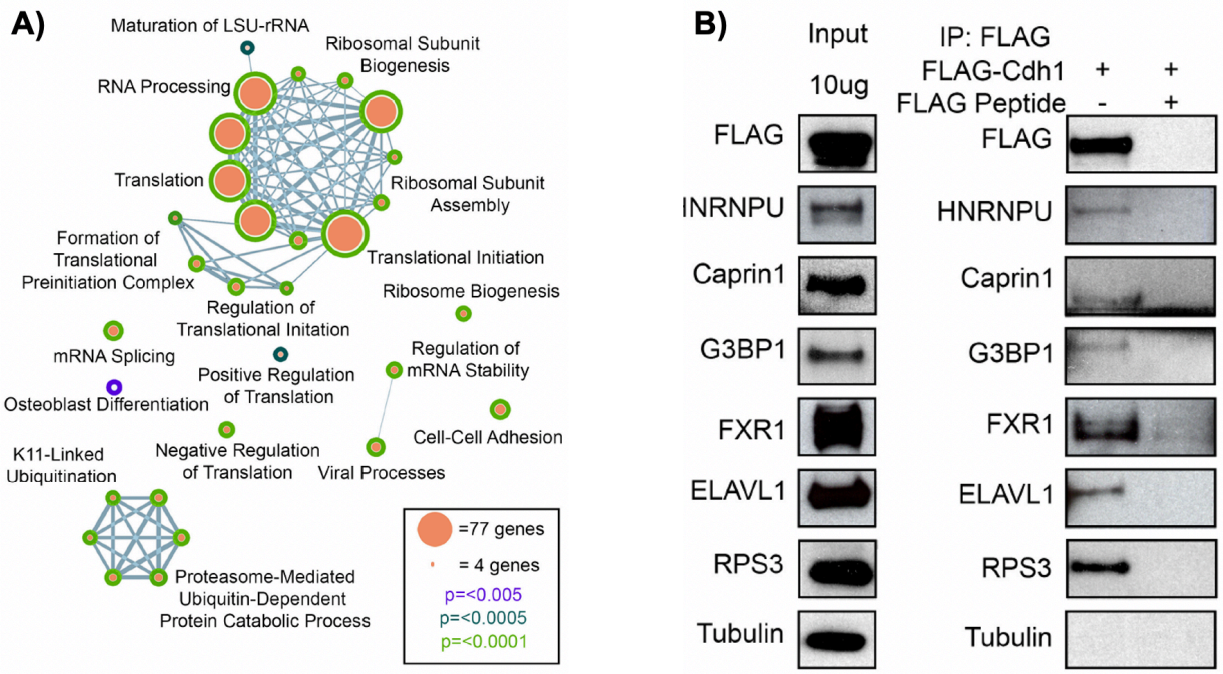

Figure 5. Cdh1 interacts with stress granule-associated proteins. (A) DAVID Biological Processes GO-term analysis following mass spectrometry. Size of the nodes indicate number of proteins in the Cdh1 interactome within a specific biological process category. The color of the border of each of the nodes represents the p-value as generated by the DAVID analysis. B) Stress granule proteins were confirmed to interact with Cdh1 in N2A cells by western blot analysis following the immunoprecipitation of FLAG-Cdh1. n=3.

Table 1. **DAVID Gene Ontology Analysis on Cdh1 interactome.**

Translational Initiation	77	43.50	2.31E-120	RPL18, RPL17, RPL19, RPL14, RPL13, RPL15, RPLP2, RPS2, LARP1, RPS3, RPS3A, RPLP0, RPL11, RPL12, RPL35A, RPS4X, EIF4G1, RPS18, RPS19, RPS16, RPS17, RPS14, RPS15, RPS12, RPS13, RPS10, RPS11, RPL27A, RPS15A, RPL36, RPS25, EIF3C, EIF3D, RPS27, RPL30, EIF3A, RPS28, EIF3B, RPS29, RPL7, RPL31, EIF3H, RPL6, RPL34, RPL9, EIF3F, RPL8, RPL3, EIF3K, EIF3L, RPL5, EIF3I, RPS20, RPL4, RPL10A, RPL7A, PABPC1, RPS21, RPS23, EIF3M, RPSA, RPL26, RPL27, RPS9, RPL24, RPL23A, RPS5, RPL28, RPS8, RPS7, EIF4E, RPL23, RPL18A, RPL13A, RPL22, RPL21, RPL37A	53.93
Ribonucleoprotein	72	40.68	1.29E-85	RPL18, RALY, RPL17, RPL19, RPL14, RPL13, SRP68, RPL15, SYNCRIP, RPLP2, RPS2, RPS3, RPS3A, RPLP0, RPL11, RPL12, RPL35A, RPS4X, HNRNPU, RPS18, RPS19, RPS16, RPS17, RPS14, RPS15, RPS12, RPS13, RPS10, RPS11, RPL27A, RPS15A, RPL36, RPS25, RPL10L, RPS27, RPL30, RPS28, HNRNPK, RPS29, RPL7, RPL6, RPL31, RPL34, RPL9, HNRNPF, RPL8, RPL3, RPL5, RPS20, RPL4, RPL7A, RPL10A, RPS21, RPS23, RPSA, RPL26, RPS9, RPL27, RPL23A, RPL24, RPS5, HNRNPA1, RPL28, RPS8, RPS7, RPL23, RPL18A, RPL13A, RPL22, RPL21, RBMXL1, RPL37A	28.28
Translation	65	36.72	4.09E-73	RPL18, RPL17, RPL19, RPL14, RPL13, RPL15, RPLP2, RPS2, RPS3, RPS3A, RPLP0, RPL11, RPL12, RPL35A, RPS4X, EIF4G1, RPS18, RPS19, RPS16, RPS17, RPS14, RPS15, RPS12, RPS13, RPS10, RPS11, RPL27A, RPS15A, RPL36, RPS25, RPL10L, RPS27, RPL30, RPS28, RPS29, RPL7, RPL6, RPL31, RPL34, RPL9, RPL8, RPL3, RPL5, RPS20, RPL7A, RPL10A, RPL4, RPS21, RPS23, RPSA, RPL26, RPS9, RPL27, RPL23A, RPL24, RPS5, RPL28, RPS8, RPS7, RPL23, RPL18A, RPL13A, RPL22, RPL21, RPL37A	24.65
Ribosomal protein	64	36.16	1.87E-86	RPL18, RPL17, RPL19, RPL14, RPL13, RPL15, RPLP2, RPS2, RPS3, RPS3A, RPLP0, RPL11, RPL12, RPL35A, RPS4X, RPS18, RPS19, RPS16, RPS17, RPS14, RPS15, RPS12, RPS13, RPS10, RPS11, RPL27A, RPS15A, RPL36, RPS25, RPL10L, RPS27, RPL30, RPS28, RPS29, RPL7, RPL6, RPL31, RPL34, RPL9, RPL8, RPL3, RPL5, RPS20, RPL7A, RPL10A, RPL4, RPS21, RPS23, RPSA, RPL26, RPS9, RPL27, RPL23A, RPL24, RPS5, RPL28, RPS8, RPS7, RPL23, RPL18A, RPL13A, RPL22, RPL21, RPL37A	40.23

Ribosome	64	36.16	1.65E-78	RPL18, RPL17, RPL19, RPL14, RPL13, RPL15, RPLP2, RPS2, RPS3, RPS3A, RPLP0, RPL11, RPL12, RPL35A, RPS4X, RPS18, RPS19, RPS16, RPS17, RPS14, RPS15, RPS12, RPS13, RPS10, RPS11, RPL27A, RPS15A, RPL36, RPS25, RPL10L, RPS27, RPL30, RPS28, RPS29, RPL7, RPL6, RPL31, RPL34, RPL9, RPL8, RPL3, RPL5, RPS20, RPL7A, RPL10A, RPL4, RPS21, RPS23, RPSA, RPL26, RPS9, RPL27, RPL23A, RPL24, RPS5, RPL28, RPS8, RPS7, RPL23, RPL18A, RPL13A, RPL22, RPL21, RPL37A	25.21
RNA-binding	54	30.51	1.15E-36	RALY, SRP68, SYNCRIP, YBX1, RPS3, LARP1, NONO, RPL11, DDX21, DHX36, RPL12, DHX30, RPL35A, G3BP1, G3BP2, YTHDF2, CDC5L, RPS4X, NCL, HNRNPU, EIF4G1, RPS18, LARP4, SERBP1, RPS11, MATR3, EIF3D, EIF3A, MOV10, EIF3B, HNRNPK, RPL7, HNRNPF, RPL8, NPM1, CSDE1, RPL5, PABPC1, UPF1, ALYREF, RPS9, ELAVL1, ILF3, RPL23A, NXF1, DDX5, LARP4B, HNRNPA1, CAPRIN1, FXR1, EIF4E, RPL22, RBMXL1, RBM14	9.44
Cytoplasmic Translation	13	7.34	4.78E-18	RPL35A, RPL7, RPL22, RPL6, RPL31, RPLP0, RPL9, RPL15, RPL8, RPL26, RPLP2, RPL36, MRTO4	49.90
Initiation Factor	12	6.78	2.10E-12	EIF3C, EIF4G1, EIF3D, EIF3A, EIF4E, EIF3B, EIF3H, EIF3F, EIF3K, EIF3L, EIF3I, EIF3M	24.06
Regulation of Translational Initiation	11	6.21	2.22E-12	EIF3C, EIF4G1, EIF3D, EIF3A, EIF3B, EIF3H, EIF3F, EIF3K, EIF3L, EIF3I, EIF3M	29.32
Positive Regulation of Ubiquitin-Protein Ligase Activity Involved in Regulation of Mitotic Cell Cycle Transition	11	6.21	5.83E-09	ANAPC1, PSMB5, ANAPC2, PSMB6, PSMA4, CDC23, CDC16, ANAPC7, SKP1, PSMA7, CDC27	13.89
Formation of Translation Preinitiation Complex	10	5.65	8.07E-13	EIF3C, EIF3D, EIF3A, EIF3B, EIF3H, EIF3F, EIF3K, EIF3L, EIF3I, EIF3M	41.72

Negative Regulation of Ubiquitin-Protein Ligase Activity Involved in Mitotic Cell Cycle	10	5.65	4.80E-08	ANAPC1, PSMB5, ANAPC2, PSMB6, PSMA4, CDC23, CDC16, ANAPC7, PSMA7, CDC27	13.51
Anaphase-Promoting Complex-Dependent Catabolic Process	10	5.65	1.24E-07	ANAPC1, PSMB5, ANAPC2, PSMB6, PSMA4, CDC23, CDC16, ANAPC7, PSMA7, CDC27	12.15
Ribosomal Small Subunit Assembly	9	5.08	7.76E-12	RPSA, RPS27, RPS28, RPS19, RPS17, RPS14, RPS15, RPS10, RPS5	45.45
Ribosomal Large Subunit Assembly	9	5.08	2.05E-11	RPL10L, RPL6, RPL3, RPL5, RPL24, RPL11, RPL23A, RPL12, MRTO4	41.12
Negative Regulation of Translation	9	5.08	1.39E-07	RACK1, EIF4E, RPL13A, SYNCRIP, ILF3, CAPRIN1, RPS3, PURA, FXR1	14.89
Ribosome Biogenesis	8	4.52	7.36E-08	NVL, RPS18, RPS28, RPLP0, RPL34, GNL3L, RPL7A, GNL3	21.32
Ribosomal Large Subunit Biogenesis	6	3.39	5.07E-06	RPL35A, RPL14, RPL7, RPL26, RPL5, RPL11	23.03
Ribosomal Small Subunit Biogenesis	6	3.39	4.49E-07	RPS28, RPS19, RPS16, RPS17, RPS15, RPS7	35.98
Positive Regulation of Translation	6	3.39	2.16E-04	NPM1, ELAVL1, PABPC1, RPS4X, LARP4B, LARP1	10.86
Proteasome, Subunit Alpha/Beta	4	2.26	7.15E-04	PSMB5, PSMB6, PSMA4, PSMA7	22.20

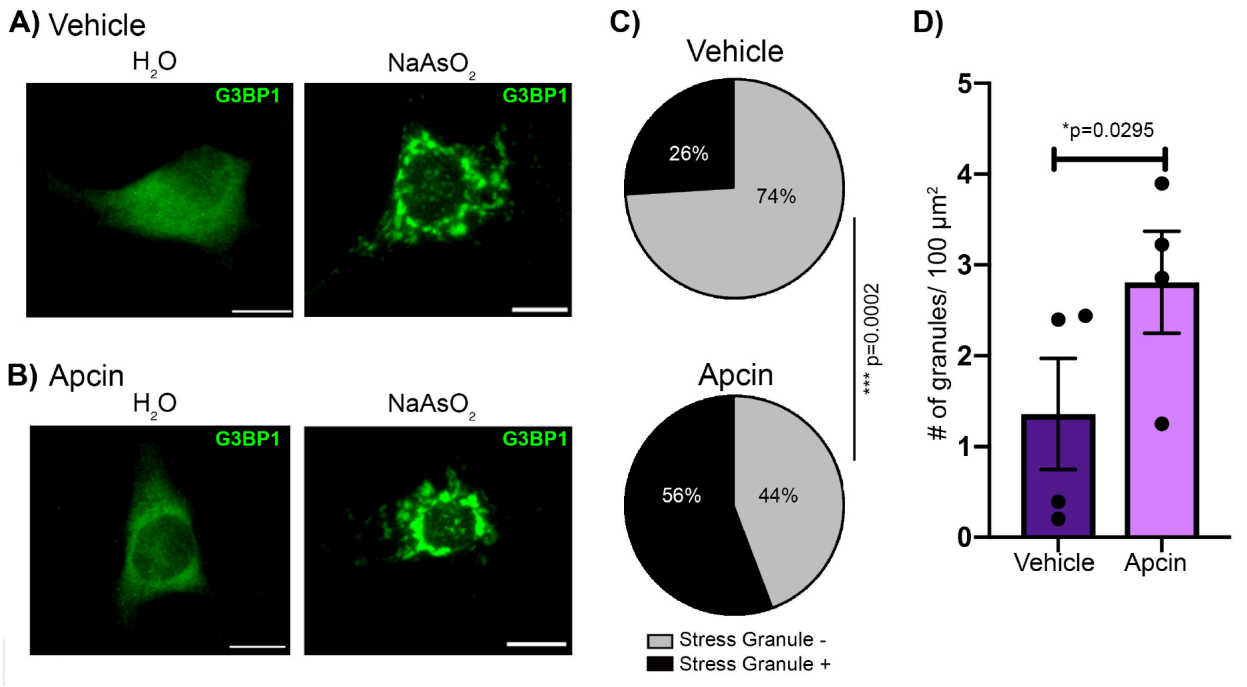

Figure 6. Apcin increases stress granule formation. (A) DIV 14-16 neurons were treated with Vehicle (DMSO) for 16-18 hours, followed by treatment with arsenite ($NaAsO_2$) or H_2O as control for 45 minutes. (B) DIV 14 cortical neurons were treated with APC inhibitor Apcin for 16-18 hours. Neurons were then treated with sodium arsenite ($NaAsO_2$) (0.5 mM) or water for 45 minutes prior to fixation. Immunofluorescence was done with antibodies against G3BP1. Scale bar indicates 10μM. **C)** User-blind scoring of neurons that were stress granule positive or stress granule negative following arsenite treatment. N=73 neurons for vehicle, 78 neurons for Apcin. **D)** Quantification of number of stress granules in the soma per 100μm^2, n=4. Statistical significance was calculated by (**C**) Z test and (**D**) Student's t test.

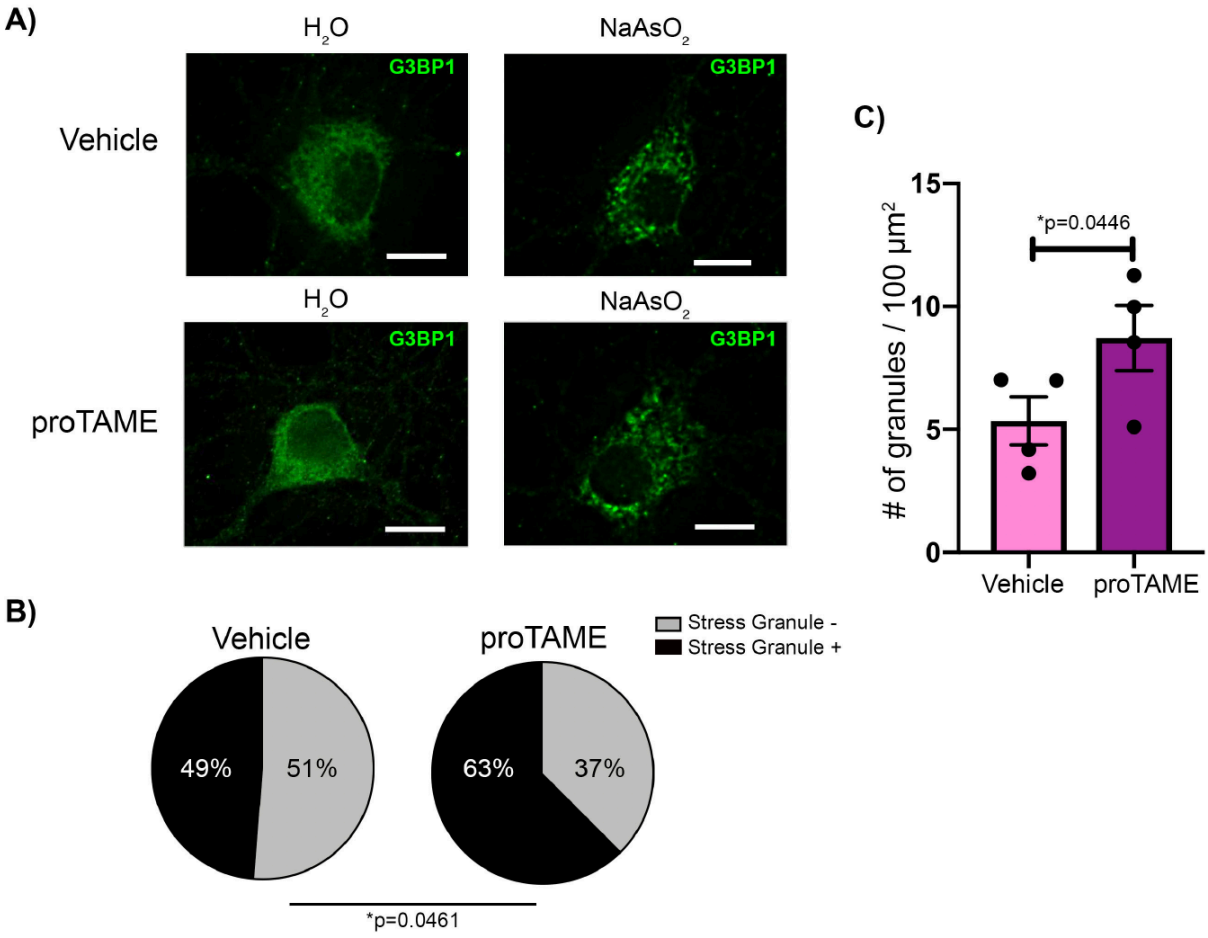

Figure 7. ProTAME increases stress granule formation. (A) DIV 14-16 neurons were treated with ProTAME or Vehicle (DMSO) for 4 hours, followed by treatment with arsenite ($NaAsO_2$) or H_2O as control for 45 minutes. **(B)** User-blind scoring of neurons that were stress granule positive or stress granule negative following arsenite treatment. N=60 neurons for both conditions. **(C)** Quantification of number of stress granules in the soma per 100μm^2. Statistical significance was calculated by Z test (**B**) and Student's t test (**C**).

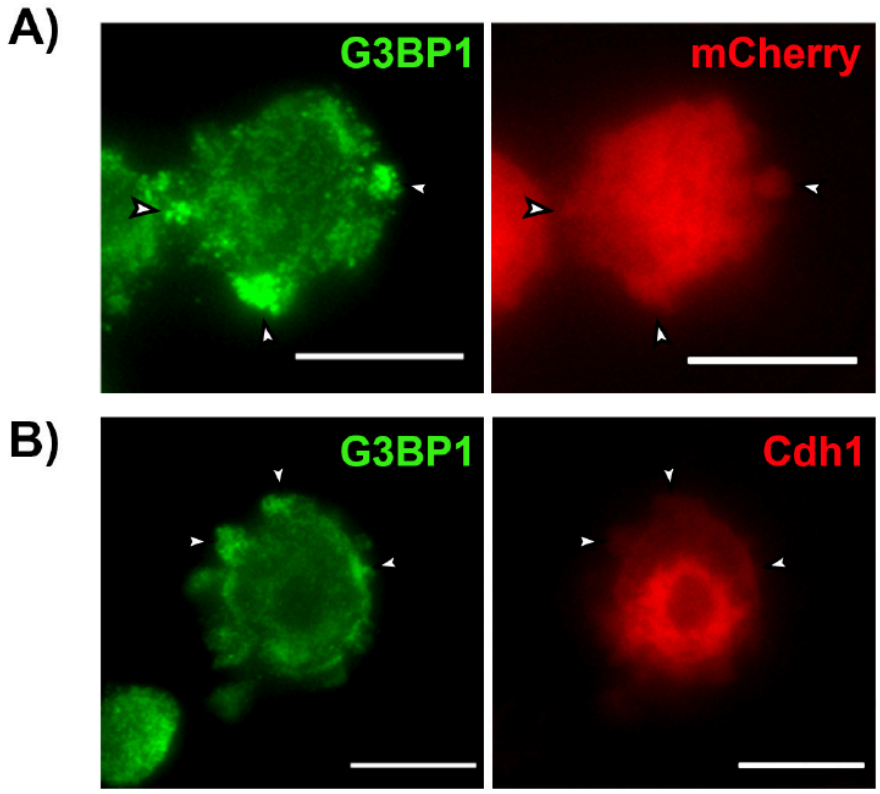

Figure 8. Cdh1 does not colocalize with G3BP1. A, B) Neuro2A cells were transfected with (**A**) mCherry or (**B**) mCherry-Cdh1. Cells were treated with sodium arsenite for 45 minutes to induce stress granules and then fixed in 4% paraformaldehyde and then immunostained for G3BP. Arrows indicate stress granule accumulation. Scale bar = 10 μm.

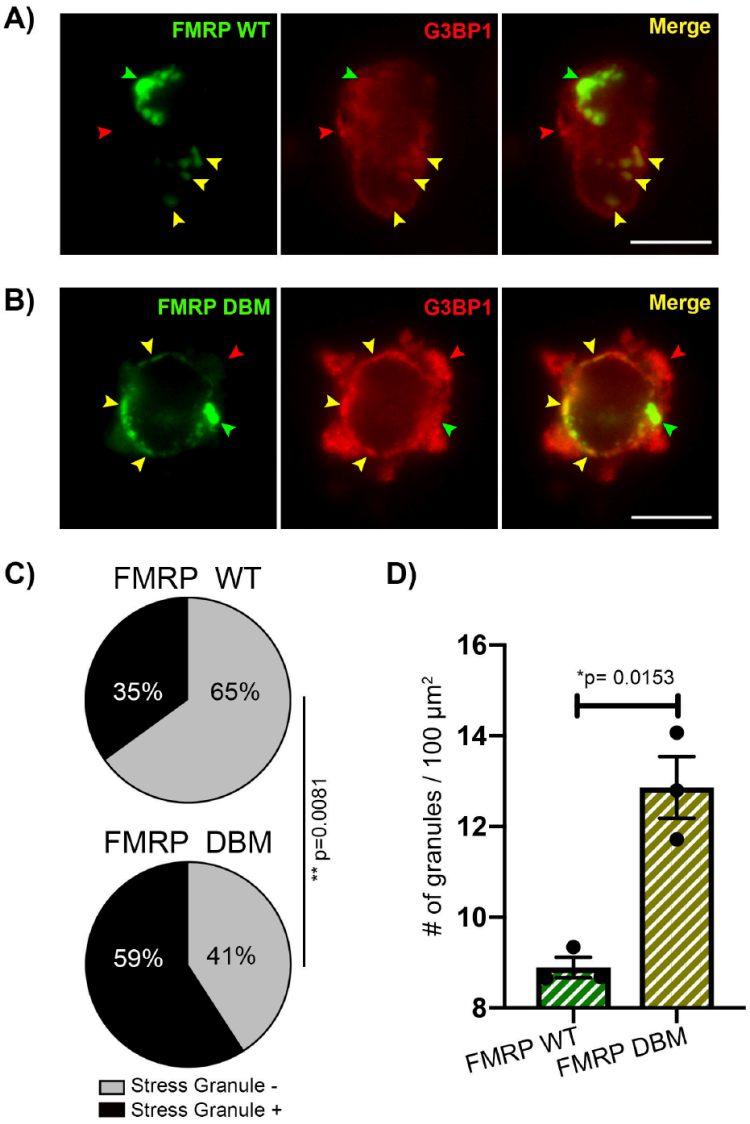

Figure 9. Cdh1-FMRP interaction regulates stress granule formation. A,B) N2A cells were transfected with **(A)** GFP-FMRP WT or **(B)** GFP-FMRP DBM,treated with sodium arsenite (amount or concentration?) and immunostained for G3BP1. Green arrowheads indicate FMRP expression. Red arrowheads indicate stress granules based upon G3BP1 staining. Yellow arrowheads indicate co-localization of FMRP with G3BP1-positive stress granules. Scale bar indicates 10μm **C)** Quantification of stress granule negative or positive cells. n=60 cells for both conditions. **D)** Quantification of number of stress granules in the soma per 100 μm^2, n=3.

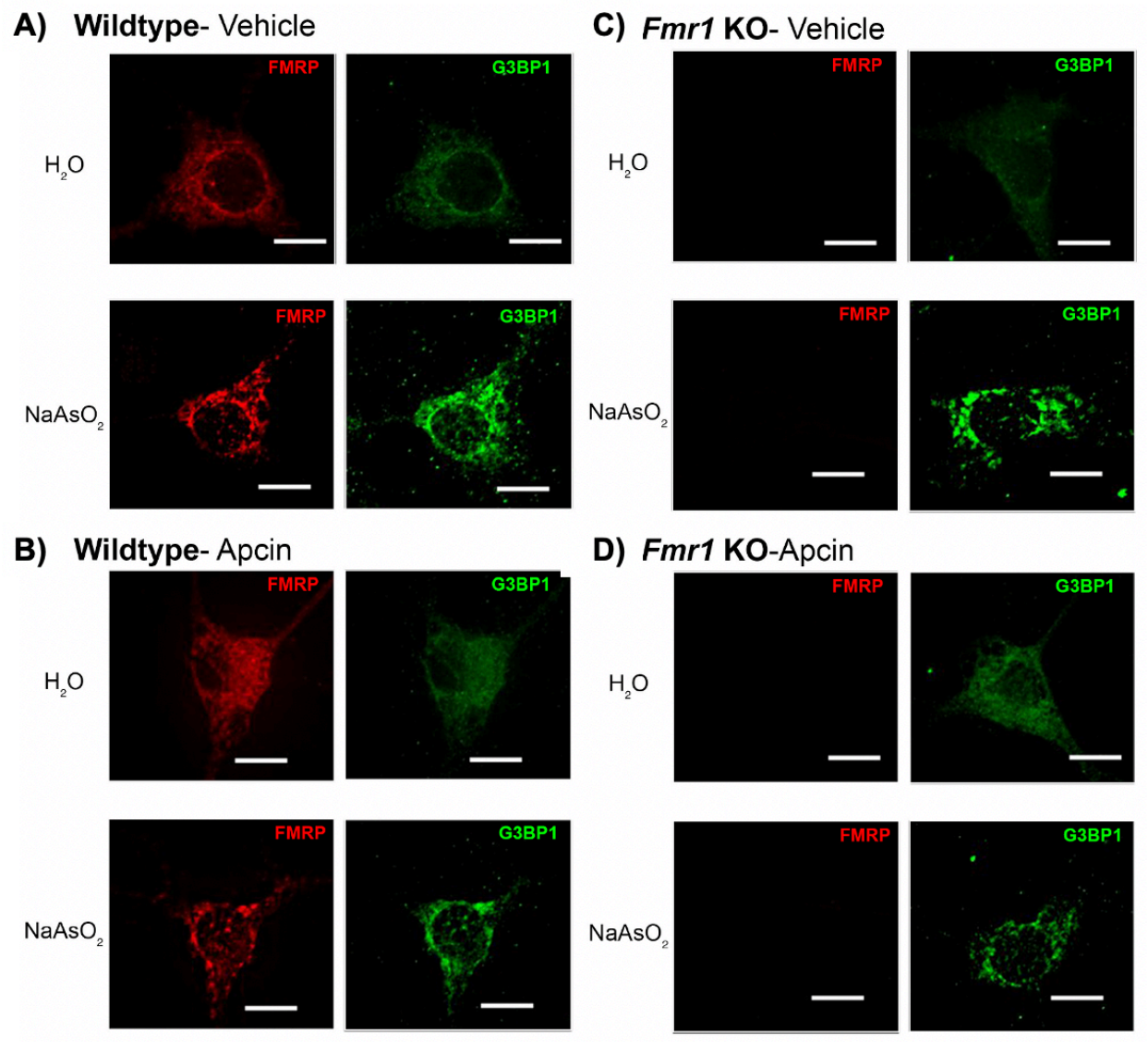

Figure 10. **Representative images of stress granule formation in wildtype and Fmr1-KO neurons. (A, B)** Postnatal wildtype DIV 14-16 cortical neurons were treated with **(A)** vehicle (DMSO) or **(B)** APC inhibitor Apcin for 16-18 hours. Following this treatment, neurons were treated with arsenite (concentration?) or H_2O as vehicle control for 45 minutes. **(C, D)** Postnatal Fmr1-KO DIV14-16 cortical neurons were treated with **(C)** vehicle (DMSO) or **(D)** Apcin for 16-18 hours, followed by 45 minute-treatment with arsenite or H_2O. All neurons were immunostained with antibodies to detect G3BP1 and FMRP. Scale bar indicates 10 μm.

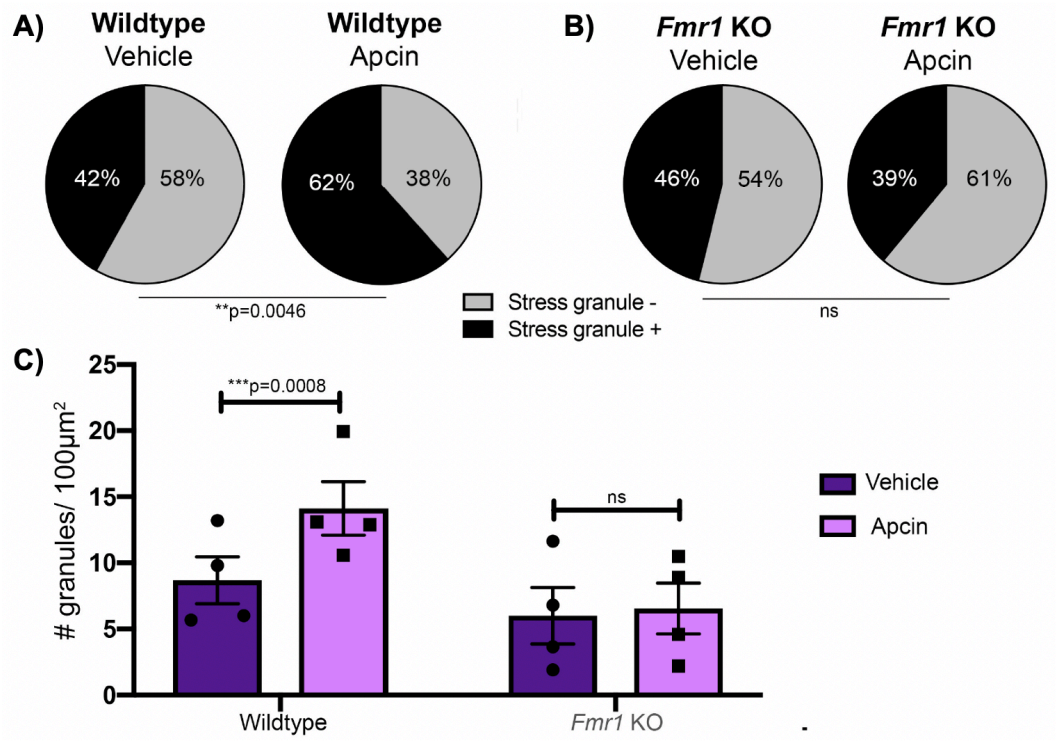

Figure 11. Quantification of stress granule formation in wildtype and *Fmr1* KO neurons after Apcin and $NaAsO_2$ treatment. A, B) Quantification of stress granule negative or positive cells. **A**) N=78 cells for Vehicle-treated and (**B**) 77 cells for Apcin-treated. (**C**) Quantification of number of stress granules in the soma per 100 μm^2 for all four conditions, n=4. Statistical significance was calculated by Z test.

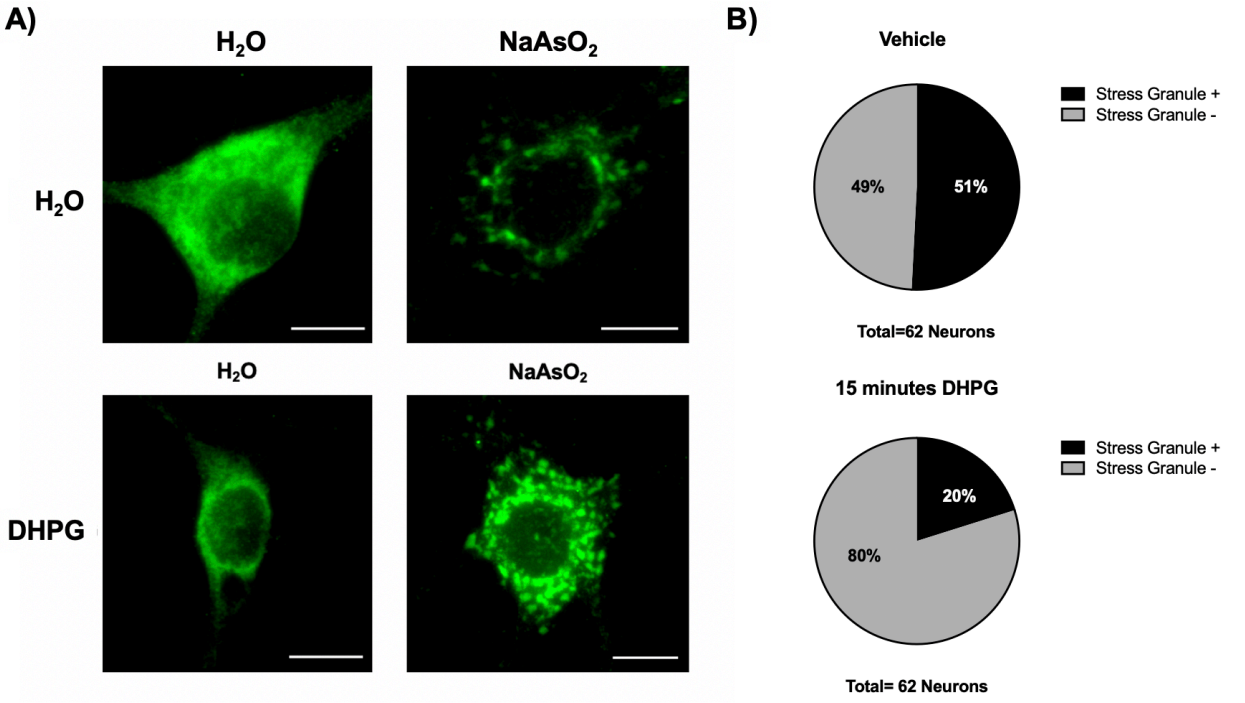

Figure 12. DHPG stimulation reduces stress granule formation. (A) DIV 14-16 neurons were treated with DHPG (1 uM) or water for 15 minutes, followed by treatment with arsenite ($NaAsO_2$) or H_2O as control for 45 minutes. Immunofluorescence was done with antibodies against G3BP1. Scale bar indicates 10μM. **B)** User-blind scoring of neurons that were stress granule positive or stress granule negative following arsenite treatment. N=62 neurons for vehicle, 62 neurons for DHPG.