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Maria Dhinojwala April 12, 2022

Altered CRABP1 Expression in Fragile-X syndrome

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Abstract

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Fragile X syndrome (FXS) is the most common heritable form of intellectual disability (ID) and monogenic cause of autism. FXS occurs due to silencing of the *FMR1* gene, which encodes the fragile X mental retardation protein (FMRP), an RNA-binding protein that is important for cell signaling and neurogenesis. While the focus has been to define direct mRNA targets of *FMR1*, non-canonical disease mechanisms remain unexplored. The Bassell lab has identified a cytoplasmic retinoic acid (RA) binding protein, CRABP1, that is significantly downregulated in human FXS patient postmortem brains and FXS patient induced pluripotent stem cell (hiPSC)-derived neural progenitor cells (NPCs) and organoids. RA plays crucial roles in cell cycle regulation and neuronal differentiation, and disrupted RA signaling has been implicated in several neurological disorders, including FXS. Given the role of RA signaling in neurogenesis, my thesis aimed to test this dysfunction of RA signaling and explore the functional consequences of CRABP1 deficiency as a direct result of reduced FMRP. RT-qPCR and western blotting reveal that CRABP1 expression trends down in *FMR1*-deficient human neuroblastoma cells, although this reduction is more robust at the mRNA level than the protein level. CRABP1 has been shown to mediate early-phase ERK 1/2 phosphorylation in response to RA stimulation, which leads to increased $p27^{KIP}$ levels and a prolonged G_0/G_1 phase of the cell cycle. To examine the potential loss of this function of CRABP1 and RA signaling in the context of FXS, either *FMR1* or *CRABP1* was depleted in human neuroblastoma cells that were subsequently stimulated with RA. In contrast to prior findings, *FMR1* and *CRABP1*-deficient cells did not show a loss of rapid ERK 1/2 phosphorylation. However, $p27^{KIP}$ was robustly reduced in *FMR1* and *CRABP1*-deficient cells, which may contribute to the neurogenic defects observed in FXS patient cells and point towards a crucial role of CRABP1 in regulating early neurogenesis. Future studies will address the underlying mechanism for how FMRP regulates CRABP1 expression in FXS-patient derived cells, and how $p27^{KIP}$ levels are regulated in FMRP and CRABP1-deficient cells to understand potential impacts of CRABP1 and $p27^{KIP}$ on early neurogenesis.

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Introduction

Fragile-X syndrome

Fragile-X syndrome (FXS) is the leading form of heritable intellectual disability (ID) and the most common monogenic cause of autism (Das Sharma et al., 2020). Individuals with FXS present with a wide range of symptoms including learning disabilities, developmental delays, attention-deficit, anxiety, mood disorders, and seizures (Lozano et al., 2016). While symptom-based treatments for FXS exist, the search for effective disease-specific therapeutics is still ongoing (Lozano et al., 2016). FXS primarily occurs due to transcriptional silencing of the X-linked fragile X mental retardation I (*FMR1*) gene, which is typically caused by an expansion of trinucleotide CGG repeats in the 5' untranslated region. Typically developing individuals have between 5-50 repeats, while pathogenic expansion in individuals can span from 55 to over 200 repeats, which is considered a full mutation. Hypermethylation of the promoter region of *FMR1* prevents the production of the Fragile X Mental Retardation Protein (FMRP), an RNA-binding protein that binds mRNA targets and regulates their stability and translation (Banerjee et al., 2018). FMRP associates with mRNA throughout its lifecycle including during splicing, trafficking, and stability (Davis & Broadie, 2017), but the non-canonical contributions of FMRP towards gene expression have yet to be explored.

FMRP plays a key role in neuronal growth and maturation, and its absence has far-reaching consequences on neurodevelopment and function. Both animal models of FXS and human patient cells show increased global protein synthesis (Gross et al., 2015; Gross & Bassell, 2012; Raj et al., 2021), hyperexcitability of neuronal circuits (Contractor et al., 2015; Gibson et al., 2008), and the dysregulation of key signaling pathways involved in neurogenesis (Bassell & Warren, 2008; Raj et al., 2021; Sharma et al., 2010). Many studies on FMRP have focused on the

regulation of synaptic transmission (Stoppel et al., 2021), specifically in response to metabotropic glutamate receptor 5 (mGluR5) activity. While exaggerated mGluR5-dependent long-term depression (LTD) occurs in FXS animal models due to increased AMPA (*α-amino-3-hydroxy-4-isoxazole* propionic acid) receptor internalization (Bear et al., 2004), clinical trials correcting for excess mGluR5 activity have thus far been unsuccessful in treating the impaired synaptic plasticity and intellectual and behavioral symptoms associated with FXS (Davis & Broadie, 2017). Thus, alternative FXS disease mechanisms such as the role of FMRP in determining cell fate and supporting neurogenesis continue to be explored as therapeutic targets.

The pharmacological inhibition of abnormal cell signaling pathways such as the phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase-1/2 (ERK 1/2) pathways were successful in correcting defects in neuronal differentiation in rodent FXS models (Gross et al., 2015, 2019; Osterweil et al., 2010). However, given most clinical FXS trials fail to meet their primary endpoints despite successful preclinical trials with animals (Kumari & Gazy, 2019), examining the molecular and cellular phenotypes of FXS in human disease-relevant cell models remains crucial for finding effective treatments. Developmental deficits in FXS human-induced pluripotent stem cell (hiPSC)-derived neural progenitor cells (NPCs) and organoids are rescued through inhibition of the PI3K pathway (Kang et al., 2021; Raj et al., 2021). Yet the link between dysregulated cell signaling due to *FMR1* silencing and the resulting consequences on neurogenesis remains elusive.

Role of FMRP in Neurogenesis

Neurogenesis is the process by which new neurons form from neural stem cells (NSCs) and intermediate neural progenitor cells (NPCs) and migrate to their final location in the brain

(Castrén, 2016). FMRP has been shown to regulate adult neurogenesis in the dentate gyrus (DG) of the hippocampus, and the loss of FMRP causes a decrease in neurogenesis and an overproduction of glial cells (Guo et al., 2011; Patzlaff et al., 2018). Moreover, restoring FMRP expression in FMRP-deficient mice rescues deficits in hippocampus-dependent learning tasks, illustrating the importance of FMRP in hippocampal neurogenesis and memory formation (Patzlaff et al., 2018).

At the fetal stage, both healthy and FXS-embryos express FMRP during the initial stages of brain development (Telias et al., 2015). Embryonic silencing of *FMR1* in FXS individuals occurs at ~ 10 gestational weeks, and neurogenesis occurs between ~8-15 gestational weeks. Thus inactivation of *FMR1* coincides with the critical period of neural development (Telias et al., 2015). Since FMRP plays a major role in neurogenesis during embryonic brain development, the absence of FMRP can alter the growth and maturation of neurons in both the developing (Telias et al., 2013) and mature brain (Guo et al., 2011). Throughout embryonic development, FMRP is widely expressed, and its expression increases during neuronal differentiation (Castrén, 2016). During corticogenesis, NSCs known as radial glial cells (RGCs) proliferate and undergo differentiation into intermediate NPCs (Casingal et al., 2020). FMRP helps regulate this transition in the embryonic brain through an actin-dependent mechanism (Saffary & Xie, 2011). Furthermore, in the mouse embryonic brain, up to 17 direct gene targets of FMRP overlapped with genes involved in neurogenesis, autism spectrum disorders (ASDs), and ID (Casingal et al., 2020), supporting the idea that *FMR1* silencing during embryonic brain development can have a widespread impact on neurogenesis in FXS. An FXS mouse model at embryonic day (E)17.5 showed delayed neuronal positioning in the cortex leading to aberrant spontaneous cortical network activity (La Fata et al., 2014). Additionally, Fragile X-human embryonic stem cells

(FX-hESCs) showed a delay in neural rosette formation and deficits in neuronal maturation along with high gliogenesis (Telias et al., 2013). Interestingly, FX-hESCs had a lower gene expression of NSC pluripotency transcription factors like *SOX1*, *PAX6*, and *NOTCH1* (Telias et al., 2013) and a reduced abundance of proliferative $Ki67^{+}$ cells in day 56 FXS patient hiPSC-derived forebrain organoids indicative of reduced NPC proliferation (Kang et al., 2021). However, FXS patient hiPSC-derived NPCs and day 28 forebrain organoids showed a higher abundance of proliferative over terminally differentiated neuronal cell types (Raj et al., 2021), suggesting that the influence of FMRP on neurogenesis may be developmental stage specific. Overall, the effects of *FMR1* silencing on early neurogenesis has deleterious consequences on subsequent neuronal maturation and activity. These impairments in early neurogenesis may lead to the development of ID and autistic-like behavior in FXS individuals.

Retinoic Acid signaling in FXS

A hallmark determinant of neuronal cell fate is retinoic acid (RA), the most active metabolite of Vitamin A. RA is a developmental morphogen which plays an essential role in directing early neuronal differentiation and neural patterning (Janesick et al., 2015). RA acts with other cell signaling pathways to posterize the neuroectoderm (Papalopulu & Kintner, 1996) and facilitate hindbrain patterning (Maden, 2007). RA exerts genomic functions by binding to nuclear RA receptors (RARs) and retinoid X receptors (RXRs) which are DNA-binding transcriptional regulators (Das et al., 2014). RA can also exert nongenomic functions through rapidly activating several kinase cascades including the p42/p44 extracellular signal-regulated kinases (ERK 1/2) in neuronal cells (Al Tanoury et al., 2013).

Given the extensive role of RA in cell cycle regulation, neurogenesis, and homeostatic synaptic plasticity in postsynaptic neurons, dysregulated RA signaling is implicated in several neurological disorders (Chatzi et al., 2011), including FXS (Park et al., 2021). Growing evidence suggests that RA signaling plays an important role in cognition and is involved in the maintenance of neural plasticity and memory performances throughout adulthood (Wołoszynowska-Fraser et al., 2021). RA modulates neurogenesis and neuronal plasticity in the adult hippocampus (Nomoto et al., 2012) and stabilizes neural networks after learning-induced changes in synaptic plasticity (Groth & Tsien, 2008). Older rodents also show reduced vitamin A metabolism (Touyarot et al., 2013) and a decrease in RA signaling (Etchamendy et al., 2003), whereas supplementing adolescent rats with RA rescues memory impairments induced by vitamin A deficiency (Etchamendy et al., 2001). These studies suggest that the regulation of RA signaling and RA-mediated gene expression is important for higher cognition, and RA deficiency may lead to symptoms of ID.

In ASDs, several links between ASD causal genes and aberrant RA signaling have been uncovered. Enhanced E3 ubiquitin (Ub) ligase UBE3A activity occurs in 1 to 3% of ASD cases worldwide, and UBE3A negatively regulates ALDH1A2, the rate-limiting enzyme of RA synthesis in mice (Xu et al., 2018). Furthermore, supplementation of RA restored RA-mediated synaptic plasticity in mice with elevated UBE3A (Xu et al., 2018). Decreased protein expression and dysregulated DNA methylation of the retinoic acid-related orphan receptor alpha (RORA) is also seen in autistic subjects (A. Nguyen et al., 2010). RORA transcriptionally regulates ASD-relevant genes like *FOXN1* and *NLGN1* (Moreno-Ramos et al., 2015; Sarachana & Hu, 2013), suggesting that aberrant genomic RA signaling may lead to ASD-associated symptoms. The transmembrane glycoprotein CD38 also received attention in ASDs due to its mediation of

oxytocin release in the brain and influence on social behavior (Riebold et al., 2011). CD38 expression is reduced in ASD subjects, and treating lymphoblastoid cell lines from ASD patients with RA raised *CD38* mRNA levels (Riebold et al., 2011). Additionally, treatment with the vitamin A precursor beta-carotene rescued ASD-associated defects in social interactions and communication in mice deficient in CD38 (Avraham et al., 2019). These findings support the concept that targeting RA signaling pathways may serve as a valuable therapeutic approach for alleviating symptoms of ID and ASD-associated behaviors.

Prior studies on the interaction between FMRP and RA signaling center around RA-dependent homeostatic synaptic plasticity. When RA is synthesized in response to reduced synaptic activity, FMRP must directly bind to the nuclear RA receptor alpha (RARα) protein for synaptic scaling to occur (Park et al., 2021). In FXS patient-derived neurons and *Fmr1* knockout (KO) neurons, FMRP re-expression could restore RA-dependent translation of the GluR1-type AMPA receptors (Soden & Chen, 2010; Z. Zhang et al., 2018). Overall, FMRP appears to be required for the form of homeostatic plasticity that depends on RA signaling and translation of GluR1.

The interaction between FMRP and RA signaling during early neurogenesis, however, has yet to be explored. RA signaling inhibits the expression of multiple pluripotency factors such as *Oct4* and *Nanog* (J. Zhang et al., 2015), as well as *Geminin*, *Zic1/2/3*, and *Notch* (Janesick et al., 2015) to repress cellular proliferation and stemness and promote neural differentiation. An absence of RA results in a depletion of intermediate NPCs in E13.5 mouse embryos, indicating that RA functions during early corticogenesis to regulate the transition of RGCs to NPCs (Haushalter et al., 2017). RA additionally increases neuronal production in the mouse postnatal

brain (T.-W. Wang et al., 2005) and endogenous NSCs (Maden, 2007), highlighting the importance of RA signaling in neuronal development.

To induce neuronal differentiation, RA increases the expression of cyclin dependent kinase (CDK) inhibitors that promote cell cycle exit for cells in the G_1 phase (Giordano & Galderisi, 2010). RA treatment increases the level of key cell-cycle related proteins such as the cyclin-dependent kinase inhibitor 1B $p27^{KIP}$ (p27) to arrest cells in the G_1 phase and reduce proliferation (Chen & Ross, 2004). Dysregulated RA signaling particularly inhibits the differentiation of GABAergic interneurons during forebrain development, and a deficiency of GABAergic neurons is associated with several neurological disorders including FXS, autism, and epilepsy (Chatzi et al., 2011). Given the essential role of RA in primary neurogenesis and observed defects in neurogenesis during brain development in FXS, investigating potential connections between FMRP and RA signaling is critically important and may define avenues for therapeutic intervention.

Cellular Retinoic Acid Binding Protein I

Recently, the Bassell lab identified a key component of RA signaling as downregulated in both human FXS postmortem brains and FXS patient hiPSC-derived cells. In an RNA sequencing study of day 28 and day 105 FXS patient iPSC-derived forebrain organoids, the cellular RA binding protein 1 (*CRABP1*) was found to be the only gene besides *FMR1* that was downregulated at different stages of neural development (Fig. 1A, Raj et al., unpublished). CRABP1 binds to RA with high affinity in the cytoplasm to regulate cytoplasmic RA levels (Napoli, 2017), and can result in RA retention (Blaese et al., 2003), limited RA activity (Liu et

al., 2015), or RA metabolism (Fiorella & Napoli, 1991). However, little is known about the role of CRABP1 in human neurodevelopment.

Work from the Bassell lab also shows that CRABP1 protein levels are reduced in FXS patient postmortem brain tissue and in FMRP-deficient hiPSC-derived neurons relative to isogenic control neurons (Figure 1B & C, Raj et al., unpublished). Single-cell RNA sequencing of control and FXS patient hiPSC-derived organoids reveal that *CRABP1* expression is most significantly downregulated in RGCs, NSCs, NPCs, and young or immature neurons (Raj et al., unpublished). Importantly, CRABP1 is highly expressed in the caudate (Consortium & The GTEx Consortium, 2020), and an enlarged caudate is one of the most consistent neuroanatomical findings in FXS individuals (Gothelf et al., 2008; Sandoval et al., 2018). Thus, reduced CRABP1 levels may underlie dysregulated RA signaling and abnormal neurogenesis in FXS patients during early neural development.

By binding to cytoplasmic RA, CRABP1 can mediate the nongenomic functions of RA. Murine CRABP1 is necessary to activate early-phase ERK 1/2 signaling to lengthen the G_1 phase of neural and embryonic stem cells to drive neuronal differentiation (Lin et al., 2017; Persaud et al., 2016). ERK 1/2 is a major signal transduction pathway in the mammalian mitogen-activated protein kinase (MAPK) family and is involved in several key cellular processes including cellular proliferation, differentiation, and cell cycle regulation (Meloche & Pouysségur, 2007; Wortzel & Seger, 2011). ERK 1/2 activation is biphasic, with an early-phase that occurs between 15 minutes to 1 hour after RA stimulation and a late-phase that is detected after 12 hours (Lin et al., 2017). ERK 1/2 signaling promotes progression from the G_1 to S phase of the cell cycle, as persistent ERK 1/2 activation in the mid-G_1 phase correlates with S-phase entry (Meloche et al., 1992). Sustained ERK 1/2 activation is also associated with an increase in the transcription and

translation of cyclin D1 in the mid-G_1 phase, a cyclin which forms a complex with cyclin-dependent kinase (CDK) 1 and 4 to allow the G_1 to S-phase transition (Meloche & Pouysségur, 2007; Villanueva et al., 2007).

There are conflicting results to explain how the loss of FMRP impacts basal or mGluR5-induced ERK 1/2 phosphorylation. Two studies show ERK 1/2 phosphorylation is elevated in the hippocampal synaptoneurosomes of *Fmr1* KO mice (Hou et al., 2006; Price et al., 2007), but other groups failed to find increased basal phosphorylated ERK 1/2 (phospho-ERK 1/2) in *FMR1* KO mice (Gross et al., 2010; Hu et al., 2008). Studies also show contrasting results in testing whether ERK 1/2 phosphorylation is absent upon mGluR5 stimulation in *Fmr1* KO mice (X. Wang et al., 2012), although lymphocytes from human FXS patients did not exhibit mGluR-induced ERK 1/2 phosphorylation (Weng et al., 2008). Cortical synaptoneurosomes from *Fmr1* KO mice show rapid dephosphorylation of early-phase ERK 1/2 due to overactive protein phosphatase 2A (PP2A) upon mGluR 1/5 stimulation (Kim et al., 2008). Furthermore, both *Fmr1* KO mouse neurons, *Fmr1* KO mouse thymocytes, and FXS patient lymphocytes showed delayed early-phase ERK 1/2 phosphorylation (Weng et al., 2008). Given the involvement of ERK 1/2 in cell proliferation and cell cycle regulation, aberrant early-phase ERK 1/2 signaling may have profound consequences on neurogenesis in FXS.

Role of p27 Cyclin-dependent Kinase Inhibitor Protein

One consequence of aberrant ERK 1/2 activation is the downregulation of p27 (Bhatt et al., 2005; Rivard et al., 1999), a cyclin dependent kinase (CDK)-inhibitor that impedes the progression from the G_1 to S phase of the cell cycle (Chen & Ross, 2004). While p27 may have a host of functions as a regulator of drug resistance and a promoter of apoptosis, p27 is a member

of the CDK interacting protein/Kinase inhibitory protein (CIP/KIP) family of CDK inhibitors (CDKIs) (Lloyd et al., 1999). CIPs/KIPs block cell cycle progression through interacting with CDK-containing complexes to inhibit kinase activity (Vidal & Koff, 2000). Multiple studies have shown p27 plays a key role in the decision to commit or withdraw from the cell cycle (Coats et al., 1996; Durand et al., 1998; Vidal & Koff, 2000). By binding to CDK2-cyclin E complexes, p27 can arrest cells in the G_1 phase (Lloyd et al., 1999; Polyak et al., 1994).

Based on recent evidence from the Bassell lab that FXS patient hiPSC-derived NPCs do not show early-phase ERK 1/2 signal activation in response to RA stimulation (Fig. 2A; Raj et al., unpublished), we hypothesize that CRABP1-mediated changes in p27 levels may drive the cell signaling defects observed in FXS. In mouse ESCs, CRABP1-mediated ERK 1/2 signaling activates PP2A to dephosphorylate p27 in the nucleus (Fig. 2B; Nagpal & Wei, 2019). Increased levels of dephosphorylated p27 lengthen the G_1 phase, which is necessary to enable neuronal differentiation (Lange et al., 2009). This model suggests that CRABP1 may act through early-phase ERK 1/2 signaling and p27 to regulate the cell cycle and promote neurogenesis.

Although p27 levels were increased in a mouse model of FXS, this was specifically in *Fmr1* KO mESCswhich exhibited accelerated neuronal differentiation (Khalfallah et al., 2017). However, determining the link from FMRP and CRABP1 expression to p27 levels may explain the increase in proliferative versus mature neuronal cell types in FXS patient hiPSC-derived NPCs and day 28 organoids (Raj et al., 2021). In further support, recent work from the Bassell lab demonstrates that CRABP1 overexpression in control NPCs increases the abundance of cells in the G_0/G_1 phase, while overexpressing CRABP1 in FXS patient-derived NPCs increases the proportion of cells in the G_0/G_1 phase to that of the control (Raj et al., unpublished). All together, these findings suggest that the role of CRABP1 in nongenomic RA signaling and

dephosphorylated p27 levels may have important implications for cell signaling and cell cycle defects seen in FXS.

Research Aims

Where previous findings demonstrated a reduction of CRABP1 expression in FXS patient-derived cells, my thesis examined whether CRABP1 is reduced as a direct consequence of acutely silencing *FMR1.* I developed a small interfering RNA (siRNA)-mediated *FMR1* knockdown (KD) model of FXS to measure CRABP1 mRNA and protein levels in SH-SY5Y human neuroblastoma cells. To explore potential consequences of altered CRABP1 expression, my thesis examines the effects of RA stimulation on early-phase ERK 1/2 phosphorylation and the levels of the CDK-cyclin inhibitor, $p27^{KIP}$ in *FMR1* KD and *CRABP1* KD human neuroblastoma cells. I hypothesize that a siRNA-mediated knockdown of *FMR1* will result in reduced CRABP1 expression at the mRNA and protein levels. As previous studies have shown that CRABP1 mediates early-phase ERK 1/2 phosphorylation, which may lead to increased p27 levels, I predict that both *FMR1* KD cells and *CRABP1* KD cells will show a loss of early-phase ERK 1/2 phosphorylation upon RA stimulation and lower p27 levels due to reduced CRABP1 expression.

My thesis includes four main research aims. The first aim was to develop an effective *FMR1* knockdown model using the human neuroblastoma cell-line, SH-SY5Y. I determined that the most effective siRNA-mediated *FMR1* knockdown was obtained with 40 to 50 nM of siRNA and a 72-hour knockdown window. This *FMR1* KD model was used for subsequent experiments to measure CRABP1 expression and effects of RA stimulation.

For the second aim, I measured CRABP1 expression as a direct consequence of FMRP depletion. I found CRABP1 mRNA and protein levels trended towards a reduction, though the reduction was not as robust as previously seen in FXS patient-derived cells or postmortem tissue.

In the third aim, I explored how early-phase ERK 1/2 phosphorylation is affected in *FMR1* and *CRABP1* KD cells upon RA stimulation, to determine if CRABP1 depletion drives changes in early-phase ERK 1/2 signaling. Unexpectedly, both *FMR1* and *CRABP1* KD SH-SY5Y cells showed rapid ERK 1/2 phosphorylation. As previous studies show a delay or loss of early-phase ERK 1/2 phosphorylation in mouse FXS cell models, the early-phase ERK 1/2 phosphorylation observed in this thesis may be due to the longer length of RA stimulation and the use of an FXS knockdown model with human neuroblastoma cells.

The fourth aim of my thesis was to determine p27 levels in response to knocking down *FMR1* and *CRABP1*, as reduced p27 levels may be responsible for the increased cellular proliferation seen in FMRP and CRABP1 depleted cells. By measuring p27 levels in both *FMR1* and *CRABP1* KD levels, we could determine whether reduced CRABP1 protein abundance mediates changes in p27. While a significant decrease in early-phase ERK 1/2 phosphorylation was not observed in cells deficient in *FMR1* or *CRABP1*, a robust decrease in p27 protein levels was found in *FMR1* KD cells and *CRABP1* KD cells compared to control.

Given the canonical role of CRABP1 in buffering cytoplasmic RA concentrations, this thesis is the first to measure CRABP1 expression and its potential influence on early-phase ERK 1/2 signaling and crucial regulators of neuronal differentiation like p27 in both control and FMRP-deficient human cells. This work also aims to examine whether *CRABP1* KD cells phenocopy *FMR1* KD cells, offering a novel insight on whether aberrant CRABP1 or p27 expression may drive abnormalities in early neurogenesis seen in FXS.

The thesis further sought to identify how potential downstream targets of RA signaling may be disrupted in Fragile-X. The results of my thesis can be used for future studies on how these target proteins impact neuronal cell fate in FXS, and potentially other neurological disorders in which dysregulated RA signaling is implicated. Given impairments in early neurogenesis can have profound consequences on learning, memory, and cognitive function seen in individuals with FXS, our results could indicate whether expression of CRABP1 and p27 is regulated by FMRP, and whether altered levels of these proteins may mediate neurogenic defects in human FXS.

Methods

Cell Culture and RNA interference

Human neuroblastoma SH-SY5Y cells were cultured in Gibco Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS), 0.5% penicillin-streptomycin (Pen-Strep), and 1% non-essential amino acid solution. Cells were passed every 4 to 5 days once they reached about a 90% confluency.

siRNA transfections (Dharmacon ON-TARGETplus SMARTpool human *FMR1* siRNA and *CRABP1* siRNA) were performed using RNAiMAX (Invitrogen) and Opti-MEM (Invitrogen) to knockdown *FMR1* and *CRABP1*. To measure CRABP1 levels in response to reduced FMRP, 40 nM of scrambled (SCR) (Dharmacon ON-TARGET*plus* Non-targeting Control) or *FMR1* siRNA were added 24 hours after seeding cells at 200k per well of a 6-well (21000 cells/cm^2) and harvested 72 hours post-transfection. To measure the effects of RA signaling on FMRP and CRABP1-deficient cells, 50 nM of SCR, *FMR1*, or *CRABP1* (Dharmacon ON-TARGETplus SMARTpool human *CRABP1*) siRNA were added 24 hours after

seeding cells at 500k cells per well in a 6-well (52600 cells/cm^2).

Acute Retinoic Acid Stimulation

72 hours post-transfection, control (SCR), *FMR1,* and *CRABP1* KD SH-SY5Y cells were stimulated with 100 nM of RA for 1 hour to determine the effects on RA-mediated early-phase ERK 1/2 signaling. Cell lysates were harvested post-stimulation.

Lysate Preparation and Immunoblotting

SH-SY5Y cells were lysed in chilled RIPA Lysis Buffer with 1x HALT protease and phosphatase inhibitors. Lysates were sonicated for 3 cycles of 5 s on/3 s off at 30% amplitude and then centrifuged at 13500 rpm for 15 minutes at 4ºC. Protein levels were quantified using a BCA assay (Pierce). Protein lysates were resolved on 4%–12% TGX gels and transferred onto 0.44 µm Nitrocellulose membranes. Membranes were incubated with primary antibodies against FMRP, CRABP1, phospho-ERK 1/2, ERK 1/2, and p27 (Cell Signaling) with GAPDH as an endogenous loading control. Blots were visualized using ChemiDoc MP imaging system and band/lane intensities were quantified with Image Studio (LI-COR).

RNA Extraction and Reverse Transcriptase - Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from cells 72 hours post-transfection using TRIzol Reagent (ThermoFisher) and the Quick RNA kit (Zymo Research) with an on-column DNase I digestion step. RT-PCR using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher) was used to obtain cDNA. To quantify relative gene expression, qPCR was performed using Taqman

assays for *FMR1* and *CRABP1* on a Quantstudio 6 Flex system (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was used to quantify relative mRNA levels normalized to average Ct values of the 60S ribosomal protein *RPLP0*.

Results

FMR1 KD SH-SY5Y cells trend towards a reduction in CRABP1 mRNA and protein levels:

To generate an FXS model of human neuroblastoma cells, I used a pool of siRNA to transiently knockdown *FMR1*. I determined a 72-hour knockdown window (n = 2, Two-Way ANOVA, p = 0.0074) (Fig. 3A) produced the most effective and consistent *FMR1* KD compared to control when using a 40 nM siRNA concentration (Fig. 3B). RT-qPCR of *FMR1* mRNA expression showed that a siRNA-mediated knockdown reduced *FMR1* by an average of 67 +/- 19% at the mRNA level (n = 3, Mann-Whitney Test, p>0.05) and an average of 64.8 +/- 0.6% at the protein level (n = 3, Mann-Whitney Test, p>0.05) (Fig. 4A & B).

CRABP1 expression in *FMR1*-deficient SH-SY5Y cells trends towards a reduction at the mRNA level by an average of 40 +/- 20% (n = 3, Mann-Whitney Test, p>0.05) and an average of 5 +/- 10% at the protein level (n = 3, Mann-Whitney Test, p>0.05) (Fig. 4C & D). CRABP1 expression is not as significantly reduced in *FMR1* KD cells as it was in FXS patient postmortem tissue and hiPSC-derived organoids and neurons seen in previous work from the Bassell lab (Fig. 1, Raj et al., unpublished). However, given that FXS patient derived samples completely lack FMRP expression, the low remaining levels of FMRP in *FMR1* KD cells may be responsible for the sustained and variable expression of CRABP1. Interestingly, I did observe that *CRABP1* mRNA was reduced by almost 70% when *FMR1* mRNA expression was reduced to less than 20%. Thus, a threshold level of FMRP reduction may be required to cause a robust decrease in

CRABP1 expression.

Acute RA stimulation of human neuroblastoma cells shows trend of early-phase ERK 1/2 phosphorylation:

To determine whether RA-induced early-phase ERK 1/2 phosphorylation occurs in SH-SY5Y human neuroblastoma cells, I stimulated the cells with RA and harvested lysates for western blotting. Early-phase ERK 1/2 activation occurs between 15 minutes to 1 hour of stimulation, and a previous study conducted a time course of RA-induced ERK 1/2 activation in SH-SY5Y cells using 10 μM of RA (Miloso et al., 2004). While some ERK 1/2 phosphorylation was observed after 1 hour, it was not a significant increase.

To determine the optimal concentration of RA, SH-SY5Y cells were stimulated with either 100 nM or 10 μM of RA for 1 hour with DMSO as the vehicle control. Though both conditions exhibited a trend in increased phosphorylated ERK 1 and ERK 2 levels (Fig. 5A), I found that stimulating cells with 100 nM of RA produced a significant early-phase ERK 1 phosphorylation ($n = 3$, Kruskal-Wallis Test with multiple comparisons, $p = 0.0447$) (Fig. 5A). The Bassell lab has previously shown that RA stimulation (100 nM) for 30 minutes produced a significant increase in ERK 1/2 phosphorylation in control hNPCs (Fig. 2A; Raj et al., unpublished). To determine the optimal timepoint of stimulation in SH-SY5Y cells, I tested both a 30 minute and 1 hour stimulation with 100 nM of RA. I found that 100 nM of RA stimulation resulted in increased ERK 1 and ERK 2 phosphorylation at both 30 minutes and 1 hour. However, given that phosphorylated ERK 1 levels were significantly induced with 1 hour of stimulation with 100 nM of RA ($n = 3$, Holm-Sidak Test, $p = 0.000026$), (Fig. 5B), this condition was chosen as the optimal RA dosage and duration for measuring ERK 1/2 phosphorylation in

FMR1 and *CRABP1* KD SH-SY5Y cells.

Interestingly, cells exhibited different degrees of ERK 1 versus ERK 2 phosphorylation, which is why expression levels of these proteins were compared separately rather than averaged together. The amino acid sequences of ERK 1 and 2 are 84% identical and both have similar protein weights of 44 kDa and 42 kDa, respectively. The ERK 1/2 isoforms are considered functionally redundant despite ERK 2's larger gene sequence and additional gene regulation sites compared to ERK 1 (Buscà et al., 2016). The results of my thesis are consistent with previous findings that ERK 2 is expressed at higher levels than ERK 1 in most mammalian tissues (Buscà et al., 2015), and that the normalized protein abundance of phospho-ERK 1 was on average ~50% of that of phospho-ERK 2's across all samples. However, given no ERK 1/2 isoform-specific agonists have yet been identified (Buscà et al., 2016), further analysis is needed to confirm whether RA induces different degrees of early-phase ERK 1 versus ERK 2 phosphorylation in human neuroblastoma cells.

RA-induced early phase ERK 1/2 activation in FMR1 KD and CRABP1 KD human neuroblastoma cells:

We hypothesized that *FMR1* KD and *CRABP1* KD cells would not respond to RA stimulation. Interestingly, *FMR1* and *CRABP1* KD human neuroblastoma cells showed an increase in early ERK 1/2 phosphorylation (n=3, Kruskal-Wallis Test with multiple comparisons, $p>0.05$) (Fig. 6). Unexpectedly, and in contrast to our earlier results, RA-stimulated non-targeting siRNA treated control cells did not exhibit ERK 1/2 phosphorylation. While it is possible that early-phase ERK 1/2 activation is not disrupted in *FMR1* and *CRABP1*-deficient neuroblastoma cells, the absence of response to RA in control cells suggests some technical

limitations in the experiment. Both temporal variability and the addition of growth factors in media can produce population heterogeneity and cell-to-cell variability in total ERK 1/2 phosphorylation (Filippi et al., 2016).

FMR1 KD and CRABP1 KD in human neuroblastoma cells shows a robust reduction in $p27^{KIP}$:

Based on previous evidence that FXS patient hiPSC-derived NPCs showed deficient early-phase ERK 1/2 phosphorylation (Fig. 2A, Raj et al., unpublished), I aimed to determine whether altered CRABP1 levels affect p27 levels in *FMR1* and *CRABP1* KD human neuroblastoma SH-SY5Y cells. CRABP1-mediated rapid ERK 1/2 activation is shown to lead to the dephosphorylation of p27, resulting in elevated nuclear p27 levels which blocks the G_1 to S phase progression (Persaud et al., 2013). I observed a reduction in p27 protein abundance in *FMR1* KD (n=3, Kruskal-Wallis Test with multiple comparisons, p = 0.0299) and *CRABP1* KD cells (n=3, Kruskal-Wallis Test with multiple comparisons, p = 0.3884) compared to control siRNA treated cells (Fig. 6C). There was no significant difference in p27 protein levels between *FMR1* KD and *CRABP1* KD cells (n=3, Kruskal-Wallis Test with multiple comparisons, p = 0.8656) (Fig. 6C). Due to the function of p27 as a G1-phase CDK-cyclin inhibitor, reduced p27 levels in FMRP and CRABP1-deficient human neuroblastoma cells may lead to increased cellular proliferation seen in FXS hiPSC-derived NPCs and organoids (Raj et al., 2021) and CRABP1 KO stem cells.

Discussion

A critical component of RA signaling, CRABP1, was recently found to be downregulated in FXS patient-derived cell types and postmortem tissue samples across several patients (Raj et

al., unpublished). These findings are particularly significant as no published study has examined CRABP1 levels in FXS patient cells or FXS cellular models. Furthermore, RA signaling plays a key role in promoting early neuronal differentiation and repressing proliferation, and these processes are compromised in FXS. An RNA sequencing study revealed *CRABP1* mRNA is the only downregulated gene besides *FMR1* in FXS patient hiPSC-derived organoids across different stages of neurodevelopment (Fig.1A, Raj et al., unpublished). Furthermore, CRABP1 protein abundance is significantly reduced in FXS patient postmortem tissue and hiPSC-derived neurons (Fig.1B & C, Raj et al., unpublished). The canonical role of CRABP1 is to bind cytoplasmic RA to regulate RA concentration. As CRABP1 mediates non-genomic RA signaling, which may have important consequences for cell cycle regulation and neuronal cell fate, my thesis sought to quantify CRABP1 levels in a human cellular model of FXS, and to determine the effects of reduced CRABP1 expression on RA-induced early-phase ERK 1/2 signaling. The findings of this thesis provide preliminary evidence regarding the role of CRABP1 in neurogenesis and potential for correcting FXS-associated defects in neurogenesis.

One main aim of my thesis was to measure CRABP1 expression as a direct consequence of FMRP reduction. In *FMR1* KD cells, the reduction in CRABP1 protein is less robust and more variable than the reduction at the mRNA level. This could be due to the involvement of CRABP1 in cell-cycle regulation, where changes in transcription and translation across stages of the cell cycle could alter the protein levels observed between cell populations. Moreover, the level of CRABP1 expression may show a dosage-dependent effect based on the degree of FMRP abundance. This idea is further supported at the mRNA level in that CRABP1 mRNA and protein did trend towards a reduction in *FMR1* KD cells, however, the degree of reduction was far less than what was previously observed in FXS patient hiPSC-derived patient cells or

postmortem tissue. Comparing the degree of *FMR1* KD versus *CRABP1* mRNA levels suggests that a threshold depletion of FMRP may be necessary to observe a more consistent and significant reduction in CRABP1 in FXS cell models. Conducting a *FMR1* siRNA dose-response at higher siRNA concentrations would be useful to determine whether both CRABP1 mRNA and protein levels change based on a large FMRP reduction in human neurons and neural progenitor cell types. Flow cytometry analysis could also demonstrate whether CRABP1 expression fluctuates throughout the cell cycle which may contribute to variable CRABP1 protein levels in proliferating *FMR1* KD SH-SY5Y cells.

In considering the role of CRABP1 in RA signaling, there is a lack of consensus on whether CRABP1-RA binding activates or represses cellular RA-dependent signaling pathways inside the cell. CRABP1 overexpression in the suprabasal layer of mouse epidermis enhanced cellular proliferation of epidermal cells upon topical RA treatment (Tang et al., 2008). However, CRABP1 decreases cellular RA levels which suppresses RA sensitivity in head and neck squamous cell carcinoma cells (Won et al., 2004) and RA-induced differentiation in the F9 teratocarcinoma stem cell line (Boylan & Gudas, 1991). Overall, studies support that CRABP1 sequesters RA in the cytoplasm and reduces the availability of RA to mediate transcriptional regulation.

Three studies from the Wei lab point towards a required role of CRABP1 in non-genomic RA signaling that affects cell cycle regulation. *CRABP1* KO ESCs derived from mice reveal CRABP1 mediates non-genomic rapid ERK 1/2 activation which activates PP2A to prolong the G_1 phase (Persaud et al., 2016) by reducing nuclear p27 levels (Persaud et al., 2013, 2016). In *CRABP1* KO NSCs, NSC proliferation in the adult mouse hippocampus is enhanced through RA-induced rapid ERK 1/2 phosphorylation (Lin et al., 2017). This was shown to improve

mouse learning and memory performance (Lin et al., 2017), highlighting how increased neuronal proliferation may have contrasting effects on cognition during early versus adult neurogenesis. In both ESCs and NSCs *in vitro*, CRABP1 increases stem cell proliferation without altering early differentiation (Lin et al., 2017). Another study supported the concept that restoring endogenous CRABP1 expression in esophageal-squamous cell carcinoma cells (ESCC) reduced cell growth through arresting cells in the G_0 to G_1 phase (Tanaka et al., 2007). Importantly, an endogenous KD of CRABP1 decreased p27 protein abundance whereas p21, another CDK-inhibitor which inhibits cellular proliferation (Schwab, 2011), was unaffected (Tanaka et al., 2007).

As FXS hiPSC-derived NPCs exhibit aberrant rapid ERK 1/2 phosphorylation (Fig. 2A; Raj et al., unpublished), there is evidence to suggest reduced CRABP1 may underlie defects in rapid ERK 1/2 phosphorylation observed in FXS patient-derived cells. Thus, this thesis examined whether aberrant CRABP1 expression affects early-phase ERK 1/2 phosphorylation and p27 levels, a CDK Kinase inhibitory protein that particularly binds CDK-cyclin complexes in early G_1 to impede the progression from G_1 to S-phase (Orford & Scadden, 2008). These findings would confirm whether CRABP1 mediates early-phase ERK 1/2 activation upon RA stimulation in human neuroblastoma cells as seen in mouse ESCs and mouse NSCs. Examining p27 levels also provides insight on the potential mechanism between CRABP1-mediated aberrant RA signaling and the observed consequences for cell cycle regulation. Both of these results would indicate whether RA-induced CRABP1 activity acts through p27 as proposed by Nagpal and Wei (2019) to prolong the G_1 phase and promote neuronal differentiation (Fig. 2B). Determining whether CRABP1 may direct neurogenesis in human cells is critical for evaluating aberrant early neurogenesis in FXS patient-derived cells and its therapeutic potential for correcting those defects.

In contrast to previous findings, I found that *FMR1* and *CRABP1* KD cells trended towards an increase, rather than a decrease, in early-phase ERK 1/2 phosphorylation. The addition of non-targeting siRNA may have altered the ERK 1/2 phosphorylation trends of the SCR control cells (Fig. 6A) versus the trends observed in untransfected cells (Fig. 5) due to the change in their extracellular stimuli. Serum-starving cells to synchronize the cell cycles and remove any basal stimulation may help reduce the variability of ERK 1/2 phosphorylation in transfected cells. In addition, stimulating cells with RA for a shorter time-period and adding RA directly to cells versus with new media may also alleviate the extrinsic noise which produces variable ERK 1/2 phosphorylation levels in genetically identical cells (Filippi et al., 2016). Given that this is the first study to examine the role of CRABP1 in RA-mediated signaling in human neuronal cells, more experimental studies will be needed to confirm whether CRABP1 mediates RA-induced early versus late ERK 1/2 signaling in human neuronal cell types.

Importantly, I found a robust decrease in p27 protein abundance in *FMR1* and *CRABP1* KD cells. This finding aligns with two previous studies that found reduced p27 levels in mouse ESCs and esophageal-squamous carcinoma cells (Persaud et al., 2013; Tanaka et al., 2007). This reduction in p27 has important implications for cell cycle regulation in FMRP-deficient cells and the role of CRABP1 in neurogenesis. Preliminary flow cytometry experiments from the Bassell lab reveal that CRABP1 overexpression in control human NPCs increases the number of cells in the G_0/G_1 phase of the cell cycle, while CRABP1 overexpression in FXS patient-NPCs normalized the level of G_0/G_1-phase cells to that of the control (Raj et al., unpublished). The reduction in p27 that I observed occurred due to an acute reduction in FMRP or CRABP1 protein levels within a short time-frame. Thus, deficient p27 levels may be responsible for the shortened G_1 phase and increased cellular proliferation observed in FXS patient NPCs and organoids (Raj

et al., 2021), especially since p27 levels are upregulated during neuronal differentiation (L. Nguyen et al., 2006).

Based on my findings, FMRP, CRABP1, and p27 can be connected together in a pathway that may be an important regulator of brain development (Fig. 7). CRABP1 may positively regulate p27 protein levels in an indirect mechanism, whereas FMRP can either be a direct or indirect positive regulator of CRABP1 and p27 expression (Fig. 7A). Thus, the depletion of CRABP1 would lead to a reduction in p27 levels (Fig. 7B). A depletion of FMRP would lead to a reduction of CRABP1, and a depletion of both FMRP and CRABP1 may lead to an even greater reduction in p27 (Fig. 7C). Future studies would be needed to uncover the mechanism underlying p27 phosphorylation due to the loss of FMRP or CRABP1.

Future Directions

My current work aimed to establish a direct link between the expression of FMRP and CRABP1 in human neural cells. Additionally, I explored a potential functional consequence of altered CRABP1 expression on signaling via the MAPK/ERK pathway. This work was based on recent findings from the Bassell lab that implicated dysregulated RA signaling in FXS. While preliminary findings from the lab suggest that CRABP1 deficiency in human FXS cells may contribute to specific defects in neurogenesis and cell signaling, there are several considerations that we hope to address in future experiments.

Given my finding of reduced p27 levels in *FMR1* and *CRABP1* KD cells, several future directions to analyze the mechanisms underlying neurogenic defects in FXS can be explored. Similar to CRABP1, future studies can measure p27 in FXS hiPSC-derived NPCs to determine if p27 mRNA and protein is reduced in cells derived from FXS patients. Control and FXS hiPSC-

derived organoids can also be used to determine whether changes in p27 and CRABP1 expression are development-stage specific. To explore the mechanism responsible for p27 phosphorylation, measuring dephosphorylated and phosphorylated p27 levels in the presence of activated or deactivated PP2A can also test if PP2A mediates p27 dephosphorylation in human neuronal precursor cells. CRABP1 can also be overexpressed to determine whether CRABP1 overexpression increases p27 levels and subsequently the number of cells in the G_0/G_1 phase as seen in flow cytometry measurements of human control NPCs in the Bassell lab. Lastly, it remains to be explored if sustained RA stimulation affects late-phase ERK 1/2 signaling and whether that may be influenced by CRABP1 in FXS patient cells. This may provide a particularly crucial insight into RA-mediated cell cycle regulation, as sustained ERK 1/2 activation allows the progression of G_1 to S phase (Meloche et al., 1992) and RA leads to a robust late-phase ERK 1/2 activation (Miloso et al., 2004).

As CRABP1 RNA and protein was found to be significantly reduced in cells derived from FXS individuals and this thesis finds that p27 is significantly reduced in a human FXS cell model, the mechanistic link between FMRP, CRABP1, and p27 can be explored. One key future experiment is to determine whether FMRP binds to *CRABP1* and *p27* mRNA. This will determine whether *CRABP1* or *p27* is a direct FMRP target which has not yet been identified from previous cross-linking and immunoprecipitation (CLIP) sequencing studies due to falling below the threshold of detection (Banerjee et al., 2018).

Two potential mechanisms driving reduced CRABP1 and p27 expression in FXS patient samples can also be explored in FXS patient samples. One is the direct or indirect control of CRABP1 and p27 translation which can be evaluated using a BioOrthogonal Non-Canonical Amino acid Tagging (BONCAT) or puromycin labeling assay to measure newly synthesized

peptide levels of CRABP1 and p27. Another mechanism is the regulation of *CRABP1* and *p27* mRNA stability by FMRP that can be assayed through measuring RNA degradation rates of *CRABP1* and *p27* RNA in FMRP-depleted cells. These future experiments would provide a key mechanistic insight on the relationship between FMRP and CRABP1 and their potential interactions with p27.

Figures

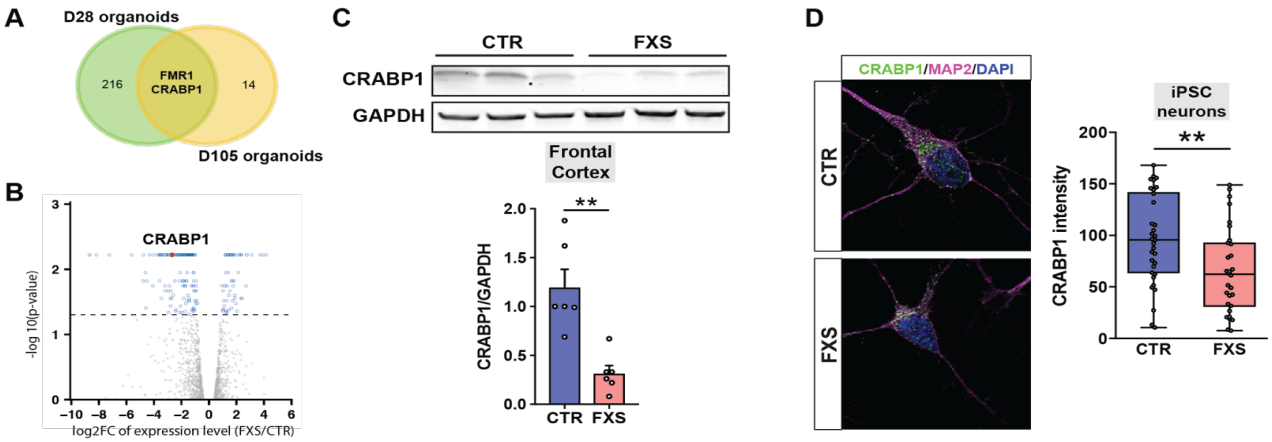


Fig. 1: (A) *FMR1* and *CRABP1* mRNA were downregulated in Day 28 and Day 105 FXS forebrain organoids.
(B) Volcano plot of differentially expressed genes (DEGs) in Day 28 forebrain FXS organoids versus control. *CRABP1* is highlighted in red.
(C) Representative western blots for CRABP1 and GAPDH protein abundance and densitometry analysis shows a significant reduction in CRABP1 in FXS patient postmortem tissue (Mann-Whitney test, **p<0.01, n=3 CTR, 3 FXS).
(D) Representative images of DIV12 control and FXS patient hiPSC-derived neurons stained for CRABP1 (green). Quantitative immunofluorescence shows significant CRABP1 reduction in FXS patient iPSC-derived neurons (Mann-Whitney test, n = 2 CTR-FXS isogenic pairs).
(Figure 1 provided by Nisha Raj).

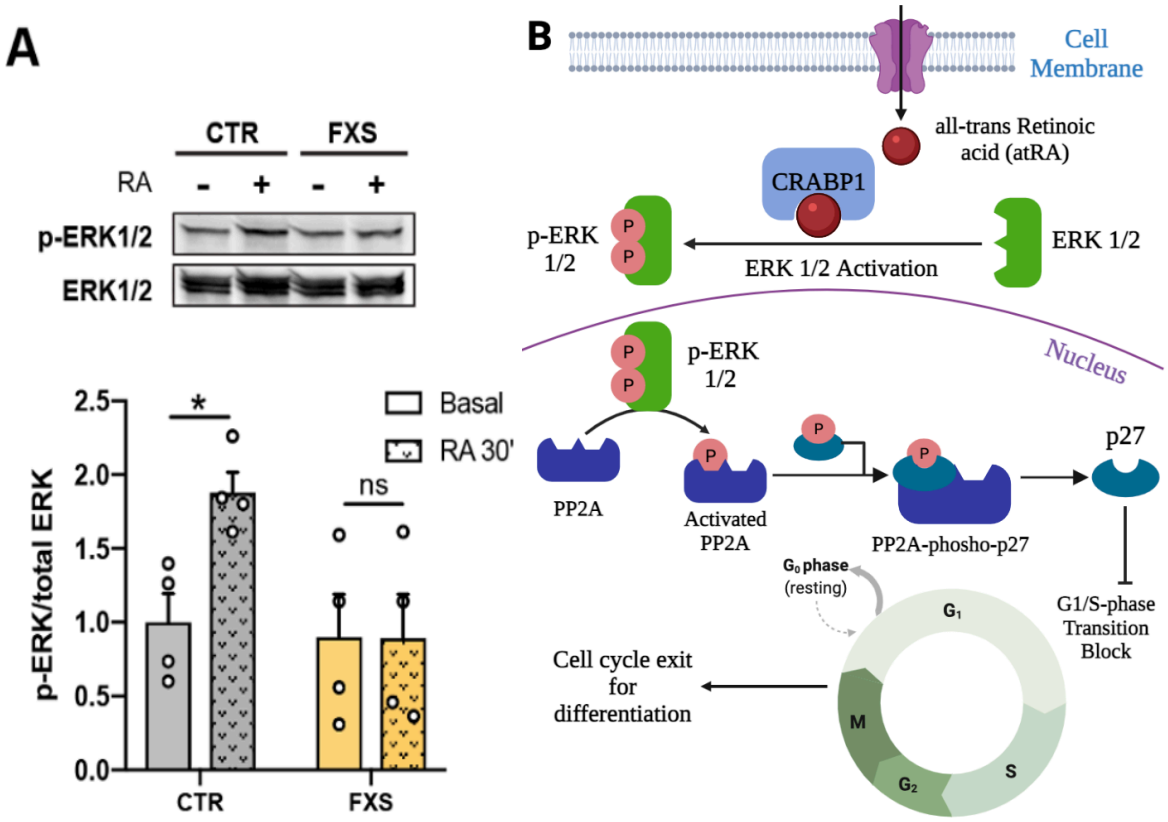


Fig. 2: (A) Early-phase ERK 1/2 activation in response to retinoic acid stimulation was lost in FXS patient hiPSC-derived neural progenitor cells (NPCs) versus control. Representative western blots for phosphor-ERK 1/2 (p-ERK 1/2) and ERK 1/2 protein and densitometry analysis show a significant reduction in p-ERK 1/2 in FXS patient hiPSC-derived NPCs (Mann-Whitney test, *$p<0.05$, n=3 CTR, 3 FXS) (Figure provided by Nisha Raj).
(B) Schematic of atRA-CRABP1 mediated early-phase ERK 1/2 activation which is hypothesized to induce dephosphorylation of p27 by protein phosphatase 2A (PP2A). Adapted from Nagpal & Wei, 2019

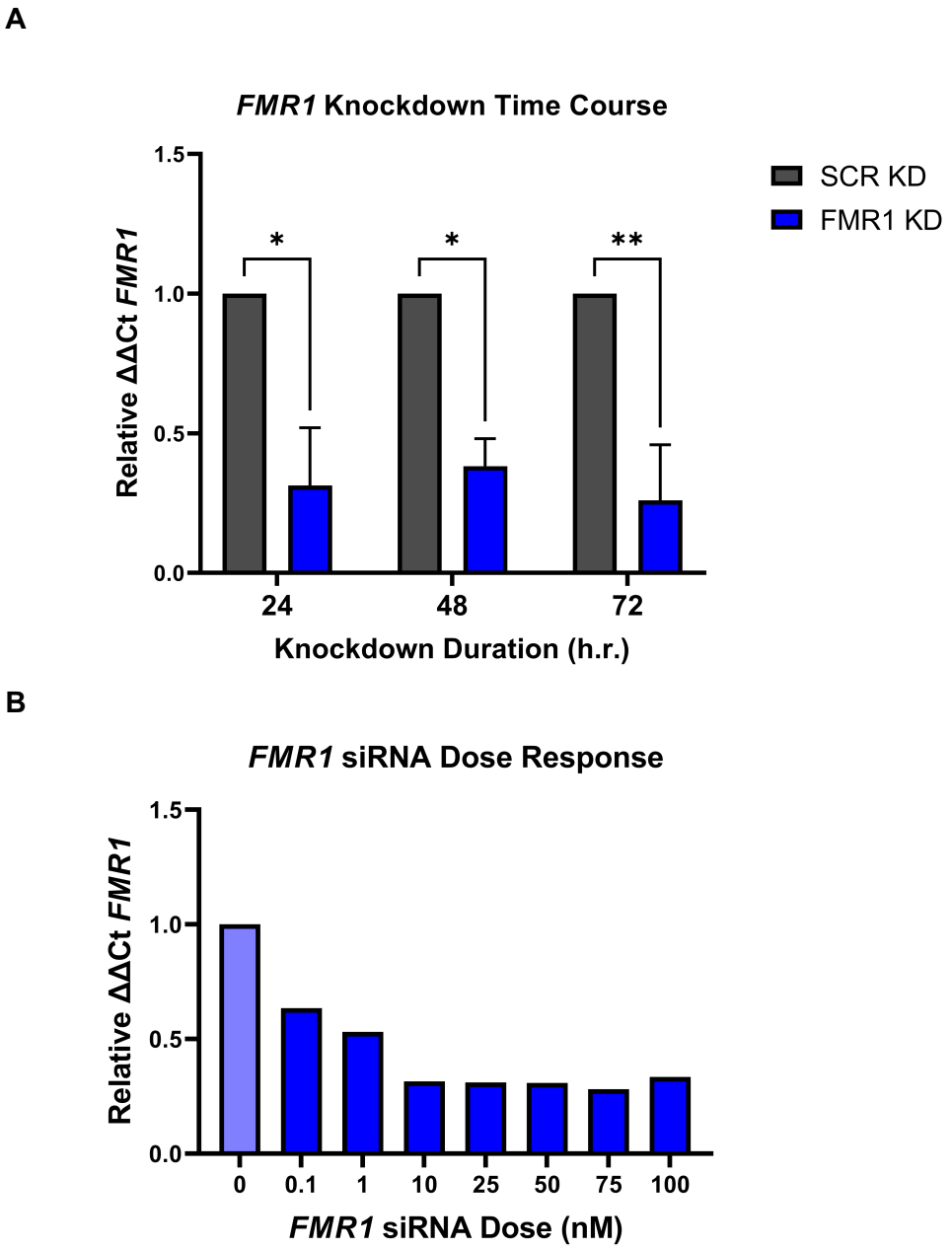


Fig. 3: (A) Human neuroblastoma SH-SY5Y cells were transfected with 40nM of scrambled (SCR) or *FMR1* siRNA (*FMR1 KD*) and harvested 24, 48, or 72 hours later. Relative mRNA expression of *FMR1* were measured using RT-qPCR normalized to ribosomal *RPLP0* (Two-Way ANOVA, *$p < 0.05$, n = 2 CTR, 2 *FMR1* KD SH-SY5Y).
(B) *FMR1* siRNA dose-response was conducted on SH-SY5Y cells. Relative mRNA expression of *FMR1* and were measured using RT-qPCR normalized to ribosomal *RPLP0* (n = 1 CTR, 1 *FMR1* KD SH-SY5Y).

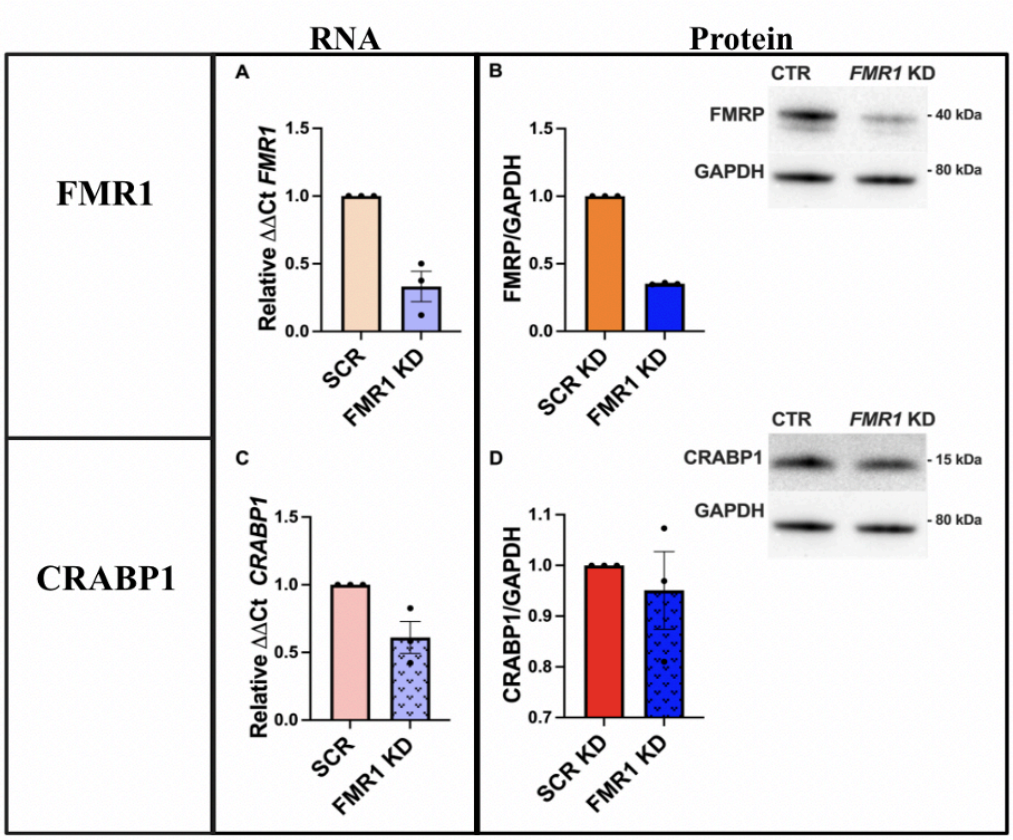


Fig. 4: Human neuroblastoma SH-SY5Y cells were transfected with 40nM of scrambled (SCR) or *FMR1* siRNA (*FMR1 KD*) and harvested 72 hours later. Relative mRNA expression of *FMR1* **(A)** and *CRABP1* **(C)** were measured using RT-qPCR normalized to ribosomal *RPLP0* (Mann-Whitney test, *p < 0.05, n = 3 CTR, 3 *FMR1* KD SH-SY5Y). Representative immunoblots and densitometry analyses for FMRP **(B)** and CRABP1 (**D)** were normalized to GAPDH (Mann-Whitney test, *p < 0.05, n = 3 CTR, 3 *FMR1* KD SH-SY5Y).

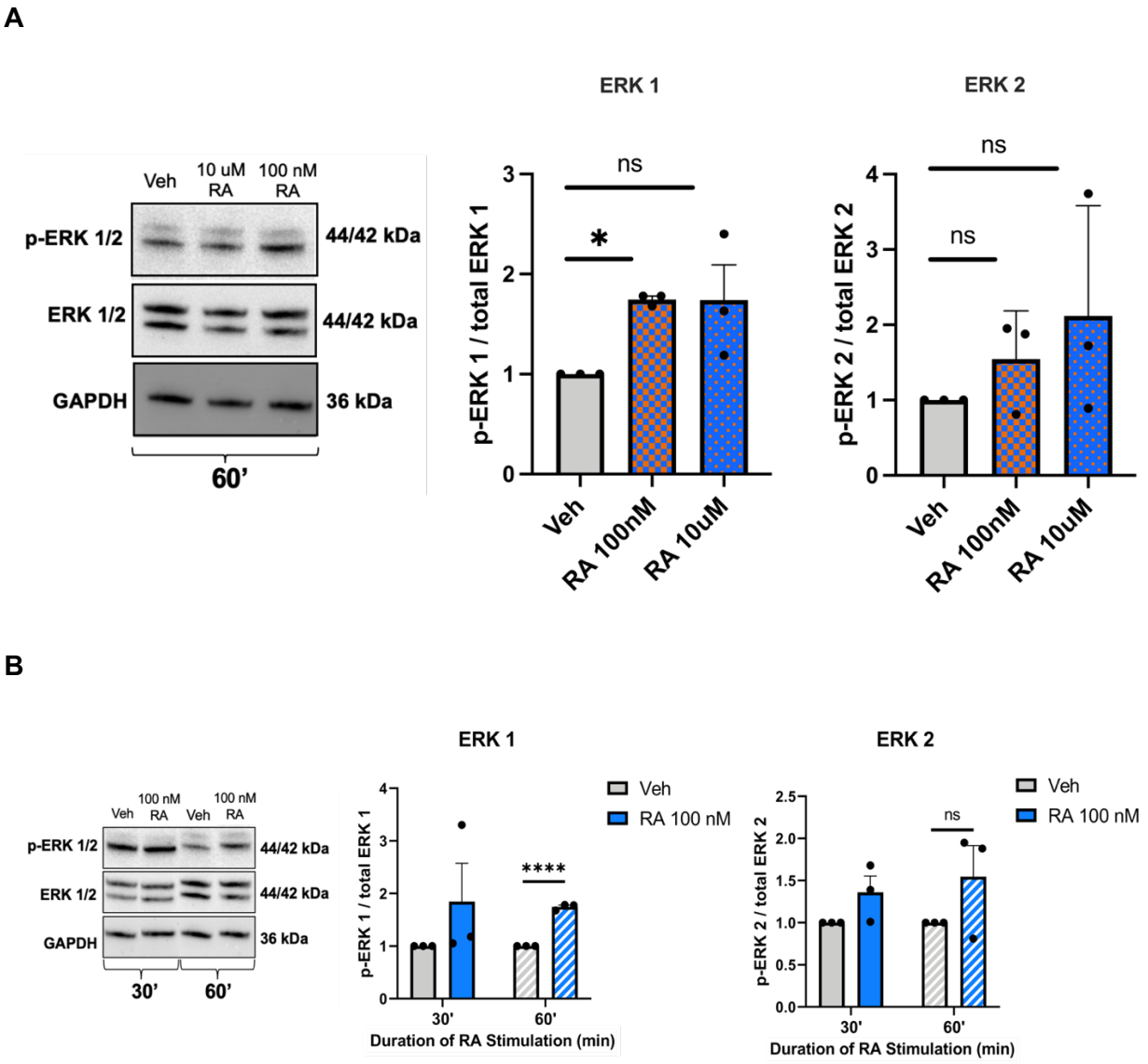
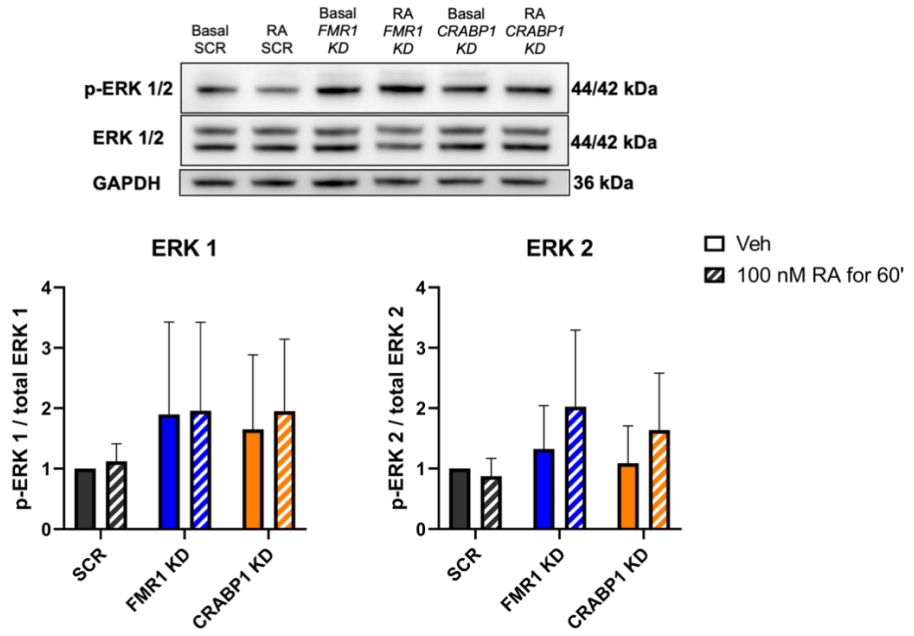


Fig. 5: (A) Human neuroblastoma SH-SY5Y cells were stimulated with 100 nM or 10 µM of retinoic acid (RA) for 1 hour. Representative immunoblot and densitometry analyses for phospho-ERK 1 (p-ERK 1, top band) and phospho-ERK 2 (p-ERK 2, bottom band) were normalized to ERK 1 (44 kDa) and 2 (42 kDa) levels, respectively. GAPDH was used as a loading control. (Kruskal-Wallis with multiple comparisons, *$p < 0.05$, n = 3 Vehicle, 3 p-ERK 1, 3 p-ERK 2).
(B) Human neuroblastoma SH-SY5Y cells were stimulated with 100 nM of retinoic acid (RA) for 30 minutes or 1 hour. Representative immunoblot and densitometry analyses for p-ERK 1 (top band) and p-ERK 2 (bottom band) were normalized to ERK 1 (44 kDa) and 2 (42 kDa) levels, respectively. GAPDH was used as a loading control. (Kruskal-Wallis with multiple comparisons, *$p < 0.05$, n = 3 Vehicle, 3 p-ERK 1, 3 p-ERK 2).

A



B

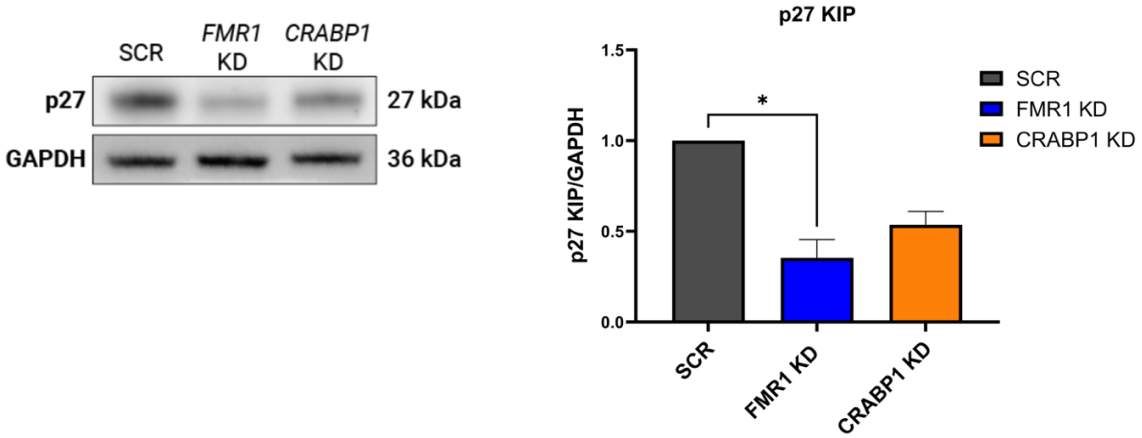


Fig. 6: Human neuroblastoma SH-SY5Y cells were transfected with 50nM of scrambled (SCR), *FMR1* siRNA (*FMR1* KD), or *CRABP1* siRNA (*CRABP1* KD) and harvested 72 hours later. **(A)** Representative immunoblot and densitometry analyses for phospho-ERK 1 (p-ERK 1, top band) and phospho-ERK 2 (p-ERK 2, bottom band) were normalized to ERK 1 (44 kDa) and 2 (42 kDa) levels, respectively, with GAPDH as a loading control (Kruskal-Wallis with multiple comparisons, *p < 0.05, n = 3 CTR, 3 *FMR1* KD SH-SY5Y, 3 *CRABP1* KD SH-SY5Y). **(B)** Representative immunoblot and densitometry analysis for p27 were normalized to GAPDH (Kruskal-Wallis with multiple comparisons, *p < 0.05, n = 3 CTR, 3 *FMR1* KD SH-SY5Y, 3 *CRABP1* KD SH-SY5Y).

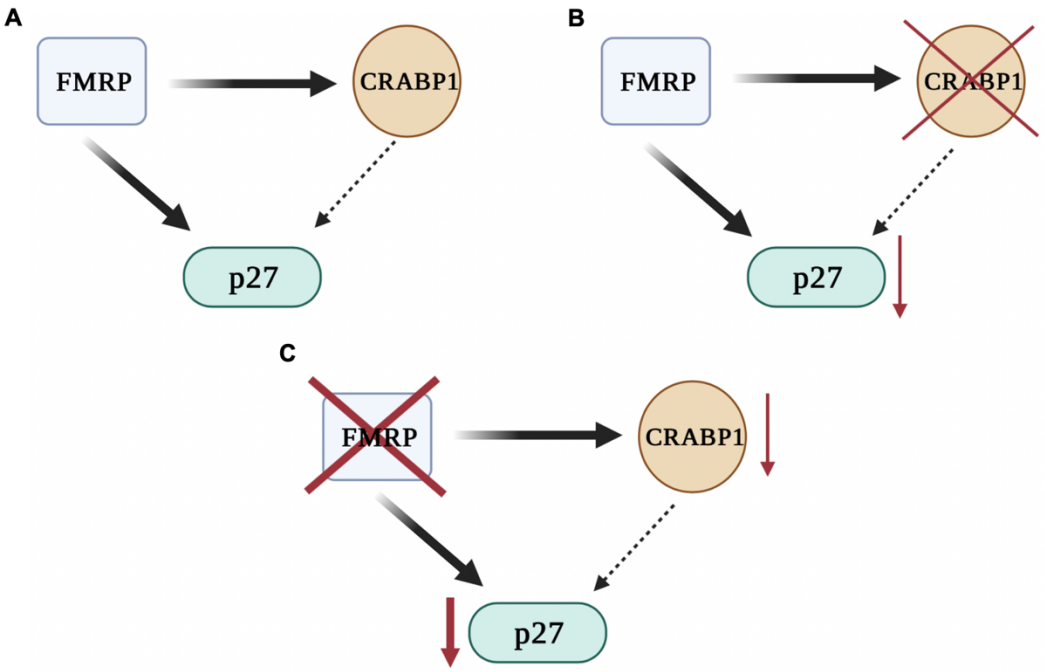


Fig. 7: Hypotheses on the connection between FMRP, CRABP1, and p27.
(A) FMRP may directly or indirectly positively regulate CRABP1 and p27 expression (solid arrow). CRABP1 likely indirectly regulates protein levels of dephosphorylated p27 (dashed arrow).
(B) If CRABP1 is depleted, then p27 protein levels may decreaase.
(C) If FMRP is depleted, then CRABP1 expression decreases. The combined effect of a decrease in FMRP and CRABP1 protein levels may lead to a more robust reduction in p27.

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