

ASD Pathogenesis and Emerging Treatments: Lessons Learned from the Monogenic Syndromic ASD

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Autism Spectrum Disorder (ASD) is a group of complex neurodevelopmental disorders characterized by social impairments and repetitive behaviors. It can be divided into two major subcategories: 1) non-syndromic (sporadic or idiopathic) ASD and 2) syndromic ASD that also manifests other characteristic medical conditions and physical features. ASD is a growing public health crisis as its prevalence increases rapidly in recent years. There is no FDA approved drug for the treatment of ASD core symptoms that define the disorder, which is a major challenge in the management of ASD. This is largely due to the lack of a good understanding of its etiology that is highly complex and heterogeneous. Many types of the syndromic ASD are caused by mutations of a single gene (monogenic), which provides an excellent tool to explore the disease mechanisms leading to the pathogenesis of the core symptoms. Here, we briefly review the recent progress in animal studies on the disease mechanisms of the fragile X syndrome and the syndromes caused by loss of function of a key negative regulator along the mTOR signaling cascade due to the deleterious mutations of the respective gene. We emphasize the disrupted signaling pathways likely shared by some non-syndromic ASD cases, and highlight druggable targets and their translation for the treatment of ASD patients.

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ASD: A GROWING PUBLIC HEALTH CRISIS

ASD is a range of complex neurodevelopmental disorders characterized by impairment of social interactions and restricted interests, stereotyped and repetitive behaviors.¹⁻⁴ In addition to the core symptoms, there are other common co-existing psychiatric and medical conditions in individuals with ASD, such as epilepsy, intellectual disability (ID), anxiety, aggression, sleep and gastrointestinal problems.⁵ The prevalence of ASD in the world is estimated to be more than 1%.⁶ The prevalence increases rapidly in recent years. According to the 2016 report prepared by the US Center for Disease Control and Prevention (CDC) (based on the nationwide data collected in 2012),⁷ the prevalence has increased by 130% from 2002 to 2012. Currently, ASD affects 1 in 68 children aged 8 years in the US.⁷ Up to two thirds of ASD individuals have low daily living skills, and need lifetime care by their family members or other caregivers.⁸ Thus, ASD is a significantly more serious challenge to the society than the Alzheimer's disease, as the latter usually affects only the last decade of the patient's life.⁹

WIDENING KNOWLEDGE GAP BETWEEN ASD GENETICS AND HOW THE AFFECTED PROTEINS CONTRIBUTE TO ASD PATHOGENESIS

There is no approved drug for the treatment of the core symptoms of ASD, which is a major challenge for its management. This is largely because ASD is a group of highly heterogeneous disorders and our current understanding of their etiologies is still very limited. Rapid advances in sequencing technologies and drastic drop of the cost have made it possible to sequence large samples of normal individuals and those with ASD, which allows to identify mutations of ASD-linked genes even with very low penetrance (< 1%).^{10,11} As of July 10, 2017, 910 ASD risk genes have been suggested based on human and animal studies (<https://gene.sfari.org/>). However, while the discovery of ASD risk genes is at an increasingly rapid pace, our understanding of how mutations of these risk genes contribute to ASD pathogenesis lags further behind.

A gene mutation can lead to at least four possible functional outcomes of the affected protein: 1) reduced or loss of function (especially when the protein fails to express); 2) increased function; 3) no change in function; and 4) gain of different (new) function. A deeper understanding of mutation-induced functional alterations of the gene products (usually proteins) and how these alterations contribute to the disease mechanisms, are prerequisites to develop novel disease-modifying pharmacotherapies for the treatment of ASD. The sequencing technology revolution makes gene mutation

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detection significantly easier than ever before, but understanding how a particular mutation changes the function of the affected protein and how this functional change contributes to ASD pathogenesis could take many years and requires collaborative efforts of many research labs in the world.

Searching for additional ASD risk genes and mutations is important in the long run to fully understand the etiology of ASD and the related disorders. However, it seems to be equally important to allocate enough resources in order to digest and integrate the overwhelming and highly informative data gathered already from the ASD genetics¹⁰⁻¹² and numerous animal models,¹² to delineate how the identified deleterious mutations of these 910 risk genes alter the function of the affected proteins and cause ASD.

MONOGENIC SYNDROMIC ASD AS A POWERFUL TOOL FOR EXPLORATION OF ASD PATHOGENESIS

ASD can be divided into two major subcategories: syndromic and non-syndromic (also called idiopathic or sporadic) ASD.¹²⁻¹³ The etiology of most non-syndromic ASD cases is complex, believed to be due to interactions between low penetrant mutations of multiple risk genes and environmental factors.^{11,14} It is practically hard to generate animal models to study the disease mechanisms in this situation.

In the syndromic ASD, in addition to the core symptoms, there are other characteristic neural and non-neural conditions (particularly dysmorphic features) not seen in the non-syndromic ASD. Many types of syndromic ASD are caused by highly penetrant mutations of a single ASD gene (monogenic, see below). This provides an excellent opportunity to explore the disease mechanisms of ASD and to identify druggable targets for the development of novel treatments. In the sections below, we review several well-studied monogenic syndromes associated with ASD and describe some common disease mechanisms that are likely shared by some non-syndromic ASD cases. We also discuss the mechanism-based investigational treatments tested in animal models and evaluated in some preliminary clinical studies. Due to space limit, we focus only on those syndromic ASD subtypes in which excessive protein synthesis is the main cause of the syndrome due to the loss of a key negative regulator.

FRAGILE-X SYNDROME

Fragile X Syndrome (FXS), an X-linked disorder, is caused by mutations in the *FMR1* gene, which results in a significantly reduced or even completely loss of the expression of Fragile X mental retardation protein (FMRP).¹⁵ It is the most common inherited form of ASD, found in ~2-6% of individuals with ASD.¹⁶⁻¹⁷ FXS is probably also the most extensively studied syndromic ASD so far. The knowledge gained from these studies is highly valuable to elucidate ASD pathogenesis in general. For this reason, we use more space to describe the disease mechanisms of FXS than those for three other syndromic ASD subtypes covered in this review.

In the full mutation group (FMRP is not expressed), the ASD phenotypes are found in nearly 70% male patients. In premutation males (FMRP expression is reduced), the ASD incidence drops quite significantly to 14%,¹⁸ indicating a causal relation between the FMRP deficiency and ASD pathogenesis. Other common medical manifestations include seizure, aggression, attention deficit hyperactivity disorder (ADHD) symptoms, anxiety, sensory hypersensitivities, self-injury, macrocephaly, and sleep disturbance. These are also comorbid conditions frequently observed in non-FXS ASD patients.^{5,19} Knocking out (KO) *Fmr1* gene in animal models produces phenotypes that manifest virtually all the major clinical and neuroanatomical features, such as cognitive impairment, autistic behaviors, macroorchidism, macrocephaly, immature spines on the dendrites¹⁶⁻²⁰ found in human patients, which further confirms the causal relation between the loss of FMRP and FXS symptoms.

FMRP AND TRANSLATION OF THE PROTEINS INVOLVED IN SYNAPSE FORMATION, MATURATION, AND FUNCTION

FMRP is a master negative regulator (repressor) of the cap-dependent protein translation. It binds to its target mRNAs and controls the initiation of the translation process.²¹⁻²² FMRP is highly expressed in neurons. It regulates local protein synthesis in a rapid and activity-dependent manner, particularly in dendritic spines where synapses are located.²³⁻²⁴

Many FMRP target mRNAs encode proteins (such as neuroligins, neurexins, shanks, MMP-9, and mGluR5) required in synapse formation, maturation, elimination, and neural plasticity.²⁵ Disruptions of these synaptic processes are linked to ASD.¹⁴ Furthermore, many protein partners involved in FMRP-required translation control and signaling pathways are themselves encoded by known ASD-linked genes, such as *eIF4E*, *MEF2C*, *PCDH10* and *CYFIP1*,²⁶⁻²⁸ and deleterious mutations of these genes have been found in non-syndromic ASD patients.²⁶⁻²⁸ This suggests that dysfunction of the FMRP-dependent translational control and signaling has a broad significance in understanding not only the pathogenesis of FXS, but also that of some non-syndromic ASD cases. We use two representative FMRP regulated proteins (**Figure 1**) to demonstrate that an excessive translation of the proteins involved either in synapse maturation or elimination is linked to ASD pathogenesis.

EF1 α OVER EXPRESSION DUE TO FMRP DEFICIENCY DISRUPTS SYNAPSE ELIMINATION

Sensory experience-dependent synapse consolidation and elimination during postnatal neurodevelopment are critical processes to shape and refine specific and mature brain circuits.²⁹ Through controlling translation of a regulatory protein, EF1 α (eukaryotic translation elongation factor 1- α), FMRP plays a key role in activity-dependent, MEF2 (myocyte enhancer factor 2)-initiated elimination of excitatory synapses.²⁷

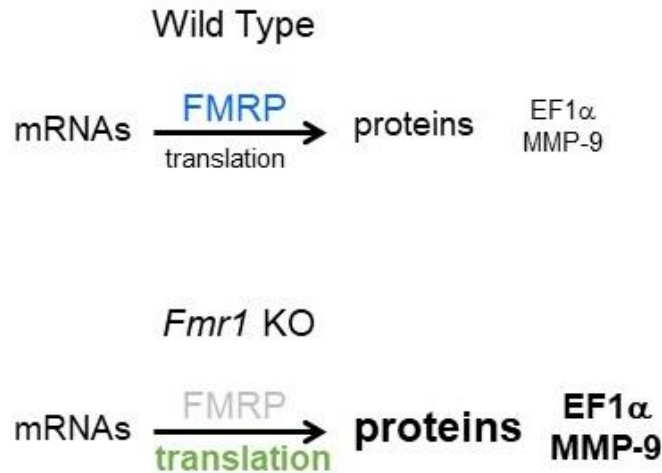


Figure 1. Loss of FMRP-dependent control in protein translation leads to an excessive translation of some proteins, such as MMP-9 and EF1a, that are critically involved in synaptic maturation and elimination.

MEF2 is a transcription factor and is activated by neural activity.³⁰ It triggers excitatory synapse elimination by promoting Pcdh10-dependent degradation of PSD-95 (a main postsynaptic scaffolding protein in excitatory synapses), which is a key step in the activity-dependent elimination of the excitatory synapse (**Figure 2A**). In wild-type (WT) neurons, MEF2 activation induces 1) translocation of Mdm2 (murine double minute 2, a specific ubiquitin E3 ligase for PSD-95) from the dendrite to the spine, and 2) transcription of *Pcdh10* gene. Pcdh10 facilitates the proteasomal deposition of PSD-95 after its ubiquitination (Ub-PSD-95 in Figure 2A) by Mdm2, leading to degradation of PSD-95 and eventual elimination of the synapse.

In *Fmr1* knockout neurons (**Figure 2B**), the loss of translational control due to FMRP deficiency causes over production of EF1 α , which binds specifically to Mdm2 and prevents it from translocating to the spine. This prevents PSD-95 ubiquitination and degradation, which stabilizes the excitatory synapse.

The discovery of this synapse elimination pathway *enriched* with multiple known ASD-linked genes (*FMRI*, *MEF2C* and *PCDH10*) has an important implication in the understanding of ASD pathogenesis. Deletion mutations of *MEF2C* and *PCDH10* genes have been identified in some rare sporadic ASD cases in human patients,³¹⁻³² but how these mutations cause ASD is unknown. From the roles of MEF2 and Pcdh10 identified in this pathway, ASD may be caused by a disruption of excitatory synapse elimination during the early neurodevelopment in these patients. It is particularly interesting that a similar disruption in excitatory synapse elimination during early neurodevelopment due to heterozygous deletion of *Tsc2* gene in the animal model of

tuberous sclerosis complex (TSC, see the corresponding section below) is also implicated in ASD pathogenesis.³³ Thus, disruption of synapse elimination is likely an important shared mechanism in the pathogenesis of both syndromic (FXS, TSC)^{27,33} and some non-syndromic ASD cases.³¹⁻³²

MMP-9 PROTEIN OVER EXPRESSION DUE TO FMRP DEFICIENCY LEADS TO ASD PHENOTYPES

MMP-9 (matrix metalloproteinase 9) is an extracellular proteinase that is translated in the dendritic spines and released from the excitatory synapses in response to neuronal activity. It is involved in the regulation of synaptic plasticity, learning and memory.³⁴ MMP-9 is required for growth and maturation of the dendritic spines and is implicated in pathogenesis of human epilepsy, FXS, and psychiatric conditions.³⁵ Translation of MMP-9 mRNA is repressed by FMRP and stimulated by eIF4E (eukaryotic translation initiation factor 4E) activation.³⁶

Indeed, FXS (*Fmr1* KO) mice display increased MMP-9 activity³⁷ and this is due to loss of repressive translational control by FMRP.³⁸ Pharmacologically down-regulating the expression³⁶⁻³⁷ or genetic removal³⁹ of MMP-9 in FXS models rescues the FXS symptoms and anatomical abnormalities, including social impairment, repetitive behavior, delayed dendritic spine maturation and even macroorchidism in the animal models of FXS. MMP-9 overexpression in wild type mice produces several FXS-like phenotypes.³⁶ This indicates that over expressed MMP-9 due to loss of translational control by FMRP plays a key role in FXS pathogenesis. Indeed, minocycline, an antibiotics that lowers MMP-9 level in FXS mice⁴⁰ also reverses synaptic and behavioral abnormalities in the FXS mice.³⁷ More importantly, it also demonstrates some clinical benefits in FXS patients.⁴¹

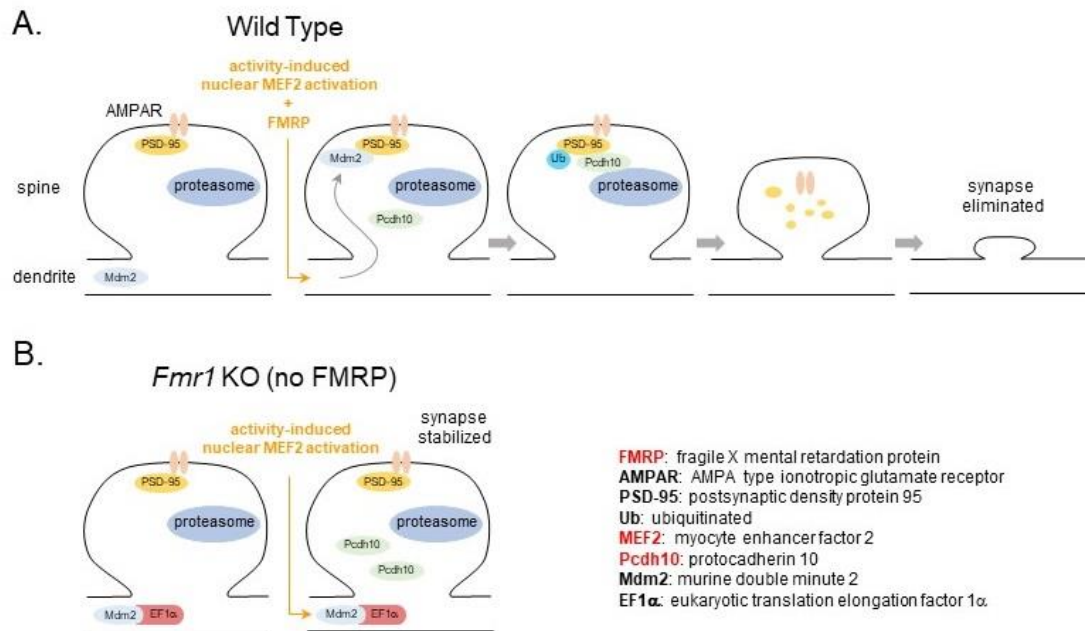


Figure 2. MEF induced, FMRP required activity-dependent elimination of excitatory synapse. **A.** In the wild type neuron, neural activity activates MEF2 that in turns stimulates Pcdh10 transcription and translation cooperatively with FMRP. MEF2 also causes translocation of Mam2 to the spine where it ubiquitinates PSD-95, allowing Pcdh10 to facilitate proteasome-mediated PSD-95 degradation and synapse elimination. **B.** The loss of FMRP-mediated translational control causes over expression of EF1α, which sequesters Mdm2 and prevents Mdm2-mediated PSD-95 ubiquitination and synapse elimination. This diagram is modified from ref. 27.

SYNDROMIC ASDS ASSOCIATED WITH OVERACTIVE MTOR, EXCESSIVE GROWTH, AND PROLIFERATION

mTOR (mammalian target of rapamycin) is a protein kinase. It usually forms two complexes, mTORC1 and mTORC2 ('C' stands for 'complex'), with two different groups of protein partners.⁴² Here we only focus on the mTORC1 since its overactivation is closely associated with three types of syndromic ASD described in the next three sections.

The PI3K (phosphatidylinositol 3-kinase) -mTOR and RAS-ERK (rat sarcoma protein-extracellular signal-regulated kinase) signaling cascades are the two major pathways for controlling neuronal survival, growth, differentiation, proliferation, activity-dependent synaptic maturation and remodeling in response to extracellular signals such as neurotransmitters and neurotropic factors (**Figure 3**).⁴²⁻⁴³ These two pathways converge to stimulate protein translation by phosphorylation of eIF4E (eukaryotic translation initiation factor 4E), 4EBP (eIF4E binding protein), and S6K (ribosomal S6 kinase). Activation of eIF4E (phosphorylated by MNK, **Figure 3**) directly stimulates the initiation of protein translation.^{36,44} 4EBP is a specific inhibitory protein of eIF4E. When it is dephosphorylated it binds to eIF4E to suppress eIF4E function. When it is phosphorylated by mTOR, 4EBP

dissociates from eIF4E, allowing for the initiation of translation by eIF4E.⁴⁴ Phosphorylation of S6K by mTOR induces protein synthesis at the ribosome, which could be a different type of protein translation from the one repressed by FMRP.⁴⁵ There is some evidence that activated (phosphorylated) S6K can also phosphorylate FMRP to enhance its function as a translational repressor⁴⁶ (**Figure 3**). The importance of this "push-pull" effect of S6K on protein synthesis is unclear, although it could render a more precise control of different translational machineries allowing specific sets of mRNAs be translated in response to selective activation of PI3K-mTOR versus RAS-ERK pathways.

Along these pathways, a number of inhibitory proteins control the activity level at several critical check points to ensure a balanced protein translation appropriate to functional need (**Figure 3**). These proteins are encoded by highly penetrant autism-linked genes. Loss of function mutations in *TSC1*, *TSC2*, *PTEN*, and *NF1* genes (encoding TSC1, TSC2, PTEN, and NF1 proteins, respectively), or gain of function mutations of *EIF4E* gene (encoding eIF4E) result in excessive protein translation and growth that disrupts synapse function, leading to syndromic and non-syndromic autisms (discussed in the next few sections below).

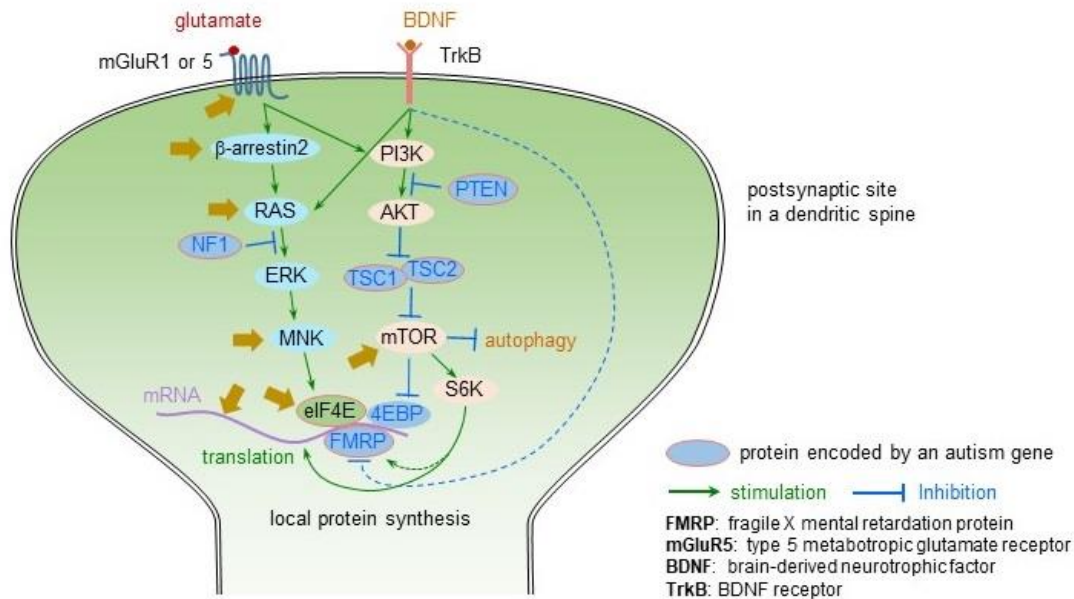


Figure 3. Neural activity activates the group1 mGluRs and TrkB receptors to stimulates local (synaptic) protein synthesis, which is stimulated mainly by the RAS-ERK and PI3K-mTOR pathways. The large arrows indicate potential drug targets.

TUBEROUS SCLEROSIS COMPLEX

Tuberous sclerosis complex (TSC) is a syndromic ASD. It is caused by autosomal dominant, loss of function mutations in *TSC1* or *TSC2* tumor suppressor genes, which results in an enhanced activation of the PI3K-mTOR pathway and dysregulation of cell growth and proliferation.⁴⁷ The abnormally elevated mTOR activity has a profound effect in neurodevelopment.^{42,48} Many of TSC children develop macrocephaly, seizure, intellectual disability, and up to 60% of the patients have the ASD phenotype.^{47,49} In animal models of TSC, which carry heterozygous genetic deletions of *Tsc1* or *Tsc2*, macrocephaly, epilepsy, and other psychiatric abnormalities, including autistic behaviors have been observed.⁵⁰⁻⁵² Furthermore, at the cellular level, disruption of synapse formation, maturation, and activity-dependent neural circuit remodeling, particularly reduction in pruning (elimination) of dendritic spines, are also observed in the *Tsc1/2* deficient mice.³³ Dendritic spine pruning is believed to be regulated by autophagy, a process negatively regulated by mTOR.⁵³ Activation of mTOR pathway leads to a suppression of autophagy (Figure 3), leading to a decreased pruning of dendritic spines and a higher synapse density.³³

The molecular genetics and studies on the *Tsc1/2* deficient mice have identified mTORC1 as a drug target for the treatment of TSC. Indeed, selective mTORC1 inhibitors and autophagy inducer,⁵³ such as rapamycin,⁴³ ameliorates many functional abnormalities as well as normalizes dendritic spine pruning in TSC animal models.^{33,54,55} The efficacy of rapamycin in animal models validates the target and indicates

a central role of mTOR overactivation in TSC pathogenesis. Interestingly, rapamycin also has beneficial effects in a mouse model of non-syndromic ASD,⁵⁶ suggesting that excessive mTOR activity may also play a key role in non-syndromic ASD.

The animal studies reviewed above indicate that mTOR can be a disease-modifying drug target for TSC. Indeed, recent clinical studies in human TSC patients show that administration of everolimus, another mTOR inhibitor, significantly reduces seizure, anxiety, depression, and autistic behaviors in children and adolescents.^{57,58} However, as mTOR inhibitors are immune suppressants, their use can increase the risk of infections.

MACROCEPHALY/ASD SYNDROME CAUSED BY PTEN DEFICIENCY

Macrocephaly/ASD syndrome is caused by heterozygous loss function mutations in the *PTEN* gene. It is an autosomal dominant disorder characterized by macrocephaly, abnormal facial features, and delayed and altered neurodevelopment resulting in autistic behavior and/or mental retardation.^{59,60}

Selective inactivation of *Pten* in the cortex and hippocampus of juvenile mice recapitulate some major Clinical abnormalities observed in the macrocephaly/ASD syndrome patients, including social interaction and cognitive impairment, anxiety, and macrocephaly.⁶¹ In the mutant mice, activity of the PI3K-mTOR pathway is significantly increased due to loss of negative control by PTEN. Furthermore, the complexity of

dendritic arborization and spine density of the affected neurons have increased,⁶¹ similar to that observed in the TSC mice.³³ This could also be related to autophagy deficit due to chronically elevated mTOR activity.^{33,53,62} PTEN deficient mice also show deficits in LTP (long-term potentiation) and LTD (long-term depression), two major forms of synaptic plasticity, and impaired spatial memory.⁶³ The anatomical, cellular, behavioral abnormalities and seizure in PTEN deficient mice can be ameliorated by specific mTOR inhibitors indicating that the mTOR pathway downstream of PTEN plays a critical role in causing these abnormalities,^{62,64} as in TSC.

NEUROFIBROMATOSIS TYPE 1

Neurofibromatosis type 1 (NF1) is a common autosomal dominant genetic disorder. It is caused by mutations of the *NF1* gene that encodes neurofibromin (NF1 protein), a GTPase-activating protein negatively regulating RAS activity.⁶⁵ Up to 80% of children with NF1 have cognitive and behavioral problems⁶⁶ and about 25% of the patients have ASD core phenotypes.⁶⁷

In *Nf1*^{-/-} knockout mice, loss of neurofibromin is associated with increased RAS-ERK pathway activity, decreased synaptic LTP and a pattern of cognitive impairment similar to the human phenotype.⁶⁸ The cognitive deficits in the NF1 animal models can be reversed by pharmacologically inhibiting RAS-ERK activity with statin drugs (e.g., lovastatin, simvastatin).⁶⁹ Translation of the results from the animal studies to NF1 patients have a mixed results.^{70,71}

LESSONS LEARNED: MECHANISMS, DRUG TARGETS, AND POTENTIAL MECHANISM-BASED TREATMENTS

Studies on the animal models of the four syndromic ASDs reviewed here have uncovered three key druggable disease mechanisms: 1) excessive translation of the FMRP-repressed mRNAs, 2), constitutively activated PI3K-mTOR pathway, and 3) constitutively activated RAS-ERK pathway. Several drug targets have been identified based on these mechanisms and are indicated by the large arrows in **Figure 3**. These targets include mGluR 1/5, β -arrestin2, RAS, MNK, mTOR, eIF4E, and the protein translation process. These drug targets have all been validated either pharmacologically^{36,44,54,55,72} with the respective inhibitors/antagonists or genetically⁷³ (in the case of β -arrestin2) in animal models of these four syndromic ASDs. For some of these targets, preliminary clinical studies on the investigational treatments through repurposing approved drugs have demonstrated beneficial effects on autistic and comorbid symptoms in the syndromic ASD patients.^{57,77}

The preclinical studies on the disease mechanisms of the syndromic ASDs could also help to explain how highly penetrant mutations of some ASD-linked genes cause non-syndromic ASD. For example, disruption in synapse elimination during postnatal neurodevelopment plays a key role in ASD pathogenesis.³³ In a key pathway required in synapse elimination, FMRP works cooperatively with MEF2 and PCDH10.²⁷ This may explain the pathogenesis of those sporadic ASD cases associated with loss of function mutations

in *MEF2C* and *PCDH10* genes.³¹⁻³² Sporadic ASD cases associated with gain of function mutations in the *EIF4E* gene⁷⁴ provide another example. In this case, the mutations reduce the effectiveness of negative regulation of eIF4E by 4EBP and FMRP (**Figure 3**), leading to an excessive eIF4E initiated protein translation. Since eIF4E is at a convergence point of the three disease mechanisms (**Figure 3**), the mechanism-based treatments for FXS and mTOR related syndromic ASDs could also be applied to the sporadic ASD cases associated with the gain of function mutations in the *EIF4E* gene.

CONCLUSIONS AND FUTURE DIRECTIONS

Syndromic ASD makes up approximately 10% of all ASD cases.⁷⁵ From the above discussion of four subtypes of monogenic syndromic ASD (together they make up more than 60% of all syndromic ASD cases),⁷⁵⁻⁷⁶ a coherent theme seems emerge: functional deficiency of key *negative* controllers (FMRP, TSC1, TSC2, PTEN, and NF1) in either translation regulation, or two fundamental signaling pathways (PI3K-mTOR and RAS-ERK) result in excessive protein synthesis, growth, dysregulated differentiation, maturation, neural remodeling and plasticity, and autistic phenotypes. The disease mechanisms could also help to explain the pathogenesis of some non-syndromic ASD cases.

While the mechanism-based investigational treatments in some preliminary clinical studies are promising, more large-scale, double blind, and placebo controlled clinical trials with a longer treatment duration are needed to confirm their efficacy in the relevant syndromic and non-syndromic ASD patients. Furthermore, a better selection of drug targets (for example, mGluR5 vs one of its downstream effectors)⁷³ for drug development may increase the therapeutic window (difference between therapeutic and toxic doses) and therefore the chance of success. Finally, as the causes of syndromic ASD are highly heterogeneous, pre-Clinical studies on the disease mechanisms of other monogenic syndromic ASDs, including many rare ones, should provide a more complete picture about the etiology of ASD in general.

CONFLICT OF INTEREST

None.

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