

## Practical use of CRISPR-Cas9 on SHANK3 and Neuroligin related to Autism Spectrum Disorder

Zhen Liu<sup>1, †</sup>, Jiangnan Xiao<sup>2, \*, †</sup>, Haoning Xue<sup>3, †</sup>

<sup>1</sup>Faculty of Medicine, Dentistry, and Health Science University of Melbourne Melbourne, Australia

<sup>2</sup>Department of Psychology University of California Los Angeles Los Angeles, United States

<sup>3</sup>Tianjin Yinghua International School Tianjin, China

\*Corresponding author: violetx@g.ucla.edu

†These authors contributed equally.

**Keywords:** Autism spectrum disorder; SHANK3; Neuroligins; CRISPER

**Abstract:** Autism spectrum disorder, is a generalized developmental disorder, whose symptoms include abnormal language skills, abnormal communication skills, narrow interests, and stubborn behavior patterns. Even though the causes of ASD remain uncertain, genetic and neuroscientific studies have identified exciting evidences for people's understanding. SHANK3 – an actin polymerization modulating gene, and neuroligin, which plays key role in the stability of synapse structure attract increasing attention as research focus and clinical treatment target. With the development of gene therapy technologies, CRISPER showed excellent advantages over traditional treatment measures. Accordingly, with going deep into the mechanism of ASD, CRISPER might be a most effective tool for it.

### 1. Introduction

Autism Spectrum Disorder (ASD) is a neurological disease comprised of classic autism and high functioning autism. People with classic autism demonstrate speech and social impairment as well as repetitive behaviors and compulsory regression, whereas people with high functioning autism are not bothered by speech defects. A multitude of risk factors contributes to ASD, including genetic factors, environmental factors, and interactions between the two. Due to its high heritability rate, a lot of researches have delved into the genetic component of ASD, confirming the existence of different defects in common pathways. Many scientists believe that it is very possible that ASD is caused by various genetic mutations working together to affect specific brain pathways [1]. Additionally, there are also significant environmental factors like maternal diabetes and maternal medication. A higher risk of ASD is also connected to birth order: the firstborn has a higher rate of ASD than later offspring. Lastly, there are also studies that look into gene-environment interactions. Chaste and Leboyer have found that although still more than the control group, the number of prenatal and perinatal complications that siblings of ASD children experienced were less than children with ASD, indicating that children with ASD may be more sensitive to environmental stimuli. The current treatment plans for ASD are non-invasive measures including behavioral and medication treatment. According to a 2016 National Survey of Children's Health, 43.3% of children with ASD were treated with behavioral treatment, 6.9% were treated with medication treatment, and 20.3% were with both treatments [2]. Behavioral treatment focuses on encouraging socially appropriate behaviors in children with ASD while decreasing stereotypical repetitive behaviors in social settings. Early and intensive behavioral modification can be quite beneficial for children in terms of their later social and communication skills. Psychotropic medications are also recommended to treat behavioral symptoms such as repetitive behaviors, social

anxiety, impulsivity, and self-injury, etc. However, none of these treatment plans is capable of eradicate ASD, and many patients need life-long plans and can never be truly free of the disorder.

This paper discusses potential gene therapy to treat ASD using CRISPR 9. More specifically, the present article explores SHANK 3 and neuroligin, which are scaffolding protein and cell adhesion and molecule that play important role in the post synaptic density

## 2. SHANK 3

SHANK 3 is a gene located in chromosome 22q13.3. It encodes for scaffolding proteins of postsynaptic density (PSD) in excitatory synapses where they bind to actin and neuroligins. SHANK 3 consists of 22 exons, encoding a multidomain protein, which is composed of various domains: ankyrin repeats (a 33-residue motif in protein), Src homology 3 (a 60 amino acid residue), and a PDZ domain (structural

motif). The autonomy of SHANK 3 also consists of multiple regions —homer binding region, SAM, and cortactin binding region [3]. This gene is predominantly

expressed in the heart, and moderately expressed in the brain and spleen. According to Uchino & Waga, SHANK 3 has been closely related to Autism Spectrum Disorder

(ASD). More specifically, it has been reported that eight non-synonymous mutations were found in patients with ASD which were absent in healthy controls [3]. Moreover, deletion of SHANK3 leads to Phelan-McDermid syndrome, caused by a deletion of 22q13.3, whose patients exhibit low muscle tone, intellectual disability,

and other characteristics similar to those of ASD [4, 5]. A analysis of SHANK3 in 134 ASD patients with manifestations similar to Phelan-McDermid

syndrome found that the mutations in SHANK3 were detected in 10% of the ASD patients. These mutations include amino acid deletion, missense alteration and

intronic insertion, as well as a repeated sequence [3].

### 2.1 The cellular and molecular mechanism of SHANK3-related diseases

SHANK3 is responsible for a variety of functions including actin polymerization, dendritic spine morphology, growth cone motility, and synaptic transmission [6]. Due to the multitude of synaptic components that SHANK 3 is capable of interacting, SHANK 3 is thought to function as a organizer of the postsynaptic density PSD. For instance, NMDA receptor is a critical PSD protein which is physically associated with SHANK 3. It is responsible of various neural development and synaptic plasticity fundamental of cognitive processes. Duffney et al. discovered that SHANK3-deficient mice exhibited a loss of NMDA receptor synaptic function and distribution in the prefrontal cortex, resulting in social deficits

and repetitive behaviors [7]. Various SHANK 3 functions and its potential brain pathways involve actin. For example, two consequences of SHANK 3 mutation are dendritic spine morphology and disturbance of actin polymerization. Dendritic spines are actin-rich structures that form the postsynaptic terminals of excitatory synapses in

the brain. The development and plasticity of spines are essential for cognitive processes, such as learning and memory. Duffney et al.'s research revealed a success in treating SHANK 3-deficient mice by targeting actin regulators found that many actin regulators associated with ASDs have a role in the regulation of synaptic structure

and/or function [7].

### 2.2 Potential theory for ASD treatment in relation to SHANK3

Based on these previous studies, many potential ASD treatment could be developed. For example, targeting specific gene responsible for producing actin, can prevent undesirable actin polymerization, dendritic spine morphology, and other activities involving action. This would greatly improve patient's cognitive processes like learning and memory.

### 3. Neuroligins

Neuroligins (NLGNs) are another cell adhesion protein located on the postsynaptic membrane [8]. They are transmembrane proteins with multiple domains, including a globular cholinesterase-like extracellular domain and an intracellular PDZ binding domain [8]. There are five NLGN genes in humans. NLGN 1 (3q26) and 2 (17p13) are both located on autosomal chromosomes, whereas NLGN 3 (Xq13), 4X (Xp22.3), and 5 (Yq11.2) are located on the sex chromosome [8]. NLGN 1 is predominantly found on excitatory synapses mediated by glutamate, whereas NLGN 2 is found on GABAergic synapses [9, 10]. In comparison, NLGN 3 is found on both excitatory and inhibitory synapses [11]. Although NLGN 4 is located on excitatory synapses [12], but there is little knowledge about NLGN 5 [8]. NLGNs 1, 2, and 3 triple knockouts (KOs) mice have the same number of synapses as normal mice [13], suggesting NLGNs KO affects synaptic function but not the gross morphology.

#### 3.1 Neuroligins function

Neuroligins are mainly interacting with presynaptic cell adhesion molecule and postsynaptic receptors. The extracellular domain binds to neurotoxins (NRXNs), which is another cell adhesion protein located on the presynaptic membrane, holding the synaptic terminals closely [5]. In addition, the PDZ domain binds to postsynaptic density protein 95 (PSD-95), which acts as a scaffold protein [14]. Thus, it provides binding sites for components such as AMPA and NMDA receptors [14]. This allows postsynaptic elements to concentrate and form the postsynaptic density area. Therefore, NLGN plays an essential role in forming and maintaining effective communication between neurons.

#### 3.2 Neuroligin 3 mutation: from genotype to phenotype

The first reported NLGNs mutation is the R451C mutation on NLGN 3 [8]. It is found on two siblings affected by autism spectrum disorder (ASD) in Sweden [8]. At the molecular and cellular level, R451C mutation results in impaired folding and membrane trafficking of NLGN3 [15-17]. This leads to unfolded protein response (UPR) and NLGN3 degradation by the proteasome [15, 18]. Furthermore, R451C mutation also decreases the binding affinity of NLGN3 to NRXN [19]. At organ level, a declining volume of the hippocampus and striatum are found in R451C mutated mice [20]. In addition, R451C knock-in (KI) mice show symptom such as repetitive behaviour and social impairment, which are characteristics of ASD [21, 22]. This could potentially be explained by the gain of function in the cerebellum excitatory synapses due to R451C mutation [23]. Furthermore, a recent study showed that R451C mutation led to neuronal deficit in the medial prefrontal cortex, which could be related to the social deficit phenotype observed in R451C KI mice [21].

### 4. CRISPR-Cas System

The clustered regularly interspaced short palindromic repeats (CRISPR) or CRISPR-associated protein (Cas) system is a immune mechanism found in bacteria and archaea that against outside infection in nature [24]. This defense mechanism was first discovered in the *Escherichia coli* genome in 1987, as the repeating sequences found in the upstream of *iap* gene with unknown function [25, 26]. In the application of CRISPR, programmable nucleases such as Cas9 are able to replace the defect DNA with exogenous DNA copy into genome [27]. Programmable nuclease of Cas9 constitutes a single guide RNA (sgRNA) and two catalytic active domains HNH and RuvC [25, 28, 29]. This single guide RNA containing of crRNA and tracrRNA enables it to recognize the targeting site and install the correct DNA copy on that specific loci [25]. With each domain of Cas9 cleaves each strand of DNA, the DNA double-strand breaks (DSB) thus are generated to be cut and pasted [29]. The DSB produced by Cas9 are generally repaired through two processes, either by homologous directed repair (HDR) or by nonhomologous end joining (NHEJ) [25]. HDR allows guide RNA to target the specific site and perform precise transgenes insertion if donor template is provided [25]. In contrast, NHEJ pathway ends with few nucleic acid deletion that induces disruption of target genes [25]. This new adaptive gene therapy leads the trend because not only it's less expensive but also for the less side effects such as insertion mutation and non-physical expression of proteins [27].

## 5. Conclusion

Unlike other approaches in gene therapy, CRISPR allows Cas9 nuclease to slice the defected genes and replace with corrected one in a more efficient way and with less side effects [27]. In case of Neural disorder associated with gene mutation, it is profound that CRISPR will lead the future. SHANK3 associated disorder, ASD, is expected to be solved via CRISPR. For example, in the research of Lu et al, the risk genes including SHANK3 is regulated by miR-873, a non-coding region located within an intro of LINGO2, which is highly expressed in brain and it is found that miR-873 is linked with learning difficulties [30]. Among the region of miR-873, potential off target genes were confirmed to be responsible for neuron morphology. After transfection with miR-873 inhibitor, the number of ramification is significantly lower than those with WT miR-873 [30]. Also, the cell differentiation was also promoted after knockout of miR-873, which may suggest that miR-873 serves as a function of suppressing neuronal differentiation [30]. Both evidence suggests miR-873 has associated with ASD syndrome [30]. Therefore, by locating the exact target side, it is possible to change the dysfunctional genes via transfection of CRISPR. We need guide RNA in the required PAM site to target the region of miR-873, to extract the genome of wild type miR-873 in order to replace the Mut miR-873 [30]. Overall speaking, there could be more factors generating different syndromes, including actin associated syndromes, but with certain approaches like CRISPR, it is logically to assume that by transfection or knockout of target genes, permanent therapies are achieved for ASD patients.

In this review, we discussed SHANK3, one of the most risk genes associated with autism spectrum disorder, and its potential pathway of causing ASD related syndromes. With specifying target genes in SHANK3, ASD might be solved via CRISPR in a more efficient way with least cost.

## Reference

- [1] Chaste P, Leboyer M. Autism risk factors: genes, environment, and gene-environment interactions. *Dialogues Clin Neurosci*. 2012;14(3):281-92.
- [2] Xu G, Strathearn L, Liu B, O'Brien M, Kopelman TG, Zhu J, et al. Prevalence and Treatment Patterns of Autism Spectrum Disorder in the United States, 2016. *JAMA Pediatr*. 2019;173(2):153-9.
- [3] Uchino S, Waga C. SHANK3 as an autism spectrum disorder-associated gene. *Brain Dev*. 2013;35(2):106-10.
- [4] Durand CM, Betancur C, Boeckers TM, Bockmann J, Chaste P, Fauchereau F, et al. Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. *Nat Genet*. 2007;39(1):25-7.
- [5] Leone P, Comoletti D, Ferracci G, Conrod S, Garcia SU, Taylor P, et al. Structural insights into the exquisite selectivity of neurexin/neuroigin synaptic interactions. *Embo j*. 2010;29(14):2461-71.
- [6] Persico AM. *Neural Circuit Development and Function in the Brain*: Academic Press; 2013.
- [7] Duffney LJ, Zhong P, Wei J, Matas E, Cheng J, Qin L, et al. Autism-like Deficits in Shank3-Deficient Mice Are Rescued by Targeting Actin Regulators. *Cell Rep*. 2015;11(9):1400-13.
- [8] Trobiani L, Meringolo M, Diamanti T, Bourne Y, Marchot P, Martella G, et al. The neuroiginins and the synaptic pathway in Autism Spectrum Disorder. *Neurosci Biobehav Rev*. 2020;119:37-51.
- [9] Song J-Y, Ichtchenko K, Südhof TC, Brose N. Neuroigin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. *Proceedings of the National Academy of Sciences*. 1999;96(3):1100-5.
- [10] Varoqueaux F, Jamain S, Brose N. Neuroigin 2 is exclusively localized to inhibitory synapses. *Eur J Cell Biol*. 2004;83(9):449-56.

- [11] Budreck EC, Scheiffele P. Neuroligin-3 is a neuronal adhesion protein at GABAergic and glutamatergic synapses. *Eur J Neurosci.* 2007;26(7):1738-48.
- [12] Marro SG, Chanda S, Yang N, Janas JA, Valperga G, Trotter J, et al. Neuroligin-4 Regulates Excitatory Synaptic Transmission in Human Neurons. *Neuron.* 2019;103(4):617-26.e6.
- [13] Varoqueaux F, Aramuni G, Rawson RL, Mohrmann R, Missler M, Gottmann K, et al. Neuroligins determine synapse maturation and function. *Neuron.* 2006;51(6):741-54.
- [14] Budreck EC, Kwon OB, Jung JH, Baudouin S, Thommen A, Kim HS, et al. Neuroligin-1 controls synaptic abundance of NMDA-type glutamate receptors through extracellular coupling. *Proc Natl Acad Sci U S A.* 2013;110(2):725-30.
- [15] De Jaco A, Comoletti D, King CC, Taylor P. Trafficking of cholinesterases and neuroligins mutant proteins. An association with autism. *Chem Biol Interact.* 2008;175(1-3):349-51.
- [16] De Jaco A, Dubi N, Comoletti D, Taylor P. Folding anomalies of neuroligin3 caused by a mutation in the alpha/beta-hydrolase fold domain. *Chem Biol Interact.* 2010;187(1-3):56-8.
- [17] Azoulay-Ginsburg S, Trobiani L, Setini A, Favaloro FL, Giorda E, Jacob A, et al. A Lipophilic 4-Phenylbutyric Acid Derivative That Prevents Aggregation and Retention of Misfolded Proteins. *Chemistry.* 2020;26(8):1834-45.
- [18] Ulbrich L, Favaloro FL, Trobiani L, Marchetti V, Patel V, Pascucci T, et al. Autism-associated R451C mutation in neuroligin3 leads to activation of the unfolded protein response in a PC12 Tet-On inducible system. *Biochem J.* 2016;473(4):423-34.
- [19] Comoletti D, De Jaco A, Jennings LL, Flynn RE, Gaietta G, Tsigelny I, et al. The Arg451Cys-neuroligin-3 mutation associated with autism reveals a defect in protein processing. *J Neurosci.* 2004;24(20):4889-93.
- [20] Ellegood J, Lerch JP, Henkelman RM. Brain abnormalities in a Neuroligin3 R451C knockin mouse model associated with autism. *Autism Res.* 2011;4(5):368-76.
- [21] Cao W, Lin S, Xia QQ, Du YL, Yang Q, Zhang MY, et al. Gamma Oscillation Dysfunction in mPFC Leads to Social Deficits in Neuroligin 3 R451C Knockin Mice. *Neuron.* 2018;97(6):1253-60.e7.
- [22] Rothwell PE, Fuccillo MV, Maxeiner S, Hayton SJ, Gokce O, Lim BK, et al. Autism-associated neuroligin-3 mutations commonly impair striatal circuits to boost repetitive behaviors. *Cell.* 2014;158(1):198-212.
- [23] Trobiani L, Favaloro FL, Di Castro MA, Di Mattia M, Cariello M, Miranda E, et al. UPR activation specifically modulates glutamate neurotransmission in the cerebellum of a mouse model of autism. *Neurobiol Dis.* 2018;120:139-50.
- [24] Zhang C, Quan R, Wang J. Development and application of CRISPR/Cas9 technologies in genomic editing. *Hum Mol Genet.* 2018;27(R2):R79-r88.
- [25] Hryhorowicz M, Lipiński D, Zeyland J, Słomski R. CRISPR/Cas9 Immune System as a Tool for Genome Engineering. *Arch Immunol Ther Exp (Warsz).* 2017;65(3):233-40.
- [26] Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the *iap* gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *J Bacteriol.* 1987;169(12):5429-33.
- [27] Xiao-Jie L, Hui-Ying X, Zun-Ping K, Jin-Lian C, Li-Juan J. CRISPR-Cas9: a new and promising player in gene therapy. *J Med Genet.* 2015;52(5):289-96.
- [28] Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science.* 2014;346(6213):1258096.

- [29] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 2012;337(6096):816-21.
- [30] Lu J, Zhu Y, Williams S, Watts M, Tonta MA, Coleman HA, et al. Autism-associated miR-873 regulates ARID1B, SHANK3 and NRXN2 involved in neurodevelopment. *Transl Psychiatry*. 2020;10(1):418.