Clinical and Molecular Assessment in a Female with Fragile X Syndrome and Tuberous Sclerosis

Carolyn M Yrigollen[1], **Laura Pacini**[2], **Veronica Nobile**[2], **Reymundo Lozano**[3], **Randi J. Hagerman**[3,4], **Claudia Bagni**[2,5,6], and **Flora Tassone**[4,7,*]

[1]Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, USA
[2]Department of Biomedicine and Prevention, University of Rome Tor Vergata, Italy [3]Department of Pediatrics, University of California, Davis, USA [4]MIND Institute, University of California, Davis, USA [5]VIB Center for the Biology of Disease and Center for Human Genetics, Leuven, Belgium [6]Department of Fundamental Neuroscience, University of Lausanne, Lausanne, Switzerland [7]Department of Biochemistry and Molecular Medicine, University of California, Davis, USA

Abstract

Objective—Fragile X syndrome (FXS) and tuberous sclerosis (TSC) are genetic disorders that result in intellectual disability and an increased prevalence of autism spectrum disorders (ASD). While the clinical presentation of each disorder is distinct, the molecular causes are linked to a disruption in the mTORC1 (mammalian Target of Rapamycin Complex 1) and ERK1/2 (Extracellular signal-Regulated Kinase) signaling pathways.

Methods—We assessed the clinical and molecular characteristics of an individual seen at the UC Davis MIND Institute with a diagnosis of FXS and TSC. Clinical evaluation of physical, behavioral, and cognitive impairments were performed. Additionally, total and phosphorylated proteins along the mTORC1 and ERK1/2 pathways were measured in primary fibroblast cell lines from the proband.

Results—In this case the phenotypic effects that result in a human with both FXS and TSC are shown to be severe. Changes in mTORC1 and ERK1/2 signaling proteins and global protein synthesis were not found to be noticeably different between four cohorts (typically developing, *FMR1* full mutation, *FMR1* full mutation and *TSC1* loss of function mutation, and *TSC1* loss of function mutation); however cohort sizes prevented stringent comparisons.

Conclusion—It has previously been suggested that disruption of the mTORC1 pathway was reciprocal in TSC and FXS double knock-out mouse models so that the regulation of these pathways were more similar to wild-type mice compared to mice harboring a $Fmr1^{-/y}$ or $Tsc2^{-/+}$ mutation alone. However, in this first reported case of a human with a diagnosis of both FXS and TSC, substantial clinical impairments, as a result of these two disorders were observed. Differences in the mTORC and ERK1/2 pathways were not clearly established when compared between individuals with either disorder, or both.

---

[*]Corresponding author: Flora Tassone, Department of Biochemistry and Molecular Medicine and MIND Institute, University of California, Davis 2805 50th Street, Sacramento, CA 95817, USA, Tel: 916-703-0463; Fax: 916-703-0464; ftassone@ucdavis.edu.

Keywords

Fragile X syndrome; Tuberous sclerosis; mTORC1; ERK1/2; Protein synthesis

---

Introduction

Fragile X syndrome

Fragile X syndrome (FXS) is a neurodevelopmental disorder that is caused by a mutation in the fragile X mental retardation 1 (*FMR1*) gene. Typically, phenotypic severity is greater in males with FXS compared to their female counterparts [1]. Approximately 1 in 5000 males and 1 in 2500 to 1 in 8000 females have FXS [2]. Individuals with FXS can suffer from an array of behavioral, cognitive, neurologic, and physical problems, with core symptoms including intellectual disability, facial dysmorphia, macroorchidism, and mood disorders [3].

FXS occurs when the *FMR1* gene is silenced by hyper methylation of the expanded trinucleotide repeat in the 5′UTR or a loss of function mutation, resulting in the absence of the encoded protein, FMRP [4,5]. The lack of FMRP, an RNA binding protein linked to translational control [6,7] results in increased translation of many mRNA targets at the synapse where it plays an important role for synaptic maturation, plasticity and function [8]. In 2004, the mGluR theory of FXS hypothesis proposed a specific example of how FMRP could play a role in the regulation of synaptic function and plasticity by regulating long-term depression (LTD) of synaptic strength in hippocampal neurons [9]. This form of LTD, involving stimulation of the metabotropic glutamate receptor 5 (mGluR5), requires protein synthesis. Accordingly, the psychiatric and neurological aspects of FXS may be the consequence of an exaggerated response to synaptic activation of the group 1 mGluRs that is coupled to local protein synthesis. The learning and memory deficits are proposed to occur through the over activation of two signaling pathways that control the synthesis of synaptic proteins, the mammalian Target Of Rapamycin Complex 1 (mTORC1) pathway and the Extracellular regulating kinase (ERK1/2) pathway [9,10]. The mTORC1 pathway is central to regulating protein synthesis, cell growth and proliferation and regulates Cap-dependent translation following inputs including growth factors, oxidative stress, and adequate energy and amino acid levels [11]. The ERK 1/2 signaling pathway controls the activity of the eukaryotic initiation factor 4E (eIF4E), a substrate that initiates translation by recruiting ribosomes to the 5′ mRNA cap. The ERK 1/2 signaling pathway is activated through extracellular inputs including mitogens and stress inducers [12]. Multiple studies have observed increased phosphorylation of mTOR targets in humans and mice with *FMR1* mutations in brain tissue and in peripheral blood cells and platelets when compared to tissues from typically developing controls or wild type mice [13,14,15,16,17]. Specifically, Sharma, Hoeffer et al. [15] reported that mTOR signaling is upregulated in the KO mouse model of FXS and increased expression of the eukaryotic initiation factor complex 4F (eIF4F). The dysregulation of the mTOR signaling observed in the FXS mouse model was also observed in human subjects with FXS where an increased phosphorylation of P70 S6K1 (Thr389), ribosomal protein S6 (Ser235/236), Akt (Ser473), and eIF4E (Ser209) was detected in brain and PBMCs of individuals with FXS compared to control samples, suggesting increased translational activity also in peripheral cells. Recently, increased

translational activity and phosphorylation of mTOR (Ser2448), ERK1/2 (Thr202/Tyr204), and P70 S6K1 (Thr389) were also reported in primary cultured fibroblast cell lines [17].

Tuberous sclerosis complex

Tuberous Sclerosis Complex (TSC) is a genetic disorder that results in the formation of noninvasive lesions within numerous tissues and organs known as hamartomas. Variations in the size and number of lesions present lead to a broad range of clinical manifestations. In the 85% of individuals with hamartomas within the central nervous system, cognitive impairments, behavioral problems and increased risk of epilepsy are common [18]. Estimated incidence rates for TSC is 1 in 6000 [19].

The genetic cause of TSC is the presence of a heterozygous loss of function mutation in either the tuberous sclerosis 1 (*TSC1*) gene on chromosome 9 or the tuberous sclerosis 2 (*TSC2*) gene on chromosome 16. The proteins encoded by *TSC1* (hamartin) and *TSC2* (tuberin) normally form a complex that regulates the mTORC1 signaling pathway directly through inhibition of the mTOR activator rheb (ras homologue expressed in brain) [20]. Therefore, the presence of loss of function mutations in either *TSC1* or *TSC2* results in increased mTORC1 activity [21,22] and subsequent increased phosphorylation of S6K1 and 4EBP1, the two downstream effectors of translation [23]. Substrates along the mTORC1 pathway including P70 S6K1 and ribosomal protein S6 show increased phosphorylation in individuals with TSC and animal models [24]. Additionally, mice with heterozygous loss-of-function mutations in *TSC1* or *TSC2* have reduced mGluR-LTD, decreased postsynaptic translation [25] and show learning and memory deficits, independently from a tumor or seizure phenotype [26].

Comorbidities across FXS and TSC

FXS and TSC are two closely associated genetic disorders. Both disorders have a high prevalence of intellectual disabilities, autism spectrum disorders (ASD) and seizures [27,28,29,30,31,32,33,34]. Interference with normal synaptic plasticity causes cognitive deficits in both syndromes and is suspected to be a core perturbation that increases the risk of developing autism [35,36].

The convergence of FXS and TSC pathophysiology on the mTORC1 signaling pathway suggests that understanding the similarities and differences in the mechanisms of these disorders could inform and lead to the development of targeted treatments. As such, a comparison of the pathophysiology in the $Fmr1^{-/y}$ mouse and $Tsc2^{-/+}$ mouse was investigated [25]. The study reported a reduction in mGluR dependent LTD and protein synthesis in the $Tsc2^{+/-}$ mice compared to the exaggerated mGluR-LTD found in the $Fmr1^{-/y}$ mice [15,37]. The cross of these mice, $Fmr1^{-/y}$ $Tsc2^{-/+}$ resulted in mGluR-LTD levels between mice with either mutation alone, and most similar to the WT mice. These double mutant mice had improved memory compared to the $Fmr1^{-/y}$ or $Tsc2^{-/+}$ mice, suggesting the mutations altered mTORC1 signaling reciprocally and this balanced the activity of the pathway when both mutations were present. These results suggested a range of activity that is optimal for the translational signaling pathway, and deviation from that range either by increasing or decreasing the activity would result in pathology. Noticeably,

gene expression profiles showed significant difference between the $Tsc2^{-/+}$ and $Fmr1^{-/y}$ mice, suggesting that cellular pathophysiology may be profoundly different in contrast to the similar phenotypic characteristics [38].

Interestingly, a unique case of a female with both FXS and TSC was clinically evaluated in the Fragile X Research Clinic at the UC Davis MIND Institute. Here we describe the clinical presentation of this participant as well as molecular consequences of the double genetic hit with respect to mTORC1 and ERK1/2 signaling in the derived fibroblast cell line from this patient and compare to those observed in subjects with the *FMR1* full mutation alone and in a subject with a *TSC1* loss of function mutation.

Methods

Participants

A total of 7 females: three typically developing controls, two harboring an *FMR1* full mutation, one with both an *FMR1* full mutation and a *TSC1* loss of function mutation, and one with a *TSC1* loss of function mutation (age range for 7 females=11–40 years) were included in this study. Participants were recruited following protocols approved by the Institutional Review Board at UC Davis and provided informed consent.

Establishing primary fibroblast cell lines

Explants of dermal biopsies (~ 3 mm) were minced and placed in a 100-mm TC-treated tissue culture dish (Corning Life Science) with 5 mL fibroblast medium (Gibco AmnioMax-C100 Basal Medium with 15% AmnioMax-C100 Supplement (Invitrogen)) and placed in 37°C humidity incubators with 5% CO_2 atmosphere. Media was replaced every 3–4 days until fibroblast outgrowths from one explant were a quarter of the size of the dish. Fibroblasts were passaged into a new dish by trypsinization, and grown in modified fibroblast medium (1 part AmnioMax-C100 medium (as described above), 1 part RPMI-1640 medium (RPMI-1640 basal medium (Invitrogen) supplemented with 1X Primocin (Invivogen), 1% non-essential amino acids, and 10% fetal bovine serum (Corning Life Science)). Media was replaced every 3–4 days and allowed to reach 90% confluence prior to passaging. Fibroblast cultures were passaged between 2–20 times prior to collection for DNA, RNA, cryopreservation, or cell activation.

Cell activation

Primary fibroblasts were seeded in triplicate onto black walled clear bottom TC-treated 96 well plates at 10,000 cells per well in modified fibroblast medium (as described above) overnight in a 37°C humidity incubator with 5% CO_2 atmosphere. The following day media was replaced with serum-starvation media (RPMI-1640 basal medium supplemented with 1X Primocin, 1% non-essential amnio-acids, and 0.5% Bovine Serum Albumin (Miltenyi Biotec)) and incubated overnight in the 37°C humidity incubator. The next day media was replaced with fresh serum-starve media and cells were stimulated with 25 ng/ml of platelet-derived growth factor (PDGF) (Cell Signaling) or 20 ng/ml or phorbol 12-myristate 13-acetate (PMA) (Tocris) for 5 minutes to 3 hours. or kept unstimulated. Cells were stimulated with PDGF in order to activate the mTOR signaling pathway through PI3 kinase, or PMA to

activate the ERK1/2 signaling pathway through protein kinase C, to measure phosphorylation of substrates along these pathways following activation. Following stimulation, fibroblast cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature (RT) for in cell Western (ICW) assays, or washed two times with ice cold PBS and lysed in lysis buffer which consisted of 1X Cell Lysis Buffer (Millipore) supplemented with Protease Inhibitor Cocktail 1 (Sigma), Phosphatase Inhibitor Cocktail 2 (Sigma), and Phosphatase Inhibitor Cocktail 3 (Sigma).

In cell western

Following fixation, cells were permeabilized by five times washes in 0.1% Triton X-100 in 1X Tris Buffered Saline (TBS). Cells were blocked for 1 hour at RT in Licor Blocking Buffer (Licor), and hybridized overnight at 4°C in primary antibody (1:250 phospho-eIF4E (Ser209) (Abcam), 1:300 phospho-Akt (Ser473) (Cell Signaling), 1:200 phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling), 1:400 phospho-4EBP1 (Thr70) (Cell Signaling), 1:500 phosphor-P70 (Thr389) (Cell Signaling), 1: 400 phosphor-S6 (Ser235/236) (Cell Signaling), and 1:400 phosphor-mTOR (Ser2448) (Cell Signaling)). Cells were washed 5 times in TBS with 0.1% Tween-20 and hybridized with 1:5000 Cell Tag (Licor) and 1:800 goat anti-mouse 800CW (Licor) or donkey anti-rabbit 800CW (Licor) for 1 hour at RT. Cells were washed 5 times in TBS with 0.1% Tween-20 and a final wash in TBS. Liquid was decanted from the 96 well plate and the plate was scanned using the Odyssey Imager (Licor) and Image Studio (Licor). We compensated for differences in cell numbers by adjusting the phosphorylation levels (800 channel) by the Cell Tag signal (680 channel). Phosphorylation levels are reported as a ratio to the mean signal in the control cells.

Infrared western blotting

Standard methodologies were used. Between 5 and 30 μg of proteins were separated by SDS-PAGE and blotted onto PVDF membranes (Millipore). Membranes were blocked for 2 hours in Licor Blocking Buffer and then hybridized overnight on 1:1000 primary antibodies. The following day membranes were washed 3 times in TBST (Bio-Rad) and hybridized with secondary antibody for 1 hour (1:50,000) (Licor). Membranes were washed in TBST with a final wash in TBS without Tween-20. Detection of immunoreactive bands, was performed by scanning the membranes using the Odyssey Imager following manufacturer's recommendations. Band's intensity was measured using Image Studio software.

Standard western blotting

Standard methodologies were used. Between 5–20 μg of total cell lysate were separated by SDS–PAGE electrophoresis and blotted on a PVDF membrane (Millipore). All the antibodies used in this study were commercially available except for FMRP (Ferrari et al. 2007) 1:1000. Primary antibodies: GAPDH (Chemi-Con) 1:10000; p-mTOR (Ser 2448) and total mTOR (Cell Signaling) 1:1000, p-ERK1/2 and total ERK1/2 (Santa Cruz) 1:1000;. p-AKT-P and total AKT (Cell Signaling) 1:1000; p-4E-BP1-P and total 4E-BP1 (Cell Signaling).1:1000. Proteins were revealed using an enhanced chemiluminescence kit (BIO-RAD) and the imaging system LAS-3000 (Fuji). Quantification was performed using the AIDA software (Raytest, version 4.27) and Coomassie staining of the membranes.

SUnSET

A protein synthesis assay was performed as previously described using the SUnSET method (Schmidt et al., 2009). Western Blot analyzed puromycin-treated samples and puromycin incorporation was detected using the mouse monoclonal antibody (1:5000, PMY-2A4 DHSB). Coomassie staining of total proteins or immunolabeling of housekeeping proteins as GAPDH was used as loading controls.

CGG sizing and methylation status

CGG repeat allele size was determined by using both PCR and Southern Blot analysis as previously described [39,40]. Methylation status was assessed on the Southern blot by densitometry using the Alpha Innotech FluorChem 8800 Image Detection System. Methylation status included percent of methylation, indicating the percent of cells carrying a methylated allele and, the activation ratio, which indicate the fraction of cells carrying the normal allele on the active X chromosome. *FMR1* mRNA expression levels measurements were performed by quantitative Real Time PCR; details are as in Tassone et al., [41].

Statistical analysis

To test whether the phosphorylation levels were significantly different in cells harboring an *FMR1* full mutation or *TSC1* mutation compared to control cells, Mann-Whitney tests were used, and p-values below 0.05 were reported as statistically significant.

Results

Female subject with heterozygous loss of function mutations in both FMR1 and TSC1: clinical history

The 12 year and 9 months old Caucasian female affected with both FXS and TSC was medically and clinically evaluated.

Dysmorphic facial features were noted including widely spaced eyes with epicanthal folds and a flattened nasal bridge, with midface flattening and mild mandibular prognthism. Possible aspects of adenoma sebaceum (facial angiofibroma) on her cheeks were present. The woods lamp exam showed the presence of a couple hypopigmented macules on the right lower leg, a couple of very small spots on her right hand, and a larger spot about 1 cm square on her lower back. Facial angiofibromas were also present as well as a left great toe ungula fibroma. These physical abnormalities support the diagnosis of tuberous sclerosis (Figure 1) [42]. Her neurosensory exam was normal as was her muscle strength. She was tanner at stage III, and her finger joints were hyperextensible with MP extension to 90 degrees.

She was adopted from Russia at 5.5 months of age, she was known to have a 34 week gestation with an Apgar score of 7 and 8 and a birth weight of 4 lbs. 6 oz. She was diagnosed with FXS during her first year. Her milestones included sitting at 9 months of age, walking at 16 months, saying words at 18 months and putting together words at 5 years of age. She had some speech regression at 18 months, coinciding with seizure episodes. She also had very low muscle tone and had difficulty maintaining an upright posture for a sustained period of time.

She reportedly had frequent staring spells as an infant and at 18 months of age she became tonic and then had a generalized seizure. By 1 year 8 months of age she had 12 reported events of unusual motor movements diagnosed as seizures. These continued to 33 months of age, including febrile convulsions. She had up to 5 or 6 seizures per day with postictal sedation and hospitalized at 2 years and 9 months of age. Mesial frontal cortical dysplasia on the right side was shown on an MRI scan along with white matter signals in the centrum semiovale suggestive of possible ischemic changes. At this time she had an Early Learning Composite on the Mullen Scales of Early Learning of 50 (1st percentile). She had cystoscopy for recurrent urinary tract infections and adenoidectomy at 18 months of age.

When she was 7 years and 4 months of age she was diagnosed with cholelithiasis. She showed mood lability, particularly around her menses, which began at age 10. She had impulsivity, inattention, hyperactivity, gaze avoidance, tactile defensiveness, significant anxiety, and perseverative language. She has pronounced imitation skills that allow her to readily model the actions and words of people around her. She also exhibits hyperarousal to sensory stimuli, hyperactivity and selective mutism. Her speech is affected by verbal and oral dyspraxia, and she fit the diagnosis of moderate ASD by Autism Diagnostic Observation Scale (ADOS 2) when tested at 12 years and 5 months of age. She also has had a history of sleep problems.

At 6 years of age she began taking Daytrana (40 mg patch). Prior to Daytrana she was also prescribed topiramate, clonazepam, and Diastat. She has continued on Daytrana (40 mg per day) and guanfacine (1 mg three times per day), which is helping with her impulsivity and hyperarousal along with ADHD symptoms. She has tics including a blinking tic and shoulder shrugs but they disappeared on the guanfacine. She was without seizures for several years but they reoccurred at age 12 and her anticonvulsant medication was changed to oxicarbamazepine and she currently has only one seizure a year. Her most recent MRI at age 15 demonstrated multiple tubers frontally and a tuber on each side of the Foraman of Munro but they are not blocking CSF flow.

Her non-pharmacological treatments have included special education intervention in school in addition to physical therapy, occupational therapy and speech and language therapy on a weekly basis. She has also utilized the CogMed program, a digital program through the internet to improve attention and concentration.

DNA molecular testing

Clinical testing of whole exome sequencing detected a 4 bp deletion in *TSC1* resulting in a frame-shift loss of function mutation. Fragile X DNA testing also revealed the presence of the *FMR1* full mutation (570, 710, 1050 CGG repeats), and of a normal allele of 29 CGG repeats, with an activation ratio of 0.68. DNA testing in fibroblast cells derived from this female revealed the presence of the same normal and methylated full mutation alleles with an activation ratio of 0.3. She had lower *FMR1* mRNA (0.61 ± 0.10) and lower FMRP (0.031) expression levels than typically developing controls (*FMR1* mRNA in controls=1.42 ± 0.26, and FMRP levels in ERK 1/2 controls=1.2 ± 0.02 [41,43].

Regulation of the mTORC1 and ERK pathways not significantly altered in fibroblast cells harboring FMR1 and TSC2 mutations

The dysregulation of the pathways controlling protein synthesis is one of the hallmarks of a number of neuropsychiatric disorders [44]. A dysregulation of protein synthesis have been observed in FXS mice [45,46,47,48] and recently have been also reported in primary cells (fibroblasts) as well as in lymphoblastoid cell lines derived from patients with FXS [17,48]. Recent experiments showed in a mouse model for Tuberous sclerosis showed an amelioration of the dysregulated pathways in FXS [25]. We investigated protein synthesis activity in fibroblasts from the proband with the double *FMR1/TSC1* mutation, three females without *FMR1* or *TSC1* mutations, 2 females with *FMR1* mutations, and 1 female with a *TSC1* loss of function mutation using SUnSET technology, a non-radioactive assay for labeling newly synthesized proteins [49].

Newly synthesized proteins were measured by puromycin incorporation. No significant difference in protein synthesis was shown between the four genotypic groups, though small increased protein synthesis was observed in the FXS cohort compared to the other three cohorts. While our findings suggest that the double mutation might indeed normalize the excessive protein synthesis in FXS, the statistical significance of these data were limited by the fact that the fibroblast cell line derived from the FXS & TSC patient represents a unique human case harboring both *FMR1* and *TSC1* mutations (compared in this study with the matched controls harboring FMR1 or TSC1 mutation) (Figure 2).

Next, we assessed the phosphorylation levels of a number of proteins along the mTORC1 and ERK1/2 signaling pathways, regulators of protein synthesis, in fibroblast cells from the four cohorts. Phosphorylation and total protein levels were measured using both In Cell Western assays and Western blots for ERK 1/2, Akt, mTOR, P70 S6K1, eIF4E, 4E-BP, and ribosomal protein S6.

Although for all proteins analyzed, the double mutation showed a tendency to a decreased phosphorylation status, no significant differences were observed (Figure 3).

Furthermore, fibroblast cells were stimulated with PDGF or PMA, or unstimulated prior to measuring mTORC1 and ERK1/2 activity. Mann-Whitney tests compared females in the TD cohort and females in the FXS cohort including the proband for each time point. No major differences were observed in the phosphorylation of ERK 1/2 ($p>0.3$), Akt ($p>0.4$), mTOR ($p>0.2$), p70 S6K1 ($p>0.7$), eIF4E ($p>0.1$), 4EBP ($p>0.4$) or RP-S6 ($p>0.2$) (Supplementary figure 1).. Detection of differences in the protein and phosphorylation levels of these data were limited by the cohort sizes, the uniqueness of the human case where no other patient is known to exist, and the FMRP expression levels of females harboring an *FMR1* full mutation are highly variable. However, we did not observe pronounced differences between any of our cohorts.

Discussion

While mTORC1 and ERK1/2 signaling dysregulation was not detected in the primary cultured fibroblast cells, the clinical evaluation of the proband harboring both the *FMR1* full

mutation and *TSC1* loss of function mutation showed pronounced deficits in learning, behavior and, typical physical disease features of FXS and NF1, but the severity of her problems were more severe than what is typically seen in females with FXS. Females with the *FMR1* full mutation are typically less severely affected than their male counterparts, in large part due to the X-inactivation that randomly silences either the full mutation or normal *FMR1* allele in females. As such, FMRP expression in females with FXS is commonly observed at a higher level than in affected males, and these higher levels can be beneficial [43].

A FXS-TSC "double-hit" mouse model has previously been reported, which showed remarkably improved phenotype compared to mouse models of FXS or TSC [26]. Although the proband described here presents with a severe phenotype compared to typical females with a FXS diagnosis, however it is unclear if this is due to the type of TSC mutation, the variability in FMRP expression levels seen in females, or species differences. The proband presented with a loss of function mutation in *TSC1*, such mutations are predicted to have a lower severity than *TSC2* loss of function mutations, modeled in the "double-hit" mouse. It is therefore difficult to compare the biological effect of this double mutation genetic background with what has been observed in the *Fmr1* (–/y) *Tsc2* (–/+) mouse [25] however we did not observe a less severe clinical presentation.

In conclusion, this study demonstrates the level of complexity underlying how these mutations alter the regulation of both the mTORC1 and ERK1/2 signaling pathways in human tissue and cells. Understanding these complexities is of importance for developing treatments for both FXS and TSC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Conflicts of Interest

RH has carried out treatment studies in FXS funded by Novartis, Roche, Alcobra, and Neuren and she has consulted with Zynerba and Roche regarding treatment studies in FXS. The other authors have no conflict of interest.

References

1. Terracciano A, Chiurazzi P, Neri G. Fragile X syndrome. American journal of medical genetics Part C, Seminars in medical genetics. 2005; 137C:32–37.

2. Tassone F, Iong KP, Tong TH, Lo J, Gane LW, et al. FMR1 CGG allele size and prevalence ascertained through newborn screening in the United States. Genome medicine. 2012; 4:100. [PubMed: 23259642]

3. Hagerman RJ, Des-Portes V, Gasparini F, Jacquemont S, Gomez-Mancilla B. Translating molecular advances in fragile X syndrome into therapy: a review. J Clin Psychiatry. 2014; 75:e294–307. [PubMed: 24813413]

4. Pieretti M, Zhang FP, Fu YH, Warren ST, Oostra BA, et al. Absence of expression of the FMR-1 gene in fragile X syndrome. Cell. 1991; 66:817–822. [PubMed: 1878973]

5. Sutcliffe JS, Nelson DL, Zhang F, Pieretti M, Caskey CT, et al. DNA methylation represses FMR-1 transcription in fragile X syndrome. Human molecular genetics. 1992; 1:397–400. [PubMed: 1301913]

6. Darnell JC, Van Driesche SJ, Zhang C, Hung KY, Mele A, et al. FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. Cell. 2011; 146:247–261. [PubMed: 21784246]

7. Darnell JC, Klann E. The translation of translational control by FMRP: therapeutic targets for FXS. Nature neuroscience. 2013; 16:1530–1536. [PubMed: 23584741]

8. Pasciuto E, Bagni C. SnapShot: FMRP mRNA targets and diseases. Cell. 2014; 158:1446–1446. e1. [PubMed: 25215498]

9. Bear MF, Huber KM, Warren ST. The mGluR theory of fragile X mental retardation. Trends Neurosci. 2004; 27:370–377. [PubMed: 15219735]

10. Santoro MR, Bray SM, Warren ST. Molecular mechanisms of fragile X syndrome: a twenty-year perspective. Annu Rev Pathol. 2012; 7:219–245. [PubMed: 22017584]

11. Huang K, Fingar DC. Growing knowledge of the mTOR signaling network. Semin Cell Dev Biol. 2014; 36:79–90. [PubMed: 25242279]

12. Joshi S, Platanias LC. Mnk kinase pathway: Cellular functions and biological outcomes. World J Biol Chem. 2014; 5:321–333. [PubMed: 25225600]

13. Berry-Kravis E, Sumis A, Hervey C, Nelson M, Porges SW, et al. Open-label treatment trial of lithium to target the underlying defect in fragile X syndrome. J Dev Behav Pediatr. 2008; 29:293–302. [PubMed: 18698192]

14. Weng N, Weiler IJ, Sumis A, Berry-Kravis E, Greenough WT. Early-phase ERK activation as a biomarker for metabolic status in fragile X syndrome. Am J Med Genet B Neuropsychiatr Genet. 2008; 147B:1253–1257. [PubMed: 18452182]

15. Sharma A, Hoeffer CA, Takayasu Y, Miyawaki T, McBride SM, et al. Dysregulation of mTOR signaling in fragile X syndrome. J Neurosci. 2010; 30:694–702. [PubMed: 20071534]

16. Hoeffer CA, Sanchez E, Hagerman RJ, Mu Y, Nguyen DV, et al. Altered mTOR signaling and enhanced CYFIP2 expression levels in subjects with fragile X syndrome. Genes Brain Behav. 2012; 11:332–341. [PubMed: 22268788]

17. Kumari D, Bhattacharya A, Nadel J, Moulton K, Zeak NM, et al. Identification of Fragile X Syndrome-Specific Molecular Markers in Human Fibroblasts: A Useful Model to Test the Efficacy of Therapeutic Drugs. Hum Mutat. 2014; 35:1485–1494. [PubMed: 25224527]

18. Curatolo P, Maria BL. Tuberous sclerosis. Handb Clin Neurol. 2013; 111:323–331. [PubMed: 23622183]

19. Curatolo P, Bombardieri R, Jozwiak S. Tuberous sclerosis. Lancet. 2008; 372:657–668. [PubMed: 18722871]

20. Hoeffer CA, Klann E. mTOR signaling: at the crossroads of plasticity, memory and disease. Trends Neurosci. 2010; 33:67–75. [PubMed: 19963289]

21. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006; 124:471–484. [PubMed: 16469695]

22. Huang J, Dibble CC, Matsuzaki M, Manning BD. The TSC1–TSC2 complex is required for proper activation of mTOR complex 2. Mol Cell Biol. 2008; 28:4104–4115. [PubMed: 18411301]

23. Inoki K, Corradetti MN, Guan KL. Dysregulation of the TSC-mTOR pathway in human disease. Nat Genet. 2005; 37:19–24. [PubMed: 15624019]

24. Huang J, Manning BD. The TSC1–TSC2 complex: a molecular switchboard controlling cell growth. Biochem J. 2008; 412:179–190. [PubMed: 18466115]

25. Auerbach BD, Osterweil EK, Bear MF. Mutations causing syndromic autism define an axis of synaptic pathophysiology. Nature. 2011; 480:63–68. [PubMed: 22113615]

26. Ehninger D, Han S, Shilyansky C, Zhou Y, Li W, et al. Reversal of learning deficits in a Tsc2+/− mouse model of tuberous sclerosis. Nat Med. 2008; 14:843–848. [PubMed: 18568033]

27. Bailey A, Bolton P, Butler L, Le Couteur A, Murphy M, et al. Prevalence of the fragile X anomaly amongst autistic twins and singletons. J Child Psychol Psychiatry. 1993; 34:673–688. [PubMed: 8340438]

28. Bailey A, Phillips W, Rutter M. Autism: towards an integration of clinical, genetic, neuropsychological, and neurobiological perspectives. J Child Psychol Psychiatry. 1996; 37:9–126.

29. Cohen, DJ., Volkmar, FR. Handbook of autism and pervasive developmental disorders. New York: J. Wiley; 1997.

30. Bolton PF. Neuroepileptic correlates of autistic symptomatology in tuberous sclerosis. Mental retardation and developmental disabilities research reviews. 2004; 10:126–131. [PubMed: 15362169]

31. Kaufmann WE, Cortell R, Kau AS, Bukelis I, Tierney E, et al. Autism spectrum disorder in fragile X syndrome: communication, social interaction, and specific behaviors. Am J Med Genet A Part A. 2004; 129A:225–234.

32. Clifford S, Dissanayake C, Bui QM, Huggins R, Taylor AK, et al. Autism spectrum phenotype in males and females with fragile X full mutation and premutation. J Autism Dev Disord. 2007; 37:738–747. [PubMed: 17031449]

33. Harris SW, Hessl D, Goodlin-Jones B, Ferranti J, Bacalman S, et al. Autism profiles of males with fragile X syndrome. Am J Ment Retard. 2008; 113:427–438. [PubMed: 19127654]

34. Jeste SS, Sahin M, Bolton P, Ploubidis GB, Humphrey A. Characterization of autism in young children with tuberous sclerosis complex. J Child Neurol. 2008; 23:520–525. [PubMed: 18160549]

35. Pignatelli M, Feligioni M, Piccinin S, Molinaro G, Nicoletti F, et al. Synaptic plasticity as a therapeutic target in the treatment of autism-related single-gene disorders. Curr Pharm Des. 2013; 19:6480–6490. [PubMed: 23432715]

36. D'Antoni S, Spatuzza M, Bonaccorso CM, Musumeci SA, Ciranna L, et al. Dysregulation of group-I metabotropic glutamate (mGlu) receptor mediated signalling in disorders associated with Intellectual Disability and Autism. Neurosci Biobehav Rev. 2014; 2:228–241.

37. Kim SH, Markham JA, Weiler IJ, Greenough WT. Aberrant early-phase ERK inactivation impedes neuronal function in fragile X syndrome. Proc Natl Acad Sci USA. 2008; 105:4429–4434. [PubMed: 18332424]

38. Kong SW, Sahin M, Collins CD, Wertz MH, Campbell MG, et al. Divergent dysregulation of gene expression in murine models of fragile X syndrome and tuberous sclerosis. Mol Autism. 2014; 5:16. [PubMed: 24564913]

39. Tassone F, Pan R, Amiri K, Taylor AK, Hagerman PJ. A rapid polymerase chain reaction-based screening method for identification of all expanded alleles of the fragile X (FMR1) gene in newborn and high-risk populations. J Mol Diagn. 2008; 10:43–49. [PubMed: 18165273]

40. Filipovic-Sadic S, Sah S, Chen L, Krosting J, Sekinger E, et al. A novel FMR1 PCR method for the routine detection of low abundance expanded alleles and full mutations in fragile X syndrome. Clin Chem. 2010; 56:399–408. [PubMed: 20056738]

41. Tassone F, Hagerman RJ, Chamberlain WD, Hagerman PJ. Transcription of the FMR1 gene in individuals with fragile X syndrome. Am J Med Genet. 2000; 97:195–203. [PubMed: 11449488]

42. Northrup H, Krueger DA. International Tuberous Sclerosis Complex Consensus G. Tuberous sclerosis complex diagnostic criteria update: recommendations of the 2012 International Tuberous Sclerosis Complex Consensus Conference. Pediatr Neurol. 2013; 49:243–254. [PubMed: 24053982]

43. Pretto D, Yrigollen CM, Tang HT, Williamson J, Espinal G, et al. Clinical and molecular implications of mosaicism in FMR1 full mutations. Front Genet. 2014; 5:318. [PubMed: 25278957]

44. Sawicka K, Zukin RS. Dysregulation of mTOR signaling in neuropsychiatric disorders: therapeutic implications. Neuropsychopharmacology. 2012; 37:305–306. [PubMed: 22157871]

45. Qin M, Kang J, Burlin TV, Jiang C, Smith CB. Postadolescent changes in regional cerebral protein synthesis: an in vivo study in the FMR1 null mouse. J Neurosci. 2005; 25:5087–5095. [PubMed: 15901791]

46. Dolen G, Osterweil E, Rao BS, Smith GB, Auerbach BD, et al. Correction of fragile X syndrome in mice. Neuron. 2007; 56:955–962. [PubMed: 18093519]

47. Bhattacharya A, Kaphzan H, Alvarez-Dieppa AC, Murphy JP, Pierre P, et al. Genetic removal of p70 S6 kinase 1 corrects molecular, synaptic, and behavioral phenotypes in fragile X syndrome mice. Neuron. 2012; 76:325–337. [PubMed: 23083736]

48. Pasciuto E, Ahmed T, Wahle T, Gardoni F, D'Andrea L, et al. Dysregulated ADAM10-Mediated Processing of APP during a Critical Time Window Leads to Synaptic Deficits in Fragile X Syndrome. Neuron. 2015; 87:382–398. [PubMed: 26182420]

49. Schmidt EK, Clavarino G, Ceppi M, Pierre P. SUnSET, a nonradioactive method to monitor protein synthesis. Nat Methods. 2009; 6:275–277. [PubMed: 19305406]
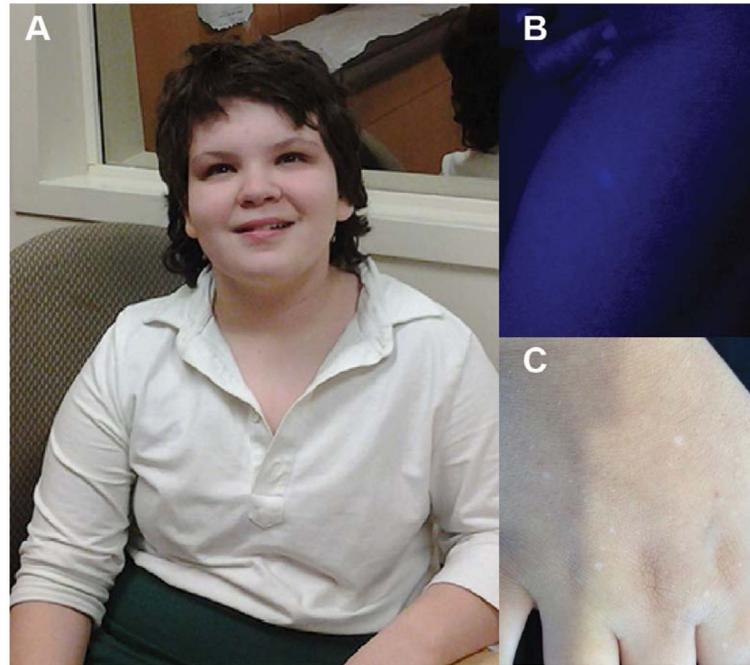
Figure 1.
Patient with *FMR1* and *TSC1* mutations. A) Note the long face and prominent ears. B) Skin macules seen with Woods lamp. C) White skin macules seen on hand.
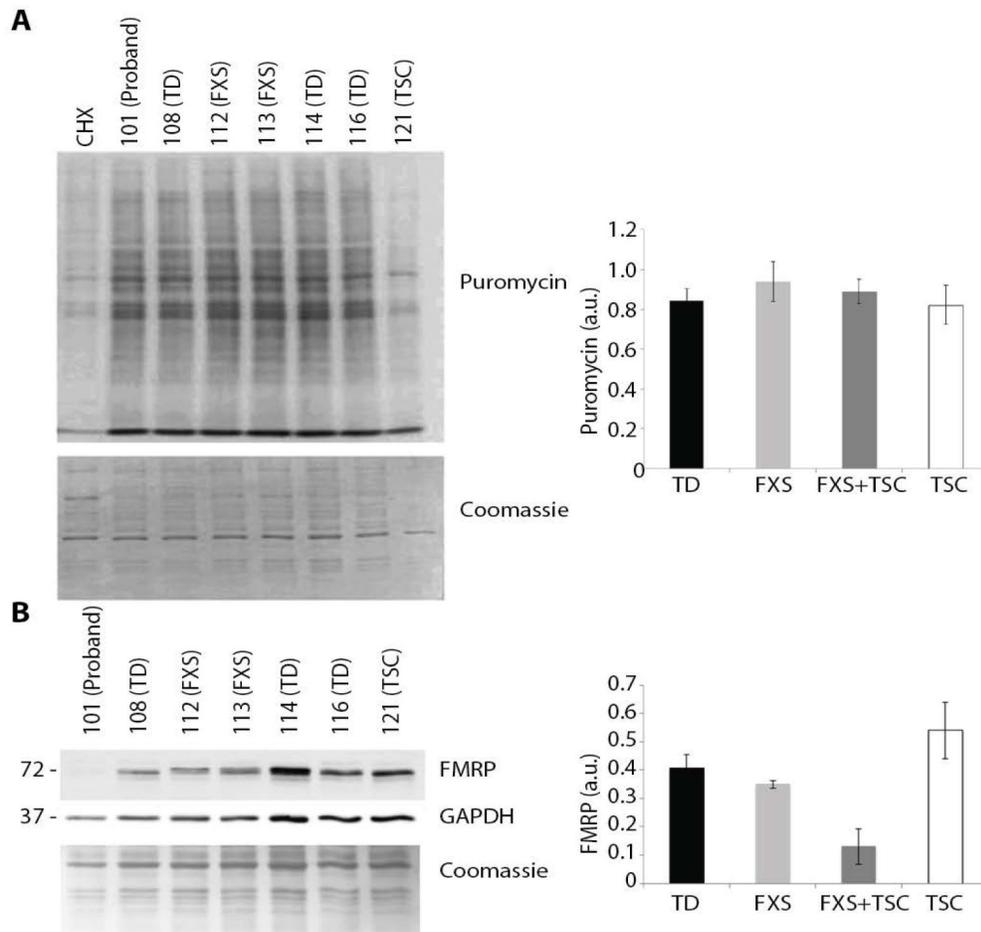
Figure 2.
Protein synthesis was not significantly different between cohorts in fibroblast cells. A) Left upper panel shows a representative WB of cells treated with puromycin and stained with antibodies against puromycin. Left lower panel shows the same blot stained with coomassie used as loading control. Right panels, levels of protein synthesis obtained normalizing the puromycin signal to the Coomassie staining. Bars represent the mean + Standard Error (n = 3), FXS (n = 2), TSC (n = 1), TSC&FXS (n = 1) technical triplicates). B) Left panel, representative WB images for FMRP from cells from controls (TD), FXS, TSC and TSC&FXS patients. Right panel, quantification of FMRP normalized to GAPDH. Bars represent the mean + Standard Error (n = 3), FXS (n = 2), TSC (n = 1), TSC&FXS (n = 1) technical triplicates).
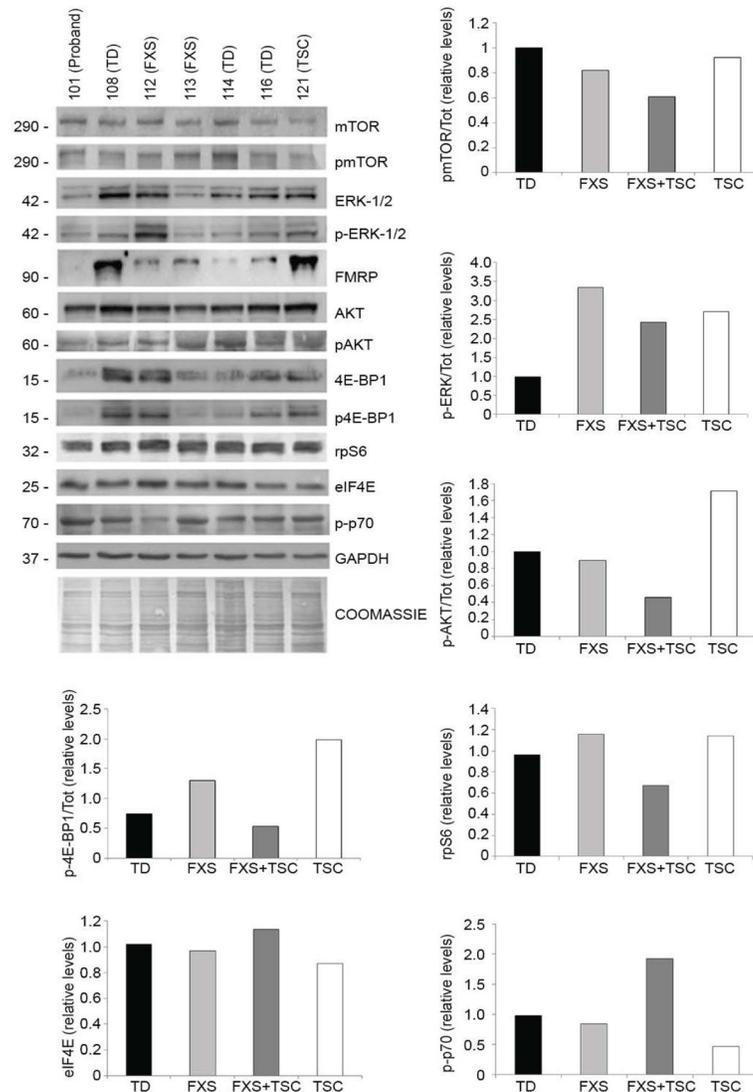
Figure 3.
Phosphorylation of substrates along the mTORC1 and ERK1/2 signaling pathways were not significantly different between cohorts in fibroblast cells. Left panels, representative WB images for mTOR, p-mTOR, ERK-1/2, p-ERK-1/2, FMRP, AKT, p-AKT, 4-EBP1, p-4EBP1, rpS6, eIF4E, p-p70, GAPDH (loading control), and coomassie stain from fibroblast lysates of controls (TD, n = 3), FXS (n = 2), TSC (n = 1), TSC&FXS (n = 1). Right panels, quantification of phosphoproteins levels over total normalized to GAPDH. Mean of technical duplicates is represented.