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Jayabhargav Annam April 05, 2016

Impairments in protein synthesis and signaling in Fragile X human cells

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Abstract

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Fragile X Syndrome (FXS) is the most common monogenic cause of an intellectual disability. The disease is caused by an inherited expanded trinucleotide repeat, which leads to methylation of the promoter a subsequent loss of the expression of the Fragile X Mental Retardation Protein (FMRP). FMRP is an mRNA binding protein that acts a negative regulator of approximately 4% of the mRNAs produced in the brain. Previous work done in FXS animal models has indicated that in the absence of FMRP, there is an increase in PI3K enzymatic activity and PI3K-regulated protein synthesis. We hypothesize that the same increases in PI3K activity and protein synthesis would be seen in FXS patient-derived cells, which could be a very useful insight into the molecular mechanisms underlying FXS in a human cellular model. To measure nascent protein synthesis in control and FXS cells, we used two methods: metabolic labeling of newly synthesized proteins via fluorescent non-canonical amino acid tagging (FUNCAT) combined with either fluorescence microscopy or flow cytometry. To measure PI3K activity, we used western blots for p110β, the catalytic subunit of PI3K. Our results indicate that there were significant increases in both in the FXS patient-derived cells, as was predicted by previous studies in animal models. In the use of flow cytometry to measure protein synthesis in FXS patient-derived cells, we developed a new application of the technique and a novel assay that can be used for future studies attempting to measure levels of abnormal protein synthesis. Furthermore, through our finding that FMRP regulates PI3K signaling through p110β and that this pathway is dysregulated in patients, a new potential therapeutic drug target and human biomarker for FXS has been found.

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Introduction

Fragile X syndrome (FXS) is one of the most common inherited forms of intellectual disability, affecting approximately 1 in 4000 males and 1 in 8000 females. FXS is most commonly caused by an expansion of the CGG trinucleotide repeat present in the 5' untranslated region of the *FMR1* gene. A typical *FMR1* gene has anywhere between 5 and 55 copies of the repeat. *FMR1* alleles containing between 55 and 200 repeats are classified as pre-mutation alleles, which may give rise to other FXS associated disorders (Nageshwaran and Festenstein, 2016). A full mutation results when an allele contains more than 200 CGG repeats. The full mutation associated trinucleotide expansion causes hypermethylation of CpG islands within the promoter, and a subsequent silencing of the gene.

FXS is an X-linked disorder that typically affects males more severely than it does females, and females can also be carriers of the mutation. Female carriers have a mutation on only one copy of *Fmr1*, while the other copy produces functional FMRP; due to the process of X-chromosomal inactivation, there is a 50% chance that the X chromosome containing the mutation is inactivated. As a result, full mutations typically cause mild to severe intellectual disability in males and mild to moderate intellectual disability in females. In addition to the cognitive impairments, those affected may also display characteristic physical features such as a long face, large ears, joint laxity, and macroorchidism. Comorbid diagnoses such as autism spectrum disorder and attention-deficit/hyperactivity disorder are also very common in individuals with FXS (Fernandez et al., 2015).

The structure of FMRP

FMRP has been identified as an mRNA binding protein that is involved in the regulation of translation, transport, and stability of large subset of mRNAs (Bassell and Warren, 2008). FMRP contains three sequence motifs that are indicative of RNA binding domains: two copies of a KH motif (KH1 and KH2) and one RGG box. It has also been shown that the amino acids located in the N-terminal can bind to RNA homopolymers (Adinolfi et al., 2003). Studies done on an atypical patient have indicated that an intragenic point mutation in the KH2 domain leads to FXS despite a normal number of CGG repeats, suggesting that altered RNA binding functions underlies the pathophysiological mechanism of FXS (De Boulle et al., 1993). The mutation in the domain resulted in the formation of abnormal FMRP-containing mRNP complexes, which prevented FMRP from associating with polyribosomes (Feng et al., 1997). Although specific mRNAs that bind to the KH2 domain have yet to be found, several mRNAs that bind to the RGG box via a G quartet have been identified to encode many crucial pre- and postsynaptic proteins (Darnell et al., 2005). The separation in sequence between the different domains supports the idea that FMRP recognizes its target mRNAs in different ways, which may either be related to the different functions of the protein in mRNA translation or may occur simultaneously to strengthen the binding (Zalfa et al., 2005).

FMRP as a Regulator of Protein Synthesis

The loss of the fragile X mental retardation protein (FMRP) leads to defects in protein synthesis, intracellular signaling, and neuronal morphology (Park et al., 2015; Gross et al., 2010). A hallmark of FXS is aberrant and excessive protein translation due to loss of FMRP's function in negatively regulating a number of mRNAs. The targets of FMRP are large in number and

diverse in function; there are approximately 842 FMRP target transcripts in the brain citation, which is equivalent to 4% of the overall mRNAs in the brain (Darnell et al., 2011).

Neuronal FMRP is primarily located within the soma, but the protein can also found in dendrites and in axons in granules during development. The majority of FMRP is associated with polyribosomes, complexes that contain multiple ribosomes that are assembling proteins from the same mRNA. Studies using CLIP have indicated that FMRP binds at a much higher frequency to coding regions of mRNA compared to the 5' and 3' UTRs, and these data suggest that FMRP could act as roadblock to deter ribosome transit and thus retard the elongation of the peptide coded by the mRNA (Darnell et al., 2011). This is one of the possible mechanisms by which FMRP functions as a translation inhibitor of its target mRNAs; other mechanisms of regulation used by FMRP include interactions with miRNA and interactions with cap-binding translation factors eIF4E and cytoplasmic FMRP-interacting protein 1 in order to regulate the initiation step of translation (Richter, Klann and Bassell, 2015).

mGluR Theory of FXS

A prevalent feature of FXS animal models is aberrant and excessive protein synthesis in neurons, due to loss of FMRP mediated repress of translation, suggesting that FMRP may be involved in regulating global translation in addition to sequence specific translation of target mRNAs. A widely accepted theory regarding FXS that addresses this is the 'mGluR theory of FXS', which states that the broad translational dysregulation seen in a FXS neuron is caused by overactive signaling by group1 metabotropic glutamate receptors (gp1 mGluR). The group 1 family of mGluRs is composed of mGluR1 and mGluR5. Although the two subtypes are known to facilitate and induce long-term depression and generally have the same function, the two vary

in location of prevalence within the brain. In this model, the mGluR activation stimulates the synthesis of FMRP, which functions to negatively regulate the downstream translation of mRNAs that are involved in the facilitation of the mGluR activated cell signaling pathway. The role of FMRP in end product inhibition signifies that in the absence of FMRP, there is exaggerated Gp1 mGluR-dependent protein synthesis even with basal level of mGluR activity. The overactive gp1 mGluR signaling might lead to epilepsy, cognitive impairment, developmental delay, and an increased density of long, thin dendritic spines-all of which are key features of fragile X syndrome (Bear et al., 2004). This theory is further supported by studies that have shown that some phenotypes of FXS can be ameliorated through the reduction of mGluR signaling. In fragile X knockout mice, the use of a mGluR5 antagonist called MPEP (2-mehyl-6-(phenylethylnyl)-pyridine) has been shown to reversibly suppress seizure phenotypes (Yan et al., 2005; Chuang et al., 2005). Another method used to demonstrate the effects of reduced mGluR signaling in suppressing FXS phenotypes has been to generate *Fmr1* knockout mice lines with reduced expression of mGluR5 by crossing two mutant lines. In these compound double mutant mice, many of the phenotypes associated with fragile X were corrected (Dolen et al., 2007). Although the use of these methods to reduce mGluR signaling and thereby rescue some of the phenotypic abnormalities has proven to be effective in mice, phase 3 clinical trials using mGluR antagonists in human patients were vastly unsuccessful. Another issue of concern is the fact that many studies have indicated that the association between mGluRs and FMRP is not a direct one, and that there may be intermediate players involved.

Dysregulation of the PI3K pathway in the absence of FMRP

Recent studies have indicated that the dysregulation of signal transduction pathways is not limited to gp1 mGluR signaling in the absence of FMRP; other membrane receptor signaling pathways that seem to be dysregulated include signaling through muscarinic acetylcholine receptors and dopamine receptors (Volk et al., 2007; Wang et al., 2008). The variety of affected signaling pathways suggest that FMRP acts on a common downstream signaling molecule rather than acting on each type of membrane receptor on its own. It is proposed that this common downstream molecule is PI3K, and that FMRP acts on the enzyme through its regulation of p110β mRNA, encoding the PI3K catalytic subunit, and the mRNA encoding PI3K enhancer (PIKE). The PI3K pathway lies downstream of mGluRs and other cell surface receptors and transduces signals from these receptors through the Akt/mTOR pathway (Lee et al., 2011). It is essential in synapse and dendritic spine development, and for forms of synaptic plasticity essential to learning and memory. Dysregulated signaling through the pathway has been shown to be a common mechanism underlying a diverse set of brain disorders such as epilepsy, intellectual disability, and autism; many of these disorders are associated with FXS as well, which could be seen as an indication of a possible association between the two (Hoeffer and Klann, 2010).

The association between the PI3K pathway and FXS is further validated by the fact that PIKE and p110β mRNAs are both targets of FMRP (Gross et al., 2015). The p110β and PIKE mRNAs are thus translationally repressed by FMRP, and in the absence of FMRP in *Fmr1*-KO mice, its activity is upregulated. The increase in the translation of both mRNAs suggests an increase in the activity of PI3K. Thus, it is believed that the excess and dysregulated general

protein synthesis in FXS is to due loss of FMRP mediated repression of the synthesis of two key components of the PI3K signaling complex, p110b and PIKE. Supportive of this model, recent studies have also shown that a PI3K antagonist can correct many of the key phenotypes seen in FXS mouse models: anomalous synaptic transmission and protein synthesis, increased immature spine density, and enhanced AMPA receptor internalization (Gross et al., 2010).

The benefits of a human-based in vitro model

Many of the studies thus far have been done in *in vivo* mouse or fly models, but these models are not truly representative of what occurs in human cells afflicted by FXS. Psychiatric disorders are uniquely human conditions, and cannot be truly interpreted in the disease context without understanding the underlying cellular and molecular events. The *Fmr1* KO mice that are used to model FXS do not express FMRP, but the FXS patients the model is trying to represent are, in many cases, actually mosaics. Their mosaicity could possibly be either due to the presence of partially silenced alleles or permutation length alleles, which are not silenced and as a result produce levels of FMRP that can vary (Wohrle et al., 1998). This variable expression of FMRP could possibly have downstream effects, which are not seen in the mouse model. Thus a human-based *in vitro* model is necessary to truly gain an understanding of the condition, and make discoveries that are translatable to human patients. Characterization of molecular phenotypes in human patient cells could be used for future drug testing, and also as biomarkers during future clinical trials, to assess if candidate drugs reduce the excess protein synthesis in FXS. Human-based *in vitro* experimental systems allow for mechanistic experimentation on patient biomaterial, consequently recapitulating human genetic diversity and overall disease phenotype.

Using patient-derived cells is even more advantageous because the disease phenotype can be studied in its natural context.

Primary human fibroblasts are a possible human cellular model that carry many benefits such as easy isolation and care. They also contain the same dysregulations as any other cell, so they are suitable for the modeling of human disease mechanisms. An added benefit of fibroblasts is that they can be made into iPSCs and subsequently into neurons. Transformation into neurons allows for further testing of the validity of fibroblasts as models of the disease by testing for commonalities between the two and also *in vitro* visualization of neuron-specific morphological changes caused by the condition. The majority of the experiments carried out in this study have been conducted on fibroblasts, but a limited number of experiments have been done with iPSC-derived neural progenitor cells, multipotent cells capable of becoming a diverse set of neurons and glia.

Hypothesis and specific aims

Studies done in the Bassell lab and others have all indicated a significant increase in PI3K activity and protein synthesis. In a FXS patient lymphoblastoid cell, protein synthesis was shown to be elevated. In the same study, protein synthesis was also found to be elevated in synaptoneurosomes from an FXS mouse model (Gross & Bassell, 2012). Both of these models arrive at the same conclusion, but as noted earlier, none of the models are truly representative of what happens in human FXS patients. In order to determine whether the same mechanistic signaling dysregulations occur in human patients, human fibroblasts have been used for this study. **We hypothesized** that there would be an increase in protein synthesis in the fragile x patient derived fibroblasts compared to controls. In order to quantitatively analyze the

differences in levels of protein synthesis in control versus FXS cells, azidohomoalananine (AHA) labeling as a tagged methionine surrogate, using click chemistry was used, coupled either to a flow cytometry based assay or immunocytochemistry. Based on previous studies, **we also hypothesized** that there would be an increase in PI3K activity in the FXS patient-derived cells.

Methods

Culturing patient fibroblasts

Frozen vials were removed from the liquid nitrogen tank and thawed in a water bath for 4 mins by gently swirling. The cells were then transferred to a 15 mL conical tube using a p1000 pipette and 6 mL DMEM/F-12 was added. The vial was rinsed with 1 mL of DMEM/F-12, and this was also transferred to the tube. This mixture was spun at 300xg for 5 mins, and the resulting pellet resuspended in FB medium that was then plated into one 10 cm dish.

The fibroblasts were passed whenever they reached ~80% confluence, which happened approximately every 4-6 days. They were generally grown in 10 cm dishes. For passaging, they were first rinsed in 1x PBS, and then 3 mL 0.05% Trypsin-EDTA was added. The dish with the trypsin was incubated 37°C for 5 minutes and then 7 mL fibroblast (FB) medium was added to neutralize the activity of the trypsin. The cells and media were transferred to a 15 mL conical tube and centrifuged at 300xg for 5 mins. After the spin, the supernatant was removed and the pellet was resuspended in 1 mL fresh FB. Cells were counted and 500,000 cells were plated per new 10 cm dish.

Culturing iPSCs

iPSC colonies are maintained in mTeSR media and were passaged every 5-7 days onto matrigel coated dishes. Ideal iPSC cultures contain little to no differentiation, and any large differentiating colonies can be removed using a p1000 pipette tip. iPSCs were passaged using ReLeSR (Stemcell Technologies).

Differentiation of iPSCs into NPCs

iPSC lines were passed into 60 mm dishes and allowed to overgrow to such an extent that the colonies were touching each other. Two days prior to making the embryoid bodies (Day -2), the medium was changed to N2 basal media consisting of DMEM/F-12 50:50, 1 M Hepes (15mM), N2 supplement (1X), GlutaMAX (1X), and Penstrep (0.2%). The factors SB431542 (10 μM) and DMH1 (5 μM) were added along with the media. The same media was used to feed the next day. On Day 0, the media was replaced and the colonies were transferred to one well of a 6-well dish and placed on a shaker overnight in an incubator set to 37°C.

Small EBs started to form by Day 1, and the EBs were fed on days 1, 3, and 5. On Day 6, the EBs were transferred to a 60 mm matrigel coated dish with NPC media, which is the same as the N2 basal media except for that it also contains B-27 supplement (1X), and bFGF (20 ng/mL). These cells were then fed every other day on days 8 and 10. At this point, the EBs plated down and began to form neural rosettes. High quality rosettes were identified and selectively hand-picked using a pipette tip. These were then transferred to a 15 mL conical tube and spun down at 200xg for 4 mins. 2 mL Accutase was then added, and the tube was allowed to incubate for 15 min at 37°C. This was followed by the addition of 1x PBS and a centrifugation step at 200xg for 4 min, which resulted in a pellet that was resuspended in NPC media and FGF in order to be plated on a PLO/laminin coated 10 cm dish. The cells that were plated were p0 NPC, which were once again fed every other day.

Immunofluorescence for iPSC pluripotency characterization

iPSCs were plated on matrigel-coated glass coverslips in 12-well plates. Cells were fixed using 0.5 ml 4% PFA per coverslips for 15 minutes at room temperature. This was followed by three 15 minute long washing steps using 1x PBS. The cells were then permeabilized using a solution of 1x PBS with 0.1% Triton-X for 10 minutes, and then blocked in a solution of 5% Donkey serum, 0.1% BSA and 0.1% Triton-X in 1X PBS for one hour at room temperature. Six primary antibodies against markers of pluripotency were used for each iPSC line and all primary antibodies were used at a dilution of 1:100 in blocking buffer Primary antibodies used were Rabbit anti Oct4 (Cell Signaling Technology), mouse SSEA4 (Cell Signaling Technology), rabbit Sox2 (Thermo Scientific), mouse Tra1-60 (Millipore), goat Nanog (RND Systems), and mouse Tra1-81 (Millipore). A humidified chamber was prepared and each coverslip was inverted onto 50 μL of the primary antibody solution and incubated in the primary antibody solutions overnight at 4°C. On the second day, the coverslips were transferred face up back into their respective wells within a 12-well plate and washed three times for 15 minutes each in 1x PBS. The following secondary antibody solutions were then prepared at 1:500 dilution in 1x PBS: Cy-2 coupled donkey anti-goat, Cy-3 coupled donkey anti-mouse IgM, Cy-2 coupled donkey anti-rabbit, and Cy-3 coupled donkey anti-mouse. Secondary antibodies were added to the coverslips in the same manner as the primary antibodies and incubated for one hour at room temperature. The coverslips were then once again returned to their respective wells within the 12-well plate and washed in 1x PBS. The coverslips were then mounted on slides using 25 μL of Prolong Gold with DAPI.

AHA labeling with Fluorescence Microscopy

The fibroblasts being used for this experiment were first grown on glass coverslips in a 12-well plate. Once ready for AHA labeling, the cells were washed in PBS, and incubated in 500 µL per well of methionine-free medium for 30 mins. Then anisomycin (final concentration of 40 µM) was added to control wells for 30 mins to inhibit protein synthesis. 10 µL AHA was then added to all wells except no AHA control wells and cells incubated for 1 hour at 37°C. The fibroblasts were then washed in PBS, and fixed in 4% PFA for 15 mins. This was followed by permeabilizing them with 0.25% Triton X-100/PBS for 15 mins and a blocking step with 3% BSA/PBS for 45 mins. After this series of steps, 50 µL of the Click-iT reaction cocktail was added to each coverslip and the coverslips were incubated for 30 mins at room temperature followed by a wash. After washing in PBS, the coverslips were then incubated with Rhodamine-labeled Phalloidin for 30 mins at room temperature, and subsequently mounted on slides.

Western Blot

Equal amounts of the fibroblast lysates were loaded into 4-20% gradient SDS polyacrylamide gels, and these gels were run at 125 V for 50 minutes. The gel was then transferred to a nitrocellulose membrane using a Bio-Rad Trans-Blot Turbo Transfer System. The system was set to 1.3 A, 25 V, and 7 minutes to allow for the complete transfer. This was then followed by a blocking step, where the membrane was immersed in Odyssey Blocking Buffer (diluted 1:1 in 1x TBS) for one hour at room temperature. The membrane was then probed using antibodies either for FMRP (mouse Anti-FMR1, 1:500, Millipore) or p110β (rabbit Anti-PI3 Kinase/p110β, 1:5000, Millipore). In both cases, β-actin (mouse beta-Actin, 1:6000, Cell Signaling Technology) was used as a loading control. The antibodies were diluted in a 1:1

mix of TBS-Tween (0.2%) and Odyssey Blocking Buffer, and the membrane was incubated with the primary antibodies on a horizontal shaker overnight at 4°C. In the subsequent day, the membrane was washed three times for 10 minutes each, in mixture of 0.1% TBS-Tween. The secondary antibodies used were Donkey anti-mouse 680LT (1:20,000) and Donkey anti-rabbit 800CW (1:10,000), both of which were diluted in 0.1% TBS-Tween; the membrane was incubated with the secondary antibodies for 1 hour at room temperature in the dark. The membrane was washed three times in 0.1% TBS-Tween for10 minutes each. The bands were visualized using the Odyssey imaging system, and the blots were quantified using *Image J.*

Bicinchoninic acid (BCA) assay

A BCA assay was used to determine protein concentrations of the samples. A standard curve was prepared with known concentrations of BSA: 0mg/mL, 0.25 mg/mL, 0.50 g/mL, 1 mg/mL, and 2 mg/mL. Cell lysates were diluted 1:10 in water for a total volume 40 µL. 10ul of the samples and the standards was pipetted per well of a 96-well plate and 200 µL of a 50:1 solution of Reagent A: Reagent B (Thermo Scientific**)** was added per well. All standards and samples were run in triplicate. Following a 30 min incubation step at 37°C, the 96-well plate was analyzed on a plate reader (BioTek Synergy HT) and the protein concentrations of the samples were calculated based on the standard curve.

Imaging and analysis

Fixed and stained coverslips were visualized with a 60x objective (Nikon, Melville, NY) on a Nikon Eclipse inverted microscope. Exposure times for each channel were unchanged across experiments and images were analyzed with Imaris (Bitplane). Rhodamine phalloidin was used as a mask and AHA fluorescent signal was visualized in the Cy-5 channel.

Results

Although in the past there have been several studies indicating differences in protein synthesis levels and PI3K activity in FXS animal models, there have been few to none done on human cells (Gross et al., 2015; Gross et al., 2010). Many studies have shown that animal models are not completely representative of the underlying disease mechanisms in humans that they are attempting to recapitulate. Due to this possible lack of concordance, it is essential that the mechanisms be tested in human cells for validation. There are two principle aims of this thesis: (1) To culture control and FXS patient derived fibroblasts and iPSCs, and generate a population of patient-derived neural progenitor cells and (2) to quantitatively analyze differences in protein synthesis levels between FXS patient and control cells using azidohomoalanine (AHA) labeling. Based on previous studies done with FXS animal models, we hypothesized that there would be an increase in both protein synthesis in the FXS patient fibroblasts as well as FXS patient iPSC-derived neural progenitor cells.

Sources of different fibroblast lines

A total of eight male fibroblast lines were used for the experiments in this study (Table 1). The patients that were included in this study have received clinical diagnoses of either FXS alone or FXS with autism. These patient fibroblasts have also been used to generate and establish induced pluripotent stem cell (iPSC) lines in our lab, which were subsequently characterized and differentiated into neural progenitor cells (NPCs) in this study.

Characterization of control and FXS patient derived iPSCs

A primary goal of this study was to characterize patient derived induced pluripotent stem cell lines and to use these iPSCs to generate neural progenitor cells (NPCs) to

model phenotypes of FXS. The patient fibroblast lines listed in Table 1 were used in our lab to generate induced pluripotent stem cells (iPSCs). In order to reprogram the fibroblasts and make them into pluripotent cells, we needed to induce high expression of four transcription factors: *Oct4, Sox2, c-Myc, Klf4.*These four transcription factors have been universally recognized of having the capacity to reprogram terminally differentiated cells into stem cells. Inducing unusually high levels of expression relative to fibroblasts was done using the Sendai virus, a single-strand RNA virus that replicates in the cytoplasm. The benefit of using this virus is that it does not integrate into the host genome, assuring that the host genome is not disrupted and making the cells useful for studying disease mechanisms (Nakanishi & Otsu, 2012). Additionally, the virus replicates independently of the cell cycle, allowing the virus to produce high copy numbers of the target genes.

In order to assess whether the cells were truly pluripotent, we characterized the iPSC lines for markers of pluripotency using immunofluorescence (Figure 1). Several molecular markers have been identified, and these can be used to verify both undifferentiated embryonic stem cells and iPSCs (Yu et al., 2007). For characterization in this study, the Yamanaka factors, Oct4 and Sox 2, were used in addition to the pluripotency markers, SSEA4, Tra-1-60, and Tra-1-81. The immunocytochemistry protocol used allowed for the visualization of two proteins at once, each of the visualized proteins appearing in either red or green depending on the secondary antibody used. In addition to the antibodies, a DAPI stain was used. DAPI is a fluorescent stain that binds to A-T rich regions in DNA, and subsequently allowed us to locate the nucleus of each cell. The results of the procedure indicate that all the markers and transcription factors are present in all the cells of the colonies shown, and the same is true of all the iPSC lines that were characterized. Before proceeding to generate NPCs, we felt that it was necessary to characterize

iPSCs for markers of pluripotency and to also confirm the lack of FMRP in FXS patient iPSC lines (Figure 3).

Generation of control and patient derived neural progenitor cells (NPCs)

Two control and four Fragile X patient iPSC lines were differentiated into neural progenitor cells (NPCs). The differentiation process from iPSCs to NPCs takes approximately 45-50 days, (Figure 2). Broadly, the steps are: adding SB431542 and DMH1 (SMAD inhibitors), isolating neural rosettes, and adding FGF2 to maintain NPCs. The drug SB431542 works by inhibiting Lefty/Activin/TGFβ pathways by blocking phosphorylation of ALK4, ALK5, and ALK7 receptors (Chambers et al. 2009). DMH1 is a highly selective small BMP-inhibitor (Neely et al. 2012). It should be noted that either treatment alone is not sufficient for the conversion into NPCs. FGF2 is added in later in the process, because it has been shown to be necessary for NPC survival when cultured at a low density. After the NPCs were generated, they were passaged for at least two passages before they were used for experiments.

Fragile X patient cells lack FMRP and show upregulation of p110b

We performed western blots for the Fragile X mental retardation protein (FMRP) in order to confirm the absence of the protein in patient lines. Although the patients included in the study have been confirmed to have a clinical diagnosis of FXS, this is a necessary step to study phenotypes at the molecular level. We used an antibody against FMRP, and β-actin as a loading control, and confirmed absence of FMRP in patient derived fibroblasts, iPSCs, and NPCs (Figure 3). Western blot analysis confirms elevation of p110b in human FXS fibroblasts, iPSC and NPCs (Figure 4), as observed in earlier reports (Gross et al 2010; 2012).

Optimization of conditions for Click-iT AHA metabolic labeling of new protein synthesis

There were two methods used for obtaining results from Click-iT AHA labeling. The first of which involved the use of quantitative fluorescence microscopy to visualize incorporation. The second method involved using a flow-cytometry based assay to analyze thousands of cells in an unbiased high throughput manner.

This is the first application of a flow-cytometry based assay using Click-iT AHA labeling for newly synthesized proteins in FXS patient cells, and thus one of the objectives was to determine the optimal conditions for the best results. In order to optimize conditions for this assay we tested different incubation times for incubation with the metabolic reagent AHA (15 mins, 30 mins, 1 hour), as well as the click-it reaction (30 mins, overnight). The incubation times selected were based on previously published literature using these reagents (Dieterich et al., 2010; Zhang et al., 2014). It can be seen that as the duration of the incubation with AHA increases, the resulting median fluorescence intensity (MFI) increases. The same can be said for the Click-iT reaction. As incubation time increases, so does the MFI. The control group for AHA labeling also contains anisomycin, which is an antibiotic that arrests protein synthesis. It is expected that there will be limited fluorescence in these cells, because there should be no new protein synthesis and thus no incorporation of AHA. The optimal conditions for these experiments appear to be hour incubation with AHA, combined with overnight click-it reaction. At these conditions there appears to be a difference in MFI indicating levels of protein synthesis between FXS and control lines.

Evidence of increased global protein synthesis in Fragile X patient fibroblasts

As stated previously, flow-cytometry was used to quantify the fluorescence produced by Click-iT AHA labeling in three control and three FXS lines. In order to determine the gating strategy, we used a forward scatter (FSC) versus side scatter (SSC) density plot to identify the viable, single cells within the sample cellular debris is FSC-low and SSC-high and cellular clumps are FSC-high so these factors were taken into account when gating the cells. The analysis was conducted only on the gated population of cells (Figure 6A). For the purpose of this assay, 25,000 events were recorded. It can be seen that there is a clear shift in peaks, with a representative FXS patient line (SO10) showing a greater fluorescence intensity compared to a representative control line (SC176) (Figure 6B). Furthermore, pooling together the control and FXS lines shows that there is an increase in protein synthesis in FXS patient fibroblasts compared to controls. This important result provides proof of concept that protein synthesis is elevated in cells from human FXS patients. Furthermore, this method could be used to measure protein synthesis as a potential biomarker in patient-derived cells, in a high throughput manner. This technique could also be extended to testing the therapeutic value of drug candidates in a patient-specific manner.

The second method used to quantify differences in fluorescence involved the use of fluorescence microscopy. Phalloidin was used to visualize F-actin, which allowed us to identify individual cells. The results of this experiment were similar to those seen through flow-cytometry, but they have not been included; the purpose of this experiment is merely to reaffirm the results we obtained from the other experiment. There is a significant difference in mean fluorescence intensities between the FXS and control cells with the FXS cells having higher fluorescence intensity.

Discussion

The goal of this study was to test our hypothesis that the excess protein synthesis seen initially only in FXS animal models can also be seen in human FXS patient-derived cells. The results of our study provide further support to the idea that the elevation of protein synthesis seen in FXS is also present in human cells and not a unique feature of mouse models. Thus, targeting dysregulated protein synthesis could be a valuable therapeutic strategy in FXS, and the developed assay using AHA labeling and flow cytometry could be potentially used for drug screening or as a biomarker (Figure 6).

The validity of the use of fibroblasts in modeling a human neuronal disease

Primary human fibroblasts are easily obtained, and do not have many requirements in terms of upkeep. Furthermore, these cells could be used to identify new therapeutic targets for conditions such as FXS and also be used to identify biomarkers to test the potency of targeted therapies in specific individuals (Kumari et al., 2014). These conditions make them an ideal *in vitro* model for many diseases. The validity of using fibroblasts in studies has been studied extensively, and its suitability as model of human diseases has been confirmed. The pathophysiological mechanisms associated with psychiatric disorders can be investigated in cell cultures because psychiatric disorder have been shown to affect the entire body, and this encompasses peripheral cells such as fibroblasts; in other words, molecular anomalies contributing to dysfunction in neurons (e.g. mitochondrial changes and signal transduction disturbances) will also be present in non-neuronal cells derived from a patient's peripheral tissues (Kálman et al., 2016; Auburger et al., 2011). Specific to FXS, studies done on fibroblasts have indicated the viability of using them for studies of FXS through the finding of the same

dysregulations in fibroblasts found in other models; this included significantly elevated protein synthesis and the same dysregulated signal pathways found in other studies (Kumari et al., 2014).

Increased levels of protein synthesis

An important finding of this study is that elevated levels of global protein synthesis were observed in FXS patient-derived fibroblasts when compared to control patient-derived fibroblasts. Previous results from a study on *Fmr1*-KO mice have indicated a significant (~20%) and anatomically widespread increase in the rate of total cerebral protein synthesis (Qin et al., 2005); however, the results from this thesis study is the first to show that the same increase in global protein synthesis also occurs in human cells. It should be noted that two slightly different but complementary methods were used to obtain these results, and these are Click-iT AHA metabolic labeling with either or flow-cytometry. Both methods made use of Click-iT AHA labeling, which is used to identify all nascent protein synthesis by replacing methionine with AHA. AHA is an amino acid analog that contains an azido moiety, and once it is incorporated into the protein, click chemistry is used to couple the protein to an alkyne-fluorophore. The difference between the methods arises in the way this is visualized. In the method, the fluorescence is observed using fluorescence microscopy and subsequently quantified. Flow cytometry is a technology that detects any fluorescent properties of all cells run through the machine. The benefits of this technology are that the machine is not biased in its quantification of fluorescence and that it is able to generate more accurate data through the analysis of a much larger sample of cells. The application of the technology to detect nascent protein synthesis in FXS patient-derived cells is a truly novel assay, which was developed in this study. Through the assay developed in this thesis, further studies can be conducted on dysregulated protein

synthesis, which has been implicated in a variety of disorders, including many autism spectrum disorders (Kelleher & Bear, 2008).

Due to the novelty of this assay, a primary objective of this study was to investigate and develop a standard protocol that optimizes the conditions. It was found that median fluorescence intensity (MFI) was greatest when the conditions included an overnight Click-iT reaction and a one hour incubation with AHA. The direct relationship between MFI and AHA incubation time is most likely due to how longer durations in time allow for more protein synthesis to occur, which in turn allows for more AHA to become incorporated and a higher resulting MFI. Ideally, AHA incubation time should correspond to the general protein synthesis rate. Previous studies are in concordance with the conclusion made from our results that one hour is the optimal time. The same direct relationship was observed with Click-iT reaction times. A longer reaction correlated to an increased MFI. It should be noted that an increased reaction time could possibly result in an increased background signal due to non-specific binding. However, background fluorescence was accounted for by the use of no-AHA control wells, which were used as a baseline to normalize MFI signal.

Increased protein synthesis may be related to increased PI3K activity in FXS patient cells

Previous studies done in the Bassell lab have pointed at the relationship between FMRP and p110β, which is the catalytic subunit of PI3K. The studies indicated that there was enhanced p110β protein expression in cultured neurons of *Fmr1* KO mice (Gross et al., 2010) as well as global increase in protein synthesis. Our data provides evidence that the same enhanced protein expression occurs in FXS patient-derived cells. Due to the fact that animal models are not always completely representative of the molecular mechanisms occurring in humans, studies done on models are not always translatable to humans. Our study shows that in this case the results do

carry over. Preliminary results using western blotting indicate that cells derived from Fragile X patients have an increased expression of p110β (Figure 4). This difference was seen across all cell types, but it was the most apparent in neurons. It is possible that other isoforms of p110 are contributing to the activity of PI3K in other cell types. In vertebrates, there are eight different catalytic subunits of PI3K and these can be divided into three different classes based on their protein structure, function, and associated regulatory subunits (Hawkins et al., 2006; Gross & Bassell, 2014). The largest and most studied of these three classes is class I, and it is the class to which p110β belongs to; other catalytic subunits included in the class are p110α, p110δ, and p110γ (Thorpe et al., 2015). Activation of class I catalytic PI3K subunits by extracellular stimuli primarily occurs via either receptor tyrosine kinases or G protein-coupled receptors, and it has been shown that the isoforms have preferences to which receptor type they associate with; P110β primarily associates with GPCRs (Guillermet-Guibert et al., 2008). Due to the abundance of this receptor type in neurons, it is possible that there is a larger quantity of the p110β mRNA present in neurons. In control cells, translation of the mRNA would be repressed, but there is nothing to stop translation of the mRNA in FXS cells. One of the hallmark characteristics of FXS animal models is exaggerated signaling through group 1 metabotropic glutamate receptors, and as posited by the mGluR theory, many of the symptoms seen in FXS can be explained by the overactive signaling gp1 mGluRs. Although mGluR antagonists such as MPEP have been shown to recover many of the FXS associated phenotypes in animal models, the same mGluR targeted treatments have been vastly unsuccessful in humans (McBride et al., 2005; Krueger & Bear, 2011). Due to the unsuccessful clinical trials, there still exists a need for identifying other potential targets for therapeutic drugs. One candidate are subunit selective p110β inhibitors which are now in clinical trials for cancer, which might be repurposed for FXS.

Studies indicate that multiple membrane receptor signaling pathways are dysregulated in the absence of FMRP, such as muscarinic acetylcholine receptors, dopamine D1/5 receptors, and tyrosine kinase receptor B (Volk et al., 2007; Wang et al., 2010; Osterweil et al., 2010). This dysregulation of multiple receptor signaling pathways suggests that FMRP acts on a common downstream signaling molecule. Activation of signaling through Gq protein-coupled receptors and dopamine receptors has been shown to induce protein synthesis; the same induction of protein synthesis was shown to occur when TrkB was activated (Wang et al., 2010; Minichiello, 2009). In FXS animal models, an increase in protein synthesis was seen through all these receptors, further supporting the idea of FMRP targeting a common downstream effector (Louhivuori et al., 2011). The increase consistent increase in protein synthesis suggests that the common effector is involved in that process. In gp1 mGluRs, protein synthesis is regulated primarily through two pathways: the ERK1/2 pathway and the PI3K/mTor pathway; making molecules in either of the two pathways possible targets of FMRP. When the different signaling pathways through these many receptors are compared, it can be seen that they all have signaling through PI3K in common (Yoshi & Constantine-Paton, 2010). This commonality makes PI3K a prime therapeutic target for regulation by FMRP, and explains why we would see an increase in activity in the absence of FMRP.

The present study provides strong evidence to show that global protein synthesis is dysregulated in a human cellular model of FXS. We also have preliminary results to suggest that PI3K signaling may be elevated in human FXS patient cells. Future directions for this work could include using the same patient derived cellular models to study whether inhibiting PI3K signaling will have a corrective effect on the dysregulated protein synthesis. Patient derived NPCs can be further differentiated into neurons, which can be used to study whether there are

any morphological differences between control and FXS patient neurons. It is anticipated that the flow cytometry based AHA labeling assays developed here to document excess protein synthesis in human FXS fibroblasts will be further developed for analysis of iPSC derived neurons from FXS patients. This work has important implications to define a technical approach to be used for drug validation and biomarker analysis in a clinical trial, not only for FXS but also more broadly for neurodevelopmental disorders having protein synthesis dysregulation as a core feature responsible for the pathophysiology.

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Figures

Table 1. Descriptions of the different Fragile X Syndrome patient-derived and control patient-derived fibroblast cell lines with line numbers and sources with a total of four control cell lines and four Fragile X patient cell lines.

LINE	DIAGNOSIS	SOURCE
SC173	Ctrl	Schwartz (UC Irvine)
SC176	Ctrl	Schwartz (UC Irvine)
CH069	Ctrl	Cubells/Hales (Emory)
CH084	Ctrl	Cubells/Hales (Emory)
SC126	FXS + Autism	Schwartz (UC Irvine)
SC128	FXS	Schwartz (UC Irvine)
CH095	FXS + Autism	Cubells/Hales (Emory)
SO10	FXS	Berry-Kravis (Rush)

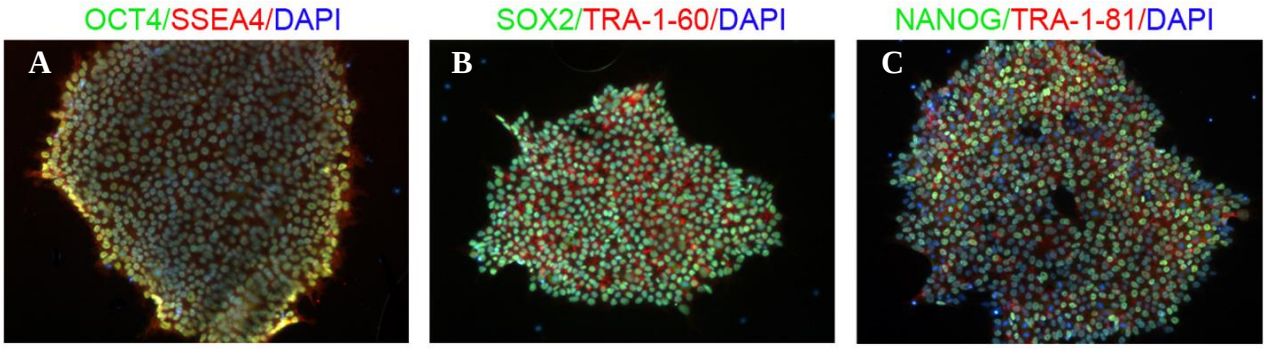

Figure 1. Characterization of control and FXS patient-derived iPSC colonies through the detection of various pluripotency markers.
Coimmunostainings of **(A)** Oct4 (green) with SSEA4 (red) **(B)** Sox2 (green) with Tra-1-60 (red) **(C)** Nanog (green) with Tra-1-81 (red) are shown. The presence of all the markers in each cell indicates that the transformation of fibroblasts into iPSCs was successful and subsequently each cell has the capacity for pluripotency.

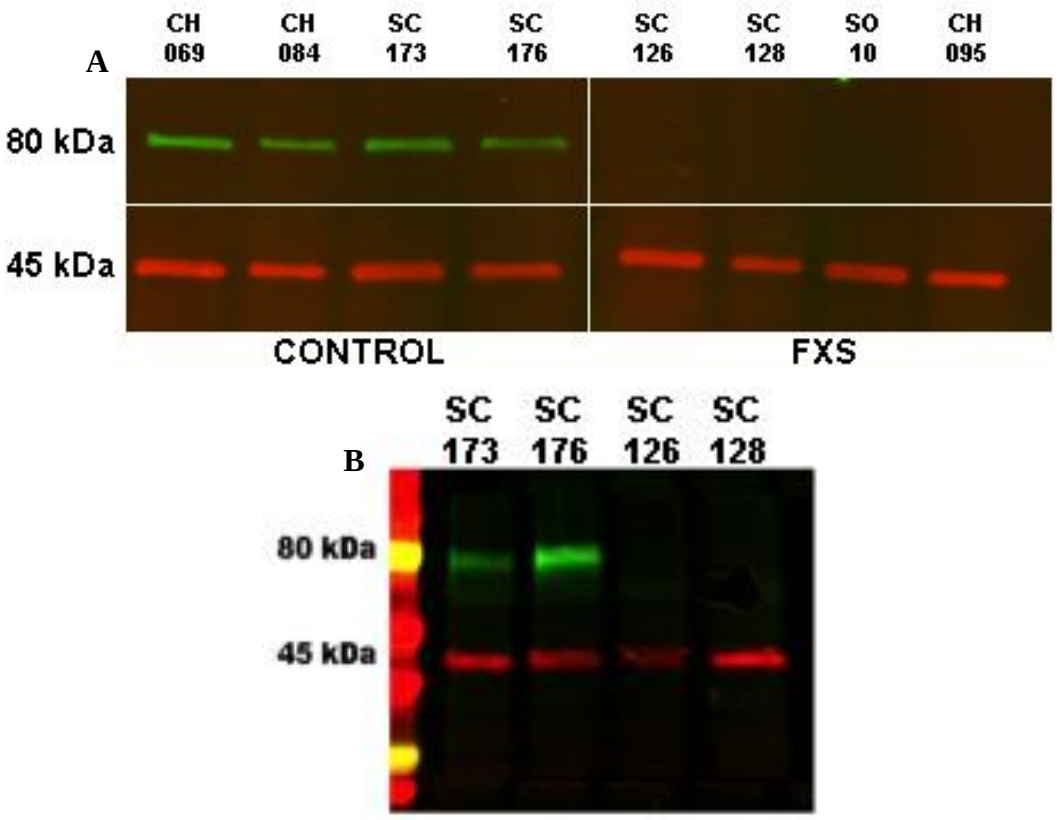

Figure 2. Western blot analysis of FMRP expression levels in FXS and control lines.
The presence of FMRP is indicated by the green bands, and the red bands indicate the presence of β-actin, which was the loading control. It be can be seen that FMRP is only present in the control lines, and not in the Fragile X lines. This is due to the methylation of *Fmr1* gene promoter caused by an expanded trinucleotide repeat present in the 5' UTR region. The lack of FMRP in FXS patient-derived cells is shows in **(A)** fibroblasts and **(B)** iPSCs.

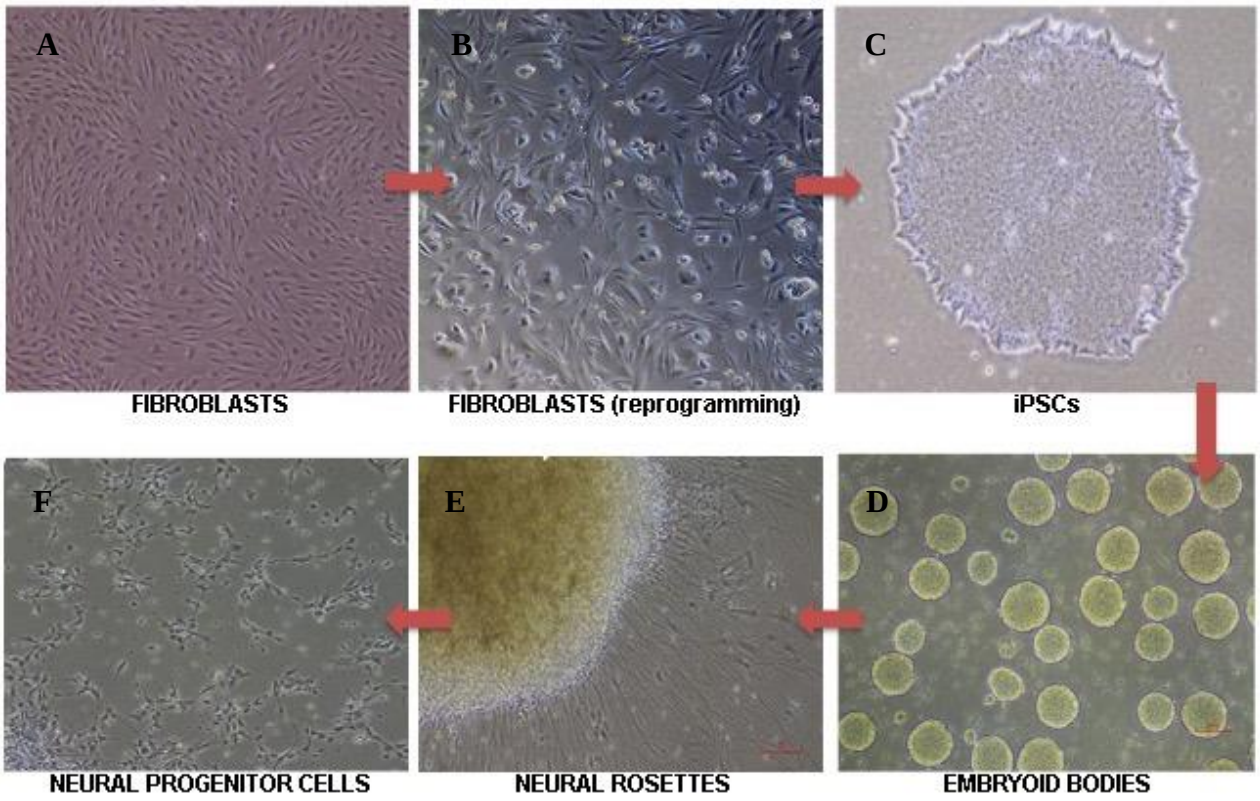

Figure 3. An overview of the steps in the differentiation process from a fibroblast into iPSC-derived NPCs.
(A) Initial fibroblasts cells grown from patient skin biopsies. **(B)** Transitioning fibroblasts 14 days after transduction with reprogramming viral cocktail. A clear reduction in number of fibroblasts is seen after transduction of virus due to cytotoxicity caused by high uptake of virus. **(C)** An iPSC colony generated after a successful transduction. (**D)** First step in the generation of NPCs from iPSCs involves the formation of Emryoid Bodies (EBs), three-dimensional aggregates of multipotent stem cells. **(E)** Neural rosettes are generated from EBs through dual-SMAD inhibition, and these cells express many of the proteins found in neuroepithelial cells in neural tube (Wilson & Stice, 2006). **(F)** Dissociated rosettes are cultured with FGF2 and maintained as NPCs.

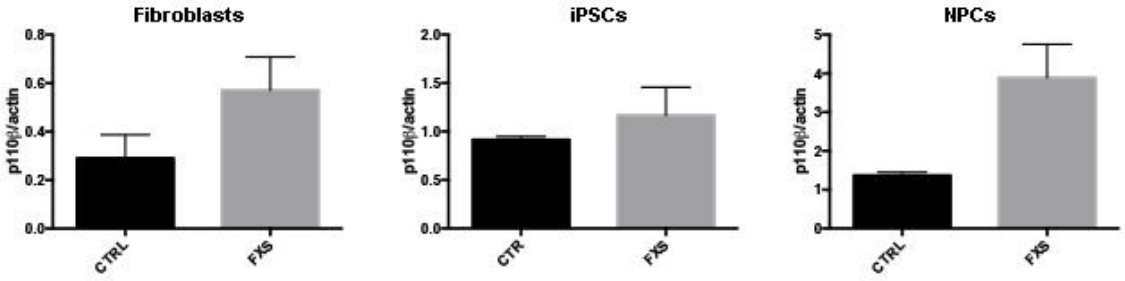

Figure 4. Preliminary western blots showing increased p110β protein levels in FXS compared to control cells.

It can be seen that there is a consistent increase in p110β protein levels acr**oss** all the cell types from the FXS lines with the biggest difference being in the NPCs. The increase in p110β suggests an increase in PI3K activity, because p110β is the catalytic subunit of the protein.

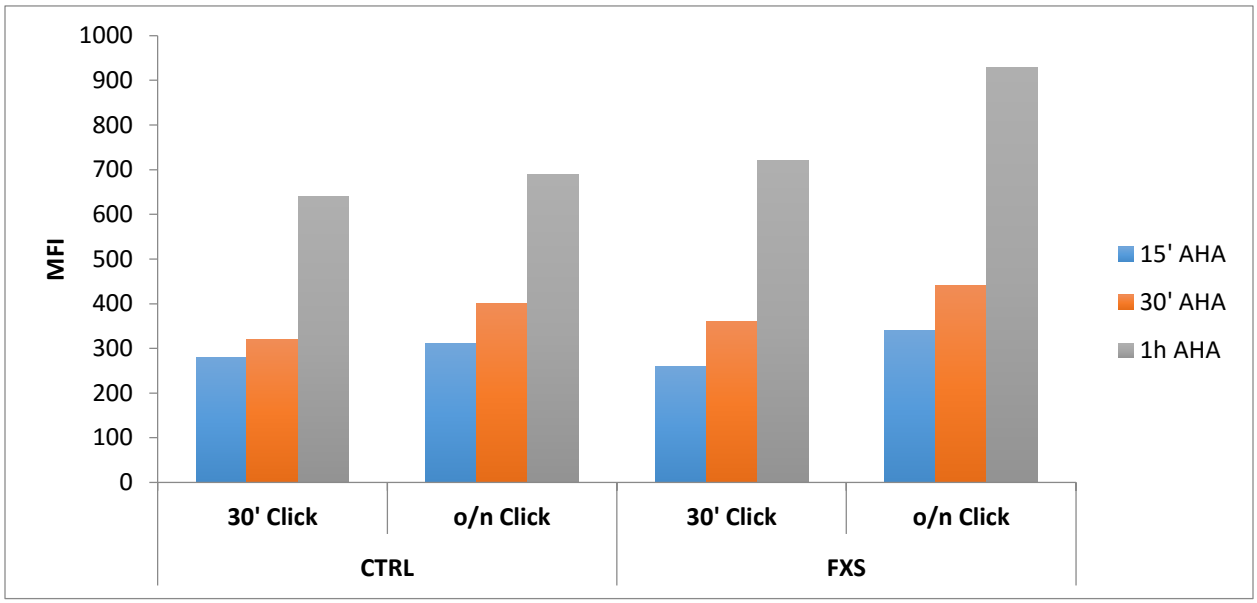

Figure 5. Analysis using flow-cytometry of different AHA labeling and Click-iT reaction incubation times for optimization purposes.
The figure indicates that the optimal conditions are a one hour incubation with AHA coupled to an overnight Click-iT reaction. It can be seen that incubation times are directly related to MFI. An increase in incubation times is leads to an increase in MFI, which is indicative of an in increase in protein synthesis.

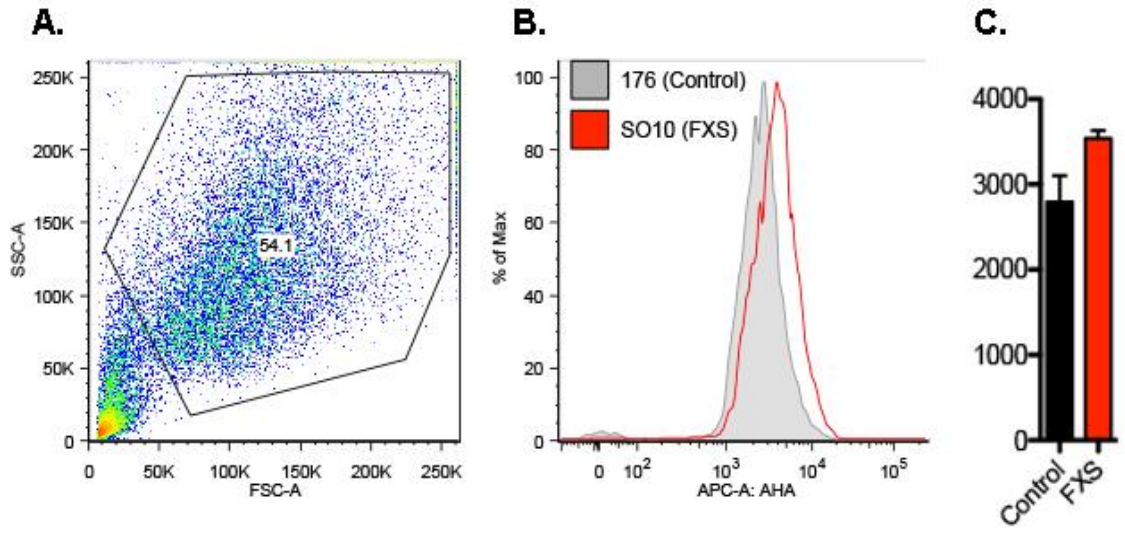

Figure 6. The results from the novel assay consisting of flow-cytometry coupled with Click-iT AHA metabolic labeling indicates an increase in fluorescence intensity in FXS fibroblasts indicating an increase in protein synthesis.
(A) The gating strategy used minimized the number of dead cells or clumps cells contributing to the median fluorescence intensity. **(B)** The shift in fluorescence peaks marks the increase in protein synthesis in FXS patient-derived fibroblasts. **(C)** Pooled data from 3 control and 3 FXS fibroblast lines show an overall increase in protein synthesis in FXS lines compared to control (n=3)