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Nisha Raj Date

Cell-type-specific profiling of defects in translation and neurogenesis in a human induced pluripotent stem cell model of Fragile X syndrome

By

Nisha Raj
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Neuroscience

_____
Gary J. Bassell, Ph.D.
Advisor

_____
Anthony W. Chan, D.V.M, Ph.D.
Committee Member

_____
Victor Faundez, M.D., Ph.D.
Committee Member

_____
Shannon L. Gourley, Ph.D.
Committee Member

_____
Stephen T. Warren, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Cell-type-specific profiling of defects in translation and neurogenesis in a human induced pluripotent stem cell model of Fragile X syndrome

By

Nisha Raj
B.A., Boston University, 2010

Advisor: Gary J. Bassell, Ph.D.

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Abstract

Cell-type-specific profiling of defects in translation and neurogenesis in a human induced pluripotent stem cell model of Fragile X syndrome

By Nisha Raj

Aberrant translation and disrupted signaling are molecular defects common to several neurodevelopmental disorders, including Fragile X syndrome (FXS), the most common monogenic cause of autism and inherited intellectual disability. Studies in animal models of FXS have shown that in the absence of the RNA-binding protein FMRP, there is an increase in global translation and dysregulation of key signaling pathways. However, the molecular pathogenesis of FXS in humans has remained understudied. Here we use induced pluripotent stem cell (iPSCs)-derived neural progenitor cells (NPCs) and differentiating neurons from multiple control and FXS patients to characterize molecular defects in a human disease-relevant model. We found that a subset of FXS patient derived NPCs show increased protein synthesis, increased proliferation and altered differentiation profiles. FXS patient derived cerebral organoids also showed an increase in KI67+/SOX2+ proliferating cells. Furthermore, genome-wide splicing analysis of patient cerebral organoids revealed alternative splicing of a key cell-cycle regulated kinase in FXS. We developed a multi-parametric flow cytometry based assay, NeuroMIP, to quantify protein synthesis and proliferation within specific neural subpopulations in our patient cells. Our results suggest that increased proliferation is a core molecular phenotype in FXS, and defects in protein synthesis may contribute to altered proliferation. The loss of FMRP also affects the differentiation profile and timing of neural progenitor cell subtypes during development, and we see increased protein synthesis in specific neural subtypes in FXS patient cells. We anticipate that this study using a human disease-relevant cellular model of FXS will provide new insight into molecular and cellular phenotypes in FXS, as well as the future development of therapeutic strategies. Our results provide strong support for an approach that involves patient stratification based on cellular and molecular phenotypes, enabling pre-clinical testing of treatments in a more tailored and precise manner.

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CHAPTER 1: General Introduction

1.1. Fragile X syndrome

It has widely been accepted that developmental disabilities such as autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), and intellectual disability appear to be more common in males compared to females (Boyle et al., 2011). However, the first report of a possible sex-linked inheritance pattern of intellectual disability came from seminal work by Julia Bell and James Purdon Martin, in which they described a family with eleven males across two generations that had severe cognitive impairments (Martin and Bell, 1943). Initially known as Martin-Bell syndrome, this disorder was later named Fragile X syndrome (FXS), after several groups identified a "fragile" or constricted site in the long arm of the X chromosome of affected patients (Harrison et al., 1983; Lubs et al., 1984; Lubs, 1969; Richards et al., 1981; Sutherland, 1977). Interestingly, this fragile site was found to be induced in cells *in vitro*, when grown under low folic acid and thymidine conditions (Sutherland, 1977).

Molecular mapping of the fragile site on the X chromosome eventually led to the identification of the *FMR1* gene (Verkerk et al., 1991), which was found to have an unstable microsatellite region containing a region of CGG repeats (Yu et al., 1991) as well as a methylated CpG island (Heitz et al., 1991). Importantly, it was found that *FMR1* was expressed in healthy individuals, but not in patients with FXS (Pieretti et al., 1991), suggesting that the disorder was a direct consequence of the loss of function of *FMR1*.

1.1.1. The Fragile X phenotype

Fragile X syndrome is the leading monogenic cause of autism and inherited intellectual disability. While early studies estimated its prevalence to be approximately 1 in 4000 males and 1 in 8000 females (Crawford et al., 2001), a more recent meta-analysis determined that FXS

affects approximately 1 in 7,000 males and 1 in 11,000 females (Hunter et al., 2014). FXS is associated with a wide range of physical, neurological, cognitive and behavioral symptoms. Since it is an X-linked disorder, affected males typically have more severe phenotypes than females. The most prominent physical feature in males with FXS is macroorchidism, or enlargement of the testes (Bowen et al., 1978; Sutherland and Ashforth, 1979). A large subset of individuals with FXS share certain dysmorphic facial features that include an enlarged forehead, a long face, a prominent jaw, and large ears (Heulens et al., 2013; Turner et al., 1980). Some patients also have connective tissue abnormalities that manifest as hyperextensible joints, flat feet and soft skin (Hagerman et al., 1984; Kidd et al., 2014; Waldstein et al., 1987).

Patients with the methylated, full mutation have mild to severe cognitive impairment (Kemper et al., 1988), and may have comorbid diagnoses including sensory hyper-reactivity, ASD, ADHD, anxiety or epilepsy (Bailey et al., 2008; Harris et al., 2008; Kaufmann et al., 2004; Kaufmann et al., 2017; Kidd et al., 2014; Musumeci et al., 1999). Autism-like impairments are frequently seen in individuals with FXS, including eye-gaze avoidance, social anxiety, self-injurious activity and other stereotyped behaviors such as hand-flapping and perseverative speech (Hernandez et al., 2009). A recent study found that approximately 50% of males with FXS met the DSM-5 criteria for ASD and this group of FXS males with ASD had a higher prevalence of seizures and other behavioral abnormalities (Kaufmann et al., 2017).

Postmortem analyses of human patients provided some of the earliest insight into the cellular and molecular phenotype in FXS. In 1985, the first neuropathological findings were reported when Golgi staining revealed dendritic spine abnormalities in a 62 year old male FXS patient with moderate cognitive impairment (Rudelli et al., 1985). It was observed that while normal dendritic spines have a short, stubby mushroom-like appearance, dendritic spines in the

FXS brain appeared to be long, thin and tortuous (Rudelli et al., 1985). Electron microcopy showed that the synaptic length or mean synaptic contact area was reduced in FXS compared to controls (Rudelli et al., 1985). Similar results were obtained when two more FXS patients were included in a subsequent analysis (Hinton et al., 1991), and FXS patients also appear to have an increased density of these immature dendritic spines. No other significant neuropathology or differences in total neuronal count were seen (Hinton et al., 1991; Reyniers et al., 1999), suggesting that the abnormal spine morphology and defects in pruning and synaptic maturation may underlie the cognitive deficits in FXS (Irwin et al., 2000; Irwin et al., 2001).

1.1.2. FMR1

Although Fragile X syndrome was first clinically described in 1943, it was only in 1991 that a team of scientists including David Nelson, Ben Oostra, and Stephen Warren identified the casual mutation to be an expansion of a trinucleotide repeat region within the *FMR1* gene (Verkerk et al., 1991). *FMR1* spans ~39 kilobases and is located at chromosome Xq27.3 (Harrison et al., 1983). The 5'UTR of *FMR1* contains the polymorphic trinucleotide $(CGG)_n$ repeat that is known to be expanded in the disease condition. The number of CGG repeats ranges from 6-54 repeats in typically developing individuals, and an intermediate repeat length of 55-200 is considered to be within the premutation range (Fu et al., 1991). The premutation allele in males is associated with an increased risk for a neurodegenerative disorder called Fragile X-associated tremor/ataxia syndrome (FXTAS) (Hagerman et al., 2001; Jacquemont et al., 2004; Jacquemont et al., 2003), while females have a higher risk of developing primary ovarian insufficiency (FXPOI) (Cronister et al., 1991; Sullivan et al., 2011).

The expansion of the CGG tract to over 200 repeats is considered the full mutation (Fu et al., 1991), and typically leads to the hypermethylation of the CpG island in the promoter and transcriptional silencing of the *FMR1* gene (Hansen et al., 1992; Pieretti et al., 1991), which results in the loss of the protein product, FMRP. The CGG repeat within *FMR1* is prone to instability; this is illustrated by the unique inheritance pattern described as the "Sherman paradox," wherein unstable premutation alleles tend to expand during oogenesis, and the propensity to expand into a full mutation is correlated with maternal repeat length (Fu et al., 1991). The instability of the CGG tract can also manifest as mosaicism across cell types in both repeat length (Loesch et al., 2004) and methylation status (Genc et al., 2000). Interestingly, there have been reports of individuals with unmethylated full mutations who appear to have typical expression of *FMR1*, and no cognitive impairments (Pietrobono et al., 2005; Smeets et al., 1995).

While a majority of FXS patients express the full mutation, several disease-causing variants in *FMR1* have been identified that result in a fragile X phenotype. The first of these to be discovered was a missense mutation in the coding sequence that resulted in severe intellectual disability, increased seizures and other FXS symptoms (De Boulle et al., 1993). Although it did not lead to loss of expression of *FMR1*, this I304N variant was found to affect the RNA-binding properties of the protein product of *FMR1* (Feng et al., 1997a; Schrier et al., 2004; Siomi et al., 1994). Shortly thereafter, two patients were identified with intragenic mutations that resulted in transcriptional silencing (Lugenbeel et al., 1995), in addition to classic clinical symptoms of FXS. Several other intragenic mutations in *FMR1* have since been described (Collins et al., 2010; Gronskov et al., 2011; Myrick et al., 2014; Quartier et al., 2017; Wang et al., 1997); however, since these patients represent a small population of individuals with FXS, more functional and thorough analyses are required to fully characterize these mutations. Thus, despite

the existence of multiple mutagenic mechanisms, the disrupted expression of *FMR1* appears to play a fundamental role in the etiology of disorder.

1.1.3. Fragile X mental retardation protein (FMRP)

The *FMR1* gene encodes for the fragile X mental retardation protein (FMRP), which is broadly expressed in several tissue types, although its expression levels are highest in the brain, placenta and testes (Devys et al., 1993; Hinds et al., 1993). The expansion of the CGG repeat tract to over 200 repeats in *FMR1* is a loss of function mutation, and patients with the full mutation do not express FMRP. Given the dramatic cognitive phenotype in these individuals, it is evident that FMRP plays a key role in learning, memory and synaptic plasticity.

Although primarily cytoplasmic, FMRP has a nuclear localization signal (NLS) and a nuclear export signal (NES), suggesting that it shuttles between these two compartments, and may play a role in nucleocytoplasmic transport of mRNA (Eberhart et al., 1996; Sittler et al., 1996). FMRP is known to associate with microtubule motor proteins, and transport mRNAs in an activity dependent manner (Antar et al., 2005; Dictenberg et al., 2008; Ferrari et al., 2007) and may also play a role in regulating mRNA stability by modulating mRNA decay (De Rubeis and Bagni, 2010; Miyashiro et al., 2003; Zalfa et al., 2007).

Importantly, there are three validated RNA-binding domains in FMRP – two K-homology domains (KH1 and KH2), and an RGG box that binds to RNA (Ashley et al., 1993; Siomi et al., 1993). Recently, a novel KH domain (KH0) was discovered in the amino terminus of FMRP that may also be capable of binding mRNA (Myrick et al., 2015). FMRP recognizes several motifs on its targets mRNAs, including G-quadruplex structures (Darnell et al., 2001; Schaeffer et al., 2001), “kissing complexes” (Darnell et al., 2005), and specific short sequence

motifs (Anderson et al., 2016; Ascano et al., 2012; Ray et al., 2013; Suhl et al., 2014). FMRP was found to associate with ribonucleoprotein (RNP) particles and actively translating polyribosomes (Corbin et al., 1997; Feng et al., 1997b; Khandjian et al., 1996; Siomi et al., 1996; Tamanini et al., 1996), suggesting that it may be involved in the control of protein synthesis. It was shown to bind approximately 4% of mRNA in the brain (Ashley et al., 1993), and several of its mRNA targets encoded for proteins that were enriched in the pre- and post-synaptic proteome (Darnell et al., 2011). Given that FMRP is heavily enriched in dendrites and in dendritic spines (Antar et al., 2004), and that defects in spine morphology are a neuropathological hallmark in FXS, it was further hypothesized that FMRP may play a role in regulating local translation of proteins required for the maintenance of synaptic structure and function (Antar and Bassell, 2003; Bagni and Greenough, 2005; Grossman et al., 2006a). Indeed, FMRP was shown to typically function as a repressor of translation, and the loss of FMRP led to excess translation of its target mRNAs (Hou et al., 2006; Laggerbauer et al., 2001; Li et al., 2001; Muddashetty et al., 2007; Narayanan et al., 2007; Zalfa et al., 2003).

1.2. Animal models of FXS

The first model of Fragile X syndrome was the *Fmr1* knockout mouse model, which was generated by disrupting exon 5, resulting in the loss of FMRP (Bakker, 1994). However, since the *Fmr1* gene is not silenced by hypermethylation as in the human condition, and there was some residual *Fmr1* transcription from the intact *Fmr1* promoter. To address this, a second knockout mouse was generated that lacked the first exon including the promoter, completely shutting down transcription of *Fmr1* as well as translation of FMRP (Mientjes et al., 2006). The mouse model has been an invaluable resource to understand the biology of FMRP and its role in

disease, as these mice recapitulate many phenotypes observed in human patients, including macroorchidism (Mientjes et al., 2006; Slegtenhorst-Eegdeman et al., 1998). Shortly after the mouse model, a fruit fly model of FXS was developed (Wan et al., 2000), followed by a zebrafish model (den Broeder et al., 2009; Tucker et al., 2006). Most recently, an *Fmr1* KO rat model was generated (Hamilton et al., 2014) and observed phenotypes remained consistent with reports from other animal models and humans with FXS (Tian et al., 2017; Till et al., 2015).

1.2.1. Insight from the FXS drosophila model

The drosophila homolog dFMRP is highly conserved to the human FMRP and has been shown to be enriched in neurons and has similar RNA-binding properties as seen in the human and mouse (Morales et al., 2002; Wan et al., 2000; Zhang et al., 2001). Loss of function of the *dFmr1* gene results in defects in neuronal morphology and synapse formation similar to those seen in the mouse and human condition (Michel et al., 2004; Morales et al., 2002; Pan et al., 2004). The *dFmr1* mutant flies also displayed impaired social interaction and cognition as measured by courtship behavior and olfactory fear conditioning paradigms (Bolduc et al., 2010; Dockendorff et al., 2002; Kanellopoulos et al., 2012). Abnormal circadian and sleep behavior have also been reported in the *dFmr1* mutant flies (Bushey et al., 2009; Dockendorff et al., 2002) that is consistent with clinical reports of sleep disturbances in human FXS patients (Berry-Kravis, 2014; Gould et al., 2000).

1.2.2. Insight from the Fmr1-knockout mouse

The behavioral phenotype in the knockout mice appears to be more subtle than in male human patients, and also varies greatly depending on the genetic background and the testing

conditions. Given that increased anxiety, hyperactivity and autism-like behaviors are common in the FXS human patient, *Fmr1* KO mice have been thoroughly evaluated for these abnormalities by several groups. There have been opposing reports of anxiety-like behaviors in the *Fmr1* KO mice – some groups see increased activity in assays such as the open-field test and the elevated plus maze, which would be indicative of reduced anxiety (Peier et al., 2000; Veeraragavan et al., 2012; Yuskaitis et al., 2010), while others see increased anxiety-like behaviors (Bilousova et al., 2009; Restivo et al., 2005). Sensorimotor processing deficits have also been shown in the knockout mouse, with reports of increased prepulse inhibition (PPI) and reduced startle response to low intensity auditory stimuli (Ding et al., 2014; Paylor et al., 2008; Spencer et al., 2011); however, divergent findings showing impairments or no difference in PPI and an enhanced startle response have also been reported (de Vrij et al., 2008; Nielsen et al., 2002). One of the more robust phenotypes that has been seen in the *Fmr1* KO mouse is an increased susceptibility to audiogenic seizures, which is consistent with the human symptoms of increased epileptic seizures and hypersensitivity to sensory stimuli (Chen and Toth, 2001; Gross et al., 2015c; Sawicka et al., 2016). The *Fmr1* knockout mice also exhibit deficits in social communication and interaction, which are common autism-like behaviors seen in individuals with FXS. These mice display impairments in social novelty discrimination tasks and novel object recognition tasks (Bhattacharya et al., 2012; Mines et al., 2010; Ventura et al., 2004). Studies have used patterns of ultrasonic vocalizations (USV) as a readout for communication deficits – one study reported a reduced rate of USV in male *Fmr1* KO mice paired with a female (Rotschafer et al., 2012), while another showed an altered pattern and duration of USV from young *Fmr1* KO pups following maternal separation (Roy et al., 2012).

Fmr1 KO mice have been shown to have various deficits in learning, memory and cognition, although once again, there have been conflicting reports. While most groups do not see significant impairments in the initial acquisition of a spatial learning and memory task such as the Morris water-maze, *Fmr1* KO mice appear to have difficulty with reversal learning (Baker et al., 2010; D'Hooge et al., 1997). There is evidence of hippocampal and amygdalar dysfunction in both FXS patients and in the knockout mice, although results from cued and contextual fear conditioning studies in mice are inconsistent (Hayashi et al., 2007; Olmos-Serrano et al., 2011; Paradee et al., 1999; Van Dam et al., 2000). FXS mouse models also exhibit specific cognitive deficits in prefrontal cortex dependent behavioral flexibility and executive functioning (Dolen et al., 2007; Gross et al., 2015c; Krueger et al., 2011).

Studies in the *Fmr1* KO mice have also further illustrated the role of FMRP as a negative regulator of translation, and several groups have shown excess protein synthesis and dysregulated signaling through multiple pathways in the FXS mouse (Dolen et al., 2007; Gross et al., 2010; Osterweil et al., 2010; Richter et al., 2015). The dendritic spine defects seen in postmortem FXS brains have also been reported in the *Fmr1* mouse model, although the results vary depending on brain region and the age of the mice. Studies have described an immature dendritic spine phenotype in hippocampal and cortical neurons of *Fmr1* KO mice mouse (Bilousova et al., 2009; Cruz-Martin et al., 2010; Galvez and Greenough, 2005; Grossman et al., 2006b; Levenga et al., 2011; McKinney et al., 2005; Nimchinsky et al., 2001; Pop et al., 2014). Some of these studies have also shown a higher density of these immature spines but these findings are less reproducible across groups (Galvez and Greenough, 2005; McKinney et al., 2005). Dendritic spine abnormalities may underlie cognitive impairments by altering synaptic strength and function, giving rise to changes in long term potentiation (LTP) and long-term

depression (LTD), which are electrophysiological correlates of learning and memory (Malenka and Bear, 2004; Sala and Segal, 2014). *Fmr1* KO mice appear to have impaired LTP and synaptic plasticity (Li et al., 2002; Nosyreva and Huber, 2005; Zhao et al., 2005) and show an increase in a form of LTD that requires the activation of metabotropic glutamate receptors (mGluR) (Huber et al., 2002; Koekkoek et al., 2003; Nakamoto et al., 2007; Nosyreva and Huber, 2005; Sidorov et al., 2013). The identification of this defect in mGluR-dependent plasticity resulted in the proposal of the "mGluR theory" of fragile X syndrome, which formed the basis of several important preclinical and clinical studies in FXS (Bear et al., 2004).

1.3. Preclinical rationale for developing targeted therapies interventions in FXS

Animal models have provided valuable insight into the normal cellular and molecular functions of FMRP, in particular, by illustrating the consequences of its absence. Its role as an RNA-binding protein that regulates protein synthesis is conserved across species, as is the dysregulation in key signaling pathways that arises from the loss of FMRP, suggesting that translational control by FMRP is critical for maintaining an overall balance in neuronal signaling. Thus, therapeutic interventions have largely been directed at correcting these defects in protein synthesis and signaling, and successful results using these approaches have led to the development of several clinical trials in FXS (Berry-Kravis et al., 2017; Berry-Kravis et al., 2011; Berry-Kravis et al., 2018; Erickson et al., 2017; Ligsay et al., 2017).

1.3.1. mGluR theory of fragile X syndrome

Glutamatergic signaling via group I metabotropic glutamate receptors (mGluRs) mediates neuronal function and synaptic plasticity (Balschun and Wetzel, 2002; Cohen and Abraham,

1996; Mannaioni et al., 2001). mGluR activation results in an irreversible form of long term depression (LTD) that requires the rapid translation of mRNA at postsynaptic site on dendrites (Huber et al., 2000; Merlin et al., 1998). As FMRP was found to be one of the proteins synthesized at synapses in response to mGluR activation (Weiler et al., 1997), and since synaptic dysfunction is a key phenotype in FXS, a logical next step was the investigation of the role of FMRP in this form of protein-synthesis-dependent mGluR-LTD. It was expected that the loss of FMRP would result in impaired LTD; however, mGluR-LTD was found to be increased in the *Fmr1* KO mice (Huber et al., 2002), and appeared to be protein-synthesis-independent (Hou et al., 2006; Nosyreva and Huber, 2006). To account for this unexpected result, it was suggested that mGluR activation triggers the synthesis of a subset of synaptic proteins, including FMRP, which typically acts as a brake on the translation of these proteins. In the absence of FMRP, this brake is removed, and there is overactive synthesis of these proteins.

The functional consequences of overactive mGluR-LTD significantly overlapped with key phenotypes seen in FXS (Bear et al., 2004), and these findings led to the proposal of the "mGluR theory of Fragile X syndrome," which postulates that exaggerated mGluR-LTD may contribute to the synaptic dysfunction and cognitive impairment in FXS, and that interventions aimed at mitigating this overactive signaling via mGluRs may have therapeutic benefits (Bear et al., 2004). Several groups have subsequently shown that mGluR-mediated protein synthesis is increased and stimulus-independent in FXS, and that inhibition of mGluR signaling corrects multiple phenotypes in animal models of FXS (Auerbach and Bear, 2010; Dolen et al., 2007; Hays et al., 2011; McBride et al., 2005; Muddashetty et al., 2007; Osterweil et al., 2010; Ronesi and Huber, 2008; Yan et al., 2005).

1.3.2. Altered signaling via membrane receptors in FXS

The seminal work investigating the role of mGluRs in FXS was subsequently expanded upon, and it was found that signaling via other cell-surface receptors was also dysregulated in FXS. Similar to mGluRs, activation of Gq-coupled M_1 muscarinic acetylcholine receptors (mAChRs) was found to induce LTD in a protein-synthesis-dependent manner, and *Fmr1* KO mice were shown to have increased mAChR-dependent protein synthesis and mAChR-LTD (Volk et al., 2007). Likewise, D1 dopamine receptor signaling and tyrosine kinase receptor B (TrkB) signaling activate protein synthesis, and induce AMPA receptor endocytosis and LTD in a translation-dependent manner, and transduction mediated by these receptors was reported to be altered in FXS (Osterweil et al., 2010; Wang et al., 2010).

The GABAergic system is compromised in FXS (Adusei et al., 2010; Curia et al., 2009; D'Hulst et al., 2009; Gatto et al., 2014; Olmos-Serrano et al., 2010; Paluszkiewicz et al., 2011a), and these defects have been thought to contribute to the overall disturbance of excitatory-inhibitory balance in FXS. FMRP binds the mRNAs of several subunits of $GABA_A$ and $GABA_B$ receptors, and their expression is reduced in FXS (Curia et al., 2009; D'Hulst et al., 2006; Gantois et al., 2006; Gatto et al., 2014; Kang et al., 2017). Electrophysiological studies have further demonstrated defects in GABAergic signaling (Centonze et al., 2008; Gibson et al., 2008; Olmos-Serrano et al., 2011; Olmos-Serrano et al., 2010; Paluszkiewicz et al., 2011b; Sabanov et al., 2017), including a delayed transition from excitatory to inhibitory signaling during development (He et al., 2014). Modulation of GABAergic signaling using specific agonists ameliorates defects in animal models of FXS (Braat et al., 2015; Braat and Kooy, 2015; Lozano et al., 2014). These preclinical studies significantly implicate a dysfunctional GABAergic system in FXS, and provide rationale for targeting specific GABA receptors as a treatment strategy.

1.3.3. Altered intracellular signaling in FXS

In addition to overactive glutamatergic signaling, the loss of FMRP has been shown to result in the overactivation of several downstream effectors of mGluR signaling. The activation of protein synthesis following the induction of mGluR-LTD engages two key signaling pathways – the mitogen-activated protein kinase/extracellular signal-regulated kinases signaling pathway (MAPK/ERK) and the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway. These two pathways have been implicated in several neurodevelopmental disorders, and converge to regulate the activity of an initiation factor eIF4E (Banko et al., 2006). Targeting eIF4E overactivity appeared to reverse spine phenotypes and specific cognitive deficits in *Fmr1* KO mice (Santini et al., 2017).

The PI3K/mTOR and MAPK/ERK signaling pathways are highly interconnected and work in concert with each other to maintain normal function; therefore, teasing out individual contributions is challenging, and while some groups have seen defects primarily in mGluR-mediated ERK 1/2 activation (Kim et al., 2008; Osterweil et al., 2010; Weng et al., 2008), others report only aberrant PI3K/mTOR signaling (Gross et al., 2010; Sharma et al., 2010). FMRP was shown to bind and regulate the translation of two mRNAs in the PI3K pathway – the catalytic subunit of PI3K, p110β, and the PI3K enhancer, PIKE (Gross et al., 2010), and genetic and pharmacological interventions to reduce PI3K signaling in FXS mice and flies corrected behavioral abnormalities, dendritic spine defects and aberrant protein synthesis (Gross et al., 2015a; Gross et al., 2010; Gross et al., 2015c; Sharma et al., 2010).

1.3.4. Development of clinical trials in FXS

Animal models of FXS have helped to understand the relationship between FMRP deficiency and phenotypes in FXS. A primary finding that seems to be consistent across model systems and research groups is the dysregulation of key signaling and molecular pathways in the absence of FMRP, and researchers have focused on targeting these pathways in an effort to correct FXS phenotypes; exciting results from these studies have led to the development of several early-phase clinical trials for FXS. The mGluR theory spurred multiple clinical trials targeting mGluR signaling, the first of which was an open-label study of an mGluR5 negative allosteric modulator (NAM), Fenobam (Berry-Kravis et al., 2009). Since this pilot study showed promising results of improved prepulse inhibition and reduced anxiety levels without any significant adverse effects, an early phase I/II trial of another mGluR5 NAM, Mavoglurant (AFQ056), was initiated. Patients showed improvements in several behavioral impairments (Jacquemont et al., 2011), however, subsequent later phase trials of AFQ056 and a similar mGluR5 inhibitor Basimglurant (RO4917523) did not appear to significantly improve behavioral deficits in FXS (Berry-Kravis et al., 2016). Inhibitors of PI3K and other key intracellular pathways have not yet been approved for use in humans, despite promising preclinical results; but lithium treatment, which is widely used to treat bipolar disorders, appeared to ameliorate some behavioral phenotypes in FXS, in addition to normalizing aberrant ERK1/2 phosphorylation levels in FXS patient lymphoctyes (Berry-Kravis et al., 2008). An mRNA target of FMRP, matrix metalloproteinase 9 (MMP-9) is increased in FXS flies and mice (Bilousova et al., 2009; Siller and Broadie, 2011) and also in plasma from FXS patients (Dziembowska et al., 2013). Two inhibitors of MMP-9 – Minocycline and Metformin - have been shown to correct defects in *Fmr1* KO mice (Gantois et al., 2017; Rotschafer et al., 2012), and are currently being

investigated in clinical trials. GABA B receptor agonists such as R-baclofen showed great potential in correcting defects in *Fmr1* KO mice, and although the *a priori* defined primary end point was not met in an early clinical trial (Berry-Kravis et al., 2012), significant behavioral improvements were reported, leading to the development of phase III trials (Berry-Kravis et al., 2017). Other modulators of the GABAergic system such as Ganaxolone, Acamprosate and Metadoxine are currently being tested for efficacy in clinical trials (Berry-Kravis et al., 2018; Erickson et al., 2013; Ligsay et al., 2017).

1.4. Induced pluripotent stem cells (iPSCs) as a tool to model neurological disorders

In 2006, Shinya Yamanaka demonstrated that human patient somatic cells can be reprogrammed into pluripotent stem cells by introducing four transcription factors, now known as the "Yamanaka factors" (Takahashi et al., 2007; Yamanaka and Takahashi, 2006). These induced pluripotent stem cells (iPSCs) could subsequently be differentiated into a variety of cell types, including neurons, making them an ideal tool for modeling neurological disorders. Early work established the feasibility of generating iPSCs from patients with known genetic mutations, and establishing protocols to differentiate these iPSCs into disease-relevant cell types (Dimos et al., 2008; Ebert et al., 2009; Park et al., 2008). The field has since made great advances in increasing the efficiency and reproducibility of reprogramming and differentiation protocols, establishing iPSCs as a valid tool to model human disorders (Ardhanareeswaran et al., 2017).

Patient-derived iPSCs provide an unprecedented opportunity to study human disorders *in vitro*, and there have been several exciting studies using iPSCs to model neurological disorders, such as Spinal Muscular Atrophy (SMA), Amyotrophic Lateral Sclerosis (ALS), Alzheimer's disease, Parkinson's disease, schizophrenia, autism and Rett's syndrome (An et al., 2012;

Brennand et al., 2011; Chen et al., 2014; Hargus et al., 2010; Kondo et al., 2013; Marchetto et al., 2010; Mariani et al., 2015; Pasca et al., 2011). These studies have added on to the invaluable insight gained from animal models, and have furthered our understanding of the etiology and pathology of these disorders in a human-specific context. Importantly, iPSC-derived neural cells have been used to investigate genetic (Corti et al., 2012; Seibler et al., 2011) and pharmacological (Shcheglovitov et al., 2013; Xu et al., 2016) interventions in neurological disorders, and hold great promise for drug discovery that could better inform the development of clinical trials. However, as with any model system, iPSCs present their own set of challenges and limitations. One of the primary concerns in the field is variability (Inoue et al., 2014) – reprogramming methods and differentiation protocols differ greatly between groups, and the multiple steps and lengthy duration of the reprogramming and differentiation process could lead to inconsistencies in results and reproducibility (Salomonis et al., 2016; Srikanth and Young-Pearse, 2014). Furthermore, the cost of generating, maintaining and differentiating iPSCs is extremely high, often limiting the number of patient lines that can be used in each of these studies. Despite these challenges, the iPSC model system shows great promise as a platform for drug discovery, and as a tool to investigate neurodevelopment and neurodegeneration in both normal and diseased states (Shi et al., 2017).

1.5. Dissertation rationale and objectives

Almost three decades after the discovery of *FMR1*, we have made great progress in our understanding of the biology of FMRP, and how to manage symptoms of fragile X syndrome; however, we still lack an effective treatment for the disorder. Despite the overwhelmingly successful preclinical studies conducted in animal models, most clinical trials in FXS failed to

meet their defined primary endpoints (Berry-Kravis et al., 2018; Erickson et al., 2017; Gross et al., 2015b). Although these results could be attributed to several factors, the importance of validating the effect, efficacy and toxicity of drugs in human patient-derived, disease-relevant cell types is becoming increasingly apparent.

The *Fmr1* KO mouse replicates several key features of the human condition, including cognitive dysfunction, behavioral abnormalities, and macroorchidism (Dolen et al., 2007; Gross et al., 2015a; Gross et al., 2015c; Udagawa et al., 2013), and studies of postmortem brains have shown distinct morphological defects in dendritic spine structure and density in FXS patient neurons (Irwin et al., 2001) that are also seen in *Fmr1* KO mice (Bhattacharya et al., 2016; Bilousova et al., 2009; Dolan et al., 2013; Gross et al., 2015a). The FXS mouse model has a high predictive value, and we have been able to identify several *in vitro* and *in vivo* phenotypes in animal models of FXS; however, our understanding of the cellular and molecular pathology in humans is extremely limited. It is important to note that animal models do not faithfully reproduce the mutation seen in human patients (Bakker, 1994; Mientjes et al., 2006), and it remains unknown whether the expanded CGG repeat contributes to moderating disease phenotypes or responses to interventions.

A few studies have used human FXS patient-derived cells to look at dysregulated signaling and protein synthesis, which are hallmark features of the FXS mouse model. FXS patient lymphoblastoid cell lines (LCLs) (Gross and Bassell, 2012) and FXS patient fibroblasts (Kumari et al., 2014) were demonstrated to have increased basal protein synthesis compared to controls. Importantly, a recent study compared protein synthesis rates in fibroblasts from 17 control and 32 FXS patients collected from three different laboratories, and showed that only a subset of FXS patients had significantly elevated protein synthesis compared to controls

(Jacquemont et al., 2018). The same group showed that both neurons and fibroblasts cultured from *Fmr1* KO mice showed a more robust and consistent phenotype of elevated translation compared to controls (Jacquemont et al., 2018), suggesting that cellular and molecular phenotypes may be more variable in human patient-derived cells. FXS patient fibroblasts and LCLs retain the human-specific expanded repeat mutation, and are relatively inexpensive and easily accessible, making them an extremely valuable tool to identify phenotypes and biomarkers of FXS; however, the loss of FMRP appears to have the most significant consequence on the nervous system, and till date there have been no studies that have systematically investigated translational dysregulation in human FXS neural cells. Thus, there is a critical need to develop human disease-relevant cellular models of FXS, and the advent of induced pluripotent stem cell (iPSC) technology has made this an attainable possibility.

Over the past decade, there have been several studies using pluripotent stem cells to model FXS, primarily directed at understanding the mechanisms of repeat expansion and instability, and the epigenetic silencing of *FMR1* (Brykczynska et al., 2016; Kaufmann et al., 2015; Kumari et al., 2015). Studies investigating morphological defects in FXS patient-derived neurons have had contrasting results, with some studies showing that FXS neurons have defects in neurite extension and early neuronal morphology (Doers et al., 2014; Sheridan et al., 2011; Urbach et al., 2010), and others showing no difference during initial neurogenesis (Bhattacharyya et al., 2008; Telias et al., 2013) or increased neurogenesis in FXS (Boland et al., 2017). Since it takes a significant amount of time to produce neurons with relatively mature morphological and electrophysiological phenotypes from human iPSCs (Srikanth and Young-Pearse, 2014), these inconsistencies may be due to differences in methods used to differentiate neurons, and the time-points at which these phenotypes were evaluated.

Studies in the *Fmr1* KO mouse have pointed towards a role for FMRP earlier in neuronal development (Li et al., 2016; Li and Zhao, 2014; Luo et al., 2010). Some mRNA targets of FMRP are known to regulate cell proliferation and neurogenesis, such as CDK4 and cyclin D1 (Luo et al., 2010) and the loss of FMRP results in dysregulation of the WNT signaling pathway (Luo et al., 2010). Embryonic as well as adult neural stem cells (NSCs) cultured from *Fmr1* KO mice were shown to have increased proliferation and impaired neurogenesis (Castren et al., 2005; Li et al., 2016; Luo et al., 2010; Tervonen et al., 2009). Recent studies have attempted to shed some light on the function of FMRP in early human neuronal development, and a growing body of evidence suggests that FXS patient derived neural progenitor cells (NPCs) exhibit aberrant expression of genes related to neural differentiation, development and migration (Boland et al., 2017; Halevy et al., 2015; Sunamura et al., 2018). One study showed that FMRP deficiency in murine NSCs affected specific transcripts involved in protein synthesis as well as neuronal differentiation (Liu et al., 2018). Thus, it is clear that FMRP plays an important role in regulating key processes during early neural development in mice and in humans. These developmental processes are also regulated by translation and signaling via the PI3K/mTOR pathway, both of which are affected by the loss of FMRP (Gross and Bassell, 2012; Gross et al., 2015c). However, the connection between dysregulated translation and aberrant neurogenesis and cell fate specification in human models of FXS remains unexplored.

This dissertation aimed to gain a better understanding of the role that FMRP plays in human neuronal development, and the consequences of its loss in fragile X syndrome. We sought to develop and characterize a human *in vitro* model of fragile X syndrome (FXS) using induced pluripotent stem cells (iPSCs) and to subsequently generate disease-relevant neural cells to identify cellular and molecular phenotypes of FXS. We aimed to examine whether hallmark

features of dysregulated signaling and aberrant protein synthesis seen in animal models of FXS are recapitulated in a human neural model, and we anticipated to elucidate novel functions of FMRP in the context of human neurogenesis.

Beyond a comparison of protein synthesis in human and mouse, we sought to develop a novel approach that would allow analysis of protein synthesis rates in a cell type specific manner. The ability to measure protein synthesis in specific neural cell types and correlate with other cellular phenotypes has not been done in any model system, and hence this work represents a novel direction. Here, we have used multiple approaches to show that FXS patient fibroblasts and neural progenitor cells have elevated global translation. Furthermore, we show for the first time in iPSC-derived neural progenitor cells and cerebral organoids that cell fate commitment and proliferation is altered in FXS. We developed a multiparametric, high-throughput, flow-cytometry based assay, NeuroMIP, that allows us to simultaneously measure protein synthesis and proliferation in specific neuronal subtypes. We believe that this assay will be a valuable tool that can be optimized and adapted for drug screens in multiple neurological disorders. To the best of our knowledge, our study is the first of its kind to identify cell-type specific dysregulation in translation in a human neural model of FXS and we propose that defects in protein synthesis may drive abnormal proliferation in FXS, altering differentiation kinetics and neuronal maturation.

CHAPTER 2: Materials & Methods

Reprogramming fibroblasts to induced pluripotent stem cells (iPSCs)

We obtained control and FXS patient fibroblasts from collaborators at three sources: Children's hospital of Orange County, Dr. Elizabeth Berry-Kravis (Rush University) and Dr. Joseph Cubells/Dr. Chadwick Hales (Emory University). All iPSC lines described here were generated from dermal fibroblasts cultured from punch biopsies of male control and FXS patients. Fibroblasts were reprogrammed using the CytoTune-iPS 2.0 Sendai virus reprogramming kit (Thermofisher), following the manufacturer protocol. Briefly, fibroblasts were transduced with a Sendai virus cocktail containing four transcription factors (hOct3/4, hSOX2, hKlf4, hc-Myc) and maintained in Fibroblast medium (DMEM, GlutaMAX, FBS; Thermofisher) for one week. Transduced fibroblasts were replated onto Vitronectin coated dishes in Essential-8 media (Thermofisher) and individual iPSC colonies began to emerge within two weeks. We manually isolated clonal iPSC colonies from each culture and expanded two clones per iPSC line for characterization.

Culturing feeder-free iPSCs

iPSCs were cultured on Matrigel (Corning) coated dishes and maintained in complete mTeSR medium (Stemcell technologies). iPSCs were passaged every 6 days using ReLeSR (Stemcell technologies) following manufacturer's protocol. For immunofluorescence characterization of iPSCs, cells were passaged onto Matrigel coated coverslips and fixed after 24 hours. For cryostorage of iPSCs, cells were treated with ReLeSR, spun down and resuspended in cryoSTOR-10 freezing media (Stemcell technologies) and samples were stored in liquid nitrogen. Isogenic lines used in this study were generated by CRISPR/Cas-9 mediated excision

of repeats from a full mutation iPSC line (Xie et al., 2016), and were a kind gift from Dr. Steve T. Warren (Emory University).

Generation of iPSC-derived NPCs

NPCs were generated using two different methods based on dual-SMAD inhibition. The EB-method used was similar to previously published methods, with minor modifications (Chailangkarn et al., 2016; Marchetto et al., 2010). Briefly, iPSCs were dissociated into single cells and cultured in suspension as EBs in neural induction media (DMEM/F-12, GlutaMAX, Hepes, N2; Thermofisher) with dual-SMAD inhibition factors SB431542 (10uM; Stemgent) and DMH1 (5uM; Tocris) for 1 week. EBs were plated on matrigel coated dishes in neural progenitor medium (DMEM/F-12, GlutaMAX, Hepes, N2, B27; Thermofisher) supplemented with bFGF (20 ng/ml; Peprotech). Within one week, neural rosettes formed and were manually isolated, spun down, and replated without dissociation on matrigel coated dishes. After another week, manual rosette selection was repeated, and rosettes were dissociated to single cells using Accutase and plated on matrigel dishes to generate a pure population of neural precursor cells (NPCs). NPCs were cultured in FGF supplemented NPC medium (DMEM/F-12, GlutaMAX, Hepes, N2, B27; Thermofisher) and passaged every 6 days.

The monolayer method followed the manufacturers recommended protocol. Briefly, iPSCs were dissociated to single cells and plated at a high density and maintained in STEMdiff SMADi Neural Induction Medium (Stemcell technologies) for up to 9 days. Cells were passaged at ~80% confluence twice in STEMdiff Neural Induction Medium (Stemcell technologies), before passaging into Complete STEMdiff Neural Progenitor Medium (Stemcell technologies). NPCs were passaged every six days and maintained in STEMdiff Neural Progenitor Medium

(Stemcell technologies) for at least another two passages. NPCs were passaged into homemade NPC medium (DMEM/F-12, GlutaMAX, Hepes, N2, B27; Thermofisher) supplemented with bFGF (20 ng/ml; Peprotech) one week prior to being used in experiments. NPCs generated using both methods can be expanded, frozen down and thawed out successfully. For cryostorage of NPCs, cells were dissociated using Accutase and resuspended in cyoSTOR-10 freezing media (Stemcell technologies).

Generation of neurons from NPCs

In order to generate neurons, NPCs were plated at a density of 15,000 cells/cm^2 onto poly-ornithine (25 ug/ml; Sigma) and laminin (3.3 ug/ml; Thermofisher) coated dishes into NPC medium (DMEM/F-12, GlutaMAX, Hepes, N2, B27; Thermofisher) supplemented with ROCK inhibitor Y-27632 (10uM; Stemgent). After 24 hours, media was changed to neuronal media (Neurobasal/B27/Glutamax; Thermofisher) supplemented with DAPT (10uM; Tocris), (BDNF (10ng/ml; Peprotech) and GDNF (10ng/ml; Peprotech). Media changes were performed every other day for the first week, and every 3-4 days subsequently. Neurons were harvested according to experimental requirements. For neurons maintained in culture beyond 7 days, DAPT was removed on day 7.

Generation of iPSC-derived organoids

iPSCs colonies were detached from Matrigel coated plates with collagenase (1mg/ml; Invitrogen) treatment for 1hr and suspended in EB medium, comprising of FGF-2-free hESC medium supplemented with Dorsomorphin (2µM; Tocris) and A-83 (2µM; Tocris), in non-treated polystyrene plates for 4 days with a daily medium change. On days 5-6, half of the

medium was replaced with induction medium consisting of DMEM/F-12, N2 Supplement (Thermofisher), 10µg/ml Heparin (Sigma), NEAA (1X), Glutamax (1X), WNT-3A (4 ng/ml; R&D Systems), CHIR99021 (1µM; Cellagentech), and SB-431542 (1µM; Cellagentech). On day 7, organoids were embedded in Matrigel (Corning) and continued to grow in induction medium for 6 more days. On day 14, embedded organoids were mechanically dissociated from Matrigel by pipetting up and down onto the plate with a 5ml pipette tip. Typically, 10 – 20 organoids were transferred to each well of a 12-well spinning bioreactor (SpinΩ) containing differentiation medium, consisting of DMEM:F12, N2 (1X), B27 (1X; Invitrogen), 2-Mercaptoenthanol (100µM), NEAA (1X), Insulin (2.5µg/ml; Sigma). Media was changed every other day until day 28 when organoids were collected for immunofluorescence analysis or RNA.

Multielectrode Array analysis

Neurons were plated on 48-well multielectrode arrays (Axion) at a density of 150,000 cells per well. Longitudinal recordings across 6 weeks of spontaneous firing, burst firing and spike activity were carried out for 10 minutes/day every 4 days using 3 control and 3 FXS patient iPSC derived neurons. Data were analyzed using Neural Metrics Tool (Axion) and represented as Mean ± SEM.

RNA extraction and quantitative reverse transcription with PCR (RT-PCR)

RNA was extracted using the Quick RNA kit (Zymo Research) with a combined on-column DNase I digestion step. Adherent cells were directly lysed in the culture dish and RNA extraction proceeded per the manufacture's protocol. cDNA was obtained via RT-PCR using the High Capacity cDNA Reverse Transcription Kit (Thermo).

To quantify relative mRNA expression for *FMR1*, qPCR was performed for each sample using Taqman gene expression assays (Thermo) on a Quantstudio 6 Flex system (Applied Biosystems). For qPCR analysis of exon 2 inclusion, a custom FAM labelled Taqman assay spanning the exon 2A-exon 3 splice junction of the 5'UTR of *AURKA* was used. Relative RNA expression was normalized to geometric mean Ct values of *RPLP0*.

RNA-seq and splicing analysis

Strand specific poly-A selected libraries were constructed and sequenced on an Illumina NextSeq500 sequencer with 30 million reads per replicate (2 x 150bp). Transcript abundances were quantified as transcripts per million (TPMs) and then collapsed to gene abundances using Salmon. Alternative splicing events were identified based on RNA-seq coverage using rMATS and PSI values of differential splicing were obtained. Stringent cutoffs for differential splicing events were set to FDR < 0.05, Inclusion level difference > -0.1, and min counts ≥ 20. Splicing scatter plots were generated using R-studio and Sashimi plots were generated for the visualization of splicing events. Pathway enrichment of genes in RNAseq were performed on gene ontology (GO) terms using Gorilla.

Lysate preparation

Adherent cells were directly lysed in ice-cold lysis buffer (50mM Tris–HCl, pH 7.4, 300mM NaCl, 1% Triton X-100, 5mM EDTA, 2% SDS) with 1x HALT protease and phosphatase inhibitors (Pierce). Lysates were sonicated at 25% amplitude for 3 cycles of 5 seconds on/5 seconds off and then cleared by spinning for 15 mins at 4C. Protein quantification was done using a BCA assay (Pierce).

Immunoblotting

For western blots, protein lysates were prepared in 4x loading buffer, heat-denatured at 70^{o}C for 15 minutes. Samples were normalized to equal amounts and resolved on 10% Bis-Tris gels (Thermo Fisher), transferred to 0.44 μm PVDF membranes, and blocked in Licor Odyssey blocking buffer (Licor) for 1 hour. The membrane was incubated overnight at 4^{o}C with primary antibodies diluted in blocking buffer. Secondary antibodies were diluted in blocking buffer and applied to membrane for 1 hour at R.T. Primary antibodies used in immunoblotting experiments included anti-FMRP (1:1000, Sigma), anti-p110β (1:1000, Millipore), anti-p-ERK1/2 (1:1000, Cell Signaling), anti-ERK1/2 (1:100, Cell Signaling), and anti-GAPDH (1:1000, Cell signaling). Densitometry analysis was done using Image Lab software. For BONCAT and puromycin blots, intensity of the lane was measured and normalized to GAPDH signal per lane. For signaling blots, density of each band was normalized to loading control signal per lane.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 10 minutes at R.T. Cells were washed 3x with 1x PBS and permeabilized for 10 min in 1x PBST (0.1% Triton-X). Cells were then blocked in 5% normal donkey serum (Jackson Labs) for 1 hour at R.T. and incubated with primary antibodies in 5% normal donkey serum overnight at 4^{o}C. The following day cells were washed 3x in PBS and incubated with secondary antibodies (Alexa Fluor, 1:1500) for 1 hour. Organoids were embedded in OCT and 12um sections were cut on a cryostat. For staining organoid sections, slides were washed 3x with 1x PBS and permeabilized for 10 min in 1x PBST (0.1% Triton-X). Sections were blocked in 10% normal donkey serum (Jackson Labs) for 1 hour at R.T. and incubated with primary antibodies in 10% normal donkey serum overnight at 4^{o}C.

The following day sections were washed and secondary antibodies were applied for 2 hours at R.T. (Alexa Fluor, 1:1500). Sections were mounted using Prolong Gold with DAPI. Antibodies used for immunofluorescence were anti-Nestin (1:500, Abcam), anti-SOX2 (1:200, Thermofisher), anti-Oct4 (1:400, Cell Signaling), anti-SSEA3/4 (1:500, Cell Signaling), anti-Nanog (1:200, R&D systems), anti-Tra1-60 (1:150, Millipore), anti-Tra-1-81 (1:150, Millipore), anti-MAP2 (1:1500, Synaptic Systems), anti-TBR1 (1:500, Abcam), anti-vGlut1 (1:500, Synaptic Systems), anti-KI67 (1:500, BD Biosciences) and anti-Synapsin (1:500, Synaptic Systems).

AHA labeling and click reaction for BONCAT and flow cytometry

Cells were incubated in methionine-free DMEM (Thermofisher) supplemented with B27 for 1 hour to deplete available methionine. Azidohomoalanine (AHA, Thermofisher) was added at a final concentration of 1mM to cells. For BONCAT, cells were incubated in AHA for two hours, washed with 1x PBS and lysed in Tris-HCl/SDS lysis buffer. Cells were scraped into 1.5ml tubes and incubated on ice for 10 minutes before sonication at 25% amplitude for 3 cycles of 5 seconds on/5 seconds off. Protein concentration was measured using BCA assay (Pierce) and each sample was made up with equal amount of protein up to a volume of 60ul. Click reaction was performed in 1.5ml tubes according to manufacturer's protocol (Protein Reaction Buffer kit, Thermofisher) to conjugate Biotin-alkyne (Thermofisher) to incorporated AHA via copper-catalyzed click chemistry. Excess biotin was removed by methanol precipitation and pellets were resolubilized using 8M Urea buffer. Newly synthesized proteins were immunoprecipitated by incubating samples with Streptavidin Dynabeads (Thermofisher) and rotating for 2 hours at R.T. Beads were resuspended in 4X laemmli buffer and western blots were

performed as described earlier. BONCAT signal was measured using an anti-Streptavidin antibody (Licor).

For flow cytometry experiments, cells were incubated in AHA media for 45 minutes and then rinsed 2X in warm media. Adherent cells were treated with accutase to lift, washed in ice-cold 1x PBS and then resuspended in 1x PBS. A Live/Dead distinguishing marker was added to cells in PBS (Live/Dead-Aqua, 1:1000, Thermofisher) for 15 mins in the dark at R.T. Cells were fixed in 4% PFA for 15 mins at R.T., and permeabilized in 0.25% Triton X-100/PBS for 15 mins. Cells were washed in 3% BSA/PBS and then the click reaction was performed according to manufactor protocol (Cell reaction buffer, Thermofisher) to label newly synthesized proteins with Alexa 647-Alkyne (Thermofisher). Cells were washed 2X with 3%BSA/PBS and analyzed on a BD LSR II flow cytometer as described below

Puromycin labeling

Cells were washed 2X in PBS and then incubated with Puromyin (10ug/ml, Sigma) for 30 mins at 37°C, following which cells were rinsed with 1x PBS. Biological negative controls of No puromycin and Puromycin+ anisomycin were included to ensure that the signal was puromcyin- and protein synthesis-dependent. Cells were lifted using accutase and resuspended in ice-cold PBS. For western blotting, cells were spun down and resuspended in lysis buffer and lysate preparation and immunoblotting was carried out as described previously. Newly synthesized proteins were visualized on a gel using anti-Puromycin antibody (1:1000, Millipore).

For flow cytometry analysis cells were resuspended in ice-cold PBS and Live/Dead marker (1:1000, Live/Dead Aqua, Live/Dead Near-IR, Thermofisher) was added for 15 mins in the dark at R.T. Cells were resuspended in TFP Fix/Perm buffer (BD Biosciences) for 15 mins in

the dark at R.T. and then washed 2X in TFP Perm/Wash buffer (BD Biosciences). For analysis of global protein synthesis in all cells, anti-Puromycin-Alexa 488 was added to cells for 1 hour in the dark at R.T., and cells were subsequently washed 1X in Perm/Wash buffer and 2X in 2% FBS/PBS and then analyzed on a cytometer. For cell type specific profiling, an expanded panel of antibodies was included as described in the NeuroMIP method below.

Flow Cytometry

Flow cytometry was conducted on a BD LSR II (BD Biosciences) and data were analyzed using FlowJO (Tree Star). Cell suspensions were run through the cytometry on the lowest speed setting and voltages were maintained across experiments. For each experiment, compensation controls and FMO controls were included in addition to biological controls. Compensation was calculated using FACS Diva at the time of sample collection and all samples were run in the same sitting. Protein synthesis and Proliferation were measured based on Mean Fluorescence Intensity (MFI) of Puromycin and KI-67 signals and percentages of cells positive for each set of markers were calculated based on predetermined gating strategy.

Neuronal Molecular Immunophenotyping (NeuroMIP)

For cell-type specific profiling of molecular phenotypes we developed optimized panels of antibodies to identify neuronal subpopulations and measure proliferation and protein synthesis. Cells were labeled using puromycin as previously described and dead cells were labeled using Live/Dead markers using fluorophores optimized for each individual panel. Markers for each panel were selected after careful evaluation of their ability to label specific subpopulations. All antibodies were titered for optimal staining and minimal spillover into

neighboring detectors. To account for any spillover in multicolor NeuroMIP panels, single-stained compensation controls were included for each color used in the panel and compensation was calculated and accounted for using BD FACS Diva software. Optimal staining of nuclear and intracellular markers was ensured by using transcription factor optimized fixation and permeabilization kits from BD Biosciences. During panel development, antibody titrations, compensation assessments and gating were all measured using consistent number of cells ($1x10^6$ cells per test) resuspended in 200ul of FACS buffer. Cytometer settings and gating strategy were designed to yield the least amount of background and maximal signal. was done with the help of controls. For each antibody within a panel, a fluorescence-minus-one (FMO) sample was included, which was stained with all other antibodies but one. This helped to discriminate between positive and negative events and establish gates for each color. The gate for identifying positive events for puromycin signal was set based on the Anisomycin negative control sample.

CHAPTER 3: Development of a human cellular model of Fragile X syndrome

Portions of this chapter were adapted from the following manuscript:

Banerjee, A., Ifrim, M.F., Valdez, A.N., **Raj, N**., and Bassell, G.J. (2018). Aberrant RNA translation in fragile X syndrome: From FMRP mechanisms to emerging therapeutic strategies. Brain research 1693, 24-36.

3.1. Introduction

Over the last two decades animal models have provided us with tremendous insight into the biology of FMRP and the potential mechanisms underlying FXS. Highly robust and promising preclinical findings in the *Fmr1*KO mouse model demonstrating the effectiveness of group 1 metabotropic receptor antagonists in the treatment of FXS led to several large clinical trials, however these trials failed to meet their primary endpoints. While this could be attributed to the need for improved trial design and better-defined clinical endpoints, a key concern is that although the *Fmr1* KO mouse allows us to investigate loss of function of FMRP, it does not capture critical features of the human condition such as the expansion of the trinucleotide repeat, and the mechanism of methylation and silencing of the *FMR1* gene.

Our knowledge of FXS in humans has largely been limited to postmortem studies of the brain; however, recent advances in neuronal differentiation using human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), have provided us with a biological model system that allows study of FXS in a disease relevant cell type. Importantly, these human pluripotent cell models allow for the investigation of molecular events underlying the CGG repeat expansion, and epigenetic silencing of *FMR1* in a developmentally regulated manner.

3.1.1. Modeling FMR1 silencing and CGG repeat expansion in FXS hPSCs

In the first characterized FXS hESC line (Eiges et al., 2007) and in chorionic villi samples from FXS fetuses (Willemsen et al., 2002), it was found that despite the presence of the full mutation of over 200 CGG repeats, the *FMR1* gene is transcribed and the promoter region is unmethylated. A subsequent study from the same group, however, showed that some FXS hESCs may be abnormally methylated, and consequently do not express *FMR1* mRNA (Avitzour et al.,

2014). Neuronal differentiation of FXS hESCs has also been implicated in the silencing of the gene (Colak et al., 2014; Telias et al., 2013; Urbach et al., 2010). One proposed mechanism for the epigenetic silencing of the *FMR1* locus is that *FMR1* mRNA transcripts interact with the expanded CGG repeat region forming RNA-DNA duplexes which in turn mediate *FMR1* silencing (Colak et al., 2014). In contrast to FXS hESCs, induced pluripotent stem cells (hiPSCs) generated from FXS patient fibroblasts are consistently hypermethylated, and lack *FMR1* mRNA expression as well as the protein product FMRP, suggesting that the repressive epigenetic marks of the original somatic cells are retained during the reprograming process (Avitzour et al., 2014; Sheridan et al., 2011; Urbach et al., 2010). Interestingly, iPSCs generated from the fibroblasts of male patients with an unmethylated full mutation acquire the hypermethylation and subsequent transcriptional silencing of *FMR1* (de Esch et al., 2014).

In another study, researchers aimed to investigate whether the hypermethylation and silencing of *FMR1* was dependent on the maintenance of the CGG repeat expansion. To this end, they employed a CRISPR/Cas9-mediated strategy to correct the expanded CGG repeat and showed that this resulted in demethylation of *FMR1* as well as restoration of *FMR1* mRNA in iPSCs as well as neural progenitor cells and neurons (Park et al., 2015; Xie et al., 2016). FXS hPSC models have given us an unprecedented opportunity to study mechanisms of *FMR1* silencing as well as CGG repeat expansion, ultimately paving the way for more accurately designed therapeutics.

3.1.2. Modeling cellular and molecular deficits in FXS hPSCs

Several labs have found that the loss of FMRP results in morphological defects, dysregulated signaling and abnormal protein synthesis in *Fmr1* KO mouse neurons. Many of

these core deficits have been shown to be rescued in the FXS mouse model by using genetic or pharmacological approaches that target key signaling pathways or cell surface receptors as described above; however, these strategies have yet to be translated to effective therapeutic approaches for human patients. The ability to reprogram somatic cells into iPSCs presents a unique opportunity to investigate the consequences of this loss-of-function mutation in a patient-specific, disease-relevant manner. Furthermore, advances in neuronal differentiation methods have opened up the possibility of large-scale, high-throughput drug screens that can be used to identify and test the efficacy of compounds to treat FXS.

In one of the earliest studies to use hPSCs to model FXS, the researchers generated clonal iPSC lines from a patient with mosaic expression of the mutation (possessing both premutation and full mutation alleles). They found that neurons differentiated from iPSC clones with the methylated full mutation did not express FMRP and exhibited deficits in neuronal morphology and differentiation as compared to FMRP-expressing neurons that were generated from iPSC clones with the unmethylated allele (Sheridan et al., 2011). FXS patient iPSC-derived forebrain neurons were also found to have fewer and shorter neurites, as well as defects in neurite initiation and extension (Doers et al., 2014). In contrast, studies from fetal cortex derived hNPCs (Bhattacharyya et al., 2008) and FXS hESC derived neurons (Telias et al., 2013) did not reveal any significant differences in neurogenesis and neurite formation compared to controls, although there appeared to be increased gliogenesis in FXS hESC derived neuronal cultures. This study further examined electrophysiological properties of these neurons and found that loss of FMRP in human neurons resulted in reduced frequency, amplitude of action potentials and poor synaptic formation (Telias et al., 2013). The same group conducted a more extensive morphological and functional analysis of FXS hESC derived neurons and found that FXS

neurons had shorter and less tortuous neurites compared to controls, as well as smaller somata (Telias et al., 2015). These neurons also exhibited deficits in action potential firing, spontaneous synaptic activity (Telias et al., 2015) and response to GABA (Telias et al., 2016) suggesting an overall immature phenotype that is in line with findings from the *Fmr1* KO mice.

The work being done using hPSCs to model FXS in neurons is exciting and provides critical preclinical validation of work done in the knockout mouse, as well as allowing for the investigation of questions that thus far were impossible to answer in other biological systems. A major obstacle in working with hPSCs is the variability in results and lack of consistency in methods, which although expected when working with human samples, needs to be optimized and standardized further across labs in order to help progress research in the field, and allow for more consistent, reproducible results.

3.1.3. Chapter 3 rationale and objectives

The failure of several clinical trials in FXS despite promising results in animal models highlighted the importance of preclinical studies in human cells that would allow us to (1) substantiate findings in animal models and (2) uncover human disease-specific biology (Berry-Kravis et al., 2018; Erickson et al., 2017; Gross et al., 2015b). Induced pluripotent stem cells (iPSCs) offer a unique opportunity to model human neurological disorders, and several groups have used iPSC-derived neural cells to identify disease-relevant phenotypes and potential therapeutic targets (Marchetto et al., 2010; Shcheglovitov et al., 2013). iPSCs are particularly well-suited to model neurodevelopmental disorders because established differentiation protocols allow for the generation of previously inaccessible human neuronal cell types across stages of development (Marchetto et al., 2010). We aimed to generate iPSCs from multiple control and

FXS patient fibroblasts that we obtained from collaborators across three centers. These iPSCs will remain a valuable resource to multiple researchers in the field. We generated neural progenitor cells (NPCs) and cerebral organoids from our iPSC lines in order to identify cellular and molecular phenotypes in a disease-relevant cellular model. NPCs offer a unique advantage in that they are an expandable population of proliferative cells that have neurogenic potential (Marchetto et al., 2017; Stiles and Jernigan, 2010). Thus, these cells can be allowed to differentiate into multiple neuronal subtypes, and we aimed to examine the consequence of FMRP deficiency in a cell-type specific manner. Cerebral organoids are 3D structures that mimic the development of the neocortex and allow for a more physiologically relevant investigation of neuronal phenotypes (Qian et al., 2016). Our overall goal was to establish a human cellular model system that would allow us to study cellular and molecular phenotypes in FXS at multiple stages of neuronal development.

Reprogramming of fibroblasts to iPSCs, characterization of iPSCs, differentiation and characterization of NPCs and neurons described in this chapter were done by **Nisha Raj**. Generation and maintenance of cerebral organoids was done by Zhexing Wen and Ying Zhou (Emory University). G-banded karyotyping analysis was done through WiCell.

3.2. Results

We have generated induced pluripotent stem cell (iPSC) lines from a total of 8 controls and 8 patients with fragile X syndrome. We have optimized protocols to differentiate these lines into neural precursor cells (NPCs), forebrain-neurons and cerebral organoids that we have used in the experiments described in subsequent chapters.

3.2.1. Generation of induced pluripotent stem cells (iPSCs) from human dermal fibroblasts

Since the pioneering work from Shinya Yamanaka's group to generate pluripotent cells from adult mouse and human fibroblasts using the retroviral transduction of "Yamanaka" factors (Takahashi et al., 2007; Takahashi and Yamanaka, 2006), there have been several advances in reprogramming methods to generate induced pluripotent stem cells (iPSCs). We used the Sendai virus (CytoTune-iPS 2.0; Thermofisher), which is a non-integrating RNA virus to transduce control and FXS patient fibroblasts with four transcription factors – Oct3/4, Sox2, Klf4 and c-Myc. Individual iPSC colonies began to emerge within two weeks, and we manually isolated and expanded multiple clonal iPSC colonies from each control and patient (**Fig. 3.1; Table 3.1**). All iPSC lines were confirmed to be karyotypically normal, and expressed markers of pluripotency by RT-PCR and immunofluorescence (**Fig. 3.2**). FXS patient lines did not express *FMR1* or FMRP (**Fig. 3.2**)

3.2.2. Differentiation of iPSCs into neural precursor cells (NPCs), neurons and cerebral organoids

iPSCs were differentiated into neural precursor cells (NPCs) using two established methods that we optimized in our lab. The first method involves an intermediate embryoid body (EB) stage (**Fig. 3.3 A**) (Marchetto et al., 2017; Marchetto et al., 2010), and the second is a monolayer-method using a commercially available kit (STEMdiff Neural System; Stemcell Technologies). NPCs generated from both methods were characterized by confirming expression of markers such as Nestin and SOX2 (**Fig. 3.3 B**), and we confirmed lack of *FMR1* expression in all FXS patient lines. There were no significant differences in the NPCs based on the

differentiation method and all experiments described in subsequent chapters used control and FXS NPC lines generated from both methods.

NPCs were differentiated into forebrain cortical neurons by withdrawing FGF and adding ROCK inhibitor Y-27632 (10uM), DAPT (10uM), BDNF (10ng/ml) and GDNF (10ng/ml) to the cultures. Neurons began to emerge from NPC cultures within 4 days and by day 21, a majority of neurons expressed neuronal markers such as MAP2, vGlut1 and TBR1 (**Fig. 3.3 C**). iPSC-derived neurons co-cultured with human fetal astrocytes (Stemcell technologies) using the Banker method (Banker and Cowan, 1977) for longer periods of time expressed more mature synaptic markers, and began to show dendritic spine-like protrusions (**Fig. 3.3 D,E**). Both control and FXS iPSC-derived neurons expressed excitatory postsynaptic markers (vGlut1) by DIV28 (**Fig. 3.3 F**). Whole cell patch clamp recordings on DIV28 iPSC-derived neurons generated using this method are functionally active and fire spontaneous action potentials (**Fig. 3.4 A-C**). We also plated iPSC-derived neurons on 48-well multielectrode arrays (MEA) (Axion biosystems) and measured spontaneous network activity over 6 weeks. We found that after ~3 weeks in culture, FXS iPSC-derived neurons appeared to have an increased mean firing rate and spontaneous network activity compared to controls (**Fig. 3.4 D-F**).

3D cerebral organoid cultures from human iPSCs have been used to model several disorders, including autism, Zika virus-induced microencephaly and Alzheimer's disease (Bershteyn, 2017; Cugola, 2016; Gonzalez et al., 2018; Iefremova, 2017; Lancaster, 2013; Lin et al., 2018; Mariani, 2015; Qian et al., 2016a; Raja et al., 2016). These cerebral organoids recapitulate many characteristics of human brain development and provide a unique opportunity to model neuronal development in a human cellular system. (Kadoshima et al., 2013; Lancaster et al., 2013; Mariani et al., 2015; Muguruma et al., 2015; Pasca et al., 2015; Qian et al., 2016a),

We generated control and FXS cerebral organoids using a spinning bioreactor method (SpinΩ) (**Fig. 3.5 A,B**) (Qian et al., 2016). By day 28, organoids began to show evidence of typical cellular organization seen in the developing human brain, such as Nestin+/SOX2+ neural precursor cells, and a layered organization of early cortical neurons (TBR1+, CTIP2+) (**Fig. 3.5 C**). Our primary goal in generating these organoids was to investigate phenotypes of proliferation and cell fate commitment in FXS, as described in subsequent chapters.

3.3. Discussion

The genetic mutation that gives rise to FXS is unique to the human condition. Attempts to introduce an expanded CGG repeat to silence *Fmr1* in mouse models have been unsuccessful, although the *Fmr1* KO mouse has been a vital resource in providing us with insight into the biology of FMRP. Therefore, a human model system that allows for experimental manipulation and expresses the actual mutation seen in patients is extremely valuable, and offers an unprecedented understanding of the disorder, and the potential to develop personalized treatments.

We have generated 8 control and 8 FXS patient iPSC lines, in addition to iPSC lines from patients with other neurodevelopmental disorders such as muscular dystrophy and DiGeorge syndrome. Additionally, cerebral organoids were generated from a separate set of 3 control and 2 FXS patient iPSCs that were not used to generate NPCs. To the best of our knowledge, this study uses the highest number of individual control and FXS patient lines, in addition to a previously published isogenic pair, in which the CGG repeat was excised to reactivate FMRP expression (Xie et al., 2016). Every experiment described in this dissertation was conducted in at least 3 individual control and 3 individual FXS patient lines. Although we isolated up 30 clones of each

iPSC line, we selected one or two clones per line at random to use in our experiments. Most experiments were conducted using a single clone per iPSC line; we reasoned that robust results across multiple individuals of varying genetic backgrounds provides stronger support for our conclusions than multiple clones from fewer individuals (Hoffman et al., 2017). The iPSC lines generated for this work have, and will continue to be shared with other researchers in the field, and we hope they will be a useful resource for the community.

A majority of the experiments described here were conducted in iPSC-derived neural precursor cells (NPCs). NPCs are multipotent cells that can be passaged every week, expanded and frozen down. On the other hand, it takes at least 3 weeks to generate immature iPSC-derived neurons, and the yield is relatively low. NPCs offer an important advantage over iPSC-derived neurons, in that they allow for more experiments to be conducted within a shorter period of time, and provide sufficient material for several types of experiments.

Furthermore, most studies in FXS have been carried out in postmitotic neurons from animal models. NPCs represent one of the earliest stages in human brain development and develop into neurons in a progressively lineage-restricted manner that can be experimentally controlled. Thus, NPCs provide us with a tool to model relatively understudied, critical developmental time-points in FXS, and to elucidate the role of FMRP at early stages of neurogenesis.

Although two NPC lines (1 control, 1 FXS) used in some of these experiments were generated using a different method, we did not find any significant differences in the neurogenic potential or expression of NPC markers between the methods. We saw some phenotypic variability across individuals in both genotypes, and across experimental replicates; however, a certain degree of variability is to be expected when working with human patient derived material

obtained from several different sources. Importantly, we identified one individual who appeared to have consistently low protein synthesis and PI3K signaling activity compared to other FXS patients. These results were consistent in experiments using fibroblasts as well as NPCs, and most likely reflect a patient-specific phenotype. Despite being a monogenic disorder, FXS patients present with a spectrum of clinical phenotypes (Berry-Kravis et al., 2018; Harris et al., 2008). This heterogeneity is likely the result of individual genetic variants and modifiers, and patient-derived iPSCs provide the ideal tool to dissect out these differences, which could help in the development of targeted treatment strategies. Thus, our work provides strong support for the use of patient iPSCs in disease-modeling, and highlights the potential for the development of patient-specific therapeutic interventions.

3.4. Figures and Tables

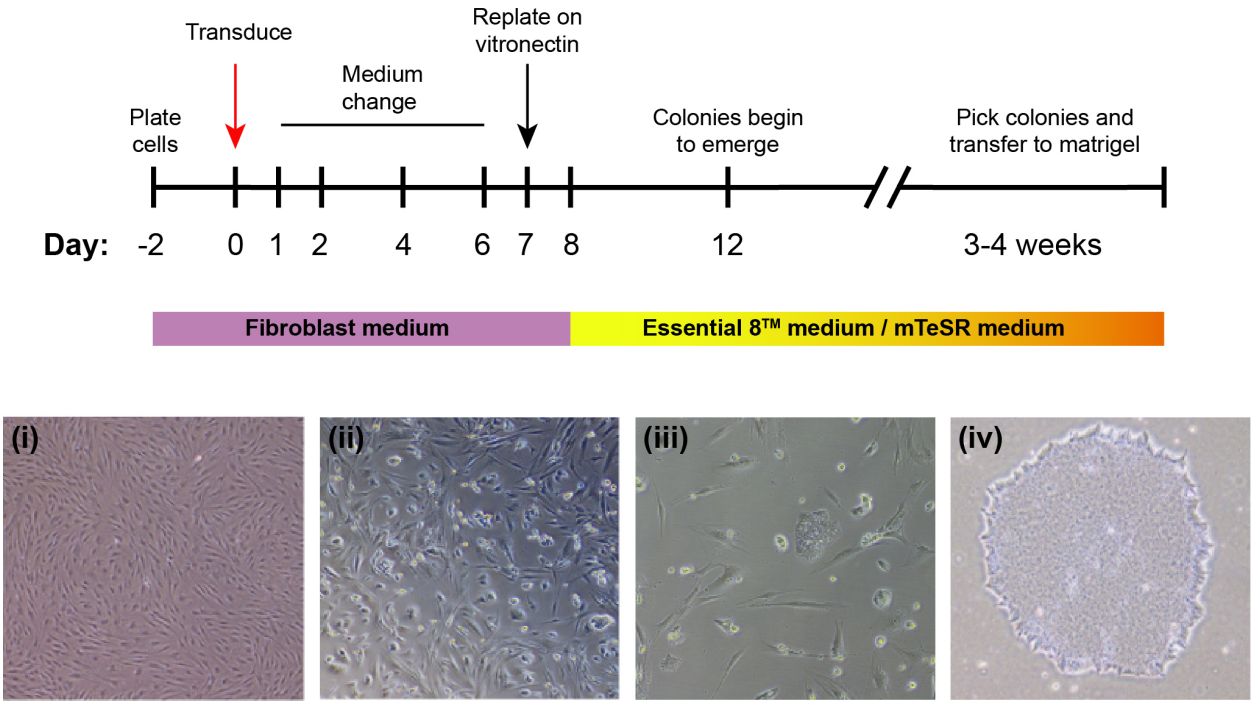


Figure 3.1. Sendai virus reprogramming of human fibroblasts to generate iPSCs

(Top) Schematic of reprogamming timeline and procedure. (Bottom) Representative images showing (i) fibroblasts before transduction (ii) fibroblasts 1 week post-transduction (iii) magnified view of a developing colony in SeV transduced fibroblast culture (iv) Representative image of a reprogrammed iPSC colony cultured on matrigel

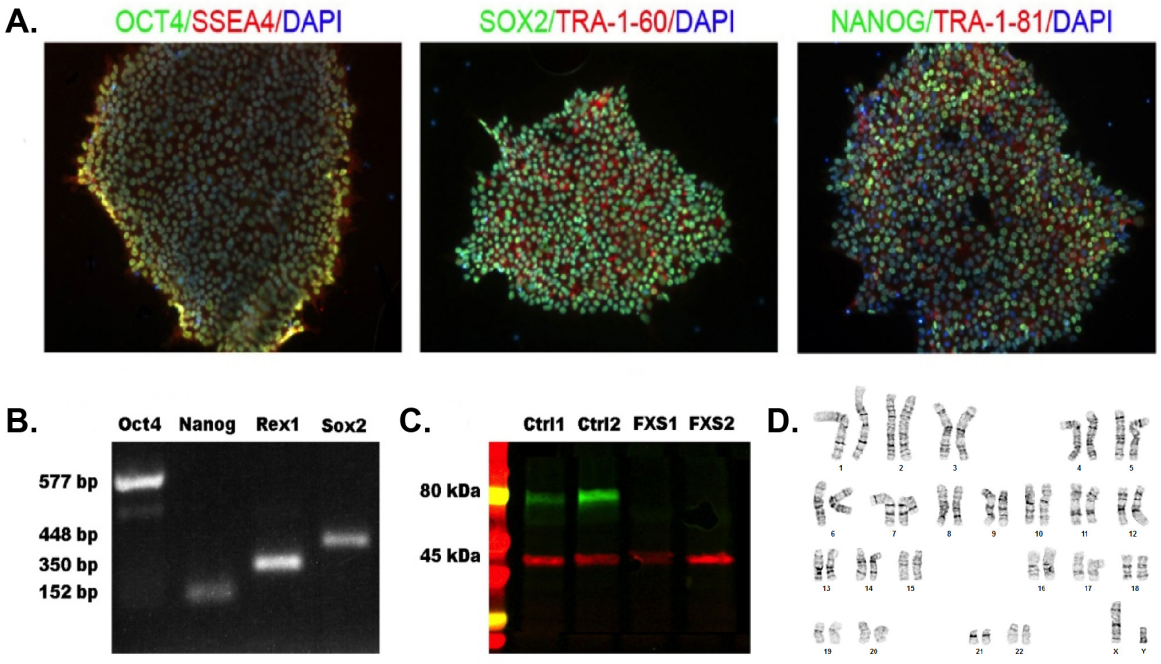


Figure 3.2. Characterization of human fibroblast derived iPSCs. All iPSC lines expressed markers of pluripotency using **(A)** immunofluorescence and **(B)** RT-PCR. **(C)** Lack of FMRP was confirmed in all FXS patient lines using western blotting; shown here is a representative blot of 2 control and 2 FXS iPSC lines **(D)** G-band karyotyping of all lines showed no karyotypic abnormalities; shown here is a representative normal karyotype

Table 3.1: Patient lines used in this study

Gender	Line ID	Diagnosis	Source	Used for NPCs	Used for Organoids
M	SC173	Control	CHOC	√	X
M	SC176	Control	CHOC	√	X
M	CHO21F	Control	Emory (J.C/C.H)	√	√
M	V44	Control	Rush (E.B.K)	√	X
M	V48	Control	Rush (E.B.K)	√	√
M	V36	Control	Rush (E.B.K)	√	X
M	C-01	Control	Emory	√	√
M	C-03	Control	Emory	√	√
M	C1-2	Isogenic Control	Emory (S.W.)	√	X
M	SC128	FXS	CHOC	√	X
M	CH095	FXS	CHOC	√	X
M	SO02	FXS	Emory (J.C/C.H)	√	X
M	SO03	FXS	Rush (E.B.K)	√	√
M	SO04	FXS	Rush (E.B.K)	√	√
M	SO05	FXS	Rush (E.B.K)	√	X
M	SO10	FXS	Emory	√	√
M	FXS	Isogenic FXS	Emory (S.W.)	√	X

FXS = fragile X syndrome, CHOC = Childrens Hospital of Orange County

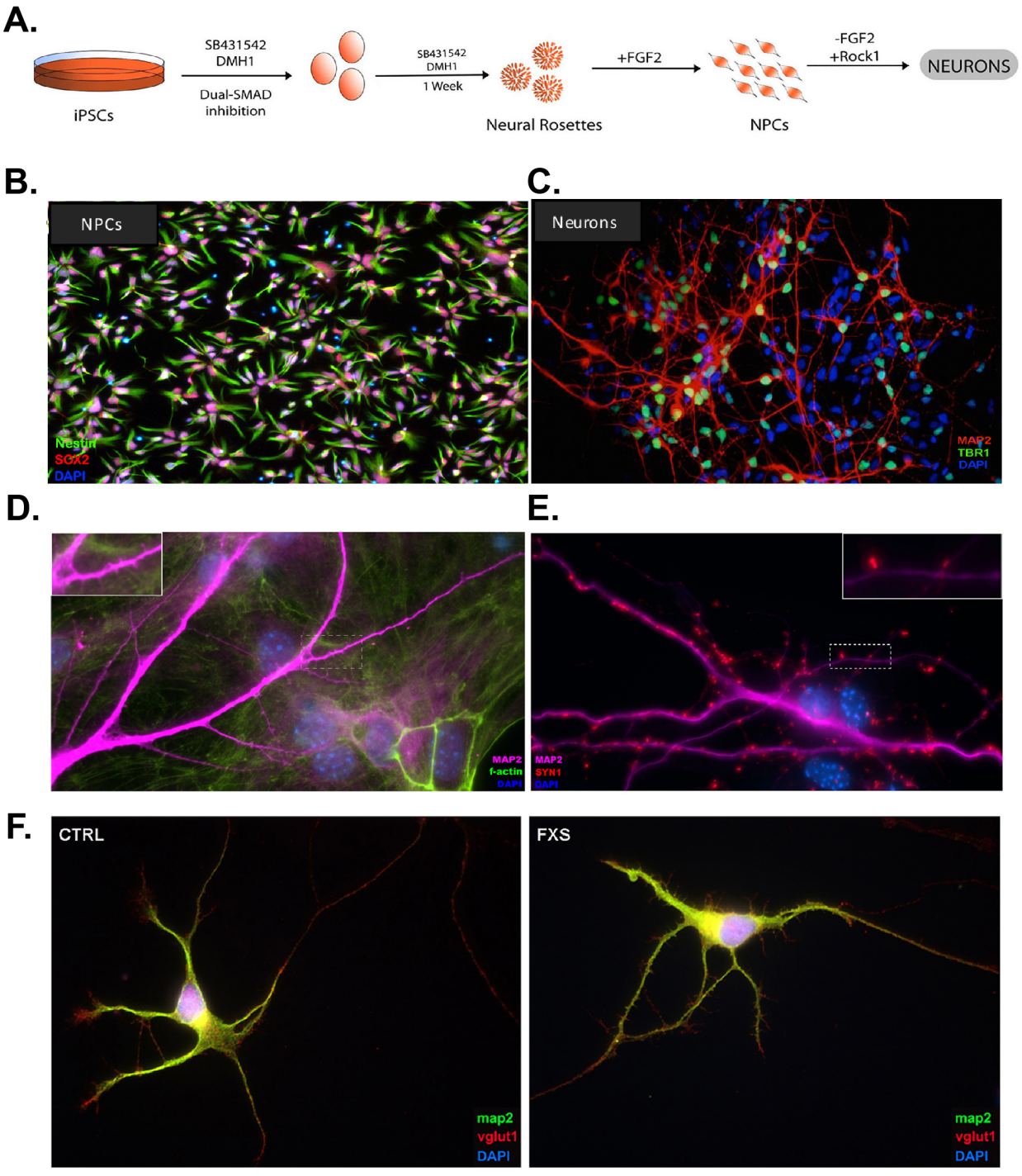

A.
iPSCs
SB431542
DMH1
Dual-SMAD
inhibition
SB431542
DMH1
1 Week
Neural Rosettes
+FGF2
NPCs
-FGF2
+Rock1
NEURONS
B.
NPCs
Nestin
SOX2
DAPI
C.
Neurons
MAP2
TBR1
DAPI
D.
MAP2
f-actin
DAPI
E.
MAP2
SYN1
DAPI
F.
CTRL
map2
vglut1
DAPI
FXS
map2
vglut1
DAPI

Figure 3.3. Characterization of iPSC derived NPCs and neurons. (A) Schematic of differentiation protocol to generate NPCs and neurons. **(B)** Representative image of NPCs stained for proliferative markers Nestin and SOX2 **(C)** Representative image of neurons stained for cortical layer marker TBR1 and neuronal marker MAP2 **(D,E)** DIV46 iPSC-derived neurons cocultured with fetal astrocytes have dendritic spine like protrusions that colocalize with synaptic markers (synapsin) **(F)** Representative image of control and FXS neurons stained with excitatory synaptic marker (vGlut1)

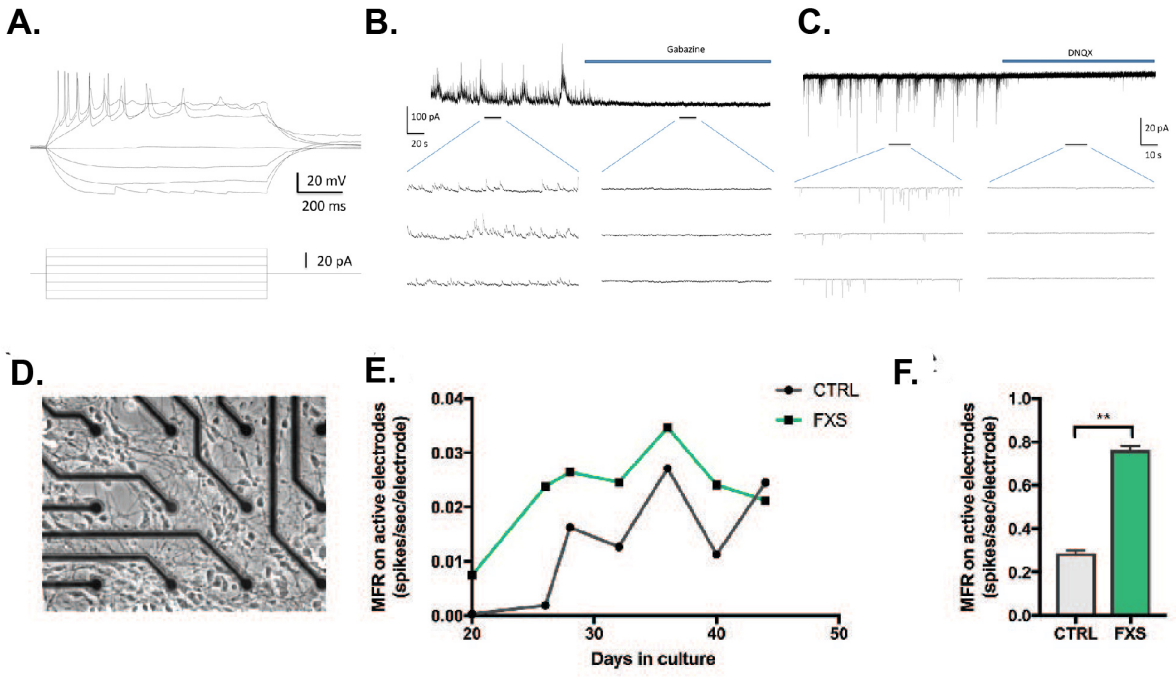


Figure 3.4. Electrophysiological characterization of iPSC-derived neurons. (A-C) Whole cell patch clamp recording from a single d28 iPSC-derived neuron. **(A)** Membrane responses of d28 iPSC-derived neuron to current injections. Action potentials are seen when depolarization currents are injected. **(B)** spontaneous inhibitory postsynaptic currents (sIPSCs) and **(C)** spontaneous excitatory postsynaptic currents (sEPSCs) were observed in d28 iPSC-derived neurons **(D-F)** iPSC-neurons plated on a 48-well microelectrode array dish. **(D)** iPSC-neurons plated on glass-bottom MEA well **(E)** Longitudinal measurements of mean firing rate (MFR) of iPSC-derived neurons from one control and one FXS patient. **(F)** Average of MFR from 3 controls and 3 FXS patients at DIV33. Data are shown as Mean±SEM (n = 3 control, 3 FXS, 5 technical replicates; **P < 0.01)

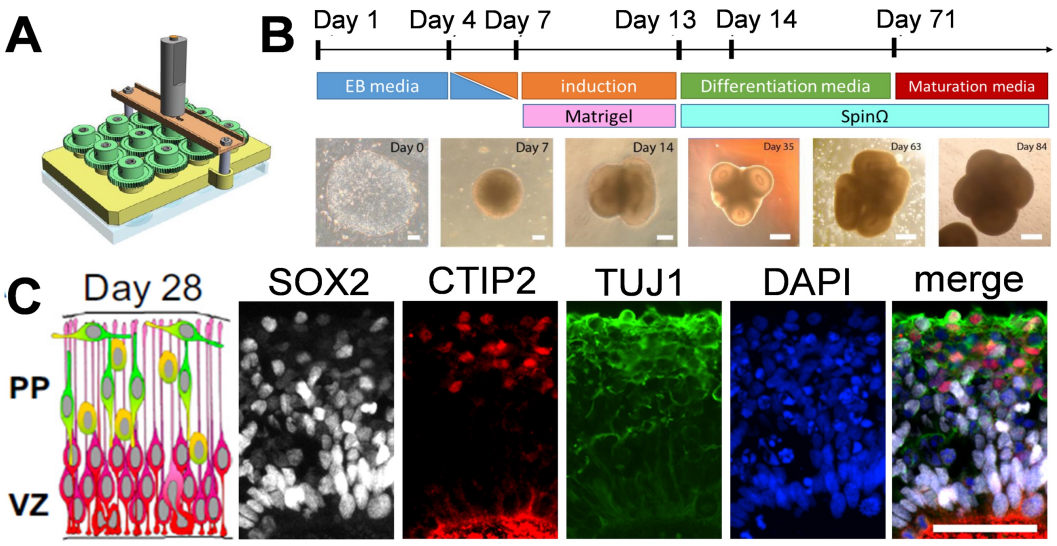


Figure 3.5. Cortical development in hiPSC-derived forebrain organoids. (A) Miniaturized bioreactor for 12-well plate. **(B)** Schematic diagram of the procedure for forebrain organoid culture and sample phase images at different stages. Scale bars: 200 µm. **(C)** Schematic diagrams and sample immunostaining images of forebrain organoids for apical neural progenitor layer (SOX2) and cortical plate layer neuron marker (CTIP2 and TUJ1) at day 28 Scale bars: 50 µm. Figure reproduced with permission from Zhexing Wen (Emory University), modified from (Qian et al., 2016)

CHAPTER 4: Dysregulated global translation in Fragile X patient derived cells

4.1. Introduction

The translation of messenger RNAs (mRNAs) into functional protein products is a highly complex, tightly-regulated, multi-step process that is critical to cellular development and function. Thus, any disruptions to this fundamental process could have widespread detrimental consequences, and defects in protein synthesis are an increasingly recurrent theme associated with neurological disorders (Scheper et al., 2007). The developing brain is particularly vulnerable to changes in the control or process of translation, and neurodevelopmental disorders like autism, schizophrenia and Rett syndrome have been linked to aberrant translation and mutations in components of the protein synthesis pathway (Berg et al., 2015; Bhattacharya et al., 2012; Gkogkas et al., 2013; Hoeffer et al., 2012; Ricciardi et al., 2011). Increased and dysregulated protein synthesis is a hallmark of fragile X syndrome (Richter et al., 2015), and multiple approaches to quantify and correct this increase have been applied in animal models of FXS. Elevated protein synthesis has been used as a readout to test genetic and pharmacological interventions in animal models of FXS; however, till date there have been no reports of this translational defect in a human disease-relevant model of FXS. Recent studies using FXS patient fibroblasts (Kumari et al., 2014) and patient-derived lymphoblastoid cell lines (Gross and Bassell, 2012) have shown that FXS patient derived non-neuronal cells exhibit increased protein synthesis in the absence of FMRP.

4.1.1 Overview of translation

Protein synthesis in eukaryotes is a dynamic process involving several regulatory factors that act in concert with transfer RNAs (tRNAs) and ribosomes, which are both key components of the translational machinery. Ribosomes are composed of ribosomal RNA (rRNA) as well as

protein, and are responsible for catalyzing the reaction to form peptide bonds between amino acids. Ribosomes provide a structure within which tRNAs can interact with and decode mRNAs in order to produce a protein. One end of each tRNA contains an anticodon that binds to the complementary codon sequence on the template mRNA, while the other end of the tRNA carries a specific amino acid corresponding to the codon. The process of translation is conventionally divided into three steps: initiation, elongation and termination. Translation initiation typically involves binding of the preinitiation complex to the 5' end of the target mRNA (Gingras et al., 1999; Hershey, 1991). The preinitiation complex consists of the 40S small ribosomal subunit, eukaryotic initiation factors (eIF3, eIF1, eIF1A & eIF5) and the ternary complex, which includes eIF2, GTP and the initiator methionyl-tRNA (Met-tRNA). This complex moves along the mRNA template from the 5' to the 3' direction until the Met-tRNA encounters an initiator codon (usually AUG) and forms a base pair (Kozak, 1989), allowing the dissociation of several initiation factors, and the subsequent binding of the 60S large ribosomal subunit to form the 80S ribosome complex.

During elongation, the ribosomal complex recruits appropriate aminoacyl-tRNAs (aa-tRNA) to its ribosomal acceptor site or A-site through base-pairing of the tRNA anticodon to the corresponding mRNA codon downstream of the initiator AUG. The ribosome catalyzes the transfer of the methionyl group from the initiator tRNA to the amino group of the newly recruited tRNA, resulting in the formation of a peptide bond. The mRNA along with the t-RNA and attached growing polypeptide then translocates into the P-site allowing another tRNA to be recruited into the A-site, and another peptide bond to be formed. This iterative process allows the nascent polypeptide chain to grow, and is mediated by several critical elongation factors (Dever and Green, 2012; Hershey, 1991). Translation of the mRNA is terminated when the ribosome

encounters a stop codon on the mRNA, and recruits a release factor that leads to the dissociation of the ribosomal complex and the subsequent release of the completed polypeptide chain (Dever and Green, 2012; Hershey, 1991).

4.1.2 Defects in signaling and translation in neurological disorders

Protein synthesis is central to the development and maintenance of cellular function in all organisms. It is unsurprising that disruption of any step or component involved in translation could lead to systemic defects, and dysregulated translation is a key emerging phenotype common to several neurodevelopmental disorders (English et al., 2015; Ricciardi et al., 2011) and neurodegenerative disorders. Mutations in proteins that regulate translation, such as 4E-BPs or eIF4E, have been linked to autism spectrum disorders (Gkogkas et al., 2013; Santini et al., 2017). Mutations in phosphatase and tensin homolog (PTEN) and tuberous sclerosis complex (TSC) have been associated with ASDs and give rise to downstream dysregulation of mTOR signaling, resulting in increased protein synthesis (Kelleher and Bear, 2008).

4.1.3 FMRP as a translational regulator

FMRP is an RNA-binding protein that is known to bind to several neuronal mRNA (Ashley et al., 1993; Darnell et al., 2011) Although there have been reports of FMRP activating translation (Bechara et al., 2009; Kwan et al., 2012), FMRP has primarily been shown to repress translation of its mRNA targets (Hou et al., 2006; Laggerbauer et al., 2001; Li et al., 2001; Muddashetty et al., 2007; Narayanan et al., 2007; Zalfa et al., 2003); however, the exact mechanism of translational control by FMRP remains unclear.

Studies have shown that FMRP regulates the initiation step of translation via interactions with eIF4E and the cytoplasmic FMRP-interacting protein 1 (CYFIP1) (De Rubeis et al., 2013; Napoli et al., 2008). FMRP is also known to associate with microRNAs and components of the RNA-induced silencing complex (RISC). Steady state levels of miR-124a were reduced in *dFmr1* mutant flies and were shown to affect dendritic arborization. MicroRNAs that have been linked to protein synthesis have also been shown to interact with FMRP, such as miR-125a, miR-125b and miR-132. One of the predominant mechanisms proposed for FMRP's role in regulating translation is by the stalling of polyribosomes. Several groups have shown that FMRP cosediments with actively translating polyribosomes, and it was found that FMRP binds to coding sequences of target mRNAs. Thus, it was proposed that FMRP acts as a physical block to impede ribosomal transit, and in the absence of FMRP, this block is relieved allowing for increased and dysregulated translation.

4.1.4 Techniques to measure protein synthesis

Traditional methods to quantify changes in protein synthesis have involved measuring the incorporation of radioactively labeled traces such as ^{35}S-methionine (Browder et al., 1992). However, several nonradioactive techniques have recently been developed that allow for the detection of protein synthesis with better spatial and temporal resolution, without the limitations of working with radioactive materials (Bowling et al., 2016; Dieterich et al., 2007; Dieterich et al., 2006; Schanzenbacher et al., 2016; Schmidt et al., 2009).

Surface sensing of translation (SUnSET) is a nonradioactive method of measuring translation that uses the antibiotic puromycin, which is a structural analog of tyrosyl-tRNA. When used at low enough concentrations, puromycin incorporates into nascent polypeptide

chains, terminating their elongation and resulting in the release of truncated puromycin-tagged peptides (Nathans, 1964; Schmidt et al., 2009). These puromycin-labeled peptides can then be detected using an antibody to puromycin, and the signal can be quantified as a measure of protein synthesis using standard techniques such as western blots or immunofluorescence (**Fig. 4.1 A**) (Schmidt et al., 2009). Another pioneering approach to visualizing and quantifying translation proteins takes advantage of "click-chemistry," which is a copper catalyzed reaction that covalently links azide and alkyne groups (Rostovtsev et al., 2002; Tornoe et al., 2002). Here, noncanonical amino acids that act as surrogates for methionine are modified to contain either an azide or an alkyne group (Beatty et al., 2005; Kiick et al., 2002). These modified amino acids become incorporated into nascent proteins and are then "clicked" onto tags bearing the corresponding alkyne or azide group (**Fig. 4.1 B**). Fluorescent noncanonical amino acid tagging (FUNCAT) utilizes fluorophore-conjugated affinity tags to allow for visualization of nascent protein synthesis (Beatty et al., 2006; Dieterich et al., 2010), while bioorthagonal noncanonical amino acid tagging (BONCAT) uses affinity tags like biotin to isolate and subsequently quantify the newly synthesized protein using immunoblots an understanding of changes in protein synthesis at a genome-wide scale. Other global profiling methods include stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002) and ribosome footprinting, which allows for the sequencing of ribosome-bound mRNAs that are being actively translated (Ingolia et al., 2009).

4.1.5. Chapter 4 rationale and objectives

The loss of translational control in FXS is believed to underlie several core deficits of the disorder and dysregulated and elevated global protein synthesis is a hallmark feature of FXS in

the *Fmr1* KO mouse (Dolen et al., 2007; Napoli et al., 2008; Qin et al., 2013; Udagawa et al., 2013). Studies using patient LCLs and fibroblasts show similar increases in global translation in the absence of FMRP (Gross and Bassell, 2012; Kumari et al., 2014), however, whether these phenotypes are recapitulated in human neural cells remains unknown. Here, we aimed to investigate whether key phenotypes seen in animal models of FXS were recapitulated in our human neuronal model. We used multiple independent approaches to quantify protein synthesis and showed consistent results across methods and patient lines. We show that fragile X patient-derived neural cells exhibit aberrant, increased protein synthesis that can be corrected using pharmacological reduction of key signaling pathways.

The experiments described in this chapter were carried out in 3 control and 3 FXS patient lines that were also used in Chapter 5. Additionally, puromycin labeling experiments were done in an isogenic pair as indicated. Isogenic iPSC lines were a gift from Steve T. Warren. Flow cytometry, immunoblotting, and protein synthesis quantification was done by **Nisha Raj.**

4.2. Results

We aimed to investigate whether key phenotypes seen in animal models of fragile X syndrome were recapitulated in our human cellular model. Using control and FXS patient fibroblasts, as well as neural progenitor cells (NPCs) described in chapter 2, we showed that signaling pathways are disrupted, and global protein synthesis is elevated in fragile X syndrome.

4.2.1. BONCAT and SUnSET show increased global translation in FXS patient cells

Various techniques to quantify protein synthesis have been applied to models of FXS, and several groups have found that global translation is increased in FXS. We used two of these

well-established biochemistry-based methods – bioorthagonal noncanonical amino acid tagging (BONCAT) and Surface sensing of translation (SUnSET) – to measure protein synthesis in control and FXS patient NPCs. Using these two independent methods, we show that FXS patient NPCs exhibit increased translation compared to controls.

BONCAT quantifies the incorporation of a methionine surrogate, azidohomoalanine (AHA), as a read-out of nascent protein synthesis. The azide group on the AHA can be covalently bound by a biotin-alkyne probe via copper catalyzed click-chemistry, and the biotin signal can be detected on a blot using a streptavidin antibody. Cells were methionine-starved for 1 hours prior to incubation with AHA for 2 hours. Using 3 control and 3 FXS patient NPC lines, we found that FXS patient NPCs had increased AHA incorporation compared to controls, suggesting that these patient NPCs had higher protein synthesis (**Fig. 4.2**).

We used puromycin labeling (SUnSET) as a second measure of protein synthesis in patient derived NPCs. A recent study showed that a subset of FXS patient cells have increased translation as measured by puromycin labeling (Jacquemont et al., 2018). At low concentrations, puromycin is incorporated into elongating polypeptides, terminating their translation, and causing them to be released from the ribosomal complex. The amount of newly synthesized protein can be quantified using an antibody against puromycin. We show that puromycin incorporation is increased in FXS patient derived NPCs compared to controls (**Fig. 4.3 A**). We also showed that puromycin incorporation was greatly increased in an FXS patient NPC line compared to its isogenic-corrected pair, further demonstrating that the loss of FMRP gives rise to increased protein synthesis in FXS patient NPCs (**Fig 4.3 B**).

4.2.2. Evidence of increased PI3K signaling in FXS

We hypothesized that signaling via the phosphoinositide-3-kinase (PI3K) and extracellular-signal-regulated kinase (ERK) pathways would be increased in FXS patient NPCs compared to control NPCs. Using 4 control and 4 FXS patient lines across two independent western blotting experiments, we found that p110β expression in FXS patient NPCs was increased (**Fig 4.5 A,B**), although there was no significant difference in ERK signaling (**Fig. 4.5 C**). These results are consistent with previous work from our lab, although it is important to note that p110β expression was variable within biological groups, and one FXS patient line had consistently low p110β expression.

4.2.3. Increased translation in FXS patient NPCs may be PI3K signaling-dependent

One of the key goals of research using human patient derived iPSCs is to investigate therapeutic strategies in a patient-specific manner. This requires the development of reproducible, scalable, high-throughput assays that allow for the reliable measurement of specific phenotypes. To this end, we adapted our established methods of quantifying translation to use in flow cytometry-based assays. We used a modified Fluorescent Non–Canonical Amino acid Tagging (FUNCAT) method (Dieterich et al., 2010), wherein control and FXS patient cells were treated with AHA, and newly synthesized proteins are "clicked" onto using a fluorophore-conjugated alkyne, instead of biotin. For the puromycin-labeling studies, we used a puromycin antibody that was directly conjugated to a fluorophore to label new proteins (Schmidt et al., 2009). In both these methods, the nascent proteins labeled with fluorescent molecules can be analyzed using a flow cytometer, and the median fluorescent intensity (MFI) value provides a quantification of protein synthesis. We show that AHA labeling is increased in FXS patient

fibroblasts (**Supp. Fig. 4.1**) as well as in FXS patient NPCs (**Fig. 4.4**). Despite the variability across individuals (**Supp. Fig. 4.2)**, these findings are reproducible and significant across multiple patient lines . Furthermore, using puromycin labeling in 4 control and 4 FXS patient NPC lines, we see a similarly robust increase in translation in FXS patient NPCs compared to controls (**Fig. 4.4**; **Supp. Fig. 4.2**). Both AHA and puromycin fluorescent signals were shown to be translation-dependent by showing that pre-treatment with a protein synthesis inhibitor (anisomycin) significantly reduced the fluorescent signal. Additionally, no-AHA and no-puro negative controls were included to account for any background fluorescence.

Given our finding that PI3K signaling is dysregulated in FXS, and since work from our lab and others suggests that these signaling defects underlie the increase in translation (Bhattacharya et al., 2016; Gross and Bassell, 2012; Gross et al., 2015a; Gross et al., 2010; Gross et al., 2015c; Sharma et al., 2010), we hypothesized that treatment with p110β inhibitors and/or S6K1 inhibitors would correct the overactive protein synthesis in FXS patient NPCs. Indeed, we found that pre-treatment with either of these inhibitors normalizes the translational defect in a subset of FXS patient NPCs (**Fig. 4.5 D**).

4.3. Discussion

We have shown that the loss of FMRP in human patient derived cells results in dysregulated signaling and an increase in global translation. We validated our findings in multiple individual control and FXS patient iPSC lines, as well as in one isogenic pair. Furthermore, we used two different strategies to label newly synthesized proteins and measured them using both biochemistry based methods as well as using our flow cytometry method. The increase in global translation is moderate, but significant, despite the expected variability that

results from using multiple individual iPSC lines. Importantly, one patient NPC line did not appear to have this phenotype of elevated protein synthesis and increased p110β expression. Upon investigation of this patient's fibroblasts, we found that once again, they showed similar levels of translation to controls. However, in subsequent experiments, this patient was found to have increased proliferation similar to other FXS patient lines, suggesting that elevated protein synthesis may not be as robust of a phenotype across FXS patients. Further investigation of this patient would provide more insight into why we see this difference.

Our results using PI3K and S6K inhibitors are promising, and future studies could be directed at testing a combination of therapeutic interventions using patient derived NPCs. Several promising interventions have been shown to have a corrective effect in FXS, and yet we have not identified an effective treatment for humans. Furthermore, targeting key signaling pathways could have diverse effects, of which we remain unaware. A potential future study could look compare the transcriptional and proteomic changes that are induced in control and FXS patient NPCs by treatment with the different drugs that have been shown to be most effective in FXS. This approach could help to identify common pathways that are altered that may be beneficial and may reveal new targets for treatment.

Alternatively, strategies to reactivate *FMR1* expression could be applied to see if the increase in protein synthesis is solely due to the loss of FMRP. Several groups have recently begun to investigate strategies to reintroduce FMRP. While there is a lot more research required into delivery and targeting methods, this approach could prove to be extremely successful, particularly if it could be done *in utero*.

4.4. Figures

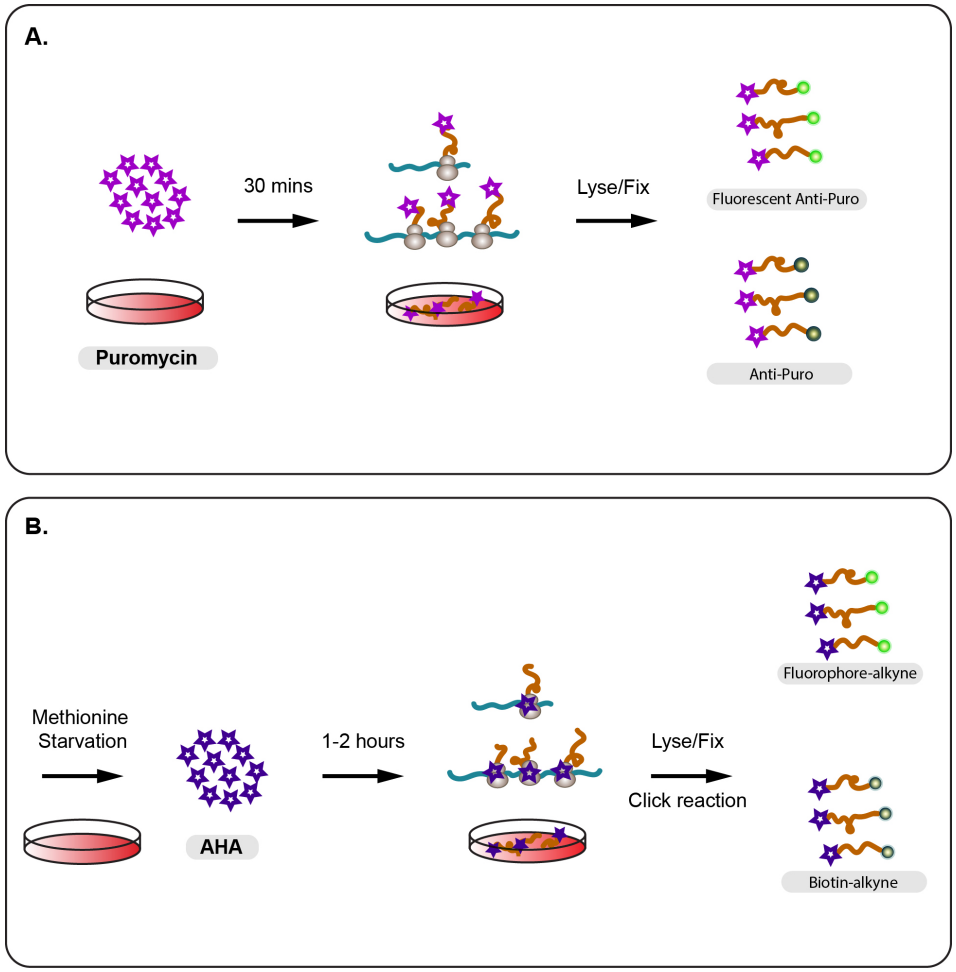


Figure 4.1. Techniques to measure protein synthesis. (A) Schematic showing SUnSET or puromycin labeling to quantify newly synthesized proteins. At low doses, puromycin is incorporated into elongating polypeptides and subsequently terminates translation, releasing labeled polypeptides. **(B)** Schematic showing azidohomoalanine (AHA) labeling method wherein methionine-starved cells incorporate AHA into newly synthesized proteins. AHA is "clicked" onto a conjugated alkyne to allow visualization of protein synthesis using biochemistry or fluorescent based methods

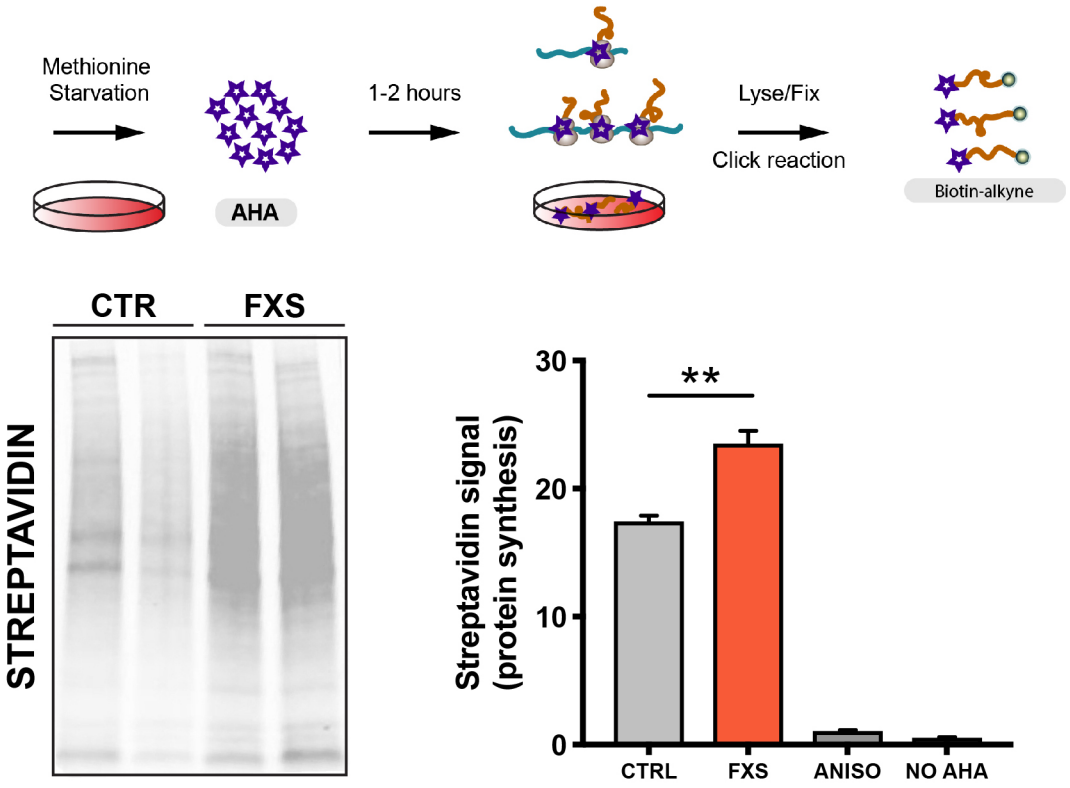


Figure 4.2. BONCAT labeling shows increased translation in FXS patient NPCs

AHA labeled lysates from 3 control and 3 FXS patient NPCs labeled with biotin-conjugated alkyne and separated via SDS-PAGE to visualize AHA incorporation. (Left) Representative blot showing 2 control 2 FXS NPC lines. (Right) Densitometry analysis of streptavidin signal normalized to GAPDH signal per lane shows increased AHA incorporation in FXS, indicative of increased protein synthesis (unpaired t-test, **$p<0.01$, n=3 controls 3 FXS, across two experiments). Data are shown as mean ± SEM.

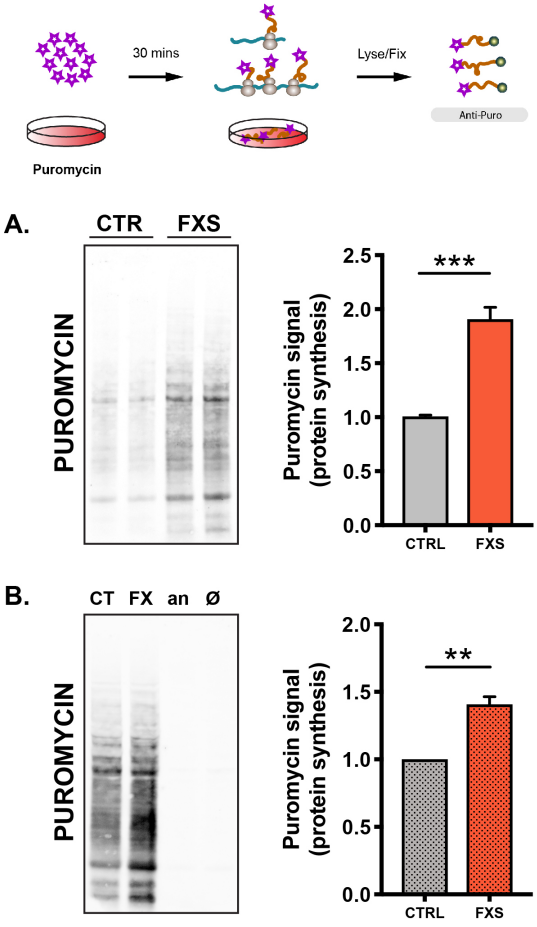


Figure 4.3. SUnSET labeling shows increased translation in FXS. (A) Increased puromycin incorporation shows increased protein synthesis in FXS patient derived NPC lines compared to controls. (Left) Representative blot showing 2 control 2 FXS NPC lines. (Right) Densitometry analysis of puromycin signal normalized to GAPDH signal per lane shows increased incorporation, indicative of elevated translation in FXS patient NPCs (unpaired t-test, ***$p<0.001$, n=3 controls 3 FXS, across two experiments). Data are shown as mean ± SEM. **(B)** Increased protein synthesis in FXS NPC line compared to isogenic control NPC line (unpaired t-test, **$p<0.01$, n= 3 experiments). Data are shown as Mean ± SEM.

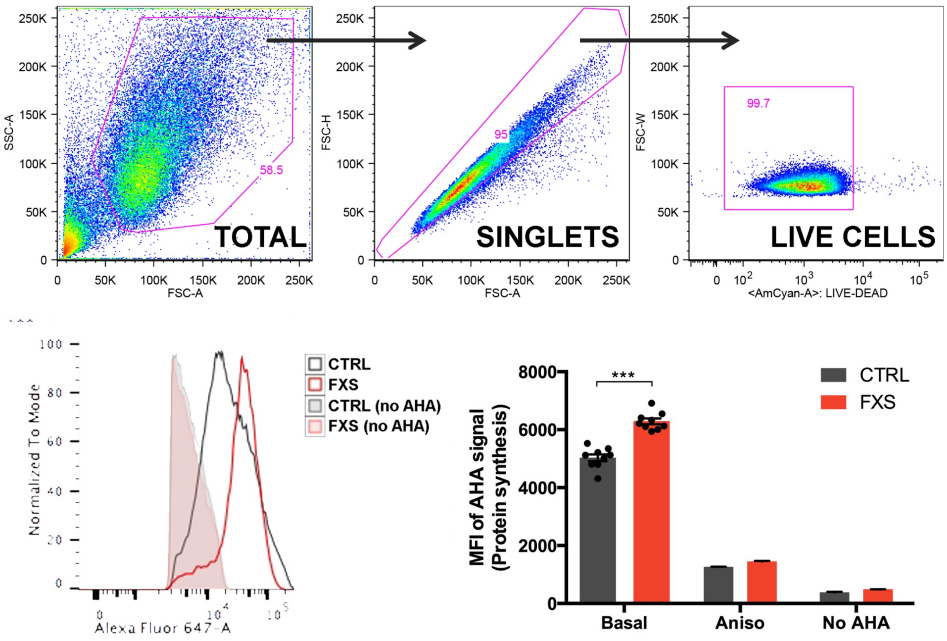


Figure 4.4. Quantification of protein synthesis using flow cytometry shows increased translation in FXS patient cells. (A) Gating strategy to isolate single, live cells for analysis **(B)** Histogram of one control and one FXS patient NPC line showing median fluorescence intensity (MFI) signal. FXS patient line is right shifted, indicating higher protein synthesis. **(C)** Basal protein synthesis is elevated in FXS NPCs compared to controls (n=3 controls, 3 FXS, across 3 experiments; ***P < 0.001). Data are shown as Mean ± SEM.

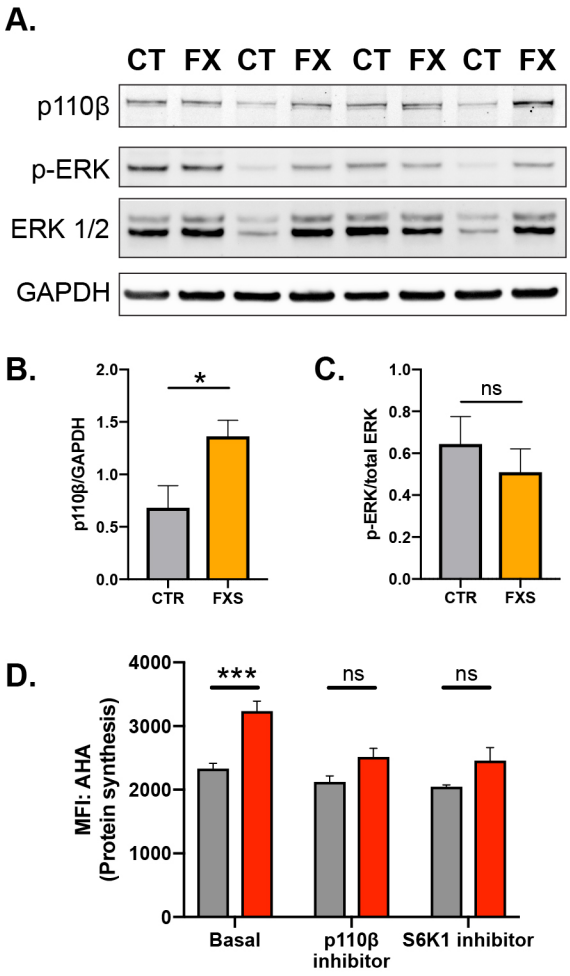
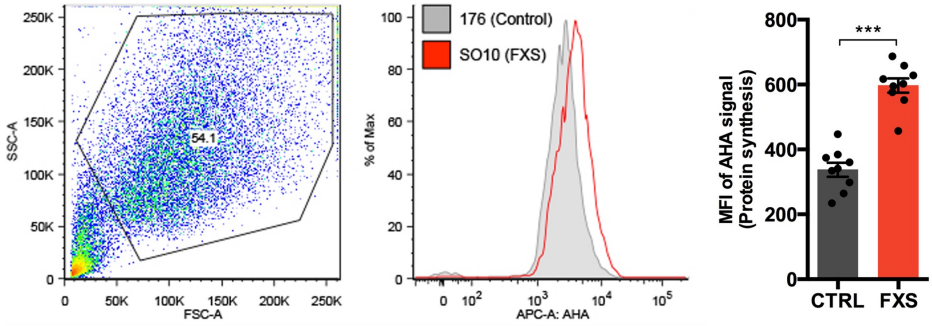
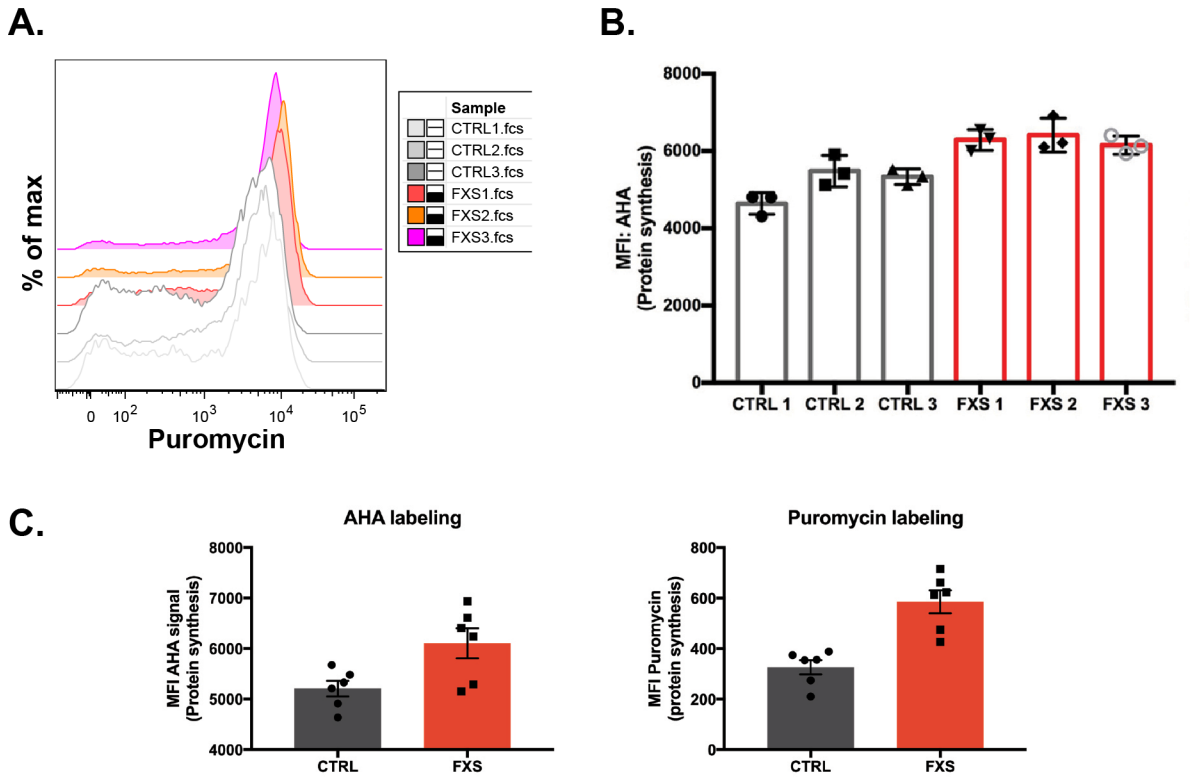


Figure 4.5. p110β expression is increased in FXS and p110β and S6K1 inhibitors correct protein synthesis defect in FXS. (A) Representative blots for p110β and phospho- and total ERK 1/2 in control and FXS patient NPCs **(B, C)** Quantification of western blots of (B) p110β and (C) phospho-/total ERK 1/2 in control and FXS patient NPCs (unpaired t-test, *p<0.05, n= 4 controls 4 FXS, 2 experiments). Data are shown as mean ± SEM **(D)** Treatment with p110β inhibitors and S6K1 inhibitor normalizes elevated translation in FXS patient NPCs (n= 3 controls 3 FXS, across two experiments; Two-way ANOVA, Sidak's test, *** adj P=0.0004). Data are shown as Mean ± SEM.



Supp. Fig 4.1. Measurement of protein synthesis using flow cytometry quantification of AHA signal shows increased translation in FXS patient fibroblasts. (Left) Total cell gate (Middle) Representative histogram showing one control and one FXS patient Mean Fluorescence Intensity (M.F.I) signal for AHA. FXS patient is right shifted indicating increased protein synthesis. (Right) Quantification of mean protein synthesis in 4 control and 4 FXS patient fibroblast lines, over two experiments, ***P < 0.001. Data are shown as Mean ± SEM.



Supp. Fig 4.2. Flow cytometry analysis of protein synthesis in control and FXS patient NPCs. (A) Representative histograms showing global protein synthesis in individual control and FXS patient NPCs. FXS patients are right shifted indicating increased translation. (B) Variability in AHA signal across and within 3 control and 3 FXS patients, with 3 technical replicates per line. (C) AHA labeling and Puromycin labeling yield similar results. Data are shown as Mean ± SEM.

CHAPTER 5: Altered cell fate and proliferation profiles in FXS patient cells

5.1. Introduction

The development of the human brain is a protracted and dynamic process that requires the precise orchestration of a sequence of complex cellular, molecular and genetic events (Stiles and Jernigan, 2010). The tight regulation of early events during neurogenesis is critical to the establishment of appropriate neural circuitry and functional connectivity in the brain. Disruptions to this process have profound consequences, and several neurodevelopmental disorders converge on molecular pathways that play a role in regulating proliferation, migration and differentiation of neural cells (Ernst, 2016).

5.1.1. An overview of cortical neurogenesis

Neural stem cells first emerge at the end of gastrulation as a single layer of neuroepithelial cells lining the ventricular cavity in what will develop into the ventricular zone (Altmann and Brivanlou, 2001). These multipotent cells produce the apical radial glial cells (aRGs) that populate the subventricular zone (SVZ) – the RG cells eventually give rise to all the neurons, neural progenitors and glia in the neocortex (Stiles and Jernigan, 2010). During early neurogenesis, these aRGs undergo symmetric divisions producing two daughter cells with each division, expanding the neural progenitor pool, and during later stages, aRGs shifts towards asymmetric division to produce a daughter aRG cell and a neuron, an intermediate progenitor (IP) cell, or a basal RG cell (Rakic, 1995). Basal IP cells undergo symmetric divisions to eventually produce postmitotic neurons, while apical IP cells directly give rise to neurons. Neurogenesis occurs in waves, with early progenitors and neurons migrating radially outwards to their final positions past layers of older cells, giving rise to the characteristic “inside-out” cortical

architecture where early-born neurons make up the deeper layers while later born neurons form the superficial layers of the cortex (Noctor et al., 2004).

5.1.2. Altered cell fate and proliferation in neurodevelopmental disorders

Despite their clinical heterogeneity and diverse etiologies, individuals with neurodevelopmental disorders share several features that may arise from common disruptions to early events in neurogenesis (Ernst, 2016). Furthermore, many of these disorders have been associated with defects in key signaling pathways that mediate critical developmental processes like cell proliferation, neural migration, neurite outgrowth and synaptogenesis (Mao et al., 2009; Sugathan et al., 2014). Microencephaly and macroencephaly are overrepresented in individuals with neurodevelopmental disorders, and these defects may stem from aberrant cell proliferation (Bernier et al., 2014; Buxbaum et al., 2007; Gilmore and Walsh, 2013; Marchetto et al., 2017; Nagamani et al., 2011). Several studies have used neural progenitor cells (NPCs) to model defects in proliferation and differentiation during development. One study using autism patient iPSC-derived NPCs showed increased proliferation in ASD patient NPCs that correlates with a macroencephaly endophenotype (Marchetto et al., 2017). The schizophrenia associated *DISC1* mutation also gives rise to altered proliferation and differentiation of cultured and iPSC-derived NPCs (Wen et al., 2014).

5.1.3. FMRP as a regulator of neurogenesis

Aberrant synaptic plasticity and neuronal connectivity are thought to underlie the severe cognitive and behavior deficits of Fragile X syndrome (FXS). The role of FMRP as a regulator of translation has been studied extensively; however, there is a growing body of evidence that

suggests that it may also be involved in the regulation of cell proliferation and other earlier neurogenic processes (Luo et al., 2010). One of the first studies to investigate the consequence of FMRP deficiency early on in development use cultured NPCs from *Fmr1* KO mice or postmortem human fetuses (Castren et al., 2005). They found that the loss of FMRP in this culture system resulted in an increase in Tuj1+ neurons and a decrease in glial cells (Castren et al., 2005). Additionally, they showed that there was increased BrdU incorporation in the SVZ of *Fmr1* KO mice compared to controls. Subsequently, FMRP deficiency was found to affect proliferation of neural progenitors in *dFmr1* mutant flies (Callan et al., 2010) as well as in *Fmr1* KO mice (Luo et al., 2010). In *dFmr1* mutant flies, dFMRP was also shown to regulate oogenesis by controlling the cell cycle progression of germ cells via the E3 ubiquitin ligase Cbl (Epstein et al., 2009). Adult hippocampal NPCs cultured from *Fmr1* KO mice showed increased proliferation, although in contrast to an earlier study, there was a reduction in postmitotic neurons and an increase in glia (Luo et al., 2010). Furthermore, FMRP was shown to specifically regulate the differentiation of glutamatergic cell lineages, since *Fmr1* KO mice exhibit abnormal accumulation of glutamatergic TBR2 expressing progenitors in the SVZ and an increased density of layer V pyramidal neurons (Tervonen et al., 2009). Aberrant activation of adult neural stem cells was linked to reduced neurogenesis and cognitive dysfunction in *Fmr1* KO mice (Li et al., 2016). However, some studies have shown that FMRP deficiency does not affect proliferation or neurogenesis, but it may give rise to defects in neuronal migration (La Fata et al., 2014) or the abnormal gene expression (Bhattacharyya et al., 2008) . More recently, studies have shown that altered calcium signaling may contribute to neurogenic defects in human iPSC-derived NPCs (Danesi et al., 2018).

Constitutive activation of the mTOR signaling pathway in tuberous sclerosis (TSC) has been linked to an increase in proliferation of NPCs and a reduction in neuronal differentiation in TSC2-deficient cells compared to controls (Sundberg et al., 2018). Inhibition of mTOR signaling by rapamycin treatment normalized these phenotypes, suggesting that these defects in proliferation and neurogenesis were mTOR-mediated (Sundberg et al., 2018). Signaling pathways known to be dysregulated in FXS have also been found to mediate neurogenesis. Components of the PI3K/Akt/mTOR signaling pathway have been implicated in cell survival, proliferation, migration, differentiation and maturation (LiCausi and Hartman, 2018; Waite and Eickholt, 2010; Yu and Cui, 2016). Furthermore, predicted and known targets of FMRP include mRNAs that are involved in cell cycle regulation and cell proliferation, such as CDK4, GSK3β and cyclin D1 (Luo et al., 2010). FMRP was shown to bind and regulate the translation of GSK3β, a key component of the WNT-/β-catenin signaling pathway, which is a critical regulator of cell fate and proliferation (Luo et al., 2010; Marchetto et al., 2017).

5.1.4. Chapter 5 rationale and objectives

Several studies have shown that elevated PI3K and mTOR signaling are linked to aberrant protein synthesis in FXS, and dysregulation in these pathways may contribute to deficits in neuronal development. The translational regulation of FMRP mRNA targets has been extensively studied in postmitotic neurons; however, relatively little is known of the regulatory role FMRP plays at an earlier developmental stage in humans.

We aimed to investigate the function of FMRP during early events in human neurogenesis. We hypothesized that the loss of FMRP would lead to altered or aberrant cell fate commitment and proliferation, similar to that seen in the mouse model (Castren et al., 2005; Luo

et al., 2010; Tervonen et al., 2009). Furthermore, we wanted to expand upon our results from Chapter 4, and explore the connection between dysregulated protein synthesis in FXS and neurogenesis. We developed a high-throughput multiparametric flow cytometry assay to simultaneously quantify proliferation and protein synthesis in a number of control and FXS patient cells. Using this assay, we showed that FMRP deficiency in actively proliferating NPCs resulted in robust defects in translation, while these defects were less pronounced in terminally differentiated cells.

We also show that FXS patient iPSC-derived neural progenitor cells (NPCs) and cerebral organoids have increased proliferation, altered cell fate and aberrant neuronal differentiation compared to controls. RNAseq analysis of control and FXS patient iPSC-derived cerebral organoids suggested that pathways linked to cell fate determination, progenitor proliferation, and neurogenesis are dysregulated in FXS. Lastly, splicing analysis revealed that FXS patient cerebral organoids and NPCs showed a novel splicing defect of increased inclusion of an exon in the 5'UTR of a cell-cycle regulated kinase, aurora kinase A (*AURKA*). Our findings definitively implicate FMRP as a mediator of neurogenesis and cell fate specification. Furthermore, we have identified a previously unknown link between translation and neurogenesis in FXS.

The NeuroMIP experiments described in this chapter were carried out in 5 control and 6 FXS lines. BrdU labeling was done in 3 control and 3 FXS NPC lines, in addition to one isogenic FXS and corrected control NPC line. The organoids were generated from 3 additional control and 3 FXS patient iPSCs not previously used to generate NPCs, and RNAseq and splicing analysis was done on organoids. The development of NeuroMIP, BrdU labeling, flow cytometry and immunofluorescence experiments described in this chapter were done by **Nisha Raj.** Generation and maintenance of organoid cultures was done by Zhexing Wen and Ying Zhou

(Emory University), and cDNA libraries were generated by Matthew Taliaferro (UC Denver) and Feiran Zhang (Emory University) Data analysis for RNAseq and alternative splicing analysis was done by Matthew Taliaferro (UC Denver).

5.2. Results

5.2.1. Development of a multiparametric assay to measure cell-type specific phenotypes

We wanted to assess the role of FMRP as a mediator of neurogenesis and proliferation, in addition to its well-established function as a negative regulator of translation. We aimed to assess cell fate commitment, and to quantify cell-type specific proliferation and protein synthesis in a high-throughput manner. To this end, we developed a multiparametric flow cytometry-based assay to characterize the differentiation profile of NPCs, and to measure specific molecular phenotypes within these defined neuronal subpopulations. We designed and optimized panels of antibodies (**Table 5.1**) that allowed us to (1) identify and define specific neural subtypes based on expression of validated markers (2) measure proliferation using an antibody to KI67, and (3) quantify protein synthesis in puromycin labeled samples using a fluorophore-conjugated antibody to puromycin. Our 8-color NeuroMIP panel includes antibodies to identify neural stem cells (NSCs), radial glia-like cells (RGs), neuroblasts, immature neurons, glia and mature neurons. For these experiments, we withdrew FGF from our NPC cultures after 6 days and harvested cells for analysis at day 10; this allowed for spontaneous differentiation that enabled us to assess cell fate commitment and investigate phenotypes in a heterogeneous population of neuronal cells.

5.2.2. Increased proliferation in FXS patient NPCs and cerebral organoids

We assessed proliferation in control and FXS patient NPCs, including one pair of isogenic NPC lines, by measuring incorporation of the thymidine analog 5-bromo-2'-deoxyuridine (BrdU). 24 hours after plating, NPCs were serum-starved to synchronize, incubated with BrdU for 16 hours and signal was detected with an α-BrdU-peroxidase antibody (Millipore). A colorimetric ELISA for BrdU incorporation showed a significant increase in BrdU incorporation in FXS patient lines over control lines (**Fig. 5.1 A**). This result was consistent in the isogenic pair, wherein the corrected NPC line showed reduced BrdU incorporation compared to the FXS NPC line (**Fig. 5.1 A**).

We further showed that FXS patient NPCs had an increased population cells that expressed KI67 and phosphohistone-H3 (**Fig. 5.1 B**). KI67 labels all actively proliferating cells, while pHH3 is expressed in cells in the late G2 and M phase of the cell cycle. Thus, FXS NPCs have more mitotically active, proliferating cells compared to controls.

Since our NPC cultures contain a heterogeneous population of cells that may include postmitotic neurons or quiescent progenitor cells, we wanted to assess proliferation in FXS patient lines in a more pure population of cells. Using flow cytometry, we selected for only actively proliferating Nestin- expressing neural progenitor cells by gating out cells expressing a markers of differentiating neurons TUJ1 (NeuroMIP1, **Table 5.1**). We found that there were more Nestin+ cells in FXS NPCs compared to controls and fewer Nestin+ cells in the isogenic NPC line compared to its paired FXS NPC line (**Fig. 5.1 C**). We measured KI67 signal within this TUJ1-/Nestin+ population and saw that FXS patient NPCs expressed more KI67 compared to controls, suggesting that this population of cells had higher rates of proliferation in FXS compared to controls (**Fig. 5.1 D**). Once again, similar results were obtained from an isogenic

pair of NPC lines (**Fig. 5.1 D**). Furthermore, this Nestin+/KI67^{hi} population of NPCs in FXS patients showed increased puromycin signal (**Supp. Fig. 5.1**). Thus, we show that FXS patient NPCs had a higher population of active proliferating cells that also had higher protein synthesis.

This defect in proliferation was also present in cerebral organoids generated from two additional FXS patient iPSC lines that were not used for the earlier NPC experiments. We stained for markers of proliferation in 28 day old organoids and found that FXS patient organoids had a higher percentage of KI67+/SOX2+ proliferative cells (**Fig. 5.1 E,F**), providing further evidence that FMRP deficiency results in a loss of control of proliferation in neural cells.

5.2.3. Altered cell fate in FXS

A possible consequence of increased proliferation in neural progenitor cells is altered cell fate commitment and abnormal neurogenesis. We hypothesized that aberrant proliferation in FXS patient NPCs alters the kinetics and profile of neurogenesis, which could lead to the subsequent delayed maturation of neurons and synaptic dysfunction in FXS. Using our NeuroMIP2 panel (**Table 5.1**) we analyzed the differentiation profiles of four control and four FXS patient NPCs. We found that FXS patient NPCs had more Nestin+/SOX2+ cells as well as more Nestin+/GFAP+ radial glia-like cells (**Fig. 5.3 A,B**). Doublecortin (DCX) positive early neuroblasts were also more abundant in FXS NPCs compared to controls (**Fig. 5.3 C**). In contrast, early-born DCX+ neurons that had acquired the expression of MAP2, as well as more mature MAP2+ neurons were less abundant in FXS NPCs compared to controls (**Fig. 5.3 D,E**). Interestingly, although there were more active glia (Nestin+/GFAP+/MAP2-) in FXS cells, there were fewer quiescent GFAP+/Nestin- glia in FXS compared to controls (**Fig. 5.3 F**). Thus, our

findings suggest that in the absence of FMRP, there is a higher abundance of proliferative cell types but fewer terminally differentiated cells in FXS.

5.2.4. Cell-type specific translational dysregulation in FXS patient NPCs

Given the altered differentiation profile in FXS patient NPCs, we wanted to further assess whether the defects we reported in protein synthesis were consistent across cell types. In puromycin-labeled control and FXS patient NPCs we measured protein synthesis within specific neuronal subtypes, identified using our NeuroMIP2 panel (**Table 5.1**). We found that translation was significantly elevated in actively proliferating NSCs and RG-like cells in FXS compared to controls (**Fig. 5.4 A,B**); however, there was no significant difference in puromycin signal between control and FXS in cells that were further lineage-restricted, such as early neuroblasts and terminally differentiated neurons and glia (**Fig. 5.4 C-F**).

5.2.5. Processes regulating neuronal fate commitment and proliferation are disrupted in FXS patient-derived cerebral organoids

We conducted bulk transcriptome analysis in 28 day old cerebral organoids from 3 control and 3 FXS patient iPSCs. We found that there were 218 differentially expressed genes (DEGS) between control and FXS day 28 organoids (**Fig. 5.6 A,B**). Downregulated genes in FXS were enriched for gene ontology (GO) terms involved in neuronal fate specification, migration, differentiation, and maturation (**Fig. 5.6 C**), while GO terms related to proliferation and regulation of MAPK/ERK signaling pathways were enriched in genes that were upregulated in FXS (**Fig. 5.6 C**).

5.2.6. Novel splicing defect in FXS patient cerebral organoids

Although FMRP is a well-established RNA-binding protein, its role in the regulation of transcription and alternative splicing (AS) has not been extensively studied. We analyzed our RNAseq data to identify alternative splicing events in day 28 cerebral organoids and found that there were 143 differentially included exons in FXS compared to controls (**Fig. 5.7 A**). One of the top AS events was the increased inclusion of a 98bp exon (exon IIa) in the 5'UTR of the cell-cycle regulated Aurora kinase A (AURKA) in all three FXS patient organoids compared to controls (**Fig. 5.7 B**). The 5'UTR of AURKA in humans has 6 splicing variants, the longest of which includes exon IIa (Lai et al., 2010). We further confirmed this result in our NPC lines using Taqman probes to the included exon, and showed that this 98bp exon was included more in FXS patient NPCs compared to control (**Fig. 5.7 C**).

5.3. Discussion

Here we show that in the absence of FMRP, there is an overabundance of proliferating progenitor cells, and a delayed commitment to mature cell fates. FXS patient NPCs have higher populations of mitotically active proliferating cells compared to controls, but fewer terminally differentiated neurons and glia. The loss of FMRP appears to be more significant in proliferative progenitor cells compared to terminally differentiated cells, suggesting that loss of translational control by FMRP in early development could have more profound consequences. This also suggests that interventions for FXS should be introduced at early stages of neurogenesis.

Our work is the first to characterize molecular phenotypes in a human neural model of FXS, in a cell-type specific manner. We developed NeuroMIP – a powerful molecular phenotyping assay that allows for the simultaneous investigation of phenotypes in multiple

neuronal subpopulations. The use of flow cytometry to study defects in signaling and proliferation is well established in the field of immunology; however, these techniques are rarely applied in neuroscience owing to the lack of well-validated antibodies and the complex morphology of neurons. Here, we have combined a technique to measure protein synthesis with an optimized a core panel of neural antibodies to allow for the measurement of protein synthesis and proliferation in multiple neuronal subpopulations. Several rounds of optimization and validation were done to confirm the specificity of antibodies. While our largest NeuroMIP panel uses 8 colors, these panels can be expanded and customized to investigate other phenotypes.

There have been very few studies investigating the global transcriptional defects in FXS, and even fewer that have been done in humans (Halevy et al., 2015; Sunamura et al., 2018). Here, we found that a majority of DEGs were downregulated in FXS and several of these downregulated genes were linked to processes that govern neuronal fate specification and differentiation, further validating our results. Our finding that FXS patients showed increased inclusion of an exon in the 5'UTR of AURKA is intriguing. It has been shown in humans, that there are six variants of the 5'UTR, although their relative frequency remains unknown (Lai et al., 2010). Of the six variants, only the longest variant (566 bps) includes exon 2a, which is the differentially included exon in our FXS patient organoids. Interestingly, one study reported that specific 5'UTR variants of AURKA appeared to mediate increased translation in response to EGF stimulation, while other variants were more vulnerable to regulation via the PI3K/mTOR/Akt pathway (Lai et al., 2010).

5.4. Figures & Tables

Table 5.1: NeuroMIP Antibody Panels

Antibody	uL/test	Fluorochrome	Company	Purpose
KI-67	0.25	PE-Cy7	BD Biosciences	Proliferation quantification
Puromycin	0.5	Alexa 647	Millipore	Protein synthesis quantification
TUJ1	1	Alexa 488	BD Biosciences	Immature Neuron
Nestin	1	V450	BD Biosciences	Neural Stem Cell/Radial Glia
SOX2	1	PerCP-Cy5.5	BD Biosciences	Neural Stem Cell
DCX	1	PE	BD Biosciences	Neuroblast/Immature Neuron
TBR2	20	PE-Cy7	BD Biosciences	Intermediate Progenitor Cell
MAP2	1	Alexa 647	BD Biosciences	Mature Neuron
GFAP	0.5	Alexa 700	BD Biosciences	Glial Cell
Puromycin	0.5	Alexa 488	Millipore	Protein synthesis quantification

NeuroMIP1 **NeuroMIP2** **Common to both panels**

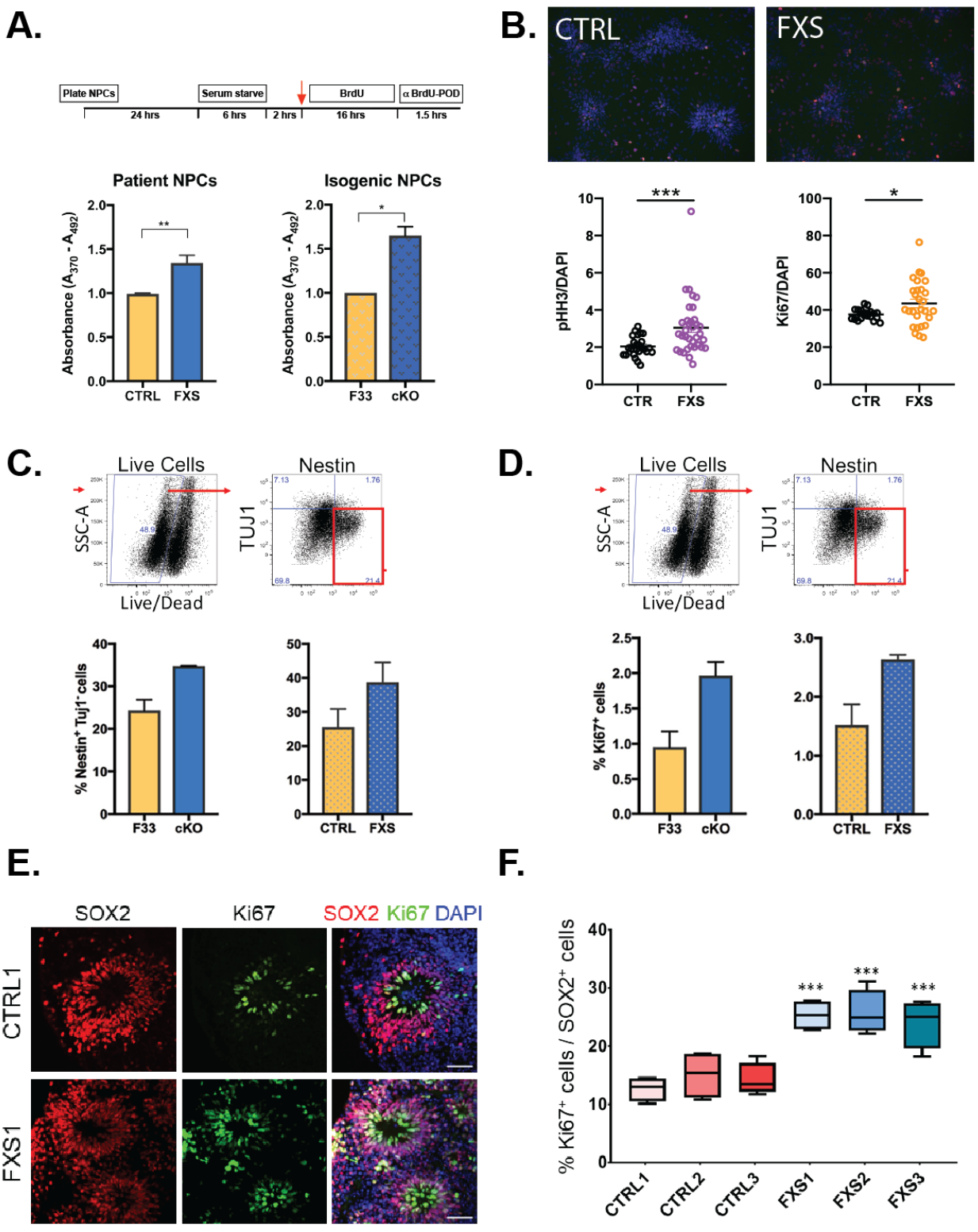

A.
Plate NPCs
Serum starve
BrdU
α BrdU-POD
24 hrs
6 hrs
2 hrs
16 hrs
1.5 hrs
Patient NPCs
Isogenic NPCs
Absorbance (A370 - A492)
CTRL
FXS
F33
cKO
B.
CTRL
FXS
pHH3/DAPI
Ki67/DAPI
CTR
FXS
C.
Live Cells
Nestin
SSC-A
Live/Dead
TUJ1
% Nestin+ Tuj1+ cells
F33
cKO
CTRL
FXS
D.
Live Cells
Nestin
SSC-A
Live/Dead
TUJ1
% Ki67+ cells
F33
cKO
CTRL
FXS
E.
SOX2
Ki67
SOX2 Ki67 DAPI
CTRL1
FXS1
F.
% Ki67+ cells / SOX2+ cells
CTRL1
CTRL2
CTRL3
FXS1
FXS2
FXS3

Figure 5.1. Increased proliferation in FXS patient cells (A) Colorimetric ELISA for BrdU incorporation shows more signal in FXS patient lines as well as in FXS isogenic line, indicating increased proliferation. **(B)** FXS patient NPCs have higher mitotic activity as evidenced by more pHH3 and KI67 positive cells. **(C)** Single, live cells are isolated, and antibodies against Nestin (NPCs) and TUJ1 (neurons) allow us to isolate pure NPCs. FXS patient derived NPCs have higher Nestin positive population, as well as **(D)** a higher KI67 (proliferative marker) population compared to controls (n=3,3). **(E)** Representative images and **(F)** quantification of the proportion of Ki67+/SOX2+ neuronal progenitor cells in both control and FXS-derived organoids at Day 28 (n = 5 cultures; ***P < 0.001, ANOVA). Scale bars, 50 μm. Data are shown as Mean ± SEM.

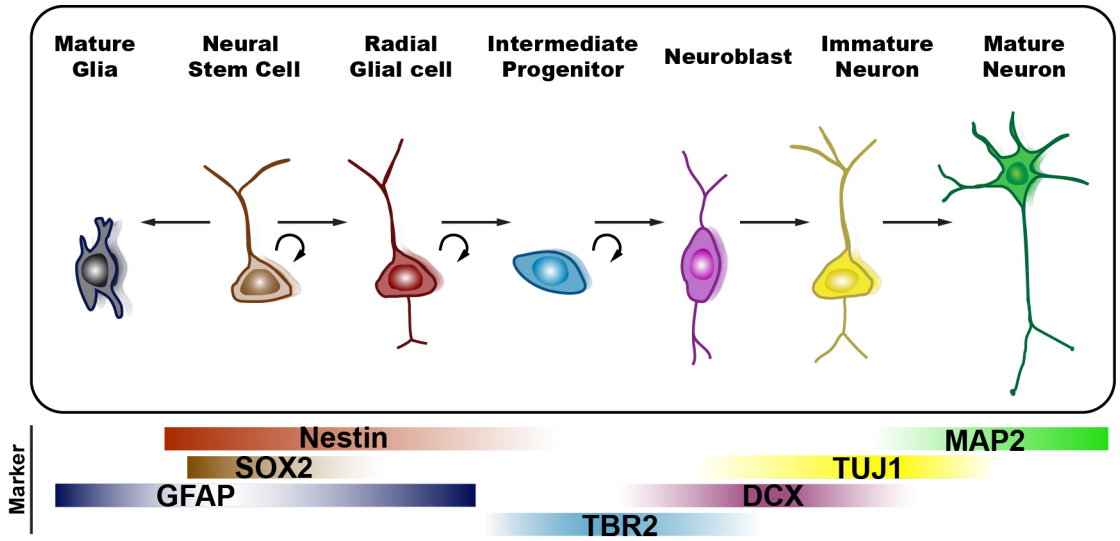


Figure 5.2. Stages of neurogenesis in human iPSC-derived NPCs. Schematic showing the acquisition of progressively lineage-restricted markers of differentiation in neural precursor cells. Neural stem cells (NSCs) and Radial glia cells (RGs) undergo several rounds of symmetric division before committing to neurogenesis or gliogenesis. TBR2+ intermediate progenitor cells undergo asymmetric divisions to produce one IPC or neuroblast cell that will eventually differentiate into a mature neuron.

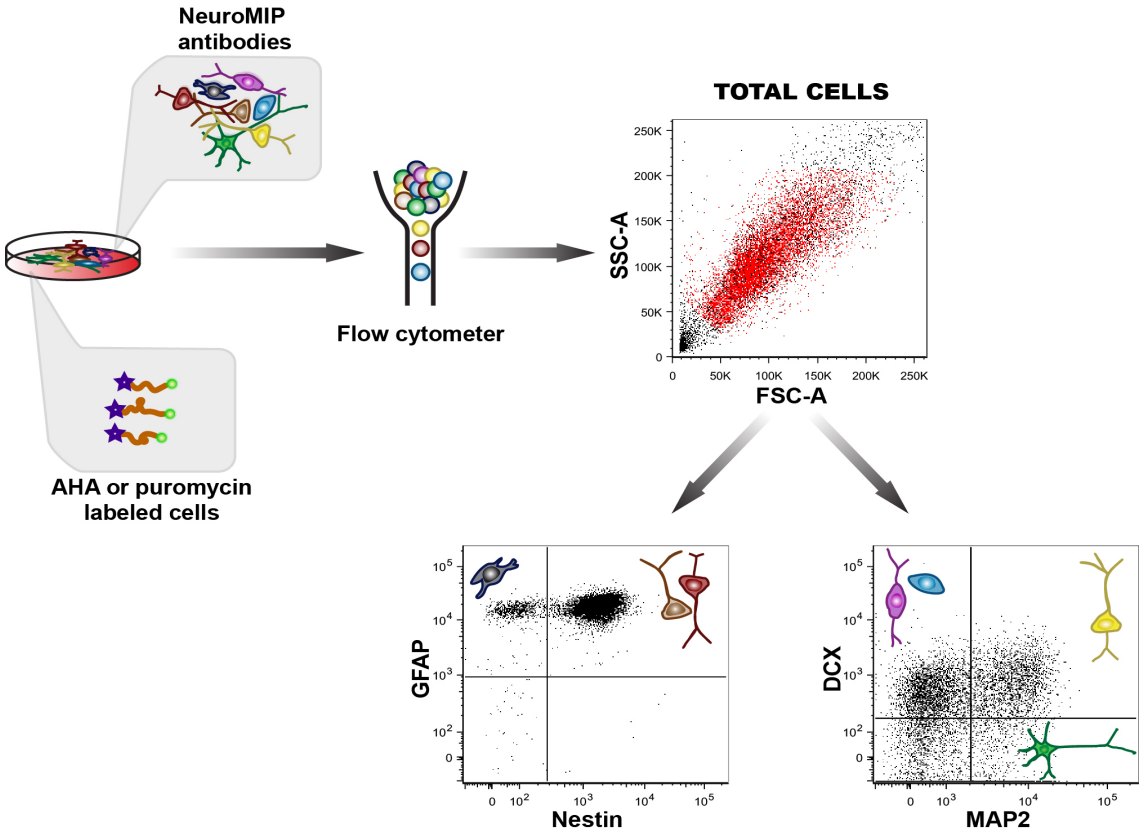


Figure 5.3. Neuronal Molecular Immunophenotyping (NeuroMIP). Schematic outlining the NeuroMIP method to simultaneously measure multiple disease-relevant phenotypes within specific neuronal subtypes. Live cells are labeled with either puromycin or azidohomoalanine (AHA) for quantification of protein synthesis and then fixed and labeled with an optimized panel of antibodies designed to identify specific neuronal subpopulations. Cells are analyzed using a flow cytometer and quantitative measures of cell-type-specific phenotypes can be obtained.

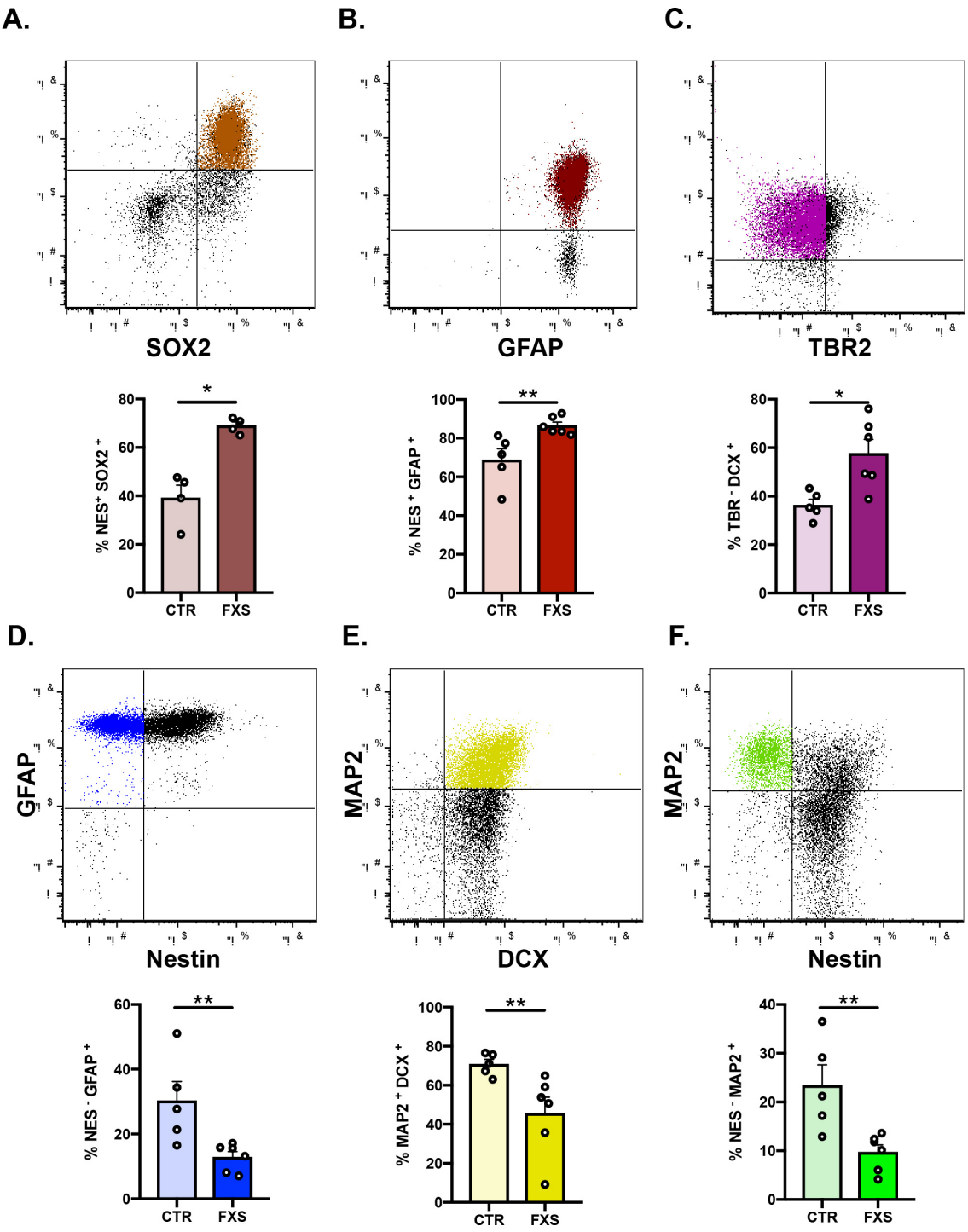

A.
B.
C.
SOX2
GFAP
TBR2
% NES+ SOX2 +
% NES + GFAP +
% TBR - DCX +
CTR
FXS
D.
E.
F.
GFAP
MAP2
MAP2
Nestin
DCX
Nestin
% NES - GFAP +
% MAP2 + DCX +
% NES - MAP2 +

Figure 5.4. Altered cell fate in FXS patient cells. NeuroMIP2 was used to assess the number of cells within each neuronal subpopulation. **(A-C)** Increased proliferative cells in FXS patients. **(A)** Nestin/SOX2 positive neural stem cells **(B)** Nestin/GFAP positive radial glia like cells and **(C)** Doublecortin positive early neuroblasts all showed increased abundance in FXS patient cultures **(D-F)** FXS patients showed fewer terminally differentiated cells. **(D)** Immature DCX-expressing neurons, **(E)** MAP2 positive neurons and **(F)** Nestin negative quiescent GFAP positive glia were all reduced in FXS cultures. (Each subpopulation was analyzed using unpaired t-tests, n=5 control, 6 FXS, $^{**}P<0.01$, $^{*}P<0.05$). Data are shown as Mean±SEM.

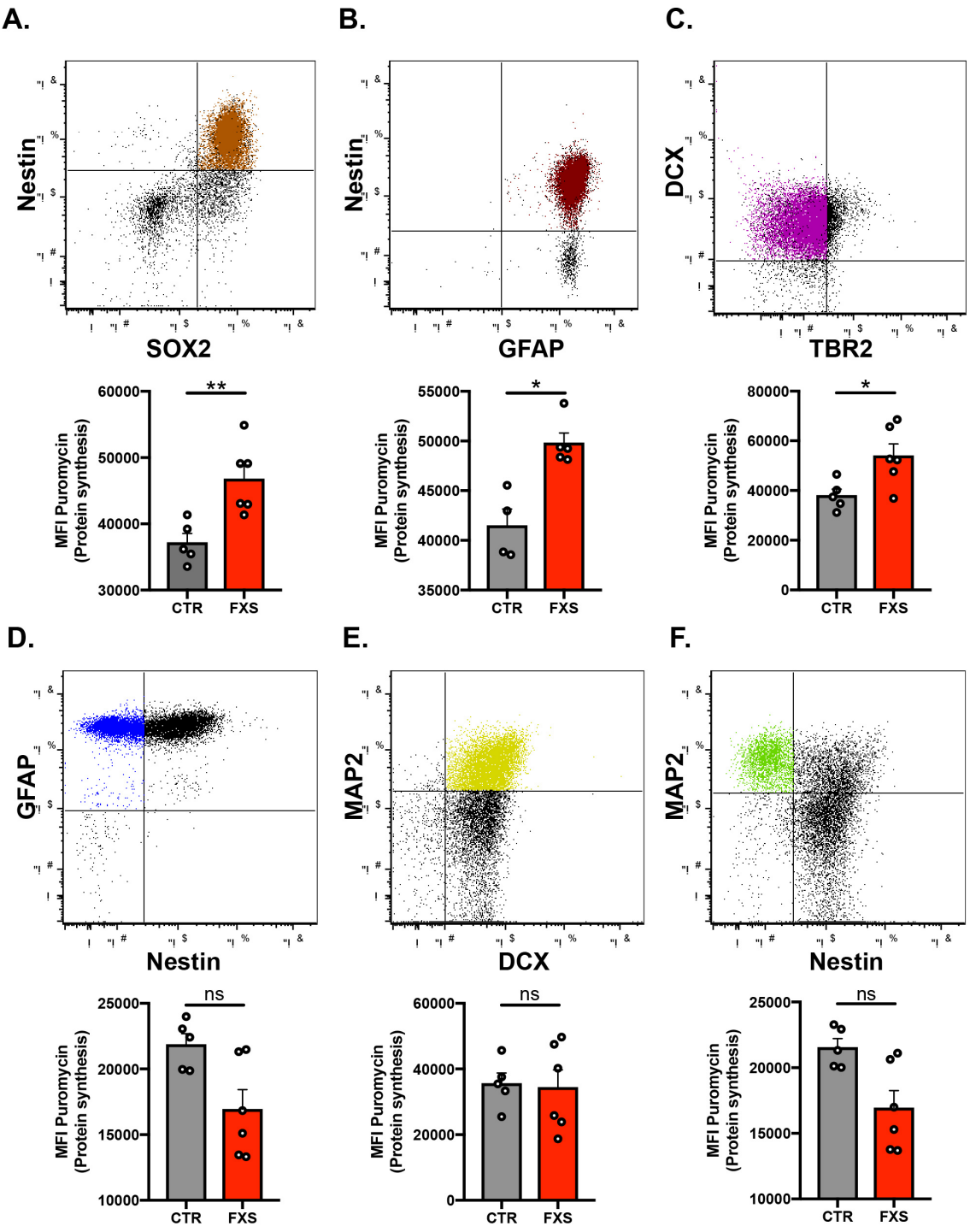

A.
B.
C.
Nestin
SOX2
Nestin
GFAP
DCX
TBR2
MFI Puromycin (Protein synthesis)
**
*
*
CTR
FXS
D.
E.
F.
GFAP
Nestin
MAP2
DCX
MAP2
Nestin
ns
ns
ns

Figure 5.5. Cell-type-specific translational dysregulation in FXS patient cells. NeuroMIP2 was used to measure translation within each neuronal subpopulation. **(A-C)** Increased translation in actively proliferating cells in FXS patients **(A)** Nestin/SOX2 positive neural stem cells **(B)** Nestin/GFAP positive radial glia like cells and **(C)** Doublecortin positive early neuroblasts all showed increased protein synthesis in FXS patient cultures **(D-F)** No significant difference in protein synthesis in terminally differentiated cells. **(D)** Immature DCX-expressing neurons, **(E)** MAP2 positive neurons and **(F)** Nestin negative quiescent GFAP positive glia showed similar levels of translation in control and FXS cultures. (Each subpopulation was analyzed using unpaired t-tests, n=5 control, 6 FXS, **P<0.01, *P<0.05). Data are shown as Mean±SEM.

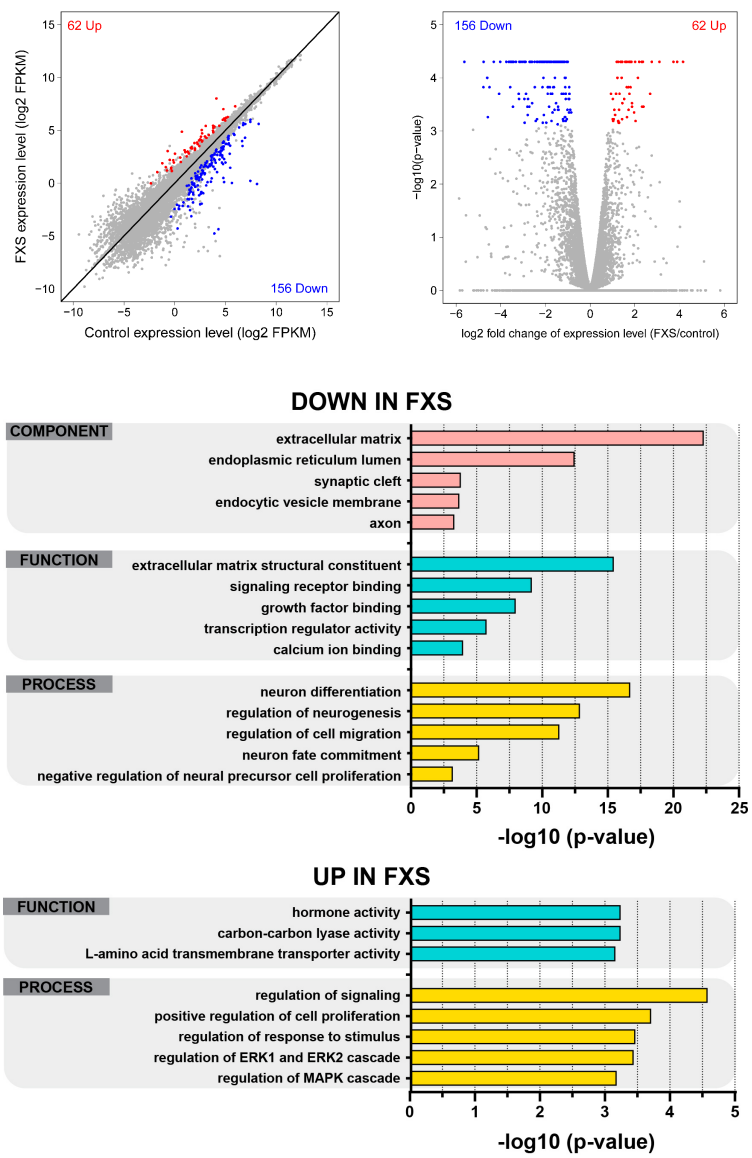


Figure 5.6. Gene Ontology analysis of RNAseq data from iPSC-derived organoids.

(A) RNAseq analysis of 28 day old control and FXS patient iPSC-derived organoids revealed 218 differentially expressed genes (DEGs) between controls and FXS organoids. **(B)** Gene ontology enrichment showed that pathways related to cell fate commitment are downregulated in FXS while signaling via MAPK/ERK pathways is up in FXS.

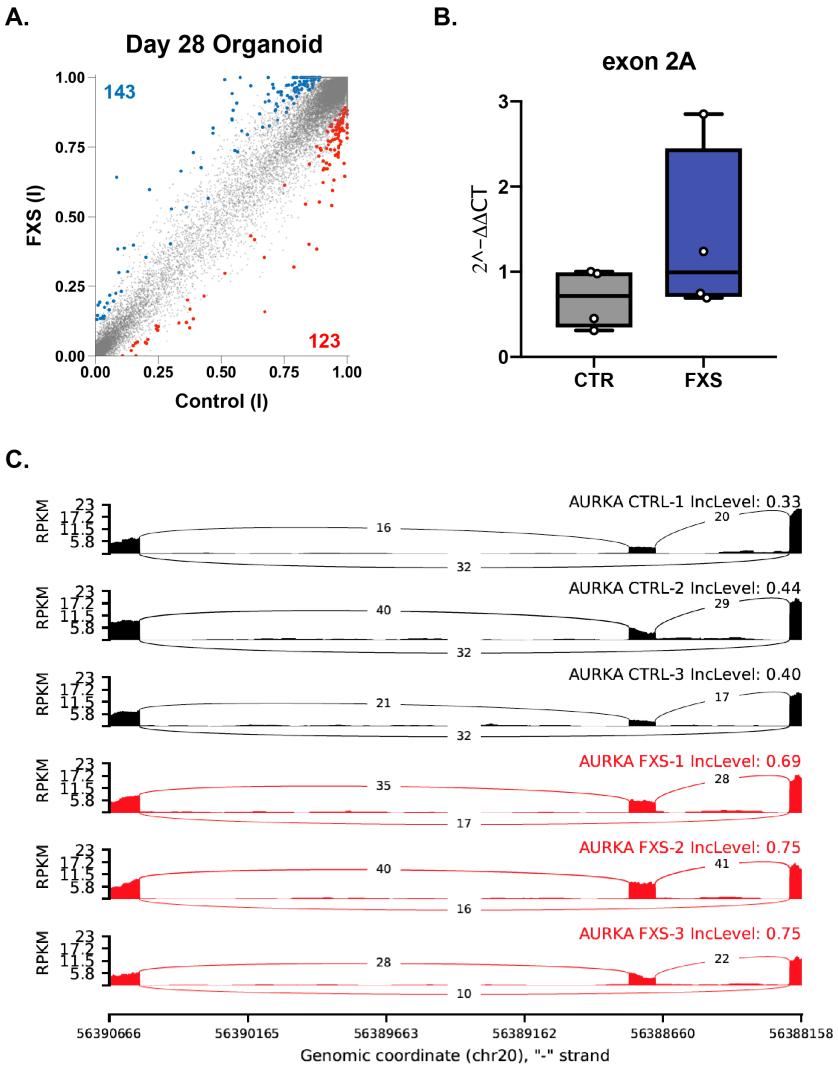
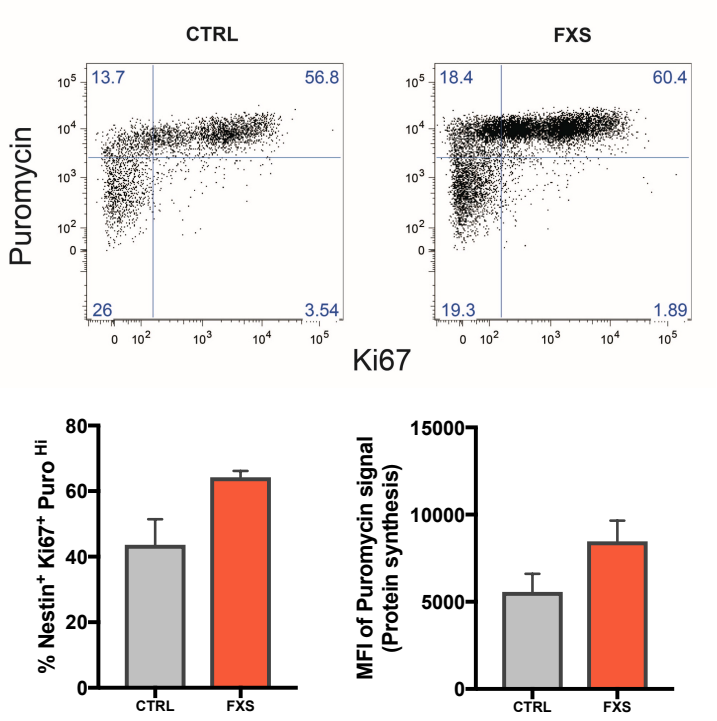


Figure 5.7. Alternative splicing of AURKA 5'UTR in FXS iPSC-derived organoids. **(A)** Differentially spliced events in controls and FXS organoids. **(B)** Taqman assay shows increased inclusion of exon 2A in FXS patient NPCs compared to controls (n=4 controls and 4 FXS) **(C)** Sashimi plots showing inclusion events in control and patient organoids.



Supp. Fig 5.1. FXS patient NPCs have an increased population of proliferative cells with high translational activity. (Top) Single, live cells are isolated, and antibodies against Nestin and KI67 (NPCs) and TUJ1 (neurons) allow us to measure protein synthesis (Puro) within a specific subpopulation (shown here is scatter plot of Puromycin versus KI67 within a Tuj1- Nestin+ subgate). (Bottom Left) Quantification of percentage of cells positive for Nestin and KI67 with High Puro and (Bottom Right) Quantification of Puro MFI showing increased global translation in FXS patient NPCs. Data are shown as Mean ± SEM.

CHAPTER 6: General Discussion

6.1. Summary

This dissertation aimed to address an unmet need in the field to validate critical findings from animal models of fragile X syndrome (FXS) in a human patient-specific, disease-relevant context. We have generated and characterized a number of control and FXS patient induced pluripotent stem cells (iPSCs) that will be a valuable resource to the FXS research community. We have optimized neuronal differentiation paradigms, and have shown that hallmark features of increased protein synthesis and dysregulated signaling seen in *Fmr1* KO mice and *dFmr1* mutant flies, are also seen in FXS patient-derived cells. Importantly, these defects in global translation are most robust in actively proliferating neural cells, a finding made possible with a new method, not previously shown in any model system.

We developed a powerful multi-parametric flow-cytometry assay, NeuroMIP, to simultaneously quantify protein synthesis and proliferation in multiple neural subtypes. This assay is an incredibly useful tool that can be broadly applied to other cellular models of neurological disease. Using iPSC-derived neural precursor cells (NPCs) and cerebral organoids, we have shown that the loss of FMRP in cells at an early stage of neurogenesis may lead to increased proliferation, altered differentiation kinetics and cell fate in FXS, and that these differences may be driven by aberrant signaling. Finally, we have identified a novel splicing defect in FXS patient derived cerebral organoids, that may be linked to dysregulated signaling and increased proliferation in FXS patient cells.

6.2. Induced pluripotent stem cell models of disease

The past 20 years has seen the burgeoning use of human pluripotent stem cells to improve our understanding and treatment of human neurological disorders. The ability to

generate multiple, specific cell types that retain individual patients' genetic information has provided us an unprecedented opportunity to dissect out precise disease-causing mechanisms and identify potential therapeutic avenues. iPSCs models of Alzheimer's disesase, Parkison's disease, ALS, schizophrenia, autism, and several other neurological disorders have helped to validate findings from animal models, and some have also been used for drug screens (An et al., 2012; Brennand et al., 2011; Chen et al., 2014; Hargus et al., 2010; Kondo et al., 2013; Marchetto et al., 2010; Mariani et al., 2015; Pasca et al., 2011).

A significant challenge in the field is the lack of reproducibility and variability of results. The process of deriving reliable iPSCs from fibroblasts and differentiating these into well-characterized neural progenitor cell lines and neurons is extremely time-consuming, and takes at least 18 weeks. Furthermore, iPSC-derived neurons tend to be much more immature than rodent cultured neurons, and some groups have had to maintain their cultures for up to an additional 80 days in order to see phenotypes (Srikanth and Young-Pearse, 2014). The multi-step process leaves considerable room for technical error, and irregularities introduced by changes in culture medium, growth factor concentrations, coating matrix, passage number etc. The reprogramming process itself could give rise to some variability in individual iPSC colonies, and to address this, we generated multiple clonal iPSC lines from each control and patient. The specific clones used in our experiments were selected at random, and we have shown that our results remain consistent even in clonal lines from the same patient. Importantly, we have used 6 individual patient lines in our experiments and our findings are reproducible across experiments.

The number of lines required in each study is an ongoing matter of debate in this field. Some believe that it is more important to have multiple replicate clones of a few control and patient iPSCs to ensure that the results are not artifacts of the reprogramming or differentiation

process. Given that we are using human patient samples from unrelated individuals with completely different genetic backgrounds, a degree of variability is to be expected. Thus, we and several others believe that increasing the number of individual lines appears to be the best way to approach this problem (Germain and Testa, 2017; Hoffman et al., 2017). An additional way to address these concerns is by including isogenic lines, wherein the genetic mutation is either corrected in a patient line, or introduced into a control. We have shown that our findings can be replicated across multiple individual patient lines, as well as in a pair of isogenic lines.

6.3. The ever-expanding functions of FMRP

FMRP is primarily known to be an RNA-binding protein that regulates translation of a subset of mRNAs. Several lines of evidence suggest that the loss of FMRP leads to "runaway" protein synthesis, which eventually results in the characteristic behavioral and cognitive impairments seen in the disorder. Defects in synaptic plasticity, neuronal morphology and neuronal circuitry in FXS have been linked to aberrant translation of FMRP mRNA targets that encode for synaptic proteins, cytoskeletal proteins and ion channels. Some studies have looked beyond mRNA targets to show that disrupting interactions of FMRP with other proteins and ion-channels may also give rise to phenotypes in FXS. FMRP has also been shown to play a key role in RNA metabolism and localization (Ifrim et al., 2015; Taliaferro et al., 2014). Since most studies that identified dysregulated synthesis of specific targets in FXS did not report any changes in the basal abundance of these mRNAs, the role of FMRP in regulation of global transcription or splicing was much less studied, although there is a renewed recent interest in this area (Gandal et al., 2018; Liu et al., 2018).

Since learning and cognition are intrinsically linked to synaptic function and neuronal connectivity, a primary focus has been to investigate the consequences of loss of function of *FMR1* in synapses of postmitotic neurons. However, it is becoming increasingly apparent that FMRP plays several critical roles earlier on in development. FMRP has been shown to regulate the proliferation and differentiation of neural stem cells in mouse and in human models (Achuta et al., 2017; Danesi et al., 2018; Khalfallah et al., 2017; Li and Zhao, 2014; Tervonen et al., 2009), and we have seen similar results.

FMRP may regulate cellular function in a developmentally-dependent, spatiotemporal manner. The *FMR1* mutation is not lethal, nor does its loss significantly affect the development of any other major systems. FMRP is expressed ubiquitously, and across human development (Willemsen et al., 1996; Willemsen et al., 2002), however, the primary insult in the absence of FMRP is to neuronal development and function. This could be explained by the timing of *FMR1* silencing - early studies using chorionic villi samples from full mutation embryos showed that the inactivation of *FMR1* did not occur until ~10 gestational weeks (GW) (Willemsen et al., 2002), by which time several critical, early developmental events have already likely occurred. However, active neurogenesis and neural migration occurs between 8-15 GWs (Stiles and Jernigan, 2010); thus, the inactivation of *FMR1* in human embryos coincides with the critical period of neuronal development.

The functional transcriptome and proteome of different cell types across development varies greatly (Ingolia et al., 2009). It stands to reason that targets of RNA-binding proteins like FMRP may also vary across cell types or stages of development. Future studies comparing the FMRP interactome and FMRP-bound transcriptome at different stages of neuronal development could provide great insight into the function of FMRP, and new avenues for therapeutic

intervention. Future work may reveal that FMRP target mRNAs in proliferating cells encode factors playing direct roles in cell proliferation. Such studies could also help us uncover the missing link between translation and neurogenesis in the context of fragile X syndrome.

6.4. Lost in Translation: cell fate and protein synthesis

Protein synthesis is a fundamental process that allows cells to grow and divide and dysregulated protein synthesis at different stages of neurogenesis may have different consequences (Jorgensen and Tyers, 2004). While the loss of translation control by FMRP is central to FXS, we hypothesize that these effects may be context dependent. Our findings that proliferative cells exhibited a more robust defect in global translation compared to terminally differentiated cells supports this theory. Previous studies showing elevated general, basal translation in *Fmr1* KO neurons compared to *WT* have demonstrated only a modest increase (Bhattacharya et al., 2012; Gross et al., 2015a; Hoeffer et al., 2012). Furthermore, FMRP regulates the translation of only about 4% of neuronal mRNA (Ashley et al., 1993). Our results do not necessarily contradict previous findings – our work was done in relatively immature neuronal cells and it is possible that they do not yet express a critical subset of FMRP target mRNA that may drive the general translational defect in postmitotic neurons. Our findings suggest that altered cell fate may be a consequence of dysregulated protein synthesis. We propose a model wherein the loss of FMRP in postmitotic cells results in defects that affect synaptic structure and function, while FMRP deficiency in mitotic, actively proliferating cells leads to aberrant cell fate acquisition, delayed maturation and prolonged and abnormal proliferation (**Fig. 6.2**).

6.5. Finding a "cure" for fragile X syndrome

Since the discovery of the gene we have made enormous strides in understanding FXS with the goal of developing effective treatments. The identification of FMRP as an RNA-binding protein propelled the search for disease-relevant direct and indirect mRNA targets that were dysregulated in FXS. As a result, several exciting studies in animal models of FXS identified components of signaling pathways, ion channels, and synaptic machinery that were disrupted with the loss of FMRP, giving rise to myriad cellular, molecular and behavioral phenotypes. Most of these studies have shown that these defects can be successfully ameliorated by targeted genetic or pharmacological treatments. Indeed, there are at least 10 different therapeutic interventions that have been effective in correcting almost all the defects in the *Fmr1* KO mouse, leading to highly promising clinical trials (**Fig. 6.1**); yet none have translated as successfully into human therapies (Berry-Kravis et al., 2018; Erickson et al., 2017; Gross et al., 2015b).

There are many possible explanations for this, the most obvious being – a mouse is not a human. The human *FMR1* CGG expansion mutation is unique to the disorder, and although mouse models have been incredibly informative, there may be human-specific as genetic polymorphisms, or miRNAs that contribute uniquely to FXS in humans. Thus, despite extensive studies in preclinical models, several promising interventions have remained unexplored due to ineffectiveness or potential adverse effects in human patients. Models that use human cells or tissue are vital to improve our understanding of the disorder, and as iPSC technology becomes more advances and easily accessible, we are likely to make significant progress in this field.

Another consideration is that most studies have used approaches to modulate protein synthesis and key signaling pathways, which are extremely dynamic processes that are regulated by several interconnected pathways. The effectiveness of these strategies could be greatly

affected by external processes and factors like nutrient availability, cellular metabolism, developmental status at the time of intervention. Furthermore, optimal cellular function depends on a delicate balance of these pathways, and correcting deficits in one component could adversely affect several others. PI3K and mTOR signaling pathways are central to multiple cellular processes and a global inhibition of these pathways could have drastic effects. Similarly, globally targeting the GABAergic or glutamatergic system could have off-target consequences and further disturb the excitatory-inhibitory balance. Despite these considerations, the number of interventions that have been successful in animal models of FXS is both heartening and intriguing. The success of multiple targeted interventions provides hope that a reversal of phenotypes is a possibility, and the interconnectedness of disrupted pathways in FXS suggests that it may come down to identifying the most accurate molecular convergence point.

Importantly, the idea of reintroduction of FMRP into individuals with FXS is highly promising, particularly since there have been reports of high-functioning males with an unmethylated expanded *FMR1* allele (Hagerman et al., 1994). Attempts to reactivate *FMR1* expression by demethylation of the promoter region have been successful in animal and human cells, and high throughput studies to identify compounds that can be used for such interventions are underway. However, the idea of reintroduction of FMRP comes with its own caveats. More research needs to be done into the timing and efficacy of reintroduction strategies. There may be a critical period of time during which *FMR1* reactivation will be successful, implying that these interventions may not be as useful for individuals already living with FXS. Additionally, reactivation of the gene with expanded repeats may result in RNA toxicity seen in FXTAS. Therefore, a combined approach to reduce the number of repeats and reactivate the gene may be required. Further studies to understand the silencing mechanism of *FMR1* will help in this area.

6.6. Future directions and concluding remarks

Here we showed elevated protein synthesis and proliferation in the majority of several FXS patient NPCs as well as cerebral organoids compared to controls. We also found that one isogenic corrected NPC line had reduced proliferation and protein synthesis compared to the FXS patient isogenic line. Interestingly, two FXS patient NPC lines consistently had lower protein synthesis rates comparable to control lines, despite having high proliferation rates. These results were reproduced in fibroblasts from these patients, suggesting that this was not simply an artifact of the differentiation process. Neurons from one of these patients consistently showed increased hyperexcitability when plated on a multielectrode array (MEA). While it is possible that these disparities arise from technical limitations of the system, it is also possible that these patients have divergent phenotypes. Even in monogenic disorders like FXS, the underlying genetic architecture is extremely complex, and it is unsurprising that there are differences in phenotypes across affected individuals. Future studies could be directed towards developing patient stratification approaches based on cellular and molecular phenotypes, which would help to develop targeted treatments in a patient-specific manner. Since clinical phenotypes within specific diagnoses are often heterogeneous (Berry-Kravis et al., 2018), this stratified approach could prove to be more beneficial for a broader group of patients who may have divergent diagnoses but share an underlying molecular pathology.

Although this dissertation primarily focused on identifying defects in iPSC-derived cells that lack FMRP, one of the unique advantages of using human FXS patient derived cells is that they retain the expanded pathogenic CGG repeat. Future studies could involve the use of these iPSC lines as a resource to further understand the biology of *FMR1* – we still do not understand the mechanism behind the CGG repeat expansion or how it mediates silencing of *FMR1* and

patient iPSCs provide an ideal system to investigate these questions. While it may be argued that FXS patient iPSCs are essentially similar to the knockout animal models since they lack both gene and protein expression, importantly, studies that are directed towards reactivating *FMR1* expression either by CRISPR/Cas-9 (Park et al., 2015; Xie et al., 2016) or by targeting the hypermethylation or other repressive epigenetic marks (Kaufmann et al., 2015; Kumari et al., 2015) would need an expanded repeat to establish a baseline measure. Additionally, factors such as CGG repeat length and methylation status could affect key cellular processes through interactions with modifiers of protein synthesis and proliferation. Human iPSCs offer the additional advantage of allowing us to model the process of neuronal development in a step-by-step manner. Thus, human iPSCs provide the perfect tool to investigate the role of FMRP across stages of development, from early stage neural stem cells to mature cerebral organoids.

The work presented in this dissertation provides novel insight into the function and biology of FMRP in translation and neurogenesis in human neurons. We believe that our collection of control and FXS patient lines as well as our optimized NeuroMIP assay can be useful to other researchers in the field.

6.7. Figures

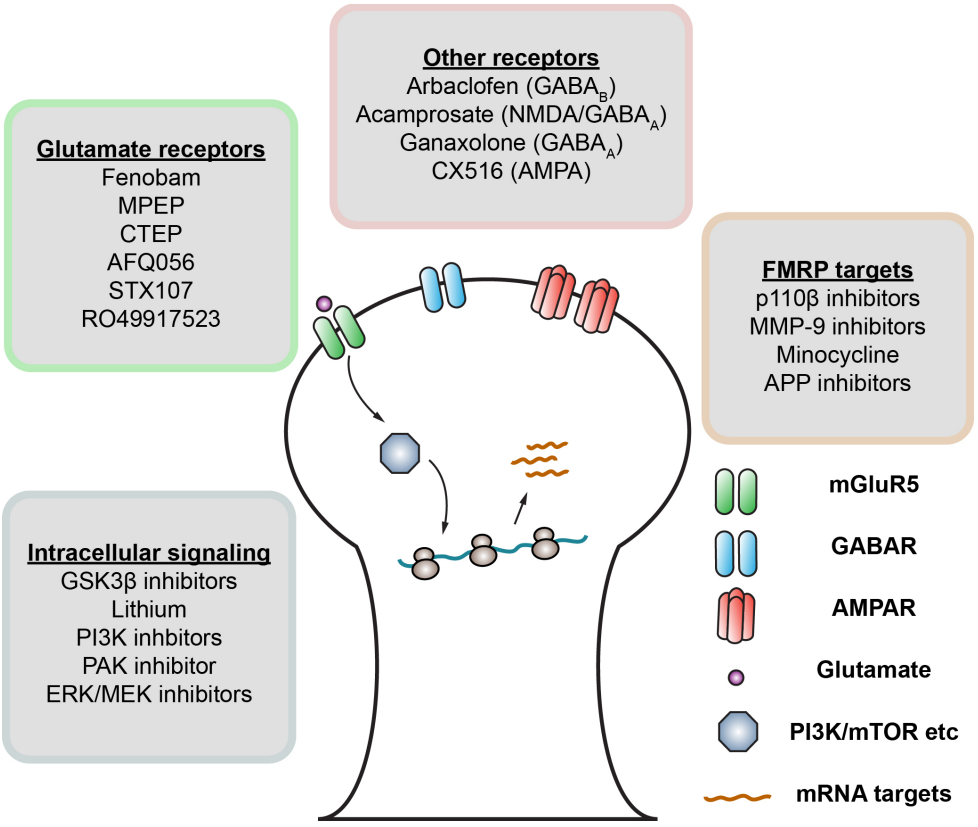


Figure 6.1. Multiple points of therapeutic intervention in FXS. Preclinical studies in animal models identified many potential therapeutic avenues to correct phenotypes in FXS, leading to the development of several clinical trials. The use of metabotropic glutamate receptor antagonists and GABA receptor agonists showed great promise, as did interventions that targeted intracellular signaling molecules and validated mRNA targets of FMRP.

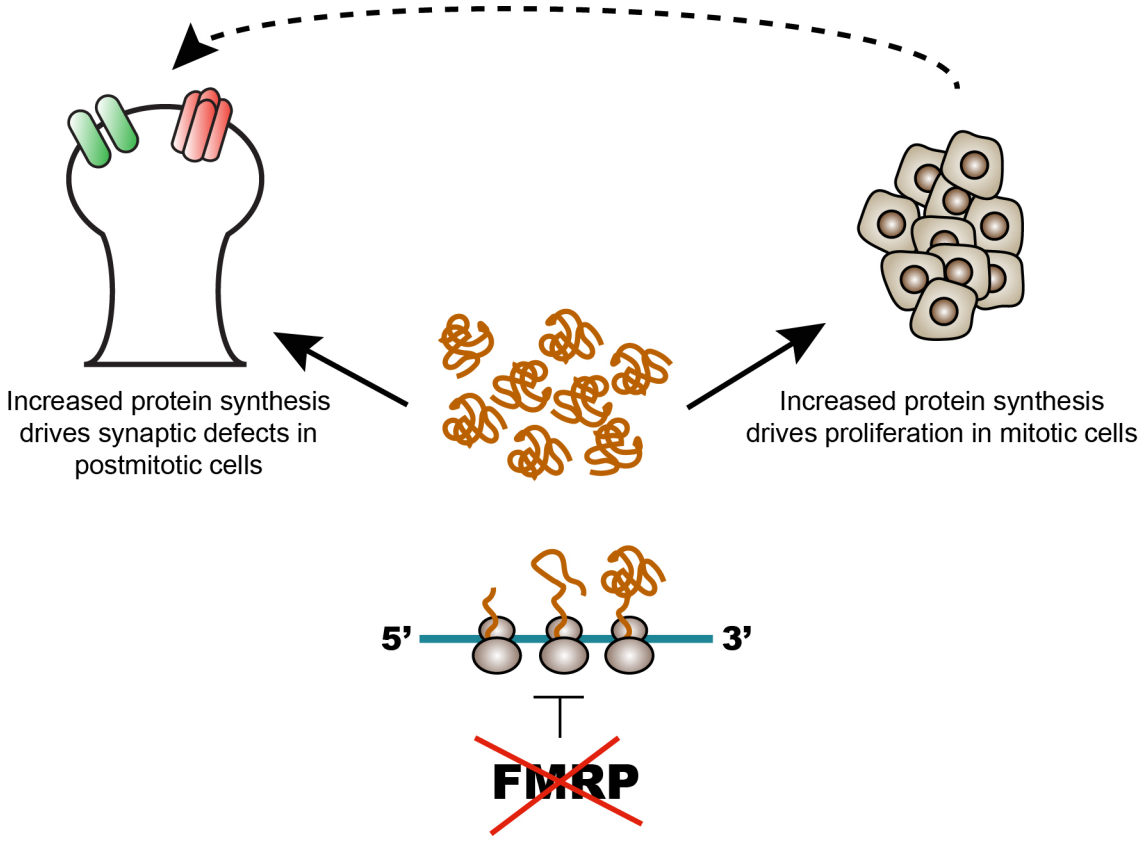


Figure 6.2. Translational defects may underlie abnormal proliferation and differentiation in FXS. We propose that the loss of FMRP in FXS results in increased and dysregulated translation, which may have different consequences at different stages of development. During early development, in proliferating cells, increased translation may give rise to overproliferation of cells in FXS. During subsequent stages of neurogenesis, FMRP deficiency may result in aberrant cell fate commitment and altered differentiation profiles. In mature postsynaptic cells, dysregulated protein synthesis may give rise to abnormal synaptic structure and function as seen in animal models.

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