The Pennsylvania State University

The Graduate School

REGULATORY FUNCTION OF AUTS2 ON VITAL SIGNALING PATHWAYS DURING BRAIN DEVELOPMENT

A Dissertation in

Biomedical Sciences

by

Zhuangzhuang Geng

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

May 2023

The dissertation of Zhuangzhuang Geng was reviewed and approved by the following:

Zhonghua Gao
Associate Professor, Department of Biochemistry & Molecular Biology
Dissertation Advisor
Chair of Committee

Sergei Grigoryev
Professor, Department of Biochemistry & Molecular Biology

Hong-Gang Wang
Lois High Berstler Professor, Department of Pediatrics
Lois High Berstler Professor, Department of Pharmacology

Yongsoo Kim
Associate Professor, Department of Neural & Behavioral Sciences

Ralph Keil
Associate Professor, Department of Biochemistry & Molecular Biology
Chair of Biomedical Sciences Program

ABSTRACT

Neurodevelopmental disorders (NDDs) describe a collection of disorders primarily associated with the abnormal function in the central nervous system (CNS), which includes but are not limited to autism spectrum disorders (ASD), attention-deficit/hyperactivity disorder (ADHD), developmental delay (DD), and intellectual disabilities (ID). *Autism Susceptibility Candidate 2* (*AUTS2*) is a high-risk ASD gene, first found in a monozygotic twin with ASD, carrying *de novo* balanced chromosomal translocation. Increasing evidence suggested the essential role of AUTS2 in brain development and neuronal differentiation. However, the underlying molecular mechanism in which AUTS2 regulates neuronal differentiation remains unclear. In this study, we have dissected the regulatory role of AUTS2 at different stages of brain development and established models of how AUTS2 functions at molecular level.

To investigate the function of AUTS2 at early-stage neuron differentiation, we used the mouse embryonic stem cells (mESC) as our model and conduct CRISPR/Cas9 genetic editing to knockout AUTS2. Phenotypic studies showed that the knockout of *Auts2* compromised the differentiation from mESC to neural progenitor cells (NPC). Next generation sequencing and biochemical assays discovered that the overactivated BMP signaling pathway in *AUTS2-/-* NPCs. The proteomic study revealed AUTS2 forms a novel protein complex with WDR68 and SKI (AWS complex) upon neural differentiation. The underlying molecular mechanism of the repressive role of AWS complex to BMP signaling was achieved by its recruitment of CUL4 E3 ubiquitin ligase, which catalyzed the poly-ubiquitination protein degradation of pSMAD1/5/9. Together, these experiments demonstrated the function of AUTS2 as BMP signaling pathway repressor via AWS complex during early-stage neuron differentiation.

The rapid development in human cerebral organoid culturing methods provides a platform for us to study the function of AUTS2 in human brain development. By genetic editing

human embryonic stem cells (hESC) to knockout AUTS2 and differentiating the cells into human cerebral organoids, biochemical and transcriptome analysis indicated the importance of AUTS2 in human brain development. To estimate the impact of AUTS2 on cell fate commitment, single-cell RNA-seq has been conducted to reveal that AUTS2 promoted the differentiation from neuro-epithelial cells to neuron-related cell types and prevented the generation of choroid plexus (ChP) epithelial cells. Pathway analysis showed that the complete deletion of AUTS2 resulted in the overactivated WNT/β-Catenin signaling pathway via AUTS2 interaction with known repressors, TCF3 and TLE3. Treating the AUTS2-/- organoid with WNT inhibitor, XAV939 can rescue the phenotypes observed in AUTS2-/- organoids, including the high expression of ChP cell marker genes and low expression of neuronal marker genes.

In summary, this study expands our understanding on the regulatory function of AUTS2 in brain development and the underlying molecular mechanisms. We discovered new AUTS2-interacting proteins, including WDR68/SKI and TCF3/TLE3, which are involved in regulating two vital signaling pathways, BMP and WNT/β-Catenin signaling pathways, during brain development. Our results showed that the phenotypes caused by the disruption of AUTS2 can be rescued by BMP/WNT inhibitor treatment, which provides insights into developing novel therapeutic approaches.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

This dissertation cannot be finished without guidance and support from my mentor, Dr. Zhonghua Gao. He provided me with the opportunity and the resources to be as creative as I like, inspiring me to do my best, and making sure I am progressing along a forward path. It is these things that have given me the most confidence in my scientific abilities.

I am grateful to have the best lab mates, Qiang, Yen-Teng, Aflah, Jessenia and Kaitlyn, who are both good coworkers and amazing friends. They made my PhD life not only more productive and efficient, but more enjoyable and interesting. I will never forget the time we spend together.

I am thankful to all my friends in Biomedical Science program and Penn State College of Medicine. To Miaolu, Kim, Franck, Xiaoming, TJ, Jiawen, Tanay and Lovely, thank you for bringing nothing but joy in the past six years.

I am sincerely thankful to my committees, Dr. Sergei Grigoryev, Dr. Hong-Gang Wang, and Dr. Yongsoo Kim. Their guidance and suggestions always inspire my research and will keep doing it in my future career.

I want to express my gratitude to our collaborators, Dr. Yuka Imamura and staffs in PSU genomic core, Dr. Ryan Hobbs, Dr. David DeGraff, and Dr. Yinsheng Wang from UC Riverside. The collaborations could not have been any easier.

Last, I want to thank my family. I could not achieve anything without the support of my parents, Cao Hong and Geng Zhilong, and my wife, Wanhui Sheng. Their love and encouragement are always there when I need them the most.

This work was funded by the National Institutes of Health R35 NIGMS, GM133496 to Dr. Zhonghua Gao. The conclusions drawn in this dissertation reflect the views of authors alone and do not represent the views of the funding agency.

Chapter 1

Introduction

Neurodevelopmental disorders and signaling pathways

Introduction of neurodevelopmental disorders

Neurodevelopmental disorders (NDD) are a collection of disorders that are characterized by the defect in brain development, which in turn, leads to impairments in emotion, learning ability, cognition, and motor. NDDs include but not limit to autism spectrum disorder (ASD), intellectual disability (ID), attention-deficit hyperactivity disorder (ADHD), and cerebral palsy (Parenti et al. 2020; Mullin et al. 2013). The occurrence of two or more NDD phenotypes, such as the combination of ASD and ID, is frequently observed in individual patient (Gilissen et al. 2014; Du et al. 2018). Although the symptoms and behaviors defects of NDDs are often mitigated as the patients grows older, some disabilities can be permanent (Parenti et al. 2020). The treatment of NDDs is challenging, often requiring the combination of professional therapy, medicines and home/school-based plans. Given the fact that over 15 percent of children aged 3 to 17 years are affected by NDDs in the United States according to the parental surveys, NDDs have become a serious society problem (David, Eva, and Christopher 2022).

The causes of NDDs include genetic risk, social deprivation, immune disorders, infectious diseases, nutritional factors, physical trauma, as well as toxic and environmental factors. Among these factors, studies on genetic risk are increasing exponentially, expanding our understanding of the neurobiological research, and motivating the finding of diagnosis and potential treatment of NDDs. Multiple large-scale studies and databases have identified numerous

risk genes with mutations, including single base mutations, amplification of trinucleotide repeats, copy number variants (CNVs), large chromosomal rearrangements, and chromosomal aneuploidy, (Abrahams et al. 2013; Fitzgerald et al. 2015; Cooley and Wright 2021; Parenti et al. 2020; Gilissen et al. 2014; Bakkaloglu et al. 2008; Sanders et al. 2011). Chromosomal aneuploidy is observed in syndromic forms of NDDs such as Down, Klinefelter, or Turner syndromes. At certain regions on chromatin, large chromosomal rearrangements and CNVs have a higher chance to occur, such as on chromosome 22q11 (velocardiofacial syndrome), 15q (Angelman and Prader-Willi syndromes), or 17p (Smith-Magenis syndrome). This expansion upstream of the *FMR1* gene impedes its expression resulting in increased translation at the synapse. Single nucleotide mutations are another example: X-linked genes, such as *MECP2*, can cause Rett syndrome, or autosomal genes, such as *CDH8* or *SHANK3*, can cause ASDs.

Tremendous progress has been made and the combination of genetic studies and animal/cell models has led to a better understanding of the pathophysiology and new treatment suggestions. Numerous of NDDs risk genes are the core components or the modulators of signaling pathways (Bandyopadhyay, Yadav, and Prashar 2013; Logan and Nusse 2004; Bae and Hong 2018; Kalkman 2012; X. Zhang et al. 2017). For instance, genetic mutations in different components of WNT signaling pathway have been reported in ASD patients (Logan and Nusse 2004; M. Katoh and Katoh 2007). Genes, including Neuroligins (*NLGN*), fragile X mental retardation 1 (*FMR1*), ubiquitin-protein ligase E3A (*UBE3A*), and *DLX*, which regulate bone morphogenetic protein (BMP) signaling were also found in ASD patients (X. Zhang et al. 2017; Kashima et al. 2016; W. Li et al. 2016; Schluth-Bolard et al. 2019). Disruption in other signaling pathways, including sonic hedgehog (SHH), transforming growth factor β, and retinoic acid are also known to contribute to the pathogenesis of NDDs. In my study, we focus on two vital signaling pathways, WNT/β-Catenin and BMP signaling pathways.

Bone morphogenetic protein signaling pathway in NDDs

BMPs constitute the largest subdivision of TGF-β superfamily and are critical in the development of the nervous system (Zi, Chapnick, and Liu 2012; Lowery and Rosen 2018). BMP regulates its target genes expression through the canonical (SMAD-dependent) and non-canonical pathways (MAPK cascade), which are shown to be dysregulated in NDDs (Graf et al. 2016; R. N. Wang et al. 2014). BMP receptors belongs to the type I or type II serine/threonine kinase receptors forms a heterotetrameric complex. The binding of the ligand to the receptor leads to the phosphorylation of the R-SMADs (SMAD1/5/9), which in turn, forms a heterodimer with co-SMAD (SMAD4) and translocates into the nucleus. By binding to the chromosome, this R-SMAD/co-SMAD heterodimer regulates its target gene expression.

At early stage of embryonic development, BMP/ TGF-β signaling pathway maintains the balance from embryonic stem cells (ESC) to three germ layers (**Fig. 1-1**) (Watabe and Miyazono 2009). The activity of BMP/ TGF-β remains at low level in the process of differentiation from ESC to ectoderm layer. On the other hand, the activation of BMP/ TGF-β allows the differentiation from ESC to primitive streak, which derives to meso/endoderm layers (Watabe and Miyazono 2009). At later stage of neuronal development, BMP signaling pathway is tightly involved in the modulation of neuronal subtype specification, promotion of dendritic and axonal growth and induction of synapse formation and stabilization (Gámez, Rodriguez-Carballo, and Ventura 2013; Bond, Bhalala, and Kessler 2012). Considering the importance of BMP signaling pathway in neuron development, the modulators of BMPs are also shown as NDD-risk genes. For instance, the deletion of FMRI results in the fragile X syndrome, which is characterized by ID and ASD. The protein product of FMRI, FMRP is a known RNA-binding protein, which target the mRNA of BMPR2 and the disruption of FMRI causes the overactivation of BMP signaling pathway, which stimulates reorganization of actin to promote neurite outgrowth and synapse

formation (Kashima et al. 2016). The overactivated BMP signaling pathway was also observed in the ASD patients with mutant DLX genes and UBE3A (W. Li et al. 2016; Schluth-Bolard et al. 2019; Sajan et al. 2011).

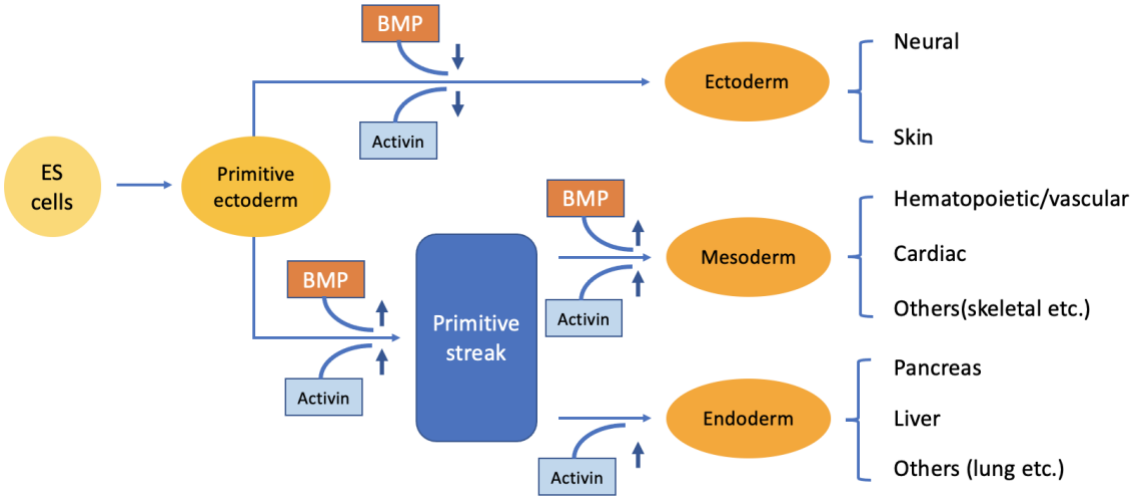

Figure 1-1: BMP/TGF-β signaling pathway in the differentiation process from ESC stage to germ layers.

Canonical WNT signaling pathway in NDDs

Canonical WNT signaling pathway is highly conserved in mammals with essential function in multiple biological processes (Lustig and Behrens 2003; Rosso and Inestrosa 2013). In mammals, in total 10 WNT cell surface receptors have been identified. Without stimulation, the WNT receptors (Frizzled and LRP5/6) capture the cadherin/β-catenin protein complex. The recruitment of β-catenin destruction complex (CK1/GSK/AXIN/APC) will then phosphorylate β-catenin and promote protein degradation. Upon the activation of canonical WNT signaling pathway, the dissociation of cadherin/β-catenin complexes in the cell membrane happens, followed by the release of β-catenin. Without the recruitment of protein degradation complex, β-catenin is able to translocate to nucleus and regulate the expression of its target genes.

WNT signaling pathway plays an important role in neurodevelopmental and post-neurodevelopmental processes, such as CNS regionalization, neural progenitor differentiation, neuronal migration, axon guidance, dendrite development, synaptogenesis, adult neurogenesis, and neural plasticity (Tang 2014; Wada and Okamoto 2009; Wodarz and Nusse 1998; Rosso and Inestrosa 2013; Fair et al. 2022b).Genetic studies displayed several core components of WNT signaling pathway, including but not limited to *WNT1*, *WNT2*, *CTNNB1*, *APC* and *PRICKLE2*, as high risk genes of ASD (Kalkman 2012; O'Roak et al. 2012; O ' Roak et al. 2012; Mulligan and Cheyette 2017). Conditional knockout of *Apc* in mice exhibits the impairment in learning and memory. In hippocampus, *Apc* knockout mice displayed the increased synaptic spine density and altered synaptic function (Mohn et al. 2014). The disruption of another core component, β-catenin in mice caused the defective brain development, such as spina bifida aperta, caudal axis bending, and tail truncation (T. Zhao et al. 2014). Several ASD-associated genes are direct or indirect modulators of WNT signaling. For example, a chromatin remodeler, *CHD8* is identified as ASD risk gene and directly regulates expression of β-catenin target genes. The *Chd8* heterozygous knockout mice were characterized with macrocephaly, craniofacial abnormalities, and behavioral deficits (Platt et al. 2017). Transcriptome analysis revealed the over-activation of WNT signaling in knockout mice, with upregulation of WNT target genes. Furthermore, the downstream target of WNT/β-catenin signaling, such as Neuroligin 3 (*Nlgn3*), is also proven to be the ASD-associated gene, and the ablation of *Nlgn3* in mice led to the deficit in synaptogenesis (Medina et al. 2018).

Genetic association between *AUTS2* and neurodevelopmental disorders

Discovery of *AUTS2* gene

Autism spectrum disorder (ASD) is a collection of neurodevelopmental disorders, characterized by the abnormalities in social interactions and commutation, restricted and repetitive behaviors, and unusual responses to sensory stimuli (Hodges, Fealko, and Soares 2020). ASD has a strong genetic basis and is highly heritable (Maenner et al. 2020; Risch et al. 1999). However, the complexity of ASD genetic is due to the unpredicted genes interactions, environmental factors, and epigenetic abnormalities (Rylaarsdam and Guemez-Gamboa 2019). Multiple family and twin studies have been conducted to dissect the impact of genetic variance on ASD (Steffenburg et al. 1989; Ritvo et al. 1985; Le Couteur et al. 1995). In 2002, a study on a monozygotic twin with ASD found a *de novo* balanced translocation involving 7q11.2 and 20p11.2 (7.20) (Sultana et al. 2002). Translocation on chromosome 7q has been reported in previous studies on the genetics of ASD (Mervis et al. 2021; Palferman et al. 2001; Ashley-Koch et al. 1999). Approximate 340 kb downstream of this translocation is a genetic region known as Williams-Beuren syndrome (WBS) critical region, whose deletion would cause a neurodevelopmental disorder characterized by the aberrant facial appearance, developmental delay, and cardiovascular problem (Bayés et al. 2003). Pieces of evidence suggested the importance of this locus, which may contain a potential autism candidate gene. Multiple bioinformatics analysis and biochemical assays predicted a protein-coding gene, which is named autism susceptibility gene 2 (*AUTS2*) (Sultana et al. 2002). Although in this study, researchers failed to establish a solid association and linkage between AUTS2 mutation with autism, due to limited patient cases number and knowledge of this gene, this study unveiled the possibility that mutation on *AUTS2* gene might contribute to autism.

Genetic association between *AUTS2* and neurodevelopmental disorders

Since the discovery of AUTS2 in a monozygotic twin, genetic studies and clinical case reports observed the association between multiple NDDs and the mutations of AUTS2 gene, including translocation, copy number variance, and single nucleotide polymorphisms (SNPs) (**Table 1-1**) (Glessner et al. 2009; M. K. Dennis et al. 2012; Kalscheuer et al. 2007; Ben-David et al. 2011; Huang et al. 2010). Although these observations suggested the potential importance of AUTS2 in NDDs, due to the small sample size, the causality role of *AUTS2* was not established.

In 2013, a large-scale study identified 24 patients carrying exonic deletions of *AUTS2* from 49,684 individuals, shared a collection of NDD-related syndromes including ID, autism/ASD, growth and feed defects, musculoskeletal abnormalities, and congenital malformations (Beunders et al. 2013; 2016). These common pathological features observed in patients were termed "*AUTS2* syndrome". From 17 well-characterized patients, the most common syndrome is ID, which was observed in 95% of patients, followed by microcephaly (70%) and short stature (60%). In this study, a scoring system has been established to evaluate the severity of *AUTS2* syndrome, based on the clinical features observed with over 10% in the cohort bearing *AUTS2* mutation. The patients with mutation on the 3' end of *AUTS2* displayed more pronounced dysmorphic features. To validate the function of AUTS2 in neuron development, they also used morpholino to knockdown *auts2* expression in zebrafish. The suppression of *auts2* in zebrafish embryo led to microcephaly, which can be rescued with different isoforms of *auts2*. Combining the results of their genetic study and observation in zebrafish, this research demonstrated the causal role of AUTS2 in NDDs and the function of AUTS2 as an essential regulator in neuron development (Beunders et al. 2013).

Two decades of studies revealed AUTS2 as one of NDD high risk genes. Both the clinical reports and functional analysis demonstrated that AUTS2 was not only tightly involved in

the brain development, but also exerted an impact on global development. From the results of patients' assessment and *Auts2*-disrupted mouse models, the main impairment is observed in the brain regions responsible for the memory, learning and cognition (Gao et al. 2014; Hori et al. 2015; Castanza et al. 2021; Beunders et al. 2013; 2016). Patients with *AUTS2* mutation also suffer dysmorphism, limb malformations, fail to thrive, short stature (Jolley et al. 2013; Beunders et al. 2016; Nagamani et al. 2013; Schluth-Bolard et al. 2019), indicating the potential role of AUTS2 regulating peripheral tissue development (**Table1-1**).

A large proportion of *AUTS2* genetic variances are copy number variance (CNV), which attributes to the chromosomal instability of this region (**Table1-1**). To artificially induce genetic mutation using a combination of compounds, the treatment of a ribonucleotide reductase inhibitor, hydroxyurea, and a DNA polymerase inhibitor, aphidicolin efficiently induced high frequency of *de novo* CNVs of certain loci in human cells, which includes *AUTS2* locus (Arlt et al. 2011). Aphidicolin alone is sufficient to induce CNVs formation in the *Auts2* locus in nonhomologous end joining mESC (Arlt et al. 2012). Moreover, the offspring from older male mice have higher risk carrying *de novo* CNVs in certain loci, including the *Auts2* locus (Flatscher-Bader et al. 2011). Besides, *AUTS2* structure variants in coding regions have been reported in patients with NDDs. Some non-coding CNVs on *AUTS2*, which lead to the improper expression of AUTS2 protein, can also cause neuronal pathological disorders. However, CNVs of *AUTS2* are reported to present in unaffected individuals, suggesting that *AUTS2* mutations are tolerated in some cases (Bakkaloglu et al. 2008; Redon et al. 2006). Not only the genetic variances of *AUTS2* are tightly associated with NDDs, epigenetic abnormality of *AUTS2* can also cause pathological consequences. A genome-wide analysis of DNA methylation on an ASD discordant and concordant monozygotic twin showed that *AUTS2* promoter region is one of top differentially methylated regions (Wong et al. 2014). The alternation of DNA methylation is known as a potential causality for ASD, as ASD patients with differential methylated methyl-

CpG binding domain protein 4 (*MBD4*) and microtubule-associated protein 2 (*MAP2*) have been reported (Loke, Hannan, and Craig 2015). In summary, genetic studies identified *AUTS2* as an NDD high-risk gene, which strongly suggested its importance in the pathological process as well as normal brain development.

Table 1-1: Summary of reports focusing on the AUTS2 mutation and NDDs.

Mutation Type	**Allele change**	**Syndrome**	**Publication**
translocation	7q11.2/p11.2	Autism	Sultana R , et al. 2002
gene variant	c.47A>G	ASD	Richler E , et al. 2006
gene variant	c.61T>G	ASD	Richler E , et al. 2006
gene variant	c.7C>T	ASD	Richler E , et al. 2006
translocation	t(7;20)(q11.2;p11.2)	Mental retardation, urogenital and limb malformations	Kalscheuer VM , et al. 2007
copy number gain	chr7: 69624365-69726112	ADHD	Elia J , et al. 2009
translocation	t(6;7)(q14.1;q11.2)	ASD, Developmental retardation, Speech/language delay	Huang XL , et al. 2010
deletion	chr7: 69,380k-69,460k	Epilepsy	Mefford HC , et al. 2010
copy number gain	-	Severe ID and epilepsy, ASD	Ben-David E , et al. 2011
copy number gain	chr7: 69,450,913–69,630,331	ASD, DD, dysmorphic features, seizures	Nagamani SC , et al. 2012
copy number gain	chr7: 69,450,913–69,630,331	ASD, DD, dysmorphic features, radioulnar synostosis	Nagamani SC , et al. 2012
deletion	chr7: 69,569,118–69,887,732	DD	Nagamani SC , et al. 2012
deletion	chr7: 69 750 889–69 884 307	DD, dysmorphic features, fail to thrive, scoliosis, atrial septal defect	Nagamani SC , et al. 2012
inversion	-		Talkowski ME , et al. 2012
copy number variance *****	*****	ID (95%), autism (33%), short stature (60%), microcephaly (70%), cerebral palsy (20%), and facial dysmorphisms, ADHD (14%) *****	Beunders G , et al. 2013 *****

copy number loss	chr7: 68,528,129-69,368,926	ASD, cognative deficit	Girirajan S , et al. 2013
deletion	chr7: 69,070,983-69,753,568	DD, ID, short stature, mild dysmorphism	Jolley A , et al. 2013
copy number loss	-	Severe speech and language disorder, history of tonic-clonic movements, and pes planus with eversion of the feet	Amarillo IE , et al. 2014
missense variant	c.778G>A	ASD, ID, congenital anomalies	Brett M , et al. 2014
stop gained	c.976C>T		Deciphering Developmental Disorders Study 2014
copy number gain	chr7: 68,391,493-68,846,056	ASD	Egger G , et al. 2014
stop gained	c.454C>T	ASD, DD	McCarthy SE , et al. 2014
deletion	-		Liu Y , et al. 2015
inversion	-	ASD	Redin C , et al. 2016
inversion	-	ASD	Redin C , et al. 2016
frameshift variant	c.1486dup	ID, DD	Bowling KM , et al. 2017
missense variant	c.349C>T	Language impairment	Chen XS , et al. 2017
missense variant	c.2368 2369delinsAT	ASD, DD, ID	Balicza P , et al. 2019
splice site variant	c.309+2T>C	ASD	Feliciano P et al. 2019
missense variant	c.2479C>G		Monies D , et al. 2019
frameshift variant	c.1464 1467del	ASD, dysmorphic features, motor DD, ID	Saeki S , et al. 2019
translocation	MC/1803t(7;10)(q21.1;q26.1)	ID, microcephaly, absent speech, facial dysmorphism	Schluth-Bolard C , et al. 2019

frameshift variant	c.983 984del	ASD, DD	Husson T , et al. 2020
frameshift variant	c.2890del	ASD, comorbid epilepsy	Lee J et al. 2020
missense variant	c.1604A>C	Mental retardation, autosomal dominant 26	Brunet T et al. 2021
frameshift variant	c.1769dup	Short stature, dysmorphic features, moderate ID and ASD	Gauld C et al. 2021
In frame deletion	c.1603 1626del	global DD, absent speech, minor craniofacial anomalies, hypoplasia of the cerebellar vermis and thinning of the corpus callosum	Palumbo P et al. 2021
missense variant	c.1611C>A	Global DD, ID	Pode-Shakked B et al. 2021
copy number gain	arr[hg19] 7q11.22 (67767963_69320956) × 3	DD and inattention	Sanchez-Jimeno C et al. 2021
copy number loss	arr[hg19] 7q11.22 (69564262-69592731) × 1	Behavioral problems, ADHD	Sanchez-Jimeno C et al. 2021
frameshift variant	c.1298del	Microcephaly, ADHD	Sanchez-Jimeno C et al. 2021
frameshift variant	c.2183del	Cognitive delay, ADHD symptoms. motor stereotypies	Sanchez-Jimeno C et al. 2021
stop gained	c.927 928delinsAT	GDD, hypotonia, failure to thrive	Sanchez-Jimeno C et al. 2021
copy number gain	-	syndromic ID/DD	Carvalho LML et al. 2022
intron variant	c.691-17364G>A		Tuncay IO et al. 2022
synonymous variant	c.3291G>A	ASD	Woodbury-Smith M et al. 2022
missense variant	NM_015570.3:c.1600A>C, p.(Thr534Pro)	Profound intellectual disability, cerebellar hypoplasia, epilepsy, and dysmorphic features	Fair SR et al. 2022

Note: * More detailed genetic and clinical information of each individual patients can be found in (Beunders et al. 2013).

Characterization of AUTS2 protein and its isoforms

AUTS2 protein

AUTS2 gene spans 1.2 Mb and contains 19 exons, which are highly conserved among vertebrates. The first six exons are separated with large introns and later 13 exons are comparatively compact (Hori and Hoshino 2017; Sultana et al. 2002; Oksenberg et al. 2013a). The protein production encoded by *AUTS2* gene contains 1259 aa, which shared over 90% amino acid similarity between mice and human and 62% amino acid conservation between human and zebrafish. Analyzing the amino acid sequence of AUTS2, results showed that AUTS2 contains known protein motifs, which may provide limited insight into the functionality of AUTS2. Two proline-rich domains (288-471, 545-646) were identified in AUTS2, which lack a rigid structure and are involved in protein-protein interactions and docking sites for signaling protein modules (Elias et al. 2020). Several nuclear localization signal sequences have been identified on the N-terminus of AUTS2 (Hori, Shimaoka, and Hoshino 2022). Other motifs include a predicted PY (ProTyr) motif (PPPY) at amino acid 515-519, several cAMP/cGMP-dependent protein kinase phosphorylation sites, putative N-glycosylation sites, and eight CAC (His) repeats. The PY motif is involved in protein-protein interactions as a potential WW-domain-binding region and is found in multiple transcription factors, indicating that AUTS2 may have a function in transcriptional regulation. A comprehensive analysis showed 86 human proteins containing five or more histidines, which is also found in AUTS2 (Salichs et al. 2009; Sultana et al. 2002). The majority of proteins are characterized with DNA/RNA-related functions and are overrepresented in the brain and/or nervous system development. No evidence was found for any signal peptide in

AUTS2, indicating that it is not secreted or exposed to the cellular membrane. No DNA-binding domains have been identified.

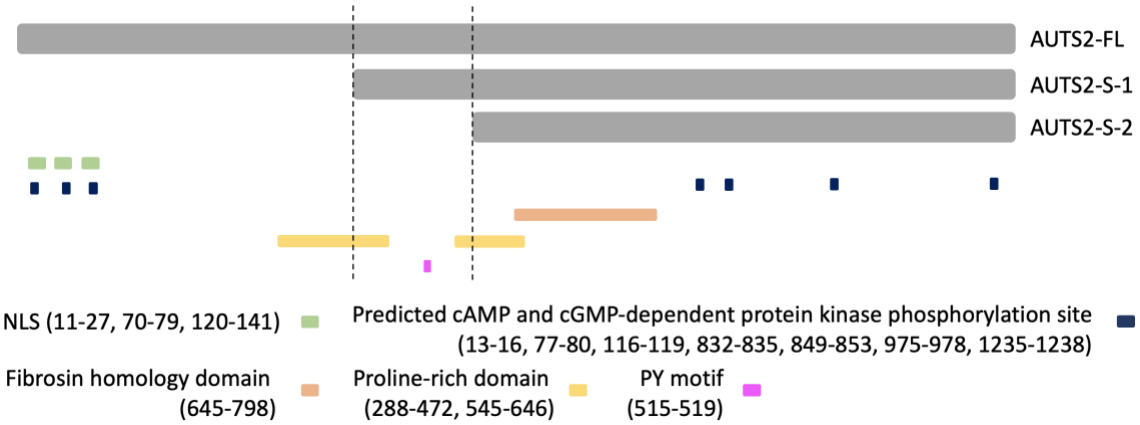

Figure 1-2: Schematic of AUTS2 isoforms and protein motifs

AUTS2 isoforms and expression pattern

Genetic analysis showed multiple alternative transcription start sites (TSS) were found in AUTS2 gene and some of them were validated using Rapid amplification of 5' cDNA ends (5' RACE) and immunoblotting (Beunders et al. 2013; Hori et al. 2014; 2020). Besides the full-length AUTS2 (AUTS2-FL), there are at least two C-terminus isoforms of AUTS2 that have been expressed in developing mouse brain. One of the isoforms translates from exon 8 (AUTS2-S-1; ~88KDa), and another one translates from exon 9 (AUTS2-S-2; ~78KDa) (Beunders et al. 2013; Hori et al. 2014; Yamashiro et al. 2020). A study on the post-translational modification of histones on AUTS2 locus revealed the intronic promoters and at least four enhancers, which are responsible for the translation initiation and regulation of these isoforms (Monderer-Rothkoff et al. 2021; Oksenberg and Ahituv 2013). In human fetal brain, an additional *AUTS2* variant with

alternative splicing skipping exon 10 and 12 was discovered (Monderer-Rothkoff et al. 2021). In zebrafish, the complexity of *AUTS2* transcripts was multiplied with 13 predicted unique transcription start sites (TSSs) and over 20 alternative transcripts (Kondrychyn, Robra, and Thirumalai 2017).

In vitro study demonstrated that the expression of Auts2 was increasing from mouse embryonic stem cells to neural progenitor cells (Monderer-Rothkoff et al. 2021). Interestingly, an expression switch between long and short isoforms of Auts2 was observed. The long isoform expressed solely at the ESC stage and the short isoform expressed at the intermedia stage. Only at NPC stage, both long and short isoforms expressed (Monderer-Rothkoff et al. 2021). In zebrafish, universal expression of *auts2* is reported in both the central and peripheral nervous system at 24 hours post fertilization (hpf). At the late stage of embryo development, auts2 expression is mainly restricted to the brain and eye (Oksenberg et al. 2013b; Beunders et al. 2013).

The expression pattern of *auts2* in zebrafish largely correlated to its expression in the mouse. *In situ* RNA staining showed that *Auts2* maintains a low expression at early stages of brain neurogenesis until E12 when *Auts2* starts to express in several brain regions related to higher cognitive functions, such as dentate gyrus, thalamus, hippocampus, and cerebellum (Bedogni, Hodge, Nelson, et al. 2010). Another independent study also showed that in mice, *Auts2* reaches its peak expression in the cerebral cortex at E15 (Gao et al. 2014). Number of neuronal cell types, including glutamatergic neurons, GABAergic neurons, and tyrosine hydroxylase (TH)-positive dopaminergic neurons were detected with abundant *Auts2* expression. Co-staining *Auts2* with other cell markers, such as *Pax6*, *Tbr2*, and *Gfap*, revealed that Auts2 was exclusively expressed in neural related cells, including precursors of Purkinje cells, Purkinje cells, and granule neurons, but not glial cells (Visel, Thaller, and Eichele 2004; Bedogni, Hodge, Nelson, et al. 2010). The expression of *Auts2* is regulated by T-box brain protein 1 (*Tbr1*), which

is an essential transcription factor involved in the development of cortical deep-layer projection neurons (Bedogni, Hodge, Nelson, et al. 2010; Bedogni, Hodge, Elsen, et al. 2010).

AUTS2 mRNA was detected in the telencephalon, ganglionic eminence, cerebellum anlagen in 8 weeks human post-mortem fetal brain (Sultana et al. 2002). In a 23-week human fetal brain, *AUTS2* expression was found in the dentate gyrus, hippocampus, caudate nucleus and putamen nuclei (Lepagnol-Bestel et al. 2008). Gene expression profiles from 10 human ocular tissue showed that *AUTS2* was one of the most abundant genes in the sclera (Wagner et al. 2013). Noticeably, not only the mRNA of *AUTS2* is enriched in human fetal brain, *AUTS2* intronic RNA also displayed a high abundance. While the functionality of the highly enriched *AUTS2* intronic RNA remain unclear, a similar observation has been found in other autism-related genes, such as *NRXN1, PCDH9,* and *MSRA* (Ameur et al. 2011). Besides the expression in the brain, *AUTS2* is also found in the skeletal muscle, kidney, lung, and leukocytes (Sultana et al. 2002; Ameur et al. 2011).

There are evidence suggesting the functional difference between long and short isoforms of AUTS2. In Hori et al., 2014, immunofluorescence staining and cell fractionation assay showed that long isoform of AUTS2 presented in both cytosol and nuclei and regulated the activity of Rac1 signaling pathway while short isoforms exclusively present in nuclei (Hori et al. 2014). A later study showed that long isoform is capable of interacting with Polycomb Repressive Complex 1 (PRC1) while short isoforms cannot (Monderer-Rothkoff et al. 2021). However, in Beunder et al., 2013 study, the phenotypes caused by suppressing *auts2* using morpholino in zebrafish embryos can be rescued by the overexpression of either long isoform or short isoform of auts2, suggesting the potential functional compensation between long and short isoforms (Beunders et al. 2013). Combined, previous studies indicated the importance of AUTS2 in brain development. However, more efforts are required to differentiate the functionality of AUTS2 isoforms.

Function of AUTS2 in neuronal differentiation and brain development

As genetic studies highlighted the causalitive role of *AUTS2* in multiple NDDs, extensive efforts have been done to dissect the biological function of AUTS2 in differentiation and development. In this section, we will summarize the known function of *AUTS2* at different stages of neuronal differentiation and how the disruption of *AUTS2* leads to developmental defects and behavioral impairment.

Essential role of AUTS2 in the early stages of neuronal differentiation

To study the function of AUTS2 at early stage of neuronal differentiation, multiple studies on AUTS2 biological function have been done using mESC, due to their capability to maintain pluripotency and stemness, as well as the potential to differentiate into other cell types (Guan, Rohwedel, and Wobus 1999; Monderer-Rothkoff et al. 2021; Q. Wang et al. 2018). The expression of AUTS2 keeps increasing in the process of mESC differentiation to neuron-related cell types (Monderer-Rothkoff et al. 2021; Q. Wang et al. 2018). As early as mouse ESC stage, Monderer ‘s study reported that knockout long isoform of *Auts2* led to the decreasing expression of pluripotency marker genes. During *in vitro* corticogenesis, heterozygote *Auts2* knockout was sufficient to promote cell death and apoptosis (Monderer-Rothkoff et al. 2021). Knockout of *Auts2* in mESC caused premature neuronal differentiation, which can be rescued by re-introducing human *AUTS2*, and the overexpression of *AUTS2* increased the expression of pluripotency marker genes and delayed differentiation (Monderer-Rothkoff et al. 2021). Our previous study showed that knockout of an essential AUTS2-interacting protein, WDR68 which compromised the integrity of AUTS2-containing protein complex, resulted in the imbalanced

expression pattern between ectoderm and meso/endoderm genes during the process of differentiation from mouse ESC to NPC. The differentiation defects were retained upon differentiating NPCs into neurons, with the low expression of neuronal marker genes, such as *Neun* and *Tubb3* (Q. Wang et al. 2018). In the field of differentiation and development, mESC is a robust model with potential to differentiate into numerous cell types and ease to manipulate. Studies on AUTS2 using mESC revealed the importance of AUTS2 in neuronal differentiation and neurogenesis. However, the gaps caused by *in vitro* culturing differentiation methods and genetic background between mouse and human require more efforts to translate to clinical application.

Given the complexity of human brain development and dramatic difference between human brain and other models, the understanding of AUTS2 in human brain development is still limited. Multiple techniques are invented to convert human pluripotency stem cells to 3D organoids, aiming to recapitulate brain development (Lancaster et al. 2013; Qian, Song, and Ming 2019; Di Lullo and Kriegstein 2017). The rapid development of technologies provides the opportunity to expand our comprehension in the function and molecular mechanism of AUTS2 in human brain organoids. Recently, the first cerebral organoid study on AUTS2 has been conducted, emphasizing the role of AUTS2 at neural progenitor cell stage (Fair et al. 2022a). Whole genome sequencing of a patient with microcephaly and ID identified a pathogenic missense variant (T534P) in exon 9 of AUTS2 (**Table1-1**). Peripheral blood mononuclear cells were reprogrammed to induced pluripotent stem cells, which were converted to human cerebral organoids. *AUTS2* mutant organoids showed defective growth trajectory and compromised NPC proliferation, which recapitulated the microcephaly clinical feature of this patient. Comparing between WT and mutant organoids, single cell RNA-seq (scRNA-seq) revealed that the AUTS2 mutation hindered the generation of NPCs with low enrichment of cells expression G1/S transition genes. Gene ontology analysis showed that the dysregulated gene expression signatures

were associated with WNT/β-Catenin signaling, which can be rescued by using CRISPR/Cas9 to correct the AUTS2 mutation. In summary, this study provides molecular insights into AUTS2 function in NPC and neural differentiation by regulating WNT signaling pathway.

As the most widely used animal model, several AUTS2 mouse models are generated to investigate the impact of AUTS2 during development process (Gao et al. 2014; Hori et al. 2014; 2020; Yamashiro et al. 2020; Castanza et al. 2021). In the study of investigating the cytosolic function of AUTS2, they generated an Auts2-deficit mouse model by deleting exon 8 (Hori et al. 2014). Immunoblotting showed that the deletion of exon 8 eliminates the expression of AUTS2 long isoform in the brain in *Auts2*$^{del8/del8}$ mouse and AUTS2 long isoform level decreased by 50% in Auts2$^{del8/+}$ mouse. However, the increasing expression of Auts2 short isoform with an alternative TSS in exon 9 was observed in both homozygous and heterozygous mice, potentially caused by compensatory effect responding to AUTS2 long isoform loss. Auts2$^{del8/del8}$ is neonatally lethal, and dissecting embryonic brains found no obvious difference in gross morphology or histology. Since previous zebrafish experiments suggested that Auts2 is involved in cell proliferation and neuronal differentiation (Beunders et al. 2013), the assessment of this mouse model in NPC proliferation showed no difference, compared with WT mice.

AUTS2 in the postmitotic neurons

Several studies on the cerebral organoids derived from autism patient demonstrated the imbalanced proportion between excitatory and inhibitory neurons, which is universal observation regardless different genetic background (Mariani et al. 2015; Astorkia, Lachman, and Zheng 2022; Velmeshev et al. 2019). By crossing Auts2$^{flox/flox}$ with Emx1$^{Cre/+}$ mice, a mouse model is generated with conditional elimination of Auts2 in mouse forebrain (Hori et al. 2020). Focusing on the pyramidal neuron in CA1 hippocampus, the number of dendritic spines, which are

protrusions known to receive input from synapse, showed the abnormal increase in Auts2 mutant mice. The immunofluorescence results indicated that the removal of Auts2 generated excessive excitatory synapse formation and exerted no influence on inhibitory synapse. Forebrain Auts2 knockout mice exhibit both cognitive and socio-behavioral dysfunction, which is potentially caused by the alterations of excitatory synapse and neuron morphology.

In neurogenesis, *Auts2*$^{del8/+}$ mice exhibit defective cortical neuron migration and exon elongation. Across WT, heterozygous and homozygous Auts2 knockout mice, neuron-related phenotypes follow a gene dose-dependent manner, which can be rescued by re-introducing Auts2 long isoform via electroporation. As one allele bearing AUTS2 mutation is sufficient to cause pathological consequences, *Auts2*$^{del8/+}$ mouse model was assessed with a battery of behavior studies to comprehend how Auts2 regulate physiological brain function (Hori et al. 2015). To estimate the capacity of this mouse model in recapitulating AUTS2-related neuropsychiatric disorder, the cognitive memory functions and normal social approach behaviors are compromised in Auts2$^{del8/+}$ mouse. On top of that, open field test and elevated plus maze test revealed reduced anxiety and abnormal emotional behaviors in Auts2 mutant mice (Hori et al. 2015). Collectively, this mouse model revealed the function of Auts2 in neuronal migration, as well as proved its ability to mimic certain AUTS2-related neuropathological syndromes.

Besides the expression of Auts2 in developing mouse forebrain, Auts2 is also enriched in cerebellum, whose function remains obscure in this region (Hori et al. 2015; Bedogni, Hodge, Nelson, et al. 2010; Yamashiro et al. 2020). To address this issue, a conditional knockout Auts2 in cerebellum mouse model was generated by crossing *Auts2*$^{flox/flox}$ with *En1*$^{Cre/+}$ mice. Compared with WT, *Auts2* conditional knockout mice displayed a decreased cerebellar size, along with Purkinje cells loss. Transcriptome profiling and immunostaining results indicated that Auts2 in cerebellum is tightly involved in synapse formation, dendrite morphology and Purkinje cells maturation. As the main functions of cerebellum are motor control and some cognitive functions,

such as attention and language, this mouse model exhibits abnormalities in locomotor activity and vocal communication (Schmahmann and Caplan 2006; Hori et al. 2020).

A recent mouse model was designed to knockout all Auts2 isoforms by placing flox sequence at the flanking regions of exon 15, which is shared by long and short isoforms of AUTS2 (Castanza et al. 2021). Crossing *Auts2*$^{flox15/flox15}$ with *Nes11-Cre* mice generate conditional Auts2 knockout mice in CNS. Both heterozygous and homozygous mutants are neonatally lethal, due to the difficulty in breathing. Breathing issue was improved in conditional *Auts2* knockout mice in cortical projection neurons (Auts2 cKO^{ctx}), which is generated by crossing *Auts2*$^{flox15/flox15}$ with *Emx1-Cre* mice. Auts2 cKO^{ctx} mice were able to survive to adulthood and fertile. Histology and immunofluorescence staining showed the defective development in cerebral cortex structure, especially the dentate gyrus hypoplasia. Electroencephalogram (EEG) displayed abnormality in cortical circuit activity, with greater power in theta, delta, and beta frequencies, similar to the observation in the brain with epilepsy. Combined, mice are proven to be a robust animal model in studying AUTS2 function. *Auts2* mutant mice recapitulate certain syndromes observed in patients, as well as provide insights into the mechanism of how Auts2 works in CNS.

The zebrafish (*Danio ririo*) is a popular animal model in the field of development and differentiation, due to its visually accessible development, genetic homology to other vertebrate, and shared CNS morphological signatures to human (Kalueff, Stewart, and Gerlai 2014). Up to date, two studies were conducted by two independent groups to investigate AUTS2 function using zebrafish (Oksenberg et al. 2013b; Beunders et al. 2013). Morphant auts2 zebrafish from both studies showed similar syndromes, including microcephaly, compromised neurogenesis, and other developmental defects. In one study, auts2 morphant zebrafish present craniofacial deformities, such as retrognathia and micrognathia, mimicking a phenotype observed in patients (Beunders et al. 2013). The other study described the defects in spinal motor neurons and sensory neurons, which have not been observed yet in humans (Oksenberg et al. 2013b). However, due to

the tendency of zebrafish gene duplication, auts2 has a homology, auts2b, which may mask the effect of morpholino targeting auts2 (Kondrychyn, Robra, and Thirumalai 2017). Moreover, the off-target effect of morpholino and lack of laminated cerebral cortex restrain the application of zebrafish in human brain development (Parker et al. 2013).

Together, animal models with the disrupted *AUTS2* have provided the evidence to reflect the importance and necessity of AUTS2 in brain development and neuronal differentiation. These animal models provide a detailed information about the function of AUTS2, starting from early stages, such as the differentiation from ESC to NPCs, to the later stages, including the axon projection and synapse formation. The results demonstrated the molecular mechanism of AUTS2 in neuronal differentiation via PRC1 complex, RNA metabolism, and Rac1 pathway regulation, which will be discussed in the next section.

AUTS2 in motor neuron differentiation

Besides the function of AUTS2 in CNS, one study unveiled the necessity of AUTS2 in the differentiation process from mESC to motor neurons (Liu et al. 2021). This study focused on the recruitment mechanism of AUTS2-containing PRC1 complex in neuron development. The ablation of Auts2 or deletion of HX repeat domain led to the misregulation of a subset of differentiation genes and compromised motor neuron development (Liu et al. 2021). The similar observation has been described in morpholino-mediated *auts2* knockout zebrafish model. Reporter zebrafish line, Tg(mnx1:GFP) displayed fewer motor neurons in the spinal cord and weaker motor neuron projections after injected with *auts2*-morpholino (Oksenberg et al. 2013a). However, it failed to recapitulate in current Auts2 mouse models (Gao et al. 2014; Hori et al. 2015; 2014; Castanza et al. 2021). More efforts are required to further understand the role of AUTS2 in motor neurons.

Molecular mechanisms of how AUTS2 regulates neuronal development

With the increasing evidence suggesting the importance of AUTS2 in brain development and differentiation, multiple studies brought insights into the molecular mechanism of how AUTS2 functions in this process (Gao et al. 2014; Q. Wang et al. 2018; Hori et al. 2014; Castanza et al. 2021). In our previous studies, we demonstrated that AUTS2 served as a component in PRC1 complex, which converted PRC1 from transcription repressor into transcription activator (Gao et al. 2014; Q. Wang et al. 2018; Gao et al. 2012). Recently, a proteomic analysis showed the potential interactions between AUTS2 and multiple RNA-binding proteins, suggesting AUTS2 might be involved in RNA metabolism. Besides its role in nuclei, AUTS2 was also discovered in cytosol, where AUTS2 mediated the activity of Rac1 signaling pathway for neuron migration and neurogenesis.

AUTS2 activates Rac1 pathway in cytosol

While AUTS2 is mainly enriched in nuclei, the immunohistochemistry assay showed the localization of AUTS2 in both nuclei and cytoplasm in the differentiated neurons (Hori et al. 2014) (**Figure1-3a**). To differentiate the cellular location of AUTS2 isoforms, the fractionation followed by immunoblotting was conducted and detected a substantial amount of AUTS2 long isoform in both cytosol and nuclei, while the AUTS2 short isoforms retain in the nuclei. To investigate the function of AUTS2, the AUTS2 long isoform was ectopically overexpressed in neuroblastoma cell line N1E-115, which resulted in the dramatic neurite outgrowth and lamellipodia. Similar observation has been reported in cells with constitutive active Rac1 (V12Rac1) (Nishimura et al. 2005). Overexpressing multiple truncated AUTS2 proteins in N1E-115 cell line showed that only the proteins containing PR1 region exhibit the similar phenotype

with cells overexpressing full length AUTS2, indicating that AUTS2 serves as an upstream activator of Rac1, via PR1 region.

During neural development, multiple protein complexes are involved in the regulation of Rac1 activity, including P-Rex1, STEF, Tiam1, and Elmo2/Dock180 (Yoshizawa et al. 2005; Buchsbaum 2007). Co-transfecting N1E-115 with AUTS2-FL with one of the dominant negative forms of these proteins, only P-Rex1 (ΔDH-P-Rex1), Elmo2 (Elmo2-ΔN), and Dock180 (Dock180-ISP) are capable to repress the phenotypes induced by the overexpression of AUTS2, suggesting these proteins may work in the same pathway with AUTS2. Moreover, the immunoprecipitation showed that Auts2 long isoform interacts with P-Rex1 and the Elmo2/Dock180 complex, which are known regulators of Rac1 activity (H. Katoh and Negishi 2003; Yoshizawa et al. 2005). The Auts2-Rac1 pathway mediated cell motility and morphogenesis by reorganization of the actin cytoskeleton. Taken together, cytosolic AUTS2 regulates neuronal migration and neuritogenesis through Rac1 pathway.

AUTS2 activates transcription as a core component in PRC1 complex

Mammalian PRC1 complexes are known to epigenetically regulate multiple biological processes, including embryonic development, stem cell self-renewal and differentiation, and carcinogenesis (Jaenisch and Young 2008; Margueron and Reinberg 2011; Sparmann and Van Lohuizen 2006; Simon and Kingston 2009; Gil and O'Loghlen 2014; J. Li et al. 2018). The composition of PRC1 is hierarchical, which contains RING1A/B as enzymatic core, one of the six Polycomb group RING finger proteins (PCGF1/2/3/4/5/6; homologous to Drosophila Psc) and other associated proteins. According to the type of PCGF proteins, PRC1 can be further categorized into 6 sub-types, named PRC1.1 – PRC1.6. A mass spectrum assay, which was designed to dissect the components of Polycomb Repressive Complex 1 (PRC1), discovered

AUTS2 as a core component in PRC1.3/5. The same complex also contains AUTS2 homologs, FBRS, FBRSL1, and other proteins, including WDR68 and CK2 (Gao et al. 2012). To test the difference of AUTS2 isoforms, yeast two hybrid (YTH) screen demonstrates that only the long isoform, but not the short isoforms is capable of interacting with PRC1 complex (Monderer-Rothkoff et al. 2021).

The canonical function of PRC1 complex on transcription is repressive, which is achieved through multiple levels. As the enzymatic core of all mammalian PRC1 complexes, E3 ubiquitin ligase RING1A/B encodes the catalytic activity for H2AK119ub1 deposition (H. Wang et al. 2004; de Napoles et al. 2004), which provides a general mechanism for PRC1-mediated transcriptional repression. PRC1 also can repress gene expression through H2AK119ub1-independent chromatin compaction (Shao et al. 1999; Francis, Kingston, and Woodcock 2004; Kundu et al. 2017; Eskeland et al. 2010). The known mechanism of AUTS2-containing PRC1 complex in transcription activation is achieved via the inhibition of Ring1a/b E3 enzymatic activity and the recruitment of transcription activator (Q. Wang et al. 2018; Gao et al. 2014; Liu et al. 2021). In contrast to canonical PRC1 complexes, AUTS2-containing PRC1 complex tends to localize to genomic loci lacking H2AK119ub1, indicating compromised mono-ubiquitination activity. In fact, the stable association of CK2 leads to the neutralization of RING1B enzymatic activity by phosphorylating RING1B at serine 168 (**Figure1-3b**) (Gao et al. 2014). In addition to the inhibition of the intrinsic inhibitory activity of PRC1, the presence of AUTS2 in the PRC1.5 complex not only recruits CK2 but interacts with P300, a histone acetyltransferase and transcriptional co-activator (Gao et al. 2014; W. Zhao et al. 2017). Knockdown or pharmacological inhibition of P300 compromises the PRC1.5-mediated induction of a luciferase reporter (Gao et al. 2014), suggesting an important role for P300 in the transcriptional activity of this complex. The involvement of P300 with PRC1.5 and PRC1.3, which share the same complex composition, in activating gene transcription has been suggested in later studies (Gao et al. 2012;

2014; W. Zhao et al. 2017; Monderer-Rothkoff et al. 2021). WDR68, an essential interacting protein of AUTS2 as well as a PRC1.3/5 component, also contributes to AUTS2-PRC1 mediated transcription activation (Q. Wang et al. 2018). In addition, a recently study suggested nuclear respiratory factor 1 (NRF1) serves as a recruiter of AUTS2-PRC1.3 complex to a collection of neurodevelopmental genes in mouse brain and during the differentiation process from mESC to motor neurons (Liu et al. 2021).

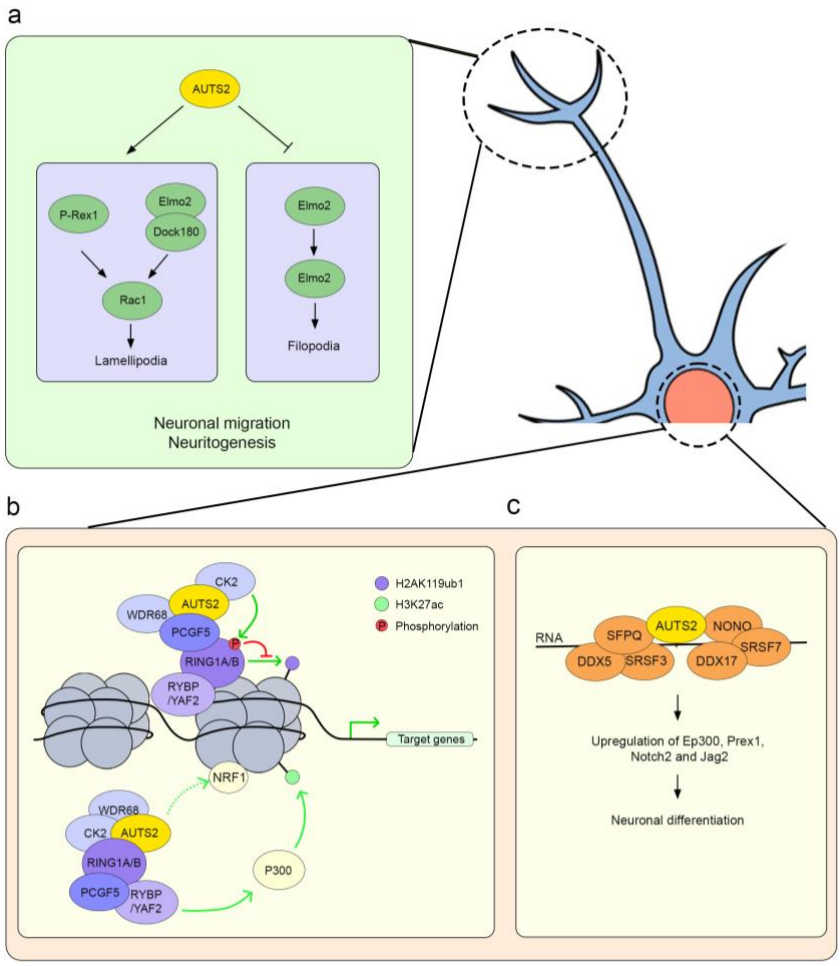

Figure 1-3: Molecular mechanism of AUTS2 in regulating neuronal differentiation

a, The mechanism of cytosolic AUTS2 as a positive regulator of Rac1/Elmo2 pathways. **b,** The molecular mechanism of nuclear AUTS2 as a component in PRC1 complex and how AUTS2-containing complex activates transcription. **c,** The potential role of AUTS2 in RNA metabolism.

AUTS2 regulates RNA metabolism

The protein sequence conservation algorithms predicted two conservative domains, in mouse AUTS2, the "AUTS2 domain" and PAT1 domain. Human AUTS2 also carries the "AUTS2 domain", which is also found in AUTS2-homologs, such as FBRS and FBRSL1 (Castanza et al. 2021). However, PAT1 domain, which is known to interact with RNA, is not found in human AUTS2. Instead, five other conservative domains are discovered in human AUTS2, in which four of them are predicted to be responsible for protein-RNA/DNA interaction. The immunoprecipitation followed by mass spectrometry in neonatal mouse cerebral cortex identified multiple novel AUTS2-interacting proteins, including 8 RNA-binding proteins (RBPs) among top 20 AUTS2-IPs (**Figure1-3c**). The interaction between AUTS2 and one of RBPs, NONO was validated by co-immunoprecipitation and immunofluorescence. To further investigate the molecular mechanism of how AUTS2-containing complexes regulate RNA, RNA immunoprecipitation and sequencing (RIP-seq) was conducted in neonatal cortex. AUTS2 occupancy was highly abundant in 1208 transcripts, which are functionally enriched in neurodevelopment processes, RNA metabolism and chromatin regulation. Comparing between conditional AUTS2 knockout mice with WT, neuronal differentiation defects were observed in dentate gyrus (DG). Together, this study provides insights in a new function of AUTS2 as a core component of RNA-binding complex, and its role in RNA metabolism to ensure brain development and neuronal differentiation (Castanza et al. 2021).

Chapter 2

Methods and Materials

Cell culturing and genetic editing

Mouse embryonic stem cell culturing

E14 mouse ES cells or their derived CRISPR-engineered cells were cultured in DMEM medium, supplemented with 15% FBS (ES certified, Atlanta Biologicals, Cat# S10250), LIF, non-essential amino acids, β-mercaptoethanol, L-glutamine, penicillin/streptomycin, sodium pyruvate, and two small-molecule kinase (MEK and GSK3) inhibitors (PD0325901, Cayman Chemical, Cat# 13034 and CHIR99021, Cayman Chemical, Cat# 13122). E14 cells were either cultured on MEF feeder cells or gelatin-coated plates.

NPC differentiation from mouse embryonic stem cell

mESC differentiation was performed as previously described (Hanafiah et al. 2020). mESCs cultured on MEF feeders were transferred onto gelatin-coated plates for two passages before differentiation. Embryonic bodies (EB) were formed by culturing cells in differentiation medium (DMEM medium, 15% FBS, non-essential amino acids, β-mercaptoethanol, L-glutamine, penicillin/streptomycin, sodium pyruvate) in ultra-low attachment plates. After four days, retinoic acid was added to the medium at 5 μM for four more days to generate neuronal progenitor cells (NPC).

Human embryonic stem cell maintenance and cerebral organoid differentiation

Stem Cell Technologies Cerebral Organoid kit (catalogue n. 08570, 08571) reagents were used, and the generation of brain organoids was performed according to the manufacturer's protocol. At day 0, EBs were generated using 9,000 hESCs per well in 96-well U-bottom suspension plates with EB Formation media supplemented with 5mM Y-27632 ROCK inhibitor. Fresh EB Formation media was added every 2 days. On day 5, EBs were transferred to 24-well ultra-low attachment plate with Induction media. After two days, EBs were embedded in 15µL Matrigel and cultured in 6-well ultra-low adherent plate with Expansion medium. On day 10, the medium was replaced with Maturation media and moved to an incubator with a shaker. The organoids were fed every 3-4 days with maturation media.

HEK293 cell culturing and plasmid transfection

HEK293 T-REx cells expressing FLAG-HA-AUTS2-S were generated by transfection with pINTO-NFH-AUTS2-S, followed by Zeocin selection. These cells and normal HEK293T cells were maintained in a standard DMEM medium containing 10% FBS (Atlanta Biologicals, Cat# S11050), L-glutamine, and penicillin/streptomycin.

CRISPR/Cas9 for gene editing

For both knock-out and knock-in experiments, sgRNA was designed with CRISPR Design (Hsu et al. 2013), targeting the regions of interests. Sequence of sgRNA can be found in Table 2-2. Oligos corresponding to candidate sgRNA sequences were purchased from IDT and cloned into pX458 as previously described (Cao, Tsukada, and Zhang 2005). Cells were transfected with the plasmids carrying sgRNA. Two days later, transfected cells were sorted

based on cell size and fluorescence signal into 96-well plate. Single cell clones were selected and genotyped with PCR. Positive clones were further confirmed using immunoblotting.

Cell-based assays

Alkaline phosphatase assay

Alkaline phosphatase (AP) staining was performed using a Stemgent AP staining kit II (ReproCELL, cat# 00-0055). Cells were fixed with fixation solution for 5 minutes at room temperature. After washing twice with PBS, the cells were incubated for 10 minutes in the dark with AP substrate solution. The reaction was terminated by removing the solution; the cells were then washed twice and covered with PBS for imaging.

MTT proliferation assay

Cell proliferation was assayed according to the manufacturer manual (Vybrant® MTT Cell Proliferation Assay Kit, Invitrogen cat# M6494)). Briefly, cells were seeded in gelatin-coated 96-well plates with the same density in replicates. Absorbance at 540 nm wavelength was determined on a microplate reader (Bio-Rad).

BMP treatment

Before treatment, cells were incubated in a serum-free starvation medium (1% BSA in DMEM) for 4 hours. BMP4 (R & R&D Systems, cat#314-BP-010) was then added with a 5 ng/ml final concentration. Cells were collected at different time points after BMP4 treatment and

subjected to immunoblotting. The image was quantified by densitometry using Image J. The mean grey value of a selected region that contained an individual band was measured and subtracted by the background.

Lentiviral infection

Lentivirus was generated in HEK293T cells through co-transfection of psPAX2, pMD136, and pLV-EF1a-IRES-Blast plasmid carrying genes of interest, using lipofectamine 2000 (Invitrogen, 11668019). Media containing lentivirus was collected, centrifuged at 4,000 g/4 °C for 15 minutes, and the resulting supernatant was concentrated on a spin column (EMD Millipore Corp, UFC910008). Concentrated lentivirus was aliquoted, fast-frozen in liquid nitrogen, and stored at -80 °C. To infect mESCs with lentivirus, 300 μl condensed lentivirus suspension was added to 6 ml ES media with 48 μg polybrene (EMD Millipore Corp, TR-1002-G). After 6 hours of incubation, media was removed and the cells were incubated overnight with a mixture of 10 ml media, 500 μl lentivirus, and 80 mg polybrene. The next day, infected mESCs were ready for further experiments.

Biochemical assays

Immunofluorescence staining

Differentiated EBs were fixed with 4% paraformaldehyde at room temperature for 20 min, followed by triple washing in PBS. Human brain organoids were fixed with 4% paraformaldehyde at room temperature for 30 min. Tissues were stocked in 10%, 20% PBS-buffered sucrose solutions at room temperature until they sink (3-6 hours). Tissues were then

placed in 30% PBS-buffered sucrose solutions overnight at 4 °C. EBs and cerebral organoids were embedded in O.C.T compound (Sakura) and snap frozen with liquid nitrogen. The O.C.T blocks were cryosectioned at 10 or 20 μM thickness.

For immunofluorescence imaging, sections were blocked and permeabilized in 0.5% Triton X-100 and 5% BSA in PBS. Sections were then incubated with primary antibody in 0.1% Triton X-100 and 1% BSA in PBS for 2 hours at room temperature (Table 2-3). After washing 3 times in PBS, sections were incubated with Alexa Fluor 488 anti-mouse antibody (Gibco) for 1 hour at room temperature. Slides were washed 3 times, stained with DAPI, and mounted for imaging. Information for antibodies can be found in Table 1.

For image quantification, the fluorescence images were loaded and converted to greyscale in Image J. EB sections were selected by using drawing tools. The integrated fluorescence intensity of sections was measured by Image J. For each image, three random regions outside EB sections were measured and averaged and used as background fluorescence intensity. The corrected fluorescence intensity of each EB was equal to the integrated intensity subtracted by background intensity.

Luciferase reporter assay

To evaluate the effect of AUTS2 on the WNT signaling, M50 Super 8x TOPFlash and pLRL-SV40 Renilla luciferase (Promega) were co-transfected into HEK293T wild type cells or HEK293T Auts2 KO cells using Lipofectamine 2000. A TOPFlash mutant, M51 Super 8x FOPFlash was used as a negative control. After 24 hours of transfection, the cells were treated with 10 μM CHIR99021. The cells were harvested after 18-20 hours of treatment and assayed with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocols.

Quantitative RT-PCR

Total RNA was extracted with TriPure reagent (Roche, Cat# 11667165001) and used to synthesize cDNA with the SuperScript III system (Invitrogen, Cat# 18080-044). The resulting cDNA was mixed with Brilliant III Ultra-Fast SYBR QPCR master mix (Agilent Cat#600883) and primers, then run on a Biorad CFX Connect real-time PCR detection system. Primers for RT-qPCR in this study can be found in Table 2-1.

Cell lysis and immunoprecipitation

Transfected cell pellet was suspended in extraction buffer (1 volume of Buffer A and 1 volume of Buffer N, supplemented with 0.1% IGEPAL, 50mM KCl, 0.2 mM PMSF, 1 μg/ml Pepstatin A, 1 μg/ml Leupeptin and 1 μg/ml Aprotinin), incubated on ice for 15 min, and then homogenized by a 2 mL dounce homogenizer for 15 strokes, using the loose pestle. After 30 min of incubation on a rotating mixer at 4 °C, the samples were centrifuged at 40, 000 x g at 4 °C for 15 min. The supernatant was collected and incubated with prewashed M2 beads. After rotation at 4 °C overnight, the M2 beads were spun down at 2000 x g at 4 °C for 2 min. The beads were washed with Buffer W (1/3 volume of Buffer A, 2/3 volume of Buffer C, 0.02% IGEPAL, 0.2 mM PMSF, 1 μg/ml Pepstatin A, 1 μg/ml Leupeptin and 1 μg/ml Aprotinin) for 5 times, and then eluted with 40 μL FLAG peptides of 250 μg/ml in Buffer W by rotating at 4 °C for 2 hours. The eluates were mixed with SDS sample buffer and analyzed by SDS-PAGE, followed by immunoblotting.

Affinity purification

Affinity purification in ESCs or NPCs was performed in triplicates previously described (Gao et al., 2012). Approximately 10^8 Cells were washed with PBS and then suspended in 10 ml Buffer A (10 mM Tris-HCl, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 1 μg/ml Pepstatin A, 1 μg/ml Leupeptin, 1 μg/ml Aprotinin), incubated on ice for 30 min, and then homogenized by a 40 ml dounce homogenizer for 10 strokes, using the loose pestle. The suspension was then subjected to 15, 000 x g centrifugation at 4 °C for 10 min. The resulting nuclear pellet was resuspended in 10 ml Buffer C (20 mM Tris-HCl, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 μg/ml Pepstatin A, 1 μg/ml Leupeptin, 1 μg/ml Aprotinin), and subjected to another 10 strokes by the dounce homogenizer. After rotation at 4 °C for 30 min, the suspension was centrifuged at 40, 000 x g at 4 °C for 30 min, and the supernatant was nuclear extract (NE). 8 ml NE was mixed with 2 ml Buffer A, 0.02% NP-40, and 200 μl pre-washed FLAG M2 beads. After rotation at 4 °C overnight, the M2 beads were spun down at 2000 x g at 4 °C for 10 min. The beads were washed with Buffer W (2/3 volume of Buffer C, 1/3 volume of Buffer A, NP-40 0.02%) 5 times and then eluted with 500 μl FLAG peptides of 250 μg/ml in Buffer W by rotating at 4 °C for 1 hour. The M2 eluate was then incubated with 30 μl HA beads at 4 °C for 4 hours. The HA beads were washed with Buffer W 5 times and eluted by 100 μl glycine (0.1 M, pH 2.0), and then neutralized by adding 6.5 μl Tris solution (1.5 M, pH 8.8), resulting in the final HA eluate.

Quantitative proteomic analysis

For quantitative proteomic analysis, the TAP enriched proteins were digested with modified MS-grade trypsin (Thermo Pierce) at an enzyme/substrate ratio of 1:100 in 50 mM

NH4HCO3 (pH 8.5) at 37 °C for overnight. The resulting peptide samples were then loaded at 3 µL/min onto a precolumn (150 µm i.d.) comprised of a 3.5-cm column packed with 5 µm C18 120 Å reversed-phase material (ReproSil-Pur 120 C18-AQ, Dr. Maisch), for LC-MS/MS analysis. The trapping column was connected to a 20-cm fused silica analytical column (PicoTip Emitter, New Objective, 75 µm i.d.) with 3 µm C18 beads (ReproSil-Pur 120 C18-AQ, Dr. Maisch). The peptides were then separated using a 180-min linear gradient of 2-45% acetonitrile in 0.1% formic acid and at a flow rate of 250 nL/min. The mass spectrometer was operated in a data-dependent scan mode. Full-scan mass spectra were acquired in the range of m/z 350-1500 using the Orbitrap analyzer with a resolution of 70,000. Up to 25 most abundant ions found in MS with a charge state of 2 or above were sequentially isolated and collisionally activated in the HCD cell with a collision energy of 27 to yield MS/MS.

Maxquant (Cox and Mann, 2008), Version 1.5.2.8, was used to analyze the LC-MS and MS/MS data for the identification and quantification of proteins in the LFQ mode. The maximum number of miss-cleavages for trypsin was two per peptide. Cysteine carbamidomethylation was set as a fixed modification. Methionine oxidation and phosphorylation on serine, threonine, and tyrosine were set as variable modifications. The tolerances in mass accuracy for MS and MS/MS were both 20 ppm. Maximum false discovery rates (FDRs) were set to 0.01 at both peptide and protein levels, and the minimum required peptide length was six amino acids.

Glycerol gradient analysis

Glycerol analysis on affinity-purified AUTS2 was conducted as previously described with certain modifications (Gao et al. 2014). Briefly, 12 ml nuclear extract (NE) from HEK293T-REx cells expressing FLAG-HA-AUTS2-S were incubated with 300 µl pre-washed FLAG M2 beads (Sigma, cat# A2220) and 3 ml Buffer A (10 mM Tris-HCl, pH 7.9, 1.5 mM MgCl2, 10 mM

KCl, 0.5 mM DTT, 0.2 mM PMSF, 1 μg/ml Pepstatin A, 1 μg/ml Leupeptin, 1 μg/ml Aprotinin), in the presence of 0.02% NP-40. After an overnight rotation at 4 °C, the M2 beads were washed once with Buffer W (2/3 volume of Buffer C (20 mM Tris-HCl, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 μg/ml Pepstatin A, 1 μg/ml Leupeptin, 1 μg/ml Aprotinin), 5 times in 1/3 volume of Buffer A, NP-40 0.02%) , then eluted with 600 μl FLAG peptides of 250 μg/ml in Buffer W by rotating at 4 °C for 1 hour. Subsequently, 500 μl M2 eluate was added to the top of a 12 ml 15 - 35 % glycerol gradient and centrifuged in an SW40Ti rotor (Beckman) at 40, 000 RPM at 4 °C for 22 hours. The resulting gradient was fractionated every 500 μl and then analyzed by immunoblotting.

***In vivo* poly-ubiquitination assay**

Examination of in vivo poly-ubiquitination of SMAD1 was performed as described previously (Abbas et al. 2008). Briefly, transfected cells with or without treatment were harvested directly in the buffer containing 1% SDS, 1mM EDTA, 2mM Na_3VO_4 in PBS. After 5 minutes of boiling, samples were passed through 26G needles 3 times and boiled for another 3 minutes. Next, the lysates were chilled and centrifuged, and the resulting supernatants were then mixed with equal volumes of Immunomix buffer (1% TX100, 1% SDS, 0.5% Deoxycholic acid, 1% BSA, 1mM EDTA, 2mM Na3VO4 in PBS) and subjected to immunoprecipitation with Anti-FLAG M2 beads, followed by SDS-PAGE and immunoblotting.

Next-generation sequencing and bioinformatics analysis

RNA-seq and analysis

RNA-seq was performed and analyzed as previously described with modifications (Q. Wang et al. 2018). Briefly, cDNA libraries were prepared using the NEXTflex™ Illumina Rapid Directional RNA-Seq Library Prep Kit (BioO Scientific) as per the manufacturer's instructions. Libraries were loaded onto a TruSeq Rapid flow cell on an Illumina HiSeq 2500 (located at the Genomic Sciences Facility at the Penn State University College of Medicine). These cells were run for 50 cycles using either a single-read or pair-end recipe according to the manufacturer's instructions. The gene expression abundance is accessed using Kallisto (raw read counts and relative gene expression/TPM) or TopHat/Cufflinks (relative gene expression/FPKM) against mm10 transcriptome reference for mESC and its derived NPC RNA-seq, and Genome Research Consortium human build 38 (GRCh38/hg38) for human cerebral organoids.

The matrixes containing raw read counts were imported into RStudio and differentially expressed genes (DEG) analysis was executed using DESeq2. DEGs were identified with cutoff of the adjusted p-value smaller than 0.5 and exact value of log2 fold change greater than 1. The z-scores of gene expressed matrix were used for heatmap plotting. The differentially expressed genes were sent for Gene Ontology (GO) analysis, which was performed using DAVID (G. Dennis et al. 2003) or R package ClusterProfiler. GO terms with lower than 0.1 adjusted p-value was selected and GO results were plotted following the rank of gene ratio for each GO terms.

Pathway analysis was performed using a web-based bioinformatics application, Ingenuity Pathway Analysis (IPA). The results of DEG analysis, containing gene name, fold change, p-value and adjusted p-value were imported into IPA. The pathway analysis was conducted using

default parameters. Results of canonical pathways were exported for downstream analysis and plotting.

Single-cell RNA-seq and analysis

Cerebral organoids were collected at day 75. Then, organoids were incubated with Papain (Worthington) at 37 °C for 30 minutes to isolate into single cells. The suspended cells were loaded onto a Fluidigm C1TM Single-Cel l Autoprep system (C1) for automatic capture and downstream processing for sequencing-ready library preparation. The DNA libraries were sequenced using Illumina NovaSeq 6000, followed by demultiplexing and trimming of the sequencing readouts.

Bowtie was used to perform alignment against Genome Research Consortium human build 38 (GRCh38/hg38) and HTseq was applied to acquire the gene abundance matrix for each cell. The raw count matrix was then imported into R. Seurat R package was used to first filtered out low/non-express gene across all cells and cells with low sequencing quality. Then, gene raw count matrix was normalized, and high variable features was identified. Linear dimensional reduction was carried using PCA, based on the identified variable features and JackStraw plot was used to determine the dimensionality. Based on the results of JackStraw plot, cells were clustered using 1:12 dimensions and resolution of 0.3. Clustered cells were visualized using tSNE. Marker genes for each cluster was identified using FindallMark function with default parameter. After identifying the exclusively high expression genes in each cluster, cell identity was determined based on the cell markers from literature and public data base (Panglao).

Trajectory analysis was performed using R package monocle3. First, gene raw count matrix was imported to monocle3 and filtered out the low expression gene and cells with low sequencing quality, using same criterial as Seurat. Dimension reduction and visualization was

performed using the program default method, UMAP. Crossing the clustering results and cell identity we acquired from Seurat, we set neuro-epithelial cells as the root for pseudo-time analysis.

Table 2-1: List of RT-qPCR primers.

Name	Sequence (F/R)
mOct4	5' - AGATCACTCACATCGCCAATCA- 3' / 5' -CGCCGGTTACAGAACCATACTC- 3'
mSox9	5' - AGCGAACGCACATCAAGA- 3' / 5' -CTGTAGTGAGGAAGGTTGAAGG- 3'
mNanog	5' - AGGCTTTGGAGACAGTGAGGTG- 3' / 5' -TGGGTAAGGGTGTTCAAGCACT- 3'
mNes	5' - AGTGCCCAGTTCTAGTGGTGTCC- 3' / 5' -CCTCTAAAATAGAGTGGTGAGGGTTG- 3'
mTbx2	5' - ATGTACATCCACCCGGACAG- 3' / 5' -GACAGCGATGAAGTCGGTCT- 3'
mDpysl2	5' - CAGAATGGTGATTCCCGGAGG- 3' / 5' -CAGCCAATAGGCTCGTCCC- 3'
mSnai1	5' - CCGATGAGGACAGTGGCAAA- 3' / 5' -CCCAGGCTGAGGTACTCCTT- 3'
mNeurod1	5' - CGAGTCATGAGTGCCCAGCTTA- 3' / 5' -CCGGGAATAGTGAAACTGACGTG- 3'
mId2	5' - CTACTCCAAGCTCAAGGAACTG- 3' / 5' -GATCTGCAGGTCCAAGATGTAA- 3'
mPax6	5' - CTTGGGAAATCCGAGACAGA- 3' / 5' -CTAGCCAGGTTGCGAAGAAC- 3'
m18SrRNA	5' - GCAATTATTCCCCATGAACG- 3' / 5' -GGCCTCACTAAACCATCCAA- 3'
mTubb2a	5' - GGAGGTGATAAGCGATGAGCATG- 3' / 5' -GGCTCCAGGTCCACTAGGATG- 3'

mHoxa6	5' - GTGACCCTACTGCCATCTTAC- 3' / 5' -GAATAATCACCGCAGGACTCT- 3'
mWwc2	5' - TCATCTGGGAGCAGTCTAGGG- 3' / 5' -TGATAGTCTGTGTCCATCTGGTC- 3'
mHand1	5' - TCGCTACACTTCCTACCTAGAG- 3' / 5' -GAAGGGAAAGGAAGGGAAAGAT- 3'
mTubb3	5' - TGAGGCCTCCTCTCACAAGT- 3' / 5' -GGCCTGAATAGGTGTCCAAA- 3'
mGata6	5' - TGCAGGATTGCATCATGACAGA- 3' / 5' -TGACCTCAGATCAGCCACGTT- 3'
mVglut1	5' - TGCTACCTCACAGGAGAATGGA- 3' / 5' -GCGCACCTTCTTGCACAAAT- 3'
mGap43	5' - TGTGCCTGCTGCTGTCACTGAT- 3' / 5' -AGGTTTGGCTTCGTCTACAGCG- 3'
mGata4	5' - TTCCTCTCCCAGGAACATCAAA- 3' / 5' -GCTGCACAACTGGGCTCTACTT- 3'
mSox1	5'-GGCCGAGTGGAAGGTCAT-3'/ 5'-ACTTGTAATCCGGGTGTTCCT-3'
mNkx2-5	5’-GATGGGAAAGCTCCCACTATG-3’/ 5’-GACACCAGGCTACGTCAATAAA-3’
mBrachyury/T	5‘-TGCACATTACACACCACTGACG-3’/ 5’-AGAACCAGAAGACGAGGACGT-5’

Table 2-2: sgRNA information for CRISPR/Cas9 gene editing.

Cell Line	Oligo Name	Sequence (5' - 3')
mAuts2-/-	mAuts2-KO-1	AGGTGCTGGCGTCGGCATGATGG
	mAuts2-KO-2	AATGCTGGGGCGCATCCCGTAGG
mAuts2-L-/-	mAuts2-L-KO-1	GCGTATATTCCCTAAACTATGGG
	mAuts2-L-KO-2	CCACAGGGTAGGGTTACCATTGG
mAuts2-S-/-	mAuts2-S-KO-1	GTCTGACATGGATGGGAGGTTGG
	mAuts2-S-KO-2	GAATGGTCACTAGCAAGCCTCGG
Ski-/-	mSki-KO-2	GGGCGGCCCGGCCGCTTTCTCGG
	mSki-KO-3	AGTCGCGCAGCACCGAGTTGAGG
NFH-mWdr68	mWdr68-KI	TCTACAAATATGAAGCGCCCTGG
HEK-hAUTS2-/-	HEK-hAUTS2-1	TGGTCTCGTCTGCTTCATTG
	HEK-hAUTS2-2	TTTTGAGGGCAGATGGAATC

Table 2-3: Antibodies used in immunoblotting, immunoprecipitation, and immune-fluorescence staining.

Antibody	Company	Category	Lot #	Application
ACTIN	Abcam	ab8277		WB
AUTS2	Gao et al., 2014			WB, IF
AUTS2	Sigma	HPA000390		WB
CLIC6	Abcam	ab204567		WB
DDB1	SANTA CRUZ BIOTECHNOLOGY	sc376860	A2318	WB
FLAG	Sigma	F3165		WB
FLAG beads	Sigma	A2220		IP
Gapdh	Thermo Fisher Scientific	MA5-15738	GA1R	WB
H3	Abcam	ab1791		WB
HA	Covance	MMS-101P		WB
HA	Abcam	ab9110	GR98618-3	WB
HA beads	Sigma	A2095		IP
NANOG	Invitrogen	PA1-41577		WB
Nestin	BD	611658		WB, IF
OCT3/4	SANTA CRUZ BIOTECHNOLOGY	sc365509	L1817	WB
PCGF3	SANTA CRUZ BIOTECHNOLOGY	sc133892		WB
PCGF3/5	Abcam	ab201510		WB
PCGF5	Abcam	ab201511		WB
pSMAD1/5/9	Cell Signaling	13820S	lot: 3	WB
pSMAD2	Cell Signaling	3108T	lot: 8	WB
RING1B	BETHYL	A302-869A	A302-869A-1	WB
SKI	SANTA CRUZ BIOTECHNOLOGY	sc33693		WB
SMAD1	Cell Signaling	6944T	lot: 5	WB
SMAD2	Cell Signaling	5339T	lot: 4	WB
SMAD4	Cell Signaling	38454T	lot: 1	WB
SMAD5	Cell Signaling	12534T	lot: 2	WB
TCF3	Cell Signaling	2883S		WB
TLE1/2/3/4	Cell Signaling	4681S		WB
TTR	Abcam	ab9015		WB, IF
WDR68	Sigma	HPA022948	A76005	WB
β-Tubulin	Abcam	ab6046		WB

Chapter 3

AUTS2 controls neuronal lineage choice through a novel PRC1-independent complex and BMP inhibition

Introduction

Neurodevelopmental disorders (NDD) result from compromised development of the central nervous system and encompass but are not limited to autism spectrum disorders (ASD), Developmental Delay (DD), and Intellectual Disabilities (ID). *Autism Susceptibility Candidate 2* (*AUTS2*) has been identified as one of the genes that are most frequently disrupted by balanced chromosome abnormalities (BCAs) in neurodevelopmental disorders (Talkowski et al. 2012). Although genomic aberrations of *AUTS2* were first identified in zygotic twins with autism, it has been repeatedly found in patients diagnosed with various NDD (Beunders et al. 2016; Oksenberg and Ahituv 2013). Disruption of *AUTS2* leads to common neurodevelopmental abnormalities, including microcephaly, developmental delay, and varying degrees of intellectual disability (Oksenberg and Ahituv 2013). The importance of *AUTS2* in the developing central nervous system (CNS) has been studied in animal models. In zebrafish, the deletion of *auts2* has been shown to affect neurogenesis and craniofacial development (Beunders et al. 2016; Oksenberg et al. 2013b). Mouse models further reveal the relationship between *AUTS2* loss of function and subsequent defects in CNS development and aberrant behaviors (Gao et al. 2014; Hori et al. 2014; 2015). Furthermore, using mouse and human embryonic stem cells (ESC), it has been shown that deleting *Auts2* leads to differentiation defects in neuronal lineage *in vitro* (Monderer-Rothkoff et al. 2021; Russo et al. 2018). However, it remains unclear how *AUTS2* regulates neuronal differentiation at the molecular level.

Expression of AUTS2 in the CNS peaks during early embryogenesis and declines after birth (Bedogni, Hodge, Nelson, et al. 2010; Gao et al. 2014). AUTS2 exists predominantly in the nucleus (Bedogni, Hodge, Nelson, et al. 2010; Gao et al. 2014), but one study suggests that its functions depend on its cytosolic expression (Hori et al. 2014). Our previous studies have shown that nuclear AUTS2 served as a core component in a type 1 Polycomb repressive complex (PRC1-AUTS2) (Gao et al. 2012) and converts PRC1 from a transcriptional repressor to an activator (Gao et al. 2014). Subsequent studies revealed additional factors, such as WDR 68 and NRF1, that are functionally important for the PRC1-AUTS2 complex (Q. Wang et al. 2018; Liu et al. 2021). On the other hand, cytosolic AUTS2 seems essential for neuronal migration and neuritogenesis in the mouse cortex through the activation of Rac1 (Hori et al. 2014). To date, two major isoforms of AUTS2 have been identified, but their functional distinctions are not well understood (Monderer-Rothkoff et al. 2021).

It has been shown recently that deletion of Pcgf5, which is the core component in the PRC1-AUTS2 complex, causes abnormal TGF-β signaling activation and a defect in mouse ESC neuronal differentiation (Yao et al. 2018). However, whether or not AUTS2 directly regulates TGF-β/BMP pathways during neuronal differentiation remains unknown. TGF-β signaling plays essential roles in cell fate determination (Massagué 2012). The differentiation of ESCs into neuroectodermal lineage requires the absence of TGF-β and its family members, bone morphogenetic proteins (BMPs), while mesoderm and endoderm differentiation rely on these signaling pathways (Watabe and Miyazono 2009). Furthermore, the inhibition of TGF-β and BMP signaling greatly enhances the efficiency of neuronal differentiation from ESCs *in vitro* (Chambers et al. 2009). The activation of the TGF-β/BMP pathway leads to the phosphorylation of regulatory SMAD (R-SMAD) proteins, among which SAMD1/5/9 responds to BMP and SMAD2/3 to TGF-

β. The phosphorylated R-SMADs translocate to the nucleus in association with SMAD4 (co-SMAD) and elicit specific transcriptional effects (Massagué 2012).

In the present study, we discovered a novel mechanism for Auts2 to control neuronal differentiation. Using CRISPR/Cas9-mediated gene editing, we generated mouse ESCs that lacked either long, short, or both isoforms of Auts2 and demonstrated that complete silencing of both Auts2 forms leads to dramatic defects in neuronal differentiation. Interestingly, this differentiation defect in Auts2-deficient cells is accompanied by an up-regulation of BMP signaling, a critical pathway involved in cellular differentiation. Mechanistically, with quantitative mass spectrometry analysis, we identified a novel protein complex comprised of AUTS2, WDR68, and SKI, which is specifically formed in neuronal progenitors and mediates the inhibition of BMP signaling during differentiation. The impact of *Auts2* on neuronal gene transcription and BMP signaling was recapitulated in cortical neurons with *Auts2* deletion. Further biochemical characterization revealed the involvement of the CUL4 E3 complex as the mediator, which promotes proteasomal degradation of BMP-specific regulatory SMAD1/5/9, thereby restricting the activation of BMP signaling during neuronal differentiation. Together, our findings provide insight into the PRC1-independent function of nucleic AUTS2 during neuronal differentiation by repressing BMP signaling pathway.

Results

Auts2 is required for neuronal differentiation in mouse embryonic stem cells

To explore how Auts2 regulates neuronal differentiation, we used an in vitro ESC neuronal differentiation model as previously described (Bibel et al. 2004). E14 cells were used to form embryoid bodies (EB), which were then induced by retinoic acid to differentiate into neuronal progenitor cells (NPC). The success of our differentiation was evidenced by the reduction of Oct4 and Nanog protein levels, two pluripotent markers, and the increase of Pax6 and Nestin, two NPC markers, during differentiation (Fig. 3-1a). Interestingly, even though protein levels remained constant in most of the tested PRC1-AUTS2 complex components, including Pcgf3, Pcgf5, Ring1b, and Wdr68, both the long and short isoforms of Auts2 (Auts2-L and Auts2-S) were increased in NPCs (Fig. 3-1a). These Auts2 isoforms have been previously observed by others (Beunders et al. 2013; Hori et al. 2014). Auts2-S is derived from an alternative transcription start site and shares the same reading frame with Auts2-L (Fig. 3-2a) (Beunders et al. 2013). Consistent with Monderer-Rothkoff's recent study, through immunoprecipitation (IP) experiments in HEK293 cells, we discovered that, unlike AUTS2-L, AUTS2-S does not associate with RING1B and PCGF5, the core components of the PRC1-AUTS2 complex (Fig. 3-1b) (Monderer-Rothkoff et al. 2021). However, both isoforms interact with WDR68 (Fig. 3-1b). Interestingly, patients with disruptions of the C-terminal region of the AUTS2 locus tend to have more severe phenotypes than those with N-terminal disruptions (Beunders et al. 2013), indicating a PRC1-independent function carried out by the C-terminal domain of Auts2-S or/and Auts2-L.

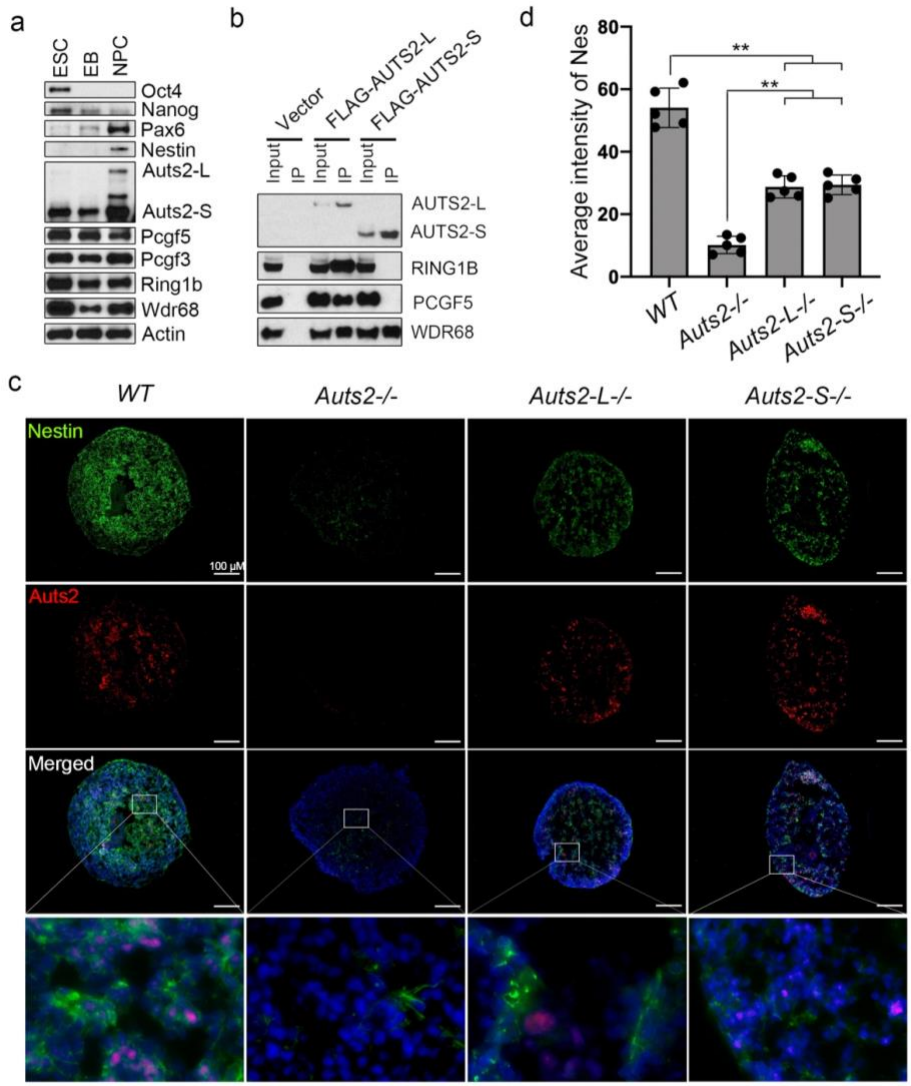

Figure 3-1: Auts2 is required for neuronal differentiation of mouse embryonic stem cells.

a, Immunoblotting of wild-type (WT) mouse embryonic stem cells (ESC), embryoid bodies (EB), and neuronal progenitor cells (NPC), using antibodies as indicated. **b,** Immunoprecipitation (IP) with FLAG antibody-conjugated M2 beads from nuclear extract (NE) of HEK293T cells transfected with N-terminal FLAG and HA-tagged AUTS2 long-form (NFH-AUTS2-L), short-form (NFH-AUTS2-S), or control plasmid (Vector). Bound proteins were resolved on SDS-PAGE and detected by immunoblotting for the antigens indicated. 5% of input was loaded in all cases unless otherwise indicated. **c,** Immunofluorescence staining of Nestin (Green) and Auts2 (Red) in mouse ESCs differentiated at NPC stage. DAPI is in Blue. The bottom panel shows the blowup of the box region as indicated. Auts2-/-, complete Auts2 knockout; Auts2-L-/-, Auts2-L specific knockout; Auts2-S-/-, Auts2-S specific knockout. **d,** Quantification of average Nestin immunofluorescence intensity by Image J. Each value is the mean of three independent measurements with error bars representing standard error. ** indicates $p<0.01$ by two-sided t-test.

To understand how these Auts2 isoforms affect neuronal differentiation, we used CRISPR/Cas9 mediated gene editing to engineer three different ESC lines, deleting Auts2-L, Auts2-S, or both (Fig. 3-2). Due to the shared coding region between Auts2-L and Auts2-S, it is impossible to use a simple CRISPR strategy that deletes or edits exons to create a null mutant specifically for Auts2-S without affecting Auts2-L. Suggested in Monderer-Rothkoff et al., 2021, there is a predicted promoter potentially for the short isoform of AUTS2. When analyzing our previous H3K4me3 ChIP-seq data, we found an H3K4me3 peak located just before exon 6 (Fig. 3-2a), indicating an internal promoter region for the alternative transcription start site. We reasoned that deletion of the intron region underlying this alternative promoter is likely to specifically silence Auts2-S (Fig. 3-2d). Indeed, using this strategy, we were able to generate an ESC line with only Auts2-S deleted (Auts2-S-/-), which was confirmed by genomic PCR, Sanger sequencing, and Immunoblotting (Fig. 3-2g, j, and k). In addition, we generated and validated ESCs with both isoforms (Auts2-/-) or only Auts2-L deleted (Auts2-L-/-) by targeting exons 9 and 7 respectively (Auts2-/-, Fig. 3-2b, e, h, and k; Auts2-L-/-, Fig. 3-2c, f, i and k).

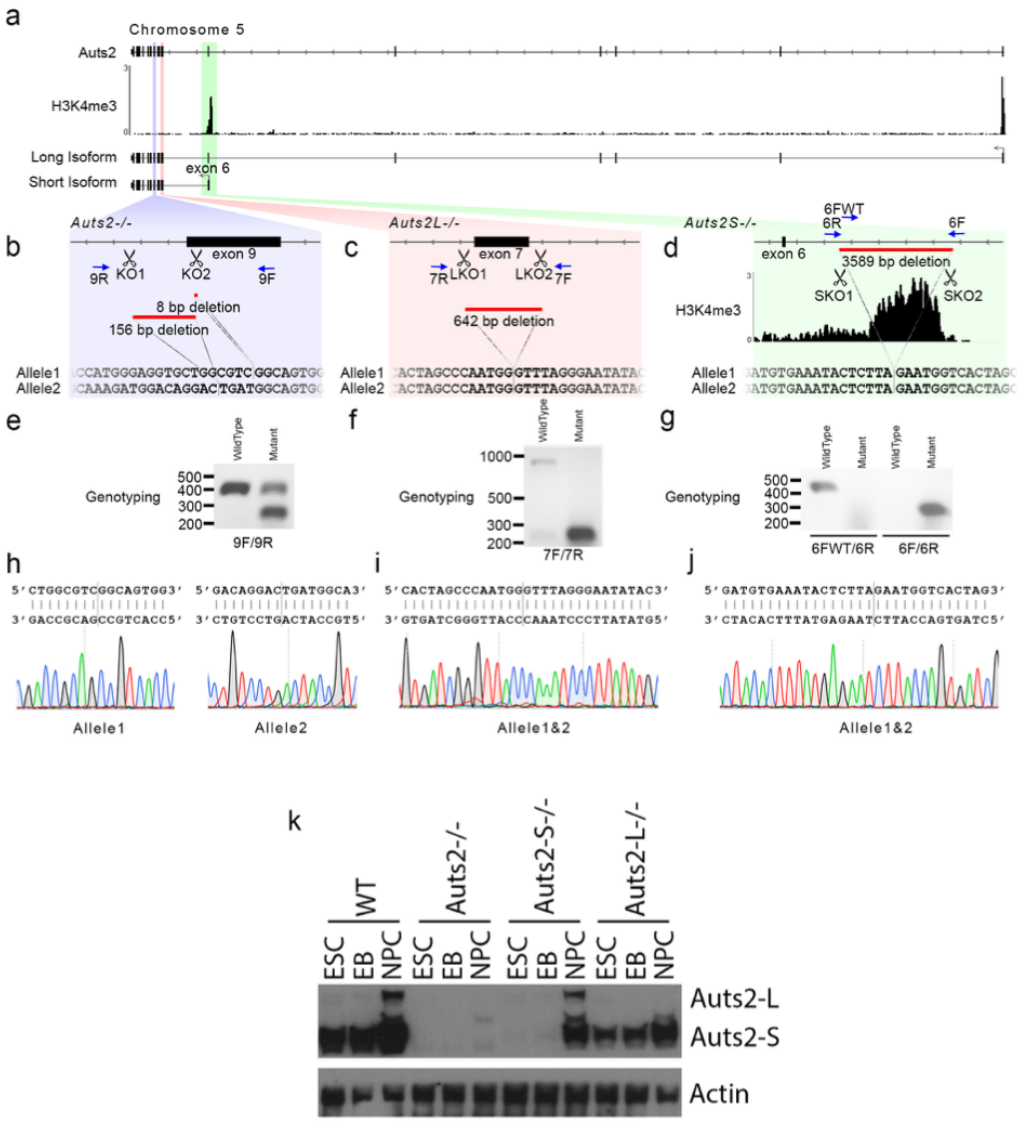

Figure 3-2: Design, generation and validation of mouse ESC lines with Auts2 deletion.

a, Schematic representation of the genetic locus of mouse Auts2. H3K4me3 peaks, indicating the two transcription start sites, were obtained from previous ChIP-seq analysis (Gao et al. 2014). Two major transcripts, one referred to as "long" and the other referred to as "short" isoforms are shown at the bottom. **b-d,** Targeting strategy and final products of CRISPR/Cas9-mediated gene editing of Auts2 locus. In each cell line, a pair of sgRNAs (labeled by scissors) were used and the deleted regions are indicated by red bars. Blue arrows indicate genotyping primers. Post-editing sequences are shown for both alleles. The two alleles in the Auts2-/- line were edited differently; one has an 8 bp frameshift deletion and the other a 156 bp deletion that spans the splicing junction (b). Identical editing events are present in both Auts2L-/- (c) and Auts2S-/- (d) lines. **e-g,** Genotyping PCR results for edited lines. **h-j,** Sanger sequencing results for edited lines. **k,** Immunoblotting to confirm the disruption of specific isoforms of Auts2 in edited lines.

To investigate the roles of Auts2-L and Auts2-S in neuronal cell fate determination, we performed immunofluorescence (IF) on the NPCs differentiated from various Auts2 deficient ESCs. We found that the protein level of Nestin, an NPC marker, is almost completely diminished in Auts2-/- cells, whereas a significant reduction in Nestin expression was observed in Auts2-L-/- or Auts2-S-/- cells (Fig. 3-1c). It is unlikely that this defect results from a change in ESC status. Using an MTT cell proliferation assay, we confirmed a lack of any noticeable difference in cell growth among all three null ESC lines compared with wild-type (WT) ESCs (Fig. 3-3a). Furthermore, the pluripotency of mESCs lacking Auts2 was not changed, as measured by their alkaline phosphatase activity (Fig. 3-3b) and protein and mRNA levels of Oct4 and Nanog (Fig. 3-3c and 2-4a). The differentiation defect observed in Auts2 null cells was further confirmed by RT-qPCR analysis. In agreement with the previously mentioned Nestin IF analysis, NPC markers such as Pax6, Nes, Neurod1, and Sox1, are significantly reduced in Auts2-/- cells and are only partially decreased in either Auts2-L-/- or Auts2-S-/- cells (Fig. 3-4b). Given the fact that only Auts2-L interacts with PRC1 components, we suspect that the neuronal differentiation defect seen in these Auts2 deficient ESCs may involve a PRC1-independent pathway. Nonetheless, both isoforms of Auts2 contribute to the regulation of the differentiation of mESCs to NPCs.

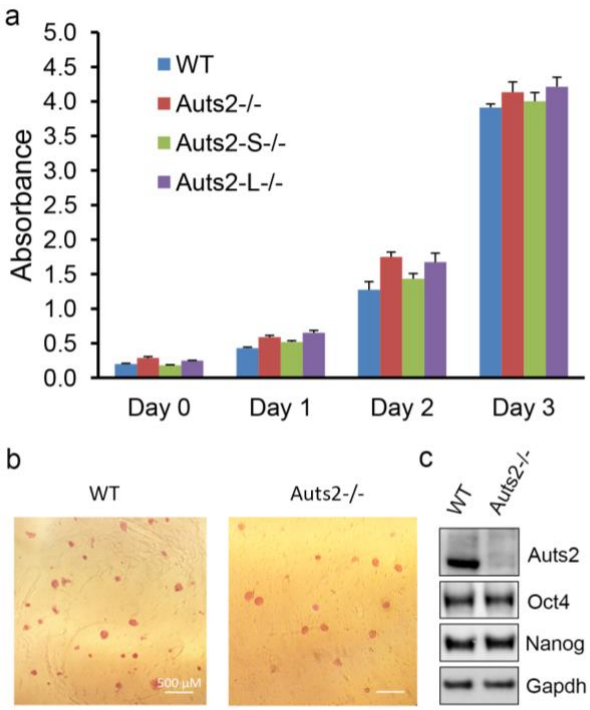

Figure 3-3: Loss of Auts2 does not affect ESC self-renewal and proliferation.

a, Alkaline phosphatase activity staining in WT and Auts2-/- ESCs. **b,** Immunoblotting shows no obvious difference in the protein level of Oct4 and Nanog, two pluripotent markers, between WT and Auts2-/- ESCs. **c,** MTT cell proliferation assay to measure the growth rate of ESCs at various days. All mean values and standard deviations were calculated from four independent measurements. No significant differences were found among ESCs of all genotypes.

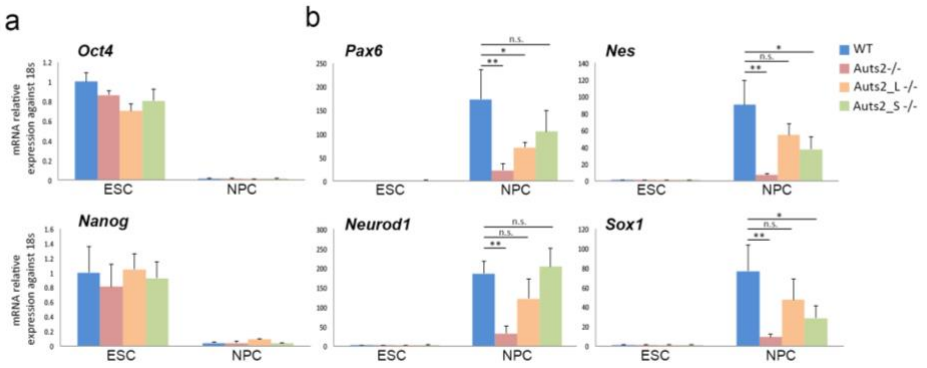

Figure 3-4: Expression of pluripotency and NPC markers in ESCs with Auts2 deletion.

a, Quantitative RT-PCR analysis of expression pluripotency markers (Oct4 and Nanog) in WT, Auts2-/-, Auts2L-/- and Auts2S-/- ESCs and NPCs. **b,** Quantitative RT-PCR analysis of NPC markers (Pax6, Nes, Neurod1 and Sox1). Expression levels are normalized relative to those in WT ESCs. All mean values and standard deviations were calculated from three independent measurements. * P<0.05, ** P<0.01, n.s., not significant, by two-sided t-test.

Auts2 deletion causes imbalanced expression of genes required for the development of three germ layers

With next-generation RNA sequencing (RNA-seq), we conducted a transcriptomic analysis of both WT and Auts2-/- in ESCs and NPCs. As shown in Fig. 3-5a, we have identified 4015 differentially expressed genes between WT and Auts2-/- NPCs. Among those, 1845 are down-regulated, and 2170 are up-regulated in Auts2-/- NPCs compared with WT. Among the down-regulated genes, many neuroectodermal marker genes were observed among the top differentially expressed genes, including *Nes*, *Sox1*, *Pax6*, *Pax2*, *Neurod1*, *Nog*, and *Tubb3* (Fig. 3-5a and b). A gene ontology (GO) analysis confirmed that these down-regulated genes in *Auts2*-/- NPCs are enriched in functional groups including "nervous system development", "axon guidance", "neuron migration", "axonogenesis", "neuron differentiation", "glial cell differentiation", "hippocampus development", and "neuron fate commitment" (Fig. 3-5b, top

panel). RNA-seq analysis also identified a group of genes that are not only up-regulated in *Auts2*-/- NPCs but, interestingly, are associated with mesoderm and endoderm development, including *Foxa1*, *Foxc1*, *Snai1*, *Tgfb2*, *Foxf1*, *Tgfb3*, *Fabp4*, *Gata4*, *Gata6*, and *Tnnt2* (Fig. 3-5a and b). Further GO analysis revealed functional aspects related to the regulation of mesoderm and endoderm lineage specification, including "angiogenesis", "heart development", "vasculogenesis", "atrial cardiac muscle tissue morphogenesis", and "liver development" (Fig. 3-5b, bottom panel).

The similarity in transcriptional signatures between cells with a dysregulated TGF-β/BMP pathway and *Auts2*-/- mESC leads to the assumption that Auts2 may regulate neuronal differentiation via the TGF-β /BMP pathway (Watabe and Miyazono 2009). To better understand the influence of TGF-β/BMP signaling on the AUTS2-mediated regulation of neuronal differentiation, we took advantage of previously identified target genes for these pathways and examined their changes during differentiation in our WT and *Auts2*-/- NPCs. As shown in Fig. 3-5c, BMP-specific SMAD1/5 target genes that are also differentially regulated between WT and *Auts2*-/- NPCs tend to show more dramatic expression changes than all up-regulated or down-regulated genes. TGF-β-specific SMAD2/3 target genes exhibit a similar pattern but to a lesser extent (Fig. 3-5c). With RT-qPCR, we further showed that BMP target genes are dysregulated in NPCs upon Auts2 deletion (Fig. 3-5d). These results strongly suggest that the essential role of Auts2 in maintaining the balanced expression of germ layers-related genes potentially through regulating BMP pathway during neuronal differentiation.

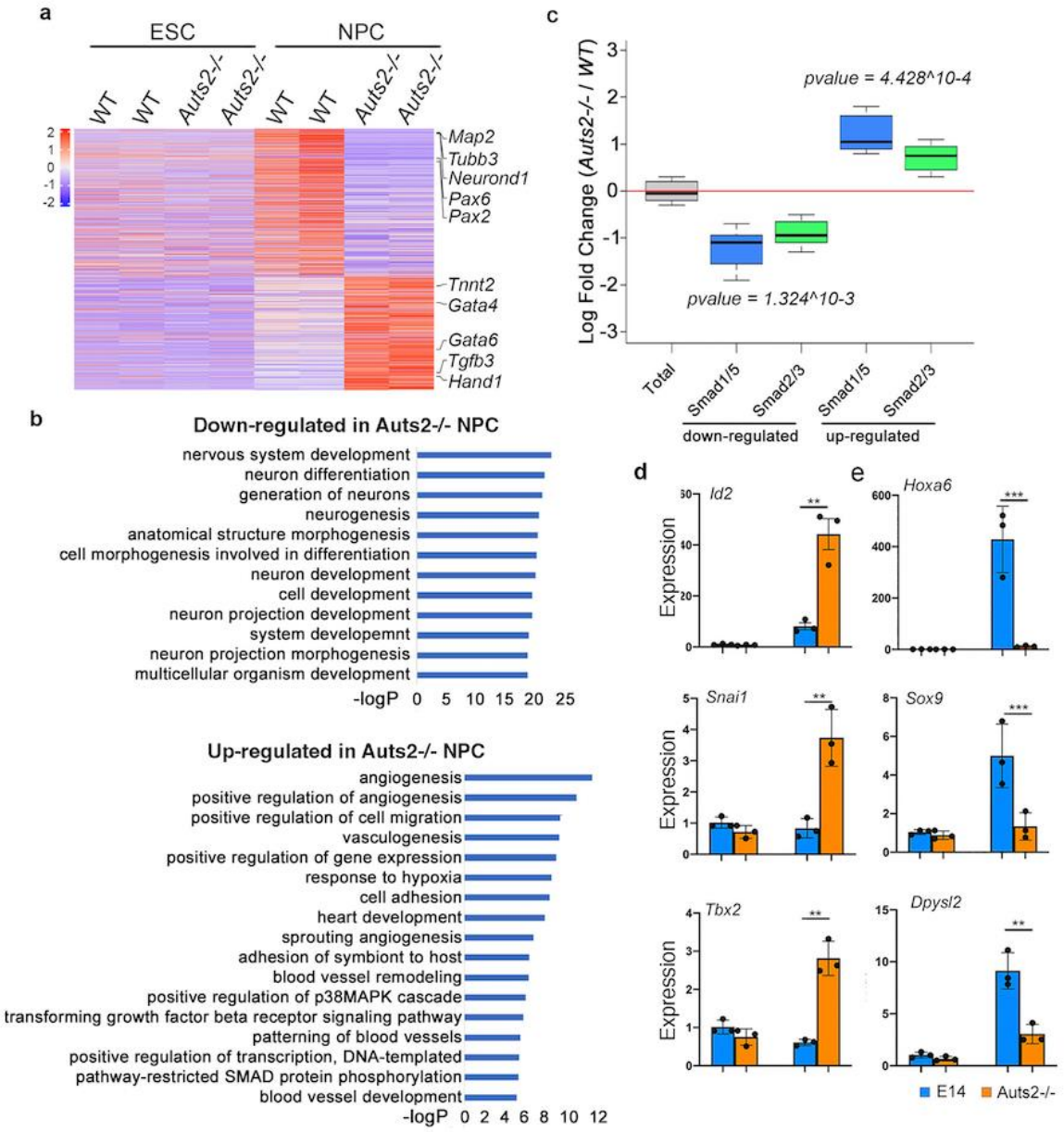

Figure 3-5: Auts2 controls the proper expression of lineage-specific genes.

a, Heatmap of transcriptomic analysis in WT and Auts2-/- ESCs and NPCs. RNA-seq analyses were performed on duplicate samples. The processed FPKM (reads per kilobase of exon per million reads mapped) values for each gene were used to calculate z scores used to generate the heatmap (See Supplemental Information for details). **b,** GO analysis of genes that are down- or up-regulated in Auts2-/- NPCs compared with WT. The x-axis (in logarithmic scale) corresponds to the binomial raw P values. **c,** Whisker-box plot shows the expression of genes targeted by SMAD1/5 or SMAD2/3. Fold change of RPKM values between WT and Auts2-/- NPCs from all expressed genes, down- or up-regulated genes, down- or up-regulated SMAD1/5-targeted genes, and down- or up-regulated SMAD2/3-targeted genes are plotted. SMAD1/5 and SMAD2/3 target

genes are obtained from (Morikawa et al., 2011). **d,** Expression of BMP responsive genes during differentiation, measured by quantitative RT-PCR. All mean values and standard deviations were calculated from three independent measurements. ** indicates $p<0.01$ by two-sided t-test.

Auts2 is required for inhibition of BMP signaling during neuronal differentiation

Upon stimulation, SMAD1/5/9, BMP-specific R-SMADs, are phosphorylated and dimerize with SMAD4, which is then translocated to the nucleus to regulate gene transcription. To understand how disruption of Auts2 may lead to the deregulation of the BMP pathway, we performed immunoblotting to examine the phosphorylation of SMADs. In keeping with our RNA-seq analysis, we found that the deletion of Auts2 results in an increase of phosphorylated Smad1/5/9 levels at the EB and NPC stages compared with WT cells, although no noticeable difference was seen in ESCs between *Auts2*-/- and WT (Fig. 3-6a). This observation further indicates that the effect of the loss of Auts2 on neuronal differentiation is due to up-regulated BMP signaling. The TGF-β pathway does not play a significant role as only a minimal increase of SMAD2 phosphorylation was found in *Auts2*-/- cells (Fig. 3-6a). To further examine the impact of Auts2 on acute cell response to BMP stimuli, we treated WT and *Auts2*-/- ESCs with BMP4 and evaluated the phosphorylation of Smad1/5/9. As expected and compared with WT cells, *Auts2*-/- cells displayed a significantly higher level of pSmad1/5/9 (Fig. 3-6b).

The BMP pathway plays an essential role in cell differentiation. Studies have shown that activated BMP signaling promotes mesoderm and endoderm differentiation at the expense of neuroectoderm lineage (Z. Li and Chen 2013). To test whether up-regulated BMP signaling in *Auts2*-/- cells contributes to cell fate switch, we treated ESCs with noggin, an inhibitor of the BMP pathway, during differentiation. We found that noggin treatment rescued the dysregulation in marker genes of three germ layers (Fig. 3-6c). Viewing these results together, it is clear that

Auts2 plays a critical role in repressing BMP signaling, serving as a crucial step for proper neuronal differentiation.

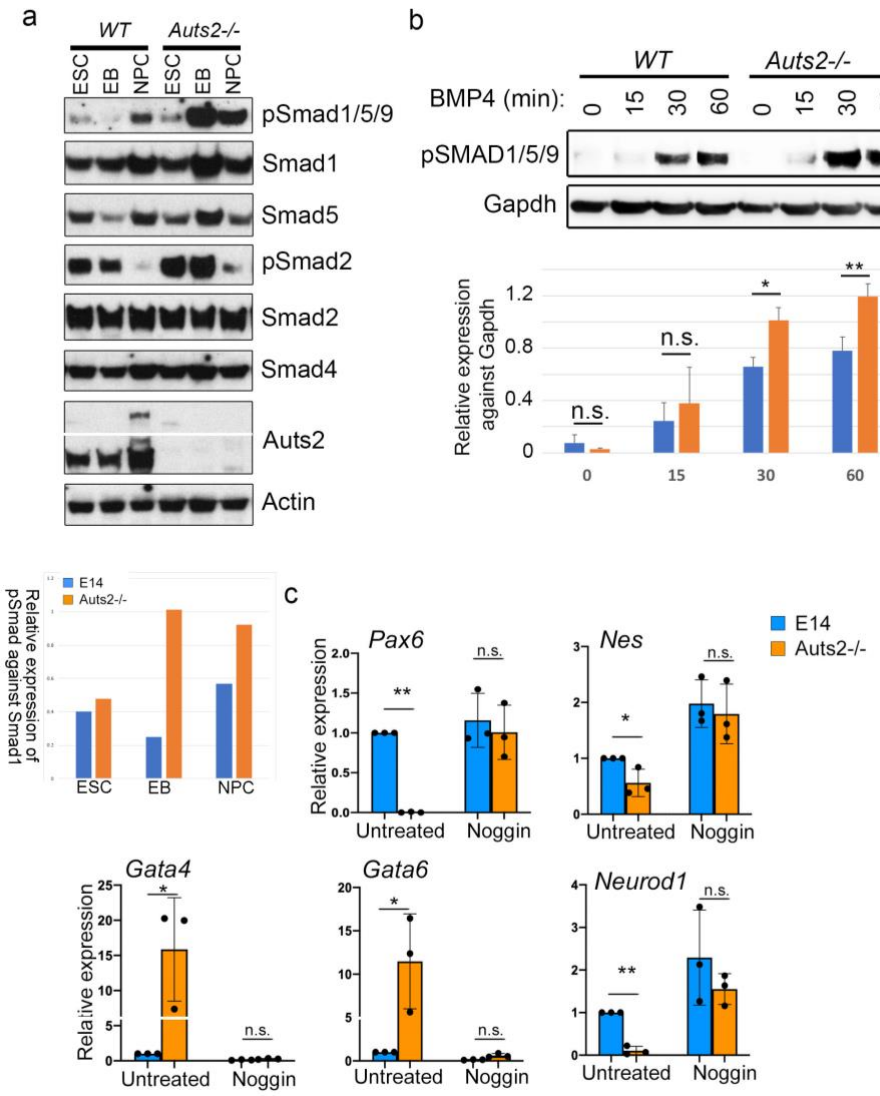

Figure 3-6: Auts2 is required for the inhibition of BMP signaling during neuronal differentiation.

a, Immunoblotting of WT and Auts2-/- mouse ESC, EB, and NPC using antibodies as indicated. **b,** WT and Auts2-/- ESCs were stimulated with BMP4 at 25 μM for the time as indicated. Activation of BMP signaling was measured by the level of Smad1/5/9 phosphorylation by immunoblotting. **c,** Expression of selected marker genes (neuroectoderm: Pax6, Nes, and Neurod1; mesoderm: Gata4; endoderm: Gata6) in Noggin-treated or untreated NPC of WT or

Auts2-/-, measured by quantitative RT-PCR. All mean values and standard deviations were calculated from three independent measurements. * $p<0.05$, ** $p<0.01$, n.s. not significant.

AUTS2 forms a neuronal lineage-specific complex with WDR68 and SKI

Both Auts2-L and Auts2-S interact with Wdr68 (Fig. 3-1b). Previously, we have shown that during NPC differentiation, the deletion of Wdr68 results in repression of the neuroectodermal markers, accompanied by the aberrant induction of mesodermal and endodermal markers (Q. Wang et al. 2018). This observation is similar to our result in *Auts2*-/- cells (Fig. 3-1). Wdr68 belongs to a large family of WD40 domain-containing proteins that generally act as adaptors or scaffold factors for other proteins or protein complexes (Li and Roberts, 2001; Stirnimann et al., 2010). These observations suggest that Auts2-L or Auts2-S and Wdr68 may form a complex with unknown proteins that, in turn, may regulate BMP signaling during neuronal differentiation. To understand the molecular mechanism of Auts2-mediated control on neuronal cell fate, we performed proteomic analysis to identify novel binding partners for Auts2 and Wdr68. For this purpose, we used CRISPR/Cas9-mediated gene editing to generate an engineered ESC line in which tandem FLAG and HA tags were placed immediately in front of the translational start site of Wdr68 (E14: NFH-Wdr68, Fig. 3-7a). Genomic PCR and immunoblotting validated successful targeting (Fig. 3-7b and c). We then performed quantitative mass spectrometry analysis on affinity-purified biological triplicate samples from E14: NFH-Wdr68 ESCs and their derived NPCs. As shown in the volcano blot in Fig. 3-8a, many known Wdr68-associated proteins (Gao et al. 2014; Q. Wang et al. 2018; Miyata et al. 2014), including Pcgf3, Pcgf5, Rnf2, and Dyrk1a, were recovered, demonstrating the success of our proteomic analysis. In addition, Sloan–Kettering Institute (Ski), a previously identified inhibitor of the TGF-β/BMP pathway (Luo et al. 1999; Stroschein et al. 1999; Sun et al. 1999), was the most enriched in NPCs (Fig. 3-8a). Auts2 also was enriched in NPCs but less abundant compared with Ski (Fig.

3-8a). Immunoprecipitation in E14: NFH-Wdr68 cells further confirmed the association of Ski as well as Auts2-L and Auts2-S with Wdr68 (Fig. 3-8b). More interestingly, Ski was only immunoprecipitated from NPCs (Fig. 3-8b). This suggests that Wdr68-Ski interaction is highly specific to this stage. Meantime, both Auts2-L and Auts2-S are more highly expressed in NPC and more abundant in the immunoprecipitation (Fig. 3-8b).

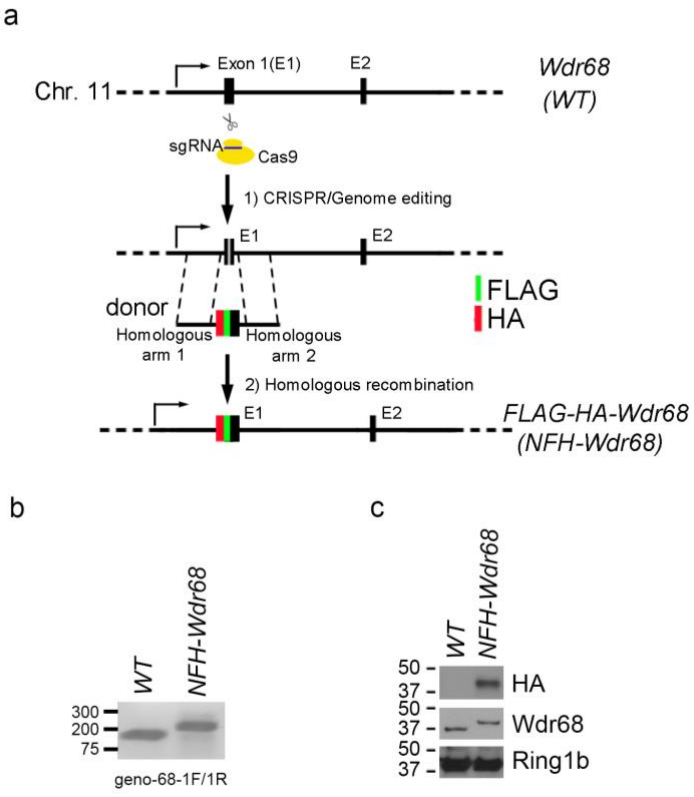

Figure 3-7: Generation of an ESC line that expresses FLAG-HA-Wdr68.

a, Schematic for the CRISPR/Cas9-mediated knock-in of FLAG-HA-Wdr68 in E14 cells. **b,** Agarose gel picture shows the PCR products of genomic DNA isolated from WT and FLAG-HA-Wdr68 cells. **c,** Immunoblotting detected the expression of FLAG-HA-Wdr68 in edited cells.

To examine if Auts2 interacts with Ski, we transfected HEK293T cells with long and short forms of N-terminal FLAG and HA-tagged AUTS2 (NFH-AUTS2-L and NFH-AUTS2-S) and performed immunoprecipitation with FLAG antibody-conjugated M2 beads. As expected,

both forms of AUTS2 interacted with SKI and WDR68, but only AUTS2-L associated with PRC1 complex component PCGF5 (Fig. 3-8c). To further pinpoint the domains of AUTS2 required for interactions with different factors, we conducted immunoprecipitation experiments with various AUTS2 constructs. Based on these results, we found that the N-terminal fragment containing the first 208 amino acids is required for binding PRC1 components such as PCGF5 and RING1B, and the internal region between 600 to 700 amino acids was essential for binding WDR68 and SKI (Fig. 3-8d and Fig. 3-9). Through glycerol gradient analysis on FLAG affinity-purified complexes from a stable HEK293T-REx line expressing FLAG-HA-SKI, we observed that SKI, AUTS2, and WDR68 are present within the same fractions (Fig. 3-8e). This indicates the presence of a stable complex consisting of these three proteins. In summary, our proteomic and biochemical analyses defined a novel protein complex formed by Auts2, Wdr68, and Ski. Hereafter we will refer to this novel complex as the AWS complex (Fig. 3-8f). While both Auts2-L and Auts2-S are capable of forming the AWS complex, only Auts2-L can form the previously identified PRC1-AUTS2 complex (Fig. 3-8f) (Gao et al. 2014).

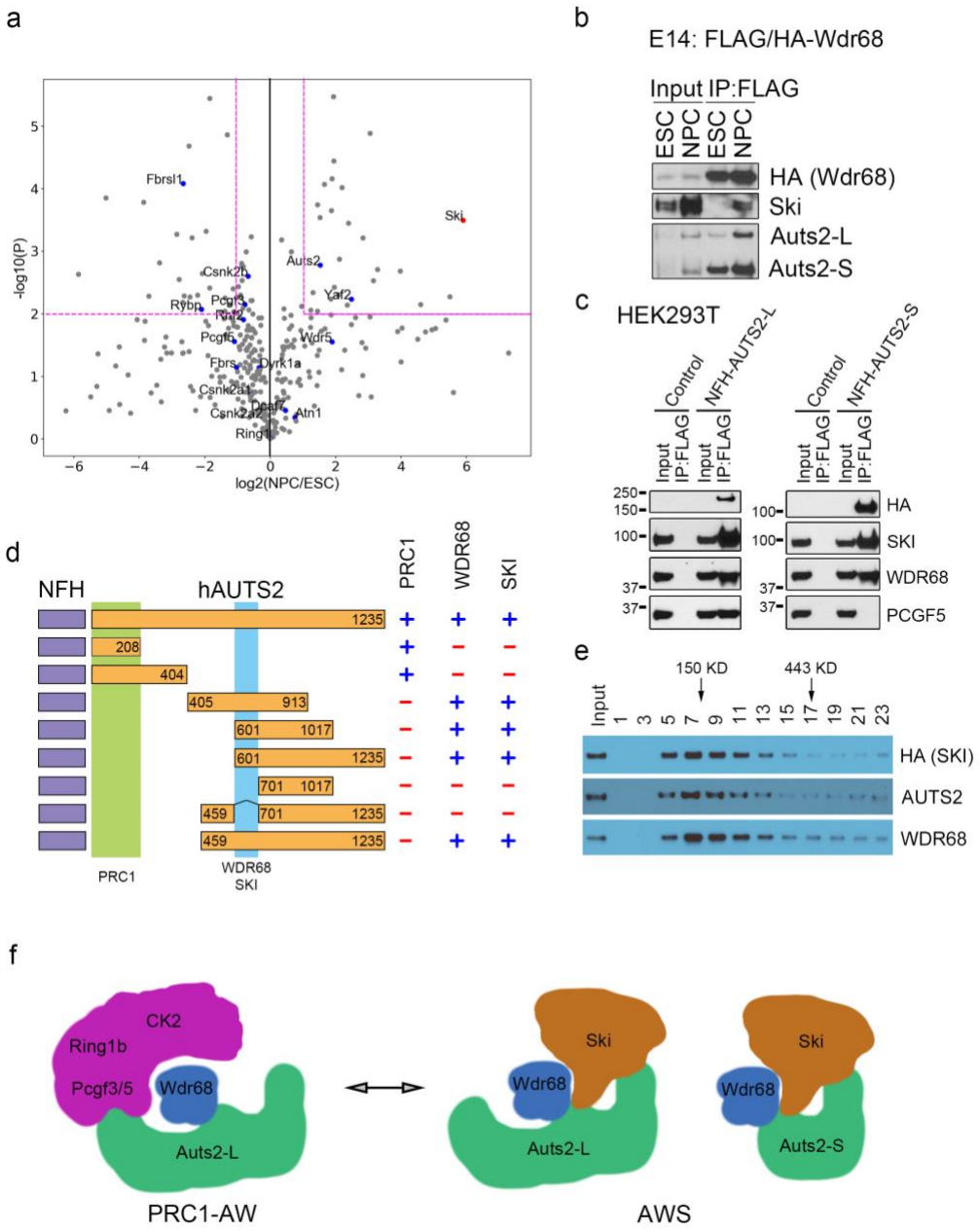

Figure 3-8: Replace this with figure caption below figure.

a, Tandem affinity purification (TAP) followed by quantitative mass spectrometry analysis (MS) revealed a novel binding partner for Wdr68 in NPC, shown by a volcano blot. An E14 cell line with engineered Wdr68 locus inserted with N-terminal FLAG and HA tags (E14: NFH-Wdr68) was either cultured at ESC or differentiated to NPC stages. TAP was performed in triplicate samples of ESCs and NPCs, followed by label-free quantitative MS analysis (see Experimental Procedures). The X-axis shows the mean ratio of peptide intensity between NPC and ESC, and the Y-axis corresponds to P-value. **b,** IP from NE of E14: NFH-Wdr68 cells at the ESC or NPC stages, using FLAG M2 beads. Bound proteins were resolved on SDS-PAGE and detected by

Western blotting for the antigens indicated. **c,** IP from NE of HEK293T cells transfected with NFH-AUTS2-L or NFH-AUTS2-S or vector control, using FLAG M2 beads. Bound proteins were resolved on SDS-PAGE and detected by Western blotting for the antigens indicated. **d,** Mapping of AUTS2 domains required for interaction with indicated proteins or protein complexes. Plasmids expressing NFH-AUTS2 with various lengths were expressed in HEK293T cells followed by IP with M2 beads to detect their interaction with PRC1 components, WDR68 and SKI, as indicated on the right (See Experimental Procedures for details). The deduced domains required for specific interaction are highlighted and indicated at the bottom. **e,** Glycerol gradient (15-35%) analysis of FLAG-purified NFH-AUTS2-S (See Experimental Procedures). Every other fraction was resolved on SDS-PAGE followed by immunoblotting for the antigens indicated. The fractions containing AUTS2, WDR68, and SKI simultaneously indicate the presence of a complex formed with these proteins (AWS). **f,** Schematic model of AUTS2-containing complexes. See text for details.

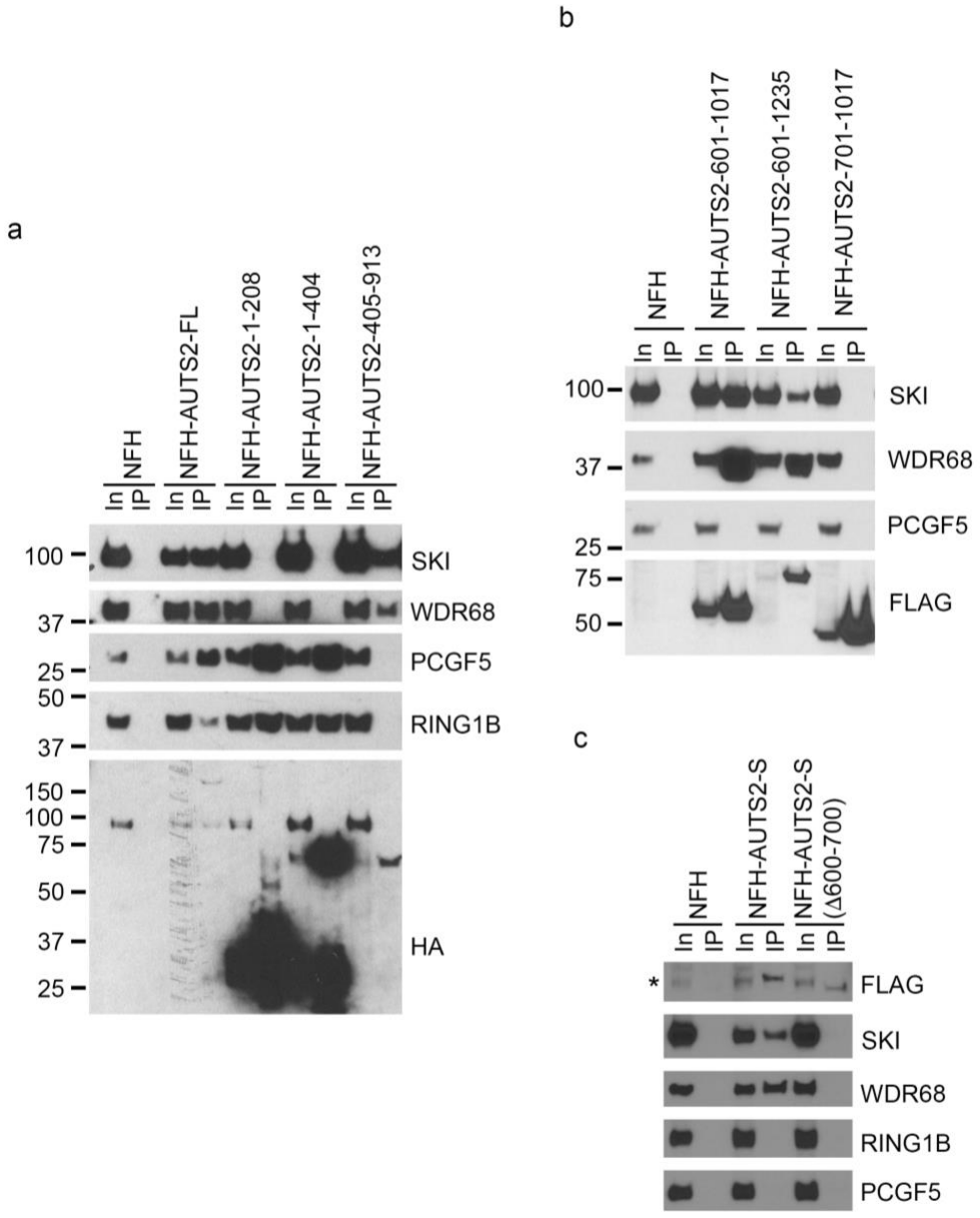

Figure 3-**9: Domain mapping of AUTS2 for interaction with various partner proteins.**

a, b and c, HEK293T cells were plasmids expressing various lengths of FLAG and HA tagged AUTS2 as indicated. IP was performed using M2 beads. Bound proteins were resolved on SDS-PAGE and detected by Western blotting for the indicated antigens.

AWS negatively regulates the stability of phosphorylated SMAD1/5/9 through CUL4-mediated poly-ubiquitination

SKI has been previously identified as an inhibitor of TGF-β/BMP signaling by directly binding to the R-SMAD/co-SMAD complex (Luo et al. 1999), thus unlikely to affect the level of R-SMAD phosphorylation level. Furthermore, transcriptional feedback does not likely explain this phenomenon as the enhancement of pSmad in *Auts2*-/- ESCs was seen shortly after 15 min of BMP stimulation (Fig. 3-6b). Another possibility is that the AWS complex may affect the activation step of SMAD signaling. However, we ruled out this explanation since Auts2 is predominantly expressed in the nucleus (Bedogni, Hodge, Nelson, et al. 2010; Gao et al. 2014).

We then asked whether the AWS complex affects the stability of SMAD1/5/9. It has been previously shown that WDR68 is present in the CUL4 E3 complex as a substrate receptor that directs specific substrate binding for polyubiquitination and subsequent proteasomal degradation (Higa et al. 2006; Jin et al. 2006). WDR68 directly interacts with Damage Specific DNA Binding Protein 1 (DDB1), an adaptor specific to the CUL4 E3 complex (Jin et al. 2006). Using immunoprecipitation, we demonstrated that AUTS2 also interacts with DDB1 (Fig. 3-10a). Therefore, we hypothesized that the AWS complex might affect the stability of the pSMAD by mediating its polyubiquitination. Indeed, when we transfected HEK293T cells with HA-ubiquitin (HA-ub) and FLAG-SMAD followed by immunoprecipitation with an HA antibody, we recovered polyubiquitinated SMAD1, which was further enhanced by treatment with MG132, a proteasome inhibitor (Fig. 3-10b). More interestingly, when these cells were treated with a pan CUL E3 inhibitor, MLN4924, polyubiquitinated SMAD1 was reduced (Fig. 3-10b), indicating that CUL E3 contributes to the polyubiquitination of SMAD1. Consistent with this observation, the addition of MLN4924 results in increased pSMAD1/5/9 upon BMP4 treatment in HEK293T cells (Fig. 3-10c). To further examine if the effect on pSMAD1/5/9 is due to CUL4 specifically,

we silenced the CUL4-specific adaptor DDB1 using siRNA and found that the DDB1 knockdown led to elevated SMAD activation compared with the control knockdown (Fig. 3-10d). Similarly, exogenous expression of a dominant-negative form of CUL4 (DN-CUL4) led to up-regulation of pSMAD1/5/9 (Fig. 3-10e). To further test our hypothesis, we transfected HEK293T cells with plasmids to express AWS components and examined their effects on the pSMAD induced by BMP treatment. As shown in Fig. 3-10f, overexpression of AUTS2 or SKI led to a reduction in pSMAD in response to BMP4 compared with the control transfection, whereas co-transfection of AUTS2 and SKI caused a slightly more substantial decrease of pSMAD. Interestingly, total SMAD levels remained unchanged (Fig. 3-10f), suggesting that the effect of AWS was specific to pSMAD. Taken together, our biochemical analyses delineated a regulatory pathway for restricting BMP-specific SMAD signaling by the AWS and CUL4 E3 complex, therefore promoting proper neuronal differentiation.

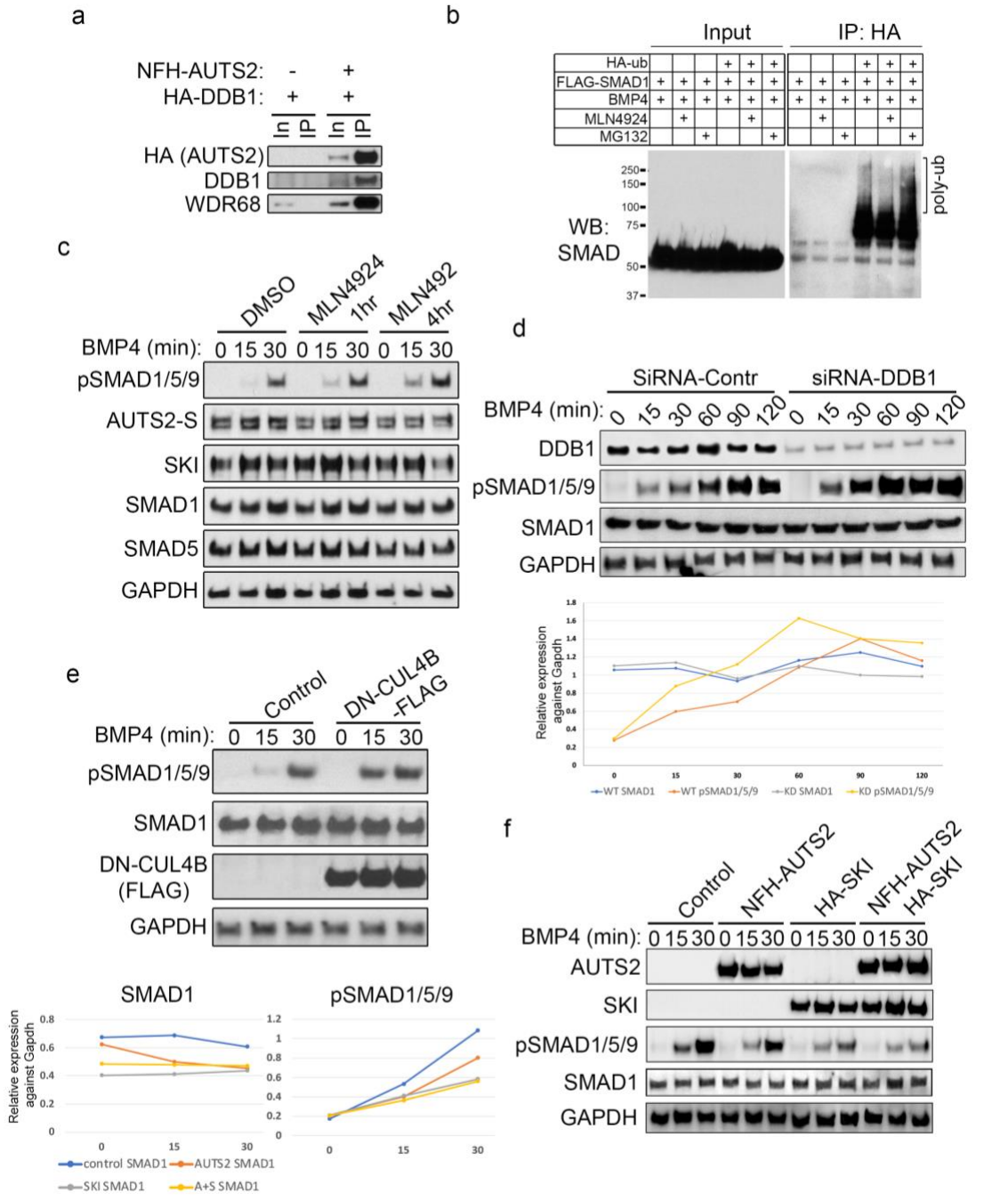

Figure 3-10: The AWS complex promotes degradation of pSMAD1/5/9 through CUL4 E3.

a, IP in HEK293T cells transfected with NFH-AUTS2 and HA-DDB1, using FLAG M2 beads. Bound proteins were resolved on SDS-PAGE and detected by Western blotting for the antigens indicated. **b,** in vivo poly-ubiquitination assay. HEK293T cells were transfected with HA-ub and FLAG-SMAD1. Two days after transfection, cells were lysed in the presence of 1% SDS and immunoprecipitated with HA beads, followed by immunoblotting with SMAD1 antibody. **c,**

Cullin E3 negatively regulates the level of pSMAD1/5/9. HEK293T cells were stimulated with BMP4 at various times as indicated with or without prior treatment with MLN4924, a pan inhibitor for CUL E3, followed by immunoblotting with antibodies as indicated. **d,** Two days after treatment of control or siRNA for DDB1, HEK293T cells were stimulated with BMP4 at various times as indicated, followed by immunoblotting. **e,** Two days after transfection with a dominant-negative form of CUL4 (DN-CUL4B), HEK293T cells were stimulated with BMP4 at various times as indicated, followed by immunoblotting. **f,** HEK293T cells were transfected with AUTS2 and SKI, individually or combinatorically, then stimulated with BMP4, followed by immunoblotting.

Auts2 control of neuronal differentiation requires the formation of the AWS complex

To understand how the interaction of Auts2 with Wdr68 and Ski affects its function during neuronal differentiation, we generated a lentivirus expressing either hAUTS2-S or a version with deleted amino acids 600-700 (hAUTS2-S-Δ). According to our domain mapping results (Fig. 3-8d), hAUTS2-S-Δ does not interact with Wdr68 and Ski and is therefore incapable of forming the AWS complex. We infected WT and *Auts2*-/- ESCs with these same lentiviruses; these ESCs were then differentiated to NPCs and scrutinized using RNA-seq analysis. We first examined whether the infection was successful by detecting RNA-seq tags of hAUTS2-S and hAUTS2-S-Δ. We found that RNA-seq signals covered all exons from 8 to 19 in hAUTS2-S infected cells, but in hAUTS2-S-Δ infected cells, there was no coverage for exons 11-14, which corresponds to translated amino acids 600-700 that were deleted (Fig. 3-11a). Next, we analyzed the global transcriptomic change between WT NPCs with mock infection (WT, mock) and *Auts2*-/- NPCs with infection of either mock, hAUTS2-S, or hAUTS2-S-Δ(*Auts2*-/-, mock/ hAUTS2-S/ hAUTS2-S-Δ). While the reintroduction of hAUTS2-S in *Auts2*-/- NPCs blocks the transcriptional deregulation in both directions compared with mock-infected WT NPCs (Fig. 3-11b, top and middle panels), expression of hAUTS2-S-Δ in *Auts2*-/- NPCs failed to do the same (Fig. 3-11b, top and bottom panels). Furthermore, down-regulation of neuroectodermal marker genes, including *Sox1, Neurod1, Pax6, Map2, and Tubb3* and up-regulation of mesodermal and

endodermal marker genes, including *Hand1, Twist2*, *Snai1*, *Gata4*, and *Gata6*, are both rescued in hAUTS2-S infected NPCs but not in hAUTS2-S-Δ infected NPCs (Fig. 3-11b). Based on our clustering analysis, the overall gene expression signature of hAUTS2-SΔ infected NPCs is closer to mock-infected WT NPCs, while hAUTS2-S-Δ infected NPCs were more similar to mock-infected *Auts2*-/- NPCs (Fig. 3-11c).

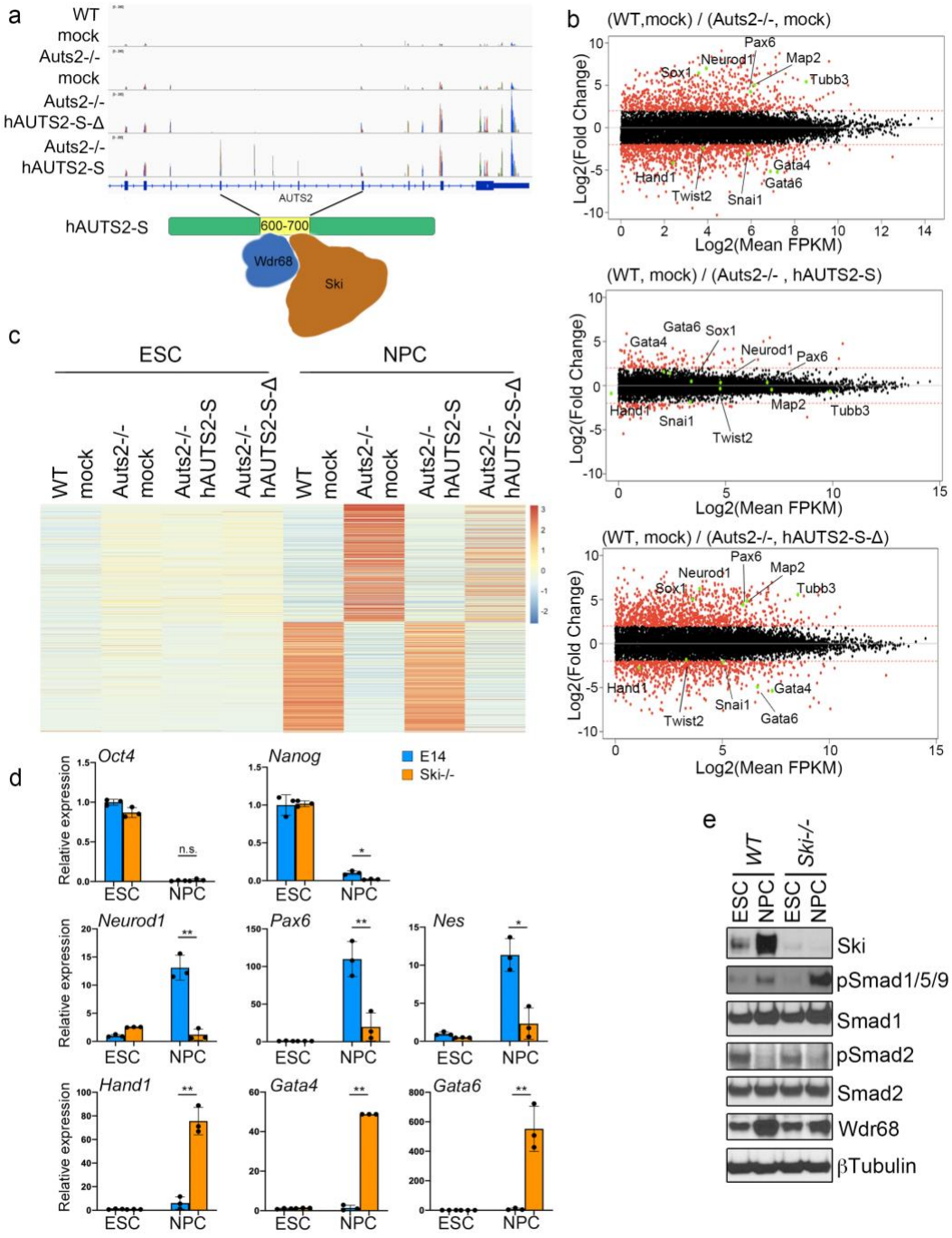

Figure 3-11: Ski mediates Auts2 regulation on neuronal differentiation.

a, RNA-seq reads confirm the expression of human-specific short form AUTS2 (hAUTS2) with or without deletion between 600 to 700 amino acids (hAUTS2-S-Δ and hAUTS2-S) through lentiviral infection in Auts2-/- mouse ESCs. A schematic of the deleted region of AUTS2 that promotes AUTS2 -WDR68 and AUTS2 -SKI interaction is shown at the bottom. **b,** Comparison of gene expression levels based on RNA-seq analysis from WT and Auts2-/- NPCs with lentiviral

infection with mock, hAUTS2-S and hAUTS2-S-Δ. The x-axis is the log2 value of the average FPKM of a gene and the y-axis is the log2 value of fold changes of FPKM of a gene between two groups. Genes with an average FPKM below one are filtered out. **c,** Heatmap of transcriptomic analysis in WT and Auts2-/- ESCs and NPCs, with lentiviral infection as indicated. Z scores were calculated using processed FPKM values for each gene as in Fig. 3-3a. **d,** Expression of selected marker genes (pluripotency: Oct4 and Nanog; neuroectoderm: Pax6, Nes, and Neurod1; mesoderm: Hand1 and Gata4; endoderm: Gata6) in ESC or NPC of WT or Ski-/-, measured by quantitative RT-PCR. All mean values and standard deviations were calculated from three independent measurements. * $p<0.05$, ** $p<0.01$, n.s. not significant. **e,** Immunoblotting of WT and Ski-/- mouse ESC and NPC using antibodies as indicated.

Ski has been previously shown to regulate brain development in mice (Berk et al. 1997), but the underlying mechanism is not fully understood. Our results suggest a novel role for Ski in regulating neuronal differentiation by promoting degradation of pSMAD1/5/9, which depends on its interaction with Auts2 and Wdr68. We generated ESC lines with deleted *Ski* (*Ski*-/-) through CRISPR to further test this hypothesis. Similar to *Auts2*-/- ESCs, *Ski*-/- ESCs showed defects in differentiation to NPCs, as evidenced by RT-qPCR analysis (Fig. 3-11d). More interestingly, this differentiation defect is accompanied by the up-regulation of pSMAD1/5/9 signaling (Fig. 3-11e). In summary, our results demonstrate that Auts2 promotes neuronal differentiation through working together with Wdr68 and Ski in a protein complex.

The AWS complex is required for proper gene expression and BMP signaling in mouse cortical neurons

To investigate the role of the AWS complex *in vivo*, we turned to a mouse model with CNS-specific deletion of both Auts2 isoforms through targeting exon 9 (Fig. 3-12a, gift from Danny Reinberg). As previously described, Auts2 is highly enriched in the neocortex of mice embryos and the cortical neurons in adult mice. Therefore, we isolated and cultured cortical neurons from WT and Auts2+/- mice at E15. Unfortunately, we were not able to obtain *Auts2*-/- mice due to potential embryonic lethality. Through RT-qPCR analysis, we found that, compared with WT, *Auts2*+/- primary cortical neurons showed lower expression of neuronal markers (Fig.

3-12). In agreement with our *in vitro* analysis on mouse ESC and NPC (Fig. 3-12a and b), mesodermal and endodermal markers were also aberrantly induced in *Auts2*+/- neurons (Fig. 3-12c).

Mechanistically, our ESC studies discovered the AWS-mediated negative regulation on BMP signaling through CUL4-mediated polyubiquitination and proteasomal degradation of pSMAD1/5/9 during NPC differentiation. To test if this mechanism plays a role in brain development, we treated the WT cortical neurons with either DMSO or MLN4924, followed by the acute stimulation by BMP4. As shown in Fig. 3-12d, pSMAD1/5/9 was increased upon the stimulation of BMP signaling with CUL inhibition (MLN4924), compared with control (DMSO). In summary, it is highly likely the AWS complex plays a role in restricting BMP signaling during neuronal differentiation *in vivo*. Furthermore, combining our mouse ESC and mouse cortical neuron studies, we uncovered a novel pathway through which AUTS2, an ASD/NDD risk factor, forms an NPC-specific complex and employs the CUL4 E3 complex to regulate BMP signaling, thereby affecting the cell fate determination in neurodevelopment (Fig. 3-12e).

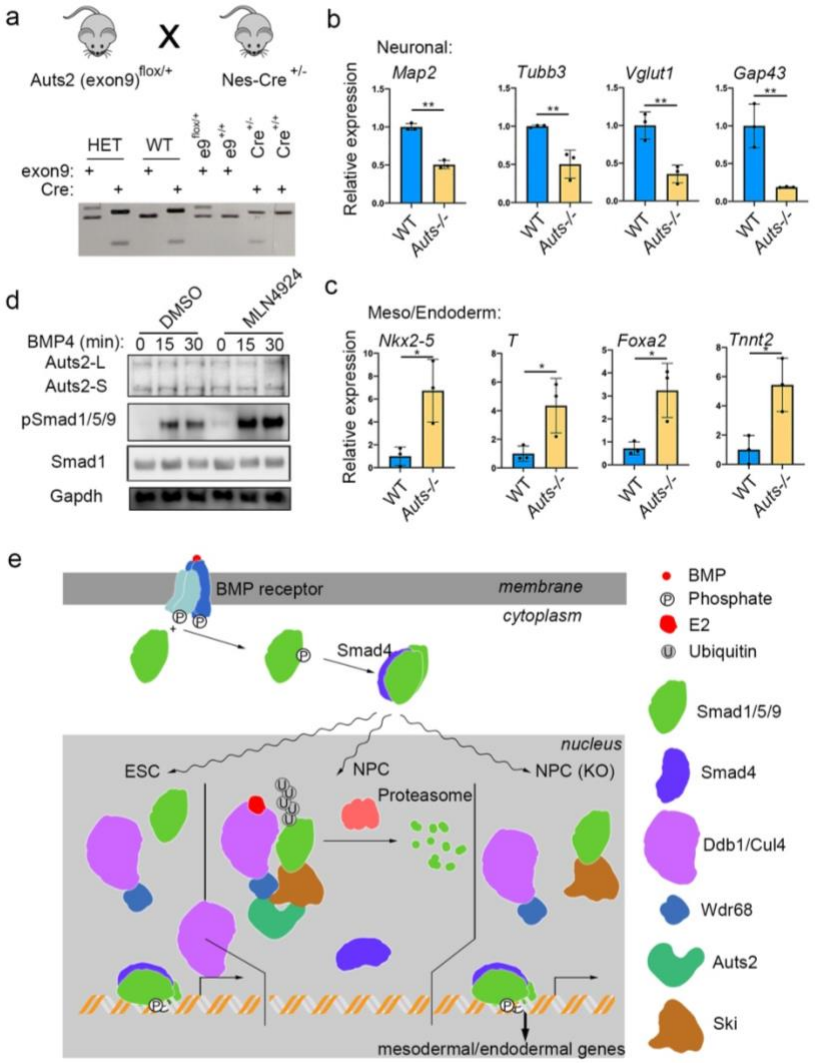

Figure 3-12: Auts2 is required for proper gene expression and BMP signaling in mouse primary cortical neurons.

a, Genotyping PCR in WT and Auts2+/- mouse primary cortical neurons. **b,** Relative expression of neuron markers between WT and Auts2+/- mouse cortical neurons. **c,** Relative expression of mesoderm and endoderm marker genes. (b and c) Each value is the mean of three independent measurements with error bars representing standard error. * $p<0.05$, ** $p<0.01$. **d,** WT cortical neurons were treated with MLN4924 or DMSO for 1 hour, then treated with BMP4 for 0, 15, and 30 minutes, followed by immunoblotting. **e,** Model of AWS/CUL4 E3-mediated regulation of BMP signaling during neuronal differentiation. See text for details.

Discussion

Our mechanistic dissection of AUTS2-mediated regulation of neuronal differentiation led to discovering a novel protein complex formed by AUTS2, WDR68, and SKI (referred to as AWS), impacting BMP signaling. To maintain proper cell fate choice during development, the BMP pathway needs to be tightly regulated; its inhibition is vital for engaging a transcriptional program that promotes neuronal differentiation (Z. Li and Chen 2013). Through biochemical analyses, we demonstrate that the AWS complex, which is formed specifically in the neural progenitor stage, targets BMP-specific SMADs to the CUL4 E3 complex for poly-ubiquitination and proteasomal degradation. Indeed, loss of Auts2 results in an up-regulation of BMP signaling and severe defects in neuronal differentiation in mouse ESCs, which is rescued by inhibition of the BMP pathway. These results uncover a novel mechanism through which a prominent NDD risk factor controls neural differentiation, providing a new opportunity for therapeutic interventions.

Although disruption of AUTS2 was initially identified in autism patients, subsequent genetic and genomic studies revealed that AUTS2 deregulation is linked to various forms of NDD (Oksenberg et al. 2013a). The phenotypic variability created by AUTS2 disruptions is accompanied by distinct transcriptional isoforms, but the underlying molecular mechanisms are not understood. It has been observed that patients with exonic deletions in the 3' region of the AUTS2 locus tend to have more severe phenotypes than those having 5' disruptions (Beunders et al. 2013). An alternative transcription start site prior to exon 6 drives the expression of a short isoform of AUTS2 (AUTS2-S), which is in frame with the long isoform (AUTS2-L). In the present study, we found that both AUTS2-L and AUTS2-S, via their shared middle region, form the AWS complex by associating with WDR68 and SKI, whereas AUTS2-L forms a specific PRC1-AUTS2 complex depending on its N-terminus (Fig. 3-8). Targeting exon 9 of Auts2 in

mouse ESCs, which results in the loss of both isoforms, clearly shows more dramatic defects in neuronal differentiation than the specific deletion of Auts2-L. These results echo the severity of dysmorphic phenotypes in patients with disruptions of AUTS2 at different regions (Oksenberg and Ahituv 2013) and indicate the potential involvement of a PRC1-independent pathway affected by these genetic altercations. Aberrations of BMP signaling have been closely linked to several craniofacial dysmorphic syndromes (Graf et al. 2016), which is highly likely the mechanism shared by both AUTS2 isoforms. However, given the many other factors associated with AUTS2 (Gao et al. 2014; Monderer-Rothkoff et al. 2021), further research is needed to elucidate the interplay between PRC1-dependent mechanism, BMP signaling, and maybe other pathways to understand how exactly AUTS2 regulates neurodevelopment.

Previously, the targeted deletion of Ski in mice led to developmental defects in the CNS, most likely by regulating the fate of neuroepithelial stem cells (Berk et al. 1997). The TGF-β/BMP pathway consists of an intricate network of factors for both stimulatory and inhibitory regulation of signal transduction (Massagué 2012). SKI has been identified as a negative regulator of TGF-β/BMP signaling, and subsequent studies suggest that SKI interacts with SMAD complexes to keep them inactive, thereby preventing transcriptional engagement of downstream target genes (Luo et al. 1999). Our studies suggest a novel mechanism by which SKI (part of the AWS complex and via recruitment of CUL4 E3) engages the BMP pathway. This occurs during neuronal differentiation and subsequently leads to the removal of pSMAD1/5/9 through ubiquitination and proteasomal degradation, a process that promotes a transcriptional program that favors a specific neuronal trajectory. It will be interesting to see how the two modes of SKI inhibition on TGF-β/BMP signaling, the SKI-SMAD inert complex and the AWS/CUL4-mediated degradation of SMADs, coordinate to regulate neuronal differentiation in a developmental stage- or cell type-specific manner. A possible molecular switch may reside in a

molecule like AUTS2 or other NDD risk factors based on our results. Future explorations into these avenues will likely provide mechanistic insight into the factors responsible for the fine-tuned regulation of these critical signaling pathways and their influence on cell fate choice during neurodevelopment. In addition, given the broad range of impact by BMP signaling on the development of various tissues, our discoveries may inspire a more specific targeting approach to correct the pathologically deregulated BMP pathway in NDD patients.

Chapter 4

AUTS2 regulates cell fate commitment through WNT/β-CATENIN signaling pathway during human brain development

Introduction

Neurodevelopmental disorders (NDDs) are a spectrum of diseases with the defective functionality of brain and peripheral neurological system, which are heavily determined by the genetics. Since a balanced translocation involving Autism Susceptibility Candidate 2 (AUTS2) was first identified in zygotic twins with autism, increasing evidence have suggested the role of AUTS2 as one of top NDD candidate genes (Talkowski et al. 2012; Beunders et al. 2013; 2016; Oksenberg and Ahituv 2013). Decades of studies on unrelated individuals carrying mutant AUTS2 show a range of defective neurological development, including intellectual disability, epilepsy, ADHD and developmental delay. Suggested by strong genetic evidence, researchers utilize both cell and animal models to validate the function of AUTS2 protein in brain development. AUTS2 expression in CNS reaches peak during early embryogenesis and declines after birth (Bedogni, Hodge, Nelson, et al. 2010; Gao et al. 2014). In zebrafish, morpholino knockdown of auts2 at early embryonic development stage led to smaller head size, neuronal reduction, and decreased mobility (Oksenberg and Ahituv 2013). Conditional knockout of Auts2 in mouse central nervous system (CNS) displayed smaller body size, impaired communication, and motor skill (Gao et al. 2014; Hori et al. 2015; 2014).

From molecular level, AUTS2 regulates neuronal differentiation and brain development via multiple pathways. Our previous studies as well others showed that AUTS2 interacted with

polycomb repressive complex 1 (PRC1) and converted PRC1 from transcriptional repressor to activator (Gao et al. 2014; Monderer-Rothkoff et al. 2021). Recent study extends our understanding of nuclei AUTS2 in regulating RNA transcription, splicing and localization via interacting with multiple RNA binding proteins (Castanza et al. 2021). In cytosol, AUTS2 works through Rac1 signaling pathway to regulate cytoskeleton and neural development (Hori et al. 2014). However, it is still elusive how AUTS2 works during human brain development. In this study, to overcome this obstacle, we applied a human pluripotent stem cell-derived three-dimensional organoid culture system established by Lancaster et al., which can recapitulate human in vivo cerebral cortex development. The brain organoid generated with this method is featured with cortex-like organization and functional cerebral cortical neurons, indicating its capability to recapitulate human brain development (Lancaster et al. 2013). On top of that, this method can mimic the pathology of patients by introducing the patient-related genetic defects to the pluripotent stem cell (Lancaster et al. 2013; Mellios et al. 2018). Although studies have shown the essential function of AUTS2 in neuronal differentiation, bias might be introduced unintentionally due to the distinct genetic background of model animals and technical limitation, which leads to failure in interpreting in vivo AUTS2 function. This state-of-the-art method provides an opportunity to dissect the function of AUTS2 in human brain development with less artificial interference.

WNT/β-catenin pathway is crucial throughout the stages of brain development. Without the stimulation of WNT signaling, the adherens junction associated-protein β-catenin is degraded in cytosol by a destruction complex, comprised of Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α). When WNT signaling pathway is activated, its receptor complex initiates a series of events, which subsequently leads to the dissociation of β-catenin destruction complex and subsequently, the accumulation of β-catenin (Logan and Nusse 2004). Once the level of β-catenin

is saturated, it translocates into nucleus and regulates WNT target gene expression as a transcriptional co-activator. The activation of WNT/β-catenin, along with retinoic acid (RA) and fibroblast growth factor (FGF) signaling pathways, regulate the development of midbrain, hindbrain, and spinal cord. Meanwhile, the absence of WNT/β-catenin pathway is required for the proper forebrain development. To ensure the proper brain development, the inhibitory machinery of WNT/β-catenin is needed to achieve its region-specific activation. Recent study revealed the involvement of Ring1 proteins, core component of PRC1, in regulating WNT signaling pathway in mouse brain (Eto et al. 2020). However, the involvement of AUTS2 in this process remains obscure.

In this study, we have demonstrated how AUTS2 regulates human brain development via WNT/β-Catenin signaling pathway. To investigate the function of AUTS2, we introduced an AUTS2 mutation found in patients with NDDs to human embryonic stem cells (hESCs) and differentiated these cells into human brain organoid. The transcriptional profiling and immunostaining showed that the absence of AUTS2 led to severe neuronal differentiation defects. Considering the heterogeneity of human brain cell composition, we took advantage of scRNA-seq technique to study the impact of AUTS2 on cell fate commitment during brain development process. Abrogation of AUTS2 results in a dramatic decrease of neuron-related cells and high abundancy of a specialized ependymal cell, choroid plexus cells (ChP), which is caused by the overactivated WNT/β-Catenin signaling pathway from pathway analysis. We revealed that the repressive role of AUTS2 is achieved by its interaction with TCF3/TLE3. In summary, our study showed the process of how aberrant AUTS2 levels lead to NDDs and reveal a novel function of AUTS2 by negatively regulating WNT/β-catenin signaling pathway, which may be a potential new therapeutic target.

Results

AUTS2 is essential for neuronal development

To unravel the function of AUTS2 in human brain development and its contribution to NDDs, we first applied CRISPR/Cas9 system to generate a total AUTS2 knockout hESC cell line by reproducing a 190kb genetic deletion observed in a patient, which removes whole exon 6 of AUTS2 gene and intronic promoter for AUTS2 short isoform (Fig. 4-2A, Fig. 4-1A, B) (Talkowski et al. 2012). Using CRISPR/Cas9 system, we have successfully generated a total AUTS2 knockout hESC line (AUTS2-/-), as well as AUTS2+/- line which will allow us to test the heterozygosity effect of AUTS2. The knockout of AUTS2 has been validated with Sanger sequencing, genotyping PCR (Fig. 4-1B, C). Immunoblotting showed that two main isoforms of AUTS2, full-length AUTS2 (above 150 KDa band) and short isoform (100 KDa band) have been removed in AUTS2-/- cell (Fig. 4-1D).

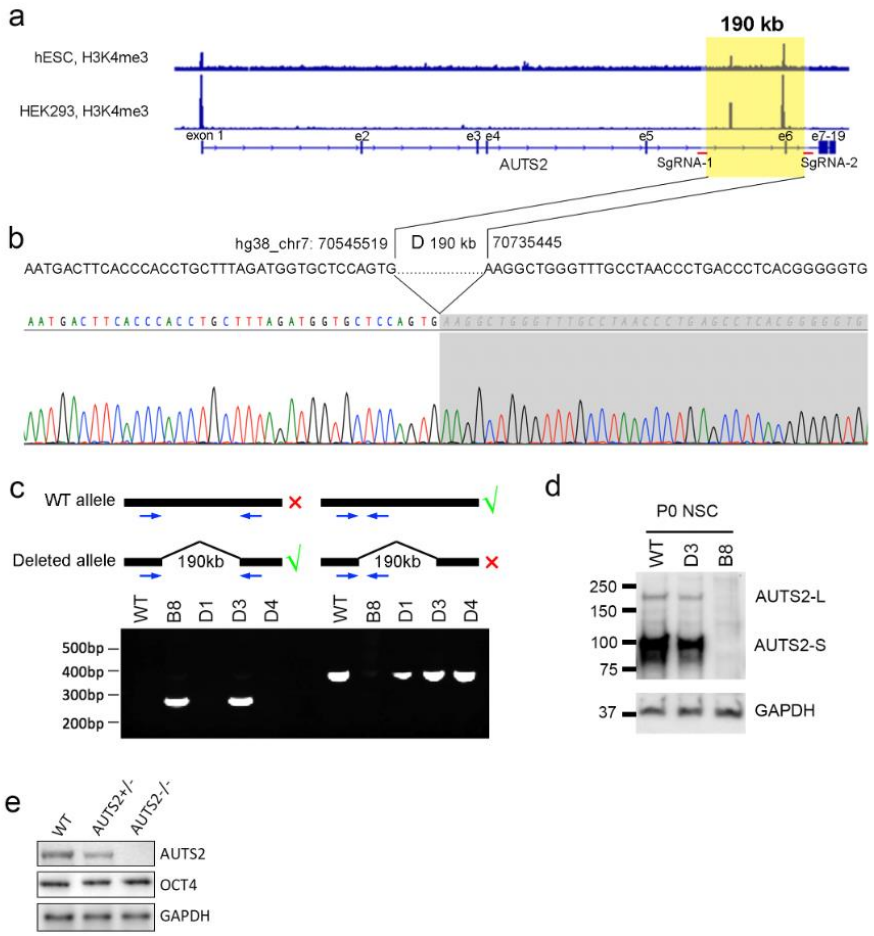

Figure 4-1: Generation of AUTS2 knockout human ESC.

a, Human genome view of H3K4me3 ChIP-seq in hESC and HEK293 cells. Both ChIP-seq tracks showed two peaks in the flanking region of exon 6 (highlighted with yellow box), indicating the intronic promoters. Red lines suggested the target region of sgRNA for the generation of AUTS2 knockout stable hESC line. **b,** Sanger sequence results showed the successful deletion of targeted 190 kb region upstream of *AUTS2* exon 6. **c,** PCR confirmed the genotypes of WT, AUTS2+/- (D3) and AUTS2-/- (B8) hESC. d, Immunoblotting confirmed the expression pattern of AUTS2 in WT, AUTS2+/- (D3) and AUTS2-/- (B8) hESC. e. Immunoblotting identified the expression level of pluripotency marker, OCT4 in WT, AUTS2+/- (D3) and AUTS2-/- (B8) hESC.

To better reflect the function of AUTS2 in human brain development, we took advantage of the in vitro 3D human cerebral organoid method described in Lancaster et al., 2013, which recapitulates human cerebral cortex development. Using the established protocol, the hESCs of heterozygous and homozygous deletion of AUTS2 as well as WT were differentiated into brain organoids. The matured organoids (60+ days) were then collected at different time points for

further experiments (Fig. 4-2A). To evaluate the importance of AUTS2 in brain development, quantitative reverse-transcription PCR (RT-qPCR) was performed on organoids day 71 and our data showed a sharp decrease of neuronal marker gene, FOXG1 in AUTS2-/- organoids, compared with WT (Fig. 4-3A). Previous study in mice demonstrated that loss of AUTS2 function leads to autistic-like behaviors due to impaired excitatory and inhibitory coordination (Hori et al. 2020). Moreover, autism patients-derived telencephalic organoids are characterized with imbalanced population between excitatory and inhibitory neurons, regardless of patients' genetic background (FOXG1-dependent dysregulation of GABA/glutamate neuron differentiation in autism spectrum disorders). Similar with aforementioned finding, we found that AUTS2-/- organoids had significant decreases of excitatory neuron marker genes (BRN2 and CTIP2) and no changes in inhibitory neuron genes (DLX1, GAD1, and VGAT) as compared to WT (Fig. 4-3A).

To further characterize the overall impact of AUTS2 in brain development, organoids were collected at day 75 and bulk RNA-seq was conducted. Compared with WT organoids, 574 genes were upregulated and 413 genes were downregulated in AUTS2-/- organoids (Fig4-2B). Pan-neuron markers, including FOXG1, TUBB3, STMN2 and NEFM were downregulated in AUTS2-/- organoids, indicating that depletion of AUST2 caused the neuronal differentiation defects. The gene ontology (GO) analysis revealed that downregulated genes in AUTS2-/- organoids were enriched in the functional terms related to neuron development, such as "axonogenensis", "peripheral nervous system development", "dendrite development", "dendritic spine development", and "positive regulation of neuron projection development" (Fig. 4-2C), further suggesting the essential role of AUTS2 in neurodevelopment. More than the involvement of AUTS2 in neuron differentiation and brain development, our result showed that the deletion of AUTS2 also negatively affect neuronal functionality, supported by the GO terms of AUTS2-/- downregulated genes, including "regulation of membrane potential", "regulation of postsynaptic membrane potential", "synapse organization", "synapse assembly", and "chemical synaptic

transmission postsynaptic". On the other hand, the upregulated genes showed the functional enrichment in the development process of tissues derived from mesoderm and endoderm, as GO terms such as "kidney development", "connective tissue development", "urogenital system development", and "cartilage development" (Fig. 4-2D). The RNA-seq of WT and AUTS2-/- organoids suggested that AUTS2 promotes neuron differentiation and represses the expression of genes functionally related to meso- and endodermal development.

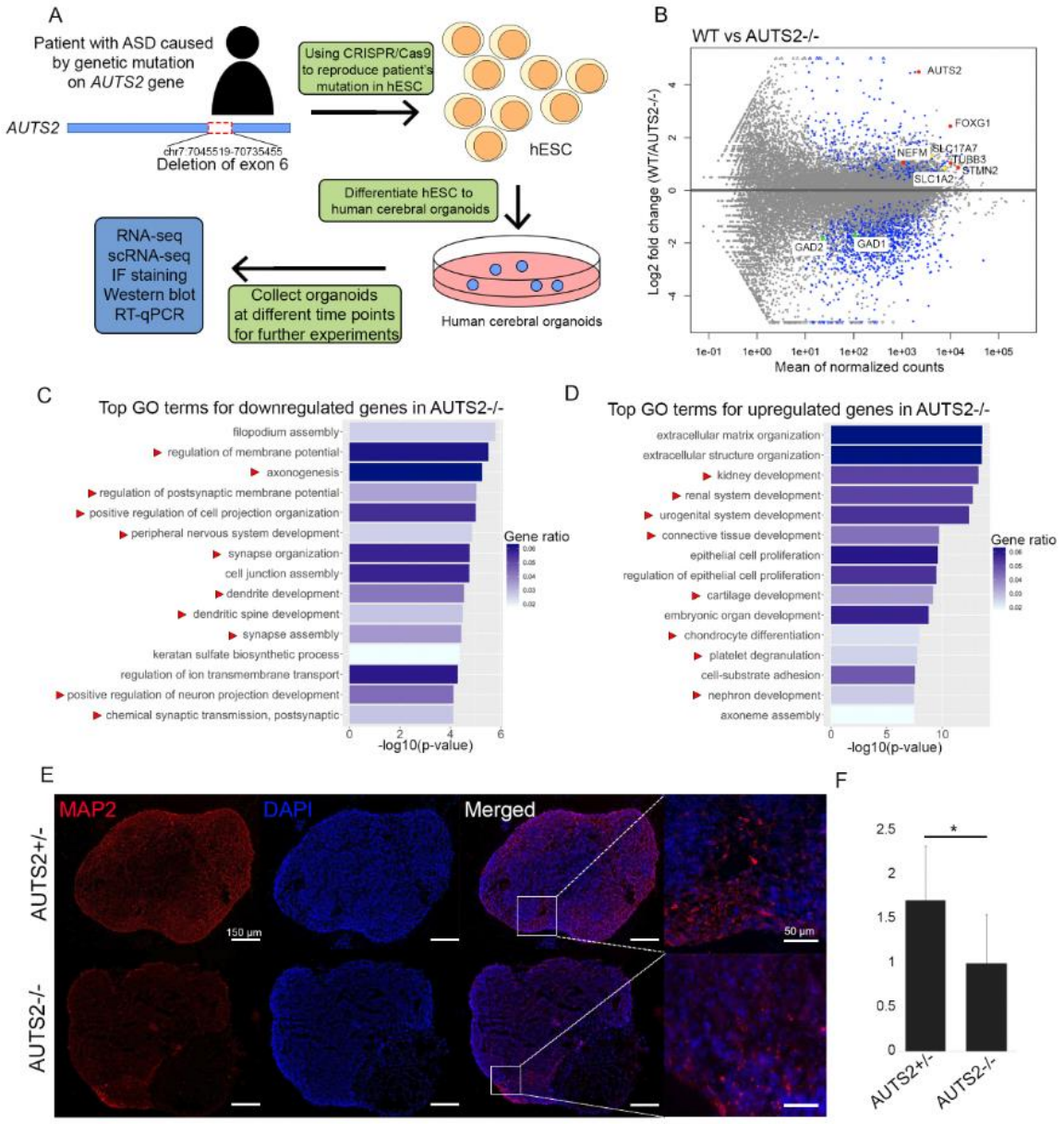

Figure 4-2: AUTS2 is essential for human brain development.

a, Schematic showed the overall experimental design of this study. **b,** MA plot of RNA-seq between WT and AUTS2-/- organoids at day 70. DEGs are labeled with blue dots and neuron-related genes are marked as red dots. **c,** Bar plot of GO analysis for the downregulated genes in AUTS2-/- organoids. Color of the bar presents the gene ratio. Neuronal differentiation and brain development related terms were highlighted with red arrow. **d,** Bar plot of GO analysis for the upregulated genes in AUTS2-/- organoids. Meso/endo-dermal-related terms were highlighted with red arrow. **e,** Immuno-fluorescence staining of neuronal marker, MAP2 in AUTS2+/- and AUTS2-/- organoids. **f,** Quantification of MAP2 immunofluorescence signal intensity between AUTS2+/- and AUTS2-/- organoids. Each bar is the mean of independent measurements with error bars representing standard error. * $p<0.05$.

As observed in patients, the mutations on one copy of AUTS2 is sufficient to cause neuron developmental diseases. Hence, we decided to compare the transcriptional profile between WT and AUTS2+/- organoids. Surprisingly, only a limited number of differential expressed genes (18 upregulated genes and 43 downregulated genes in AUTS2+/- organoids) were identified (Fig. 4-3B). Meanwhile, comparing with AUTS2+/- organoids, 932 downregulated genes and 1045 upregulated genes were identified in AUTS2-/- organoids, which were largely overlapped with the differential expressed genes between WT and AUTS2-/- (Fig. 4-3C). GO analysis of AUTS2+/- and AUTS2-/- organoids showed that the differential expressed genes were functionally enriched in the similar terms as it in the comparison between WT and AUTS2-/- (Fig. 4-3D, E).

Combining the RNA-seq results from WT, AUTS2+/- and AUTS2-/- organoids, it showed that the complete homozygous AUTS2 knockout severely compromised brain developmental process, while heterozygous knockout of AUTS2 showed no obvious difference with WT from transcriptional level. Therefore, we would focus on the comparison between AUTS2+/- and AUTS2-/- organoids in later experiments.

Next, we performed immunofluorescence staining of pan-neuron marker MAP2 on AUTS2+/- and AUTS2-/- organoids. Consistent with our findings in RNA-seq that suggested the necessity of AUTS2 in neuronal differentiation, AUTS2-/- organoids showed lower *MAP2* level, compared with AUTS2+/- (Fig. 4-2E, F). Together, our data highlighted the importance of AUTS2 in brain development. The complete deletion of AUTS2 leads to severe neuronal differentiation defects, while using cerebral organoids, the heterozygosity effect of *AUTS2* was hard to detect from transcriptome level.

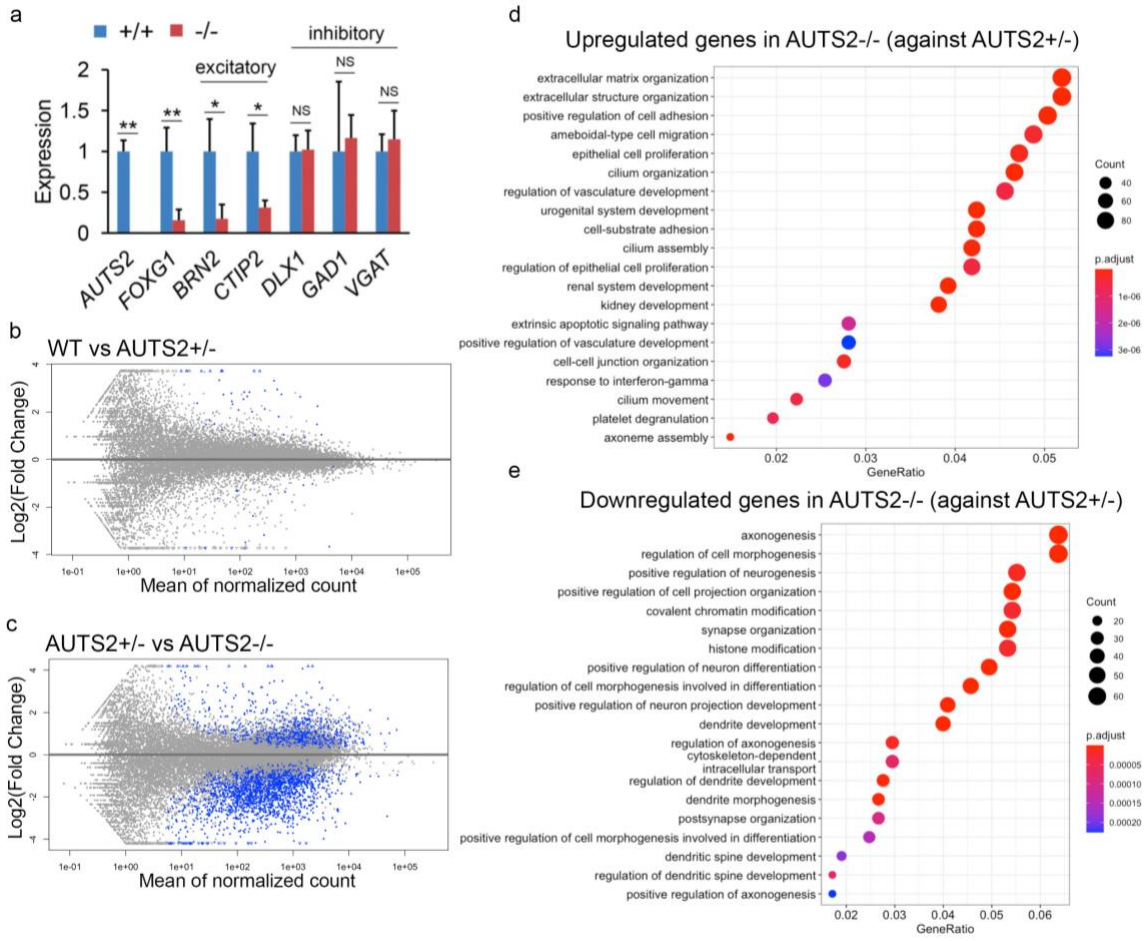

Figure 4-3: Comparison of bulk RNA-seq among the organoids from three genotypes.

a, RT-qPCR of AUTS2, inhibitory and excitatory neuron maker genes between WT and AUTS2-/- organoids. **b,** MA plot of the bulk RNA-seq between WT and AUTS2+/- organoids. DEGs are labeled with blue dots. **c,** MA plot of the bulk RNA-seq between WT and AUTS2+/- organoids. **d,** Bar plot of GO analysis for the upregulated genes in AUTS2-/- organoids, compared with AUTS2+/- organoids. **e,** Bar plot of GO analysis for the upregulated genes in AUTS2-/- organoids, compared with AUTS2+/- organoids. Each value is the mean of three independent measurements with error bars representing standard error. * $p<0.05$, ** $p<0.01$

AUTS2 regulates cell fate commitment by promoting neuron differentiation and repressing ChP generation

To further understand the extent to which AUTS2 play distinct roles in regulating cell fate choice during neuronal differentiation, we performed single cell RNA sequencing (scRNA-

seq) using Fluidigm C1TM Single-Cell Autoprep System (C1). A total of 400 cells from AUTS2-/- organoids and 800 cells from AUTS2+/- organoids were harvested at day 78 for this purpose. After filtering out cells with poor sequencing quality, there were 358 cells from AUTS2-/- organoids and 753 cells from AUTS2+/- organoids. Based on the transcriptional profiles, cells were divided into 9 subpopulations using unsupervised cluster algorithm (Fig. 4-4A). First, we identified the differentially expressed genes from each cluster and systematically compared the gene expression signatures with literatures (Quadrato et al. 2017; Franzén, Gan, and Björkegren 2019). We were able to determine five clusters that successfully recapitulated the cell identities discovered before using same differentiation method (C1, C2, C4, C6, and C8). Forebrain markers, FOXG1, a master regulator for cortex development, was highly expressed in C1 cells (Fig. 4-4C). Several forebrain and neuronal markers, including TUBB3, NEUROD6 and POU3F3, also showed high expression in C1. Based on this observation, we identified C1 cells as neurons (Fig. 4-4D). The expression of AUTS2 was positively correlated to neuron markers, supported by the high abundancy of AUTS2 in C1 cluster (Fig. 4-4C). C2 cells were characterized with high expression of retinal cell marker genes, such as RCVRN, NEUROD1 and NEUROD4 (Fig. 4-4C, D). Therefore, this cluster was assigned as retinal cell. Both forebrain and proliferation markers were highly expressed in C4 cells, suggesting its identity as radial glial cells (Fig. 4-4D). A small number of cells were clustered in C8, exclusively expressing neuroepithelial cells marker genes (Fig. 4-4D). Majority of cells from both C0 and C6 clusters highly expressed TTR (Fig. 4-4C). With the awareness of TTR as a canonical choroid plexus cell marker, we initially suspected both clusters were choroid plexus (ChP) cells. However, further analysis on other ChP markers, such as CLIC6, KRT18 and IGFBP7 has successfully revealed the transcriptional difference between C0 and C6, as these markers showed high expression level in all C6 cells but an inconsistent expression pattern across C0 cells (Fig. 4-5A). Therefore, we defined C6 as ChP cells and C0 as ChP-like cells.

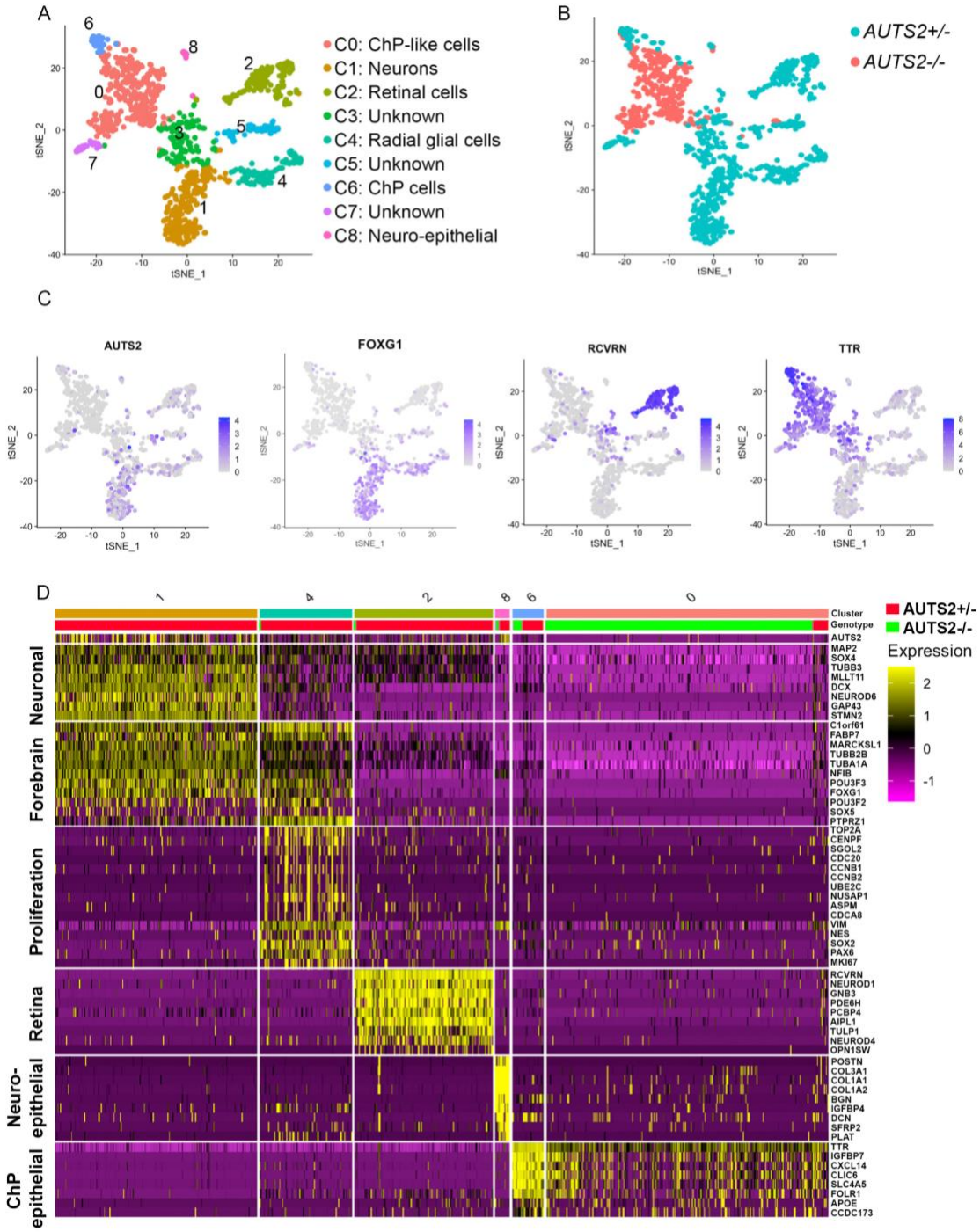

Figure 4-4: AUTS2 regulates cell fate commitment by promoting neuron differentiation and repressing ChP generation

a, tSNE plot of 1111 cells from AUTS2+/- and AUTS2-/- organoids at day 70, colored by the annotated cell types (C0-C12). **b,** tSNE plot of AUTS2+/- and AUTS2-/- organoids at day 70,

colored by the genotypes. **c,** Features plot of markers of AUTS2, forebrain marker (*FOXG1*), retinal neurons (*RCVRN*), and ChP epithelial (*TTR*). **d,** Heatmap of selected marker genes for neurons, forebrain, proliferation, retina, neuro-epithelial cells, and ChP epithelial cells. Clusters are labeled on top of the heatmap. Genotypes are color-labeled on the second row.

Noticeably, labeling the cells based on genotype, the results showed a strong separation between AUTS2+/- and AUTS2-/- cells, with more than 85% of cells (308/356) from AUTS2-/- organoids in C0 cluster (Fig. 4-4B, Fig. 4-5A). This observation suggested that complete deletion of AUTS2 leads to a skewed cell composition and the differentiated organoid lost cell diversity. In AUTS2+/- organoids, more than half of the cells were identified as neuron-related cells, including neurons and retinal cells (Fig. 4-5A). Over 20% of cells were radial glial cells and neuroepithelial cells. A small number of cells were defined as ChP cells, which agreed with the observation reported in other studies. On the other hand, AUTS2-/- organoids showed a biased cell composition with low proportion of neuron-related cell types (neurons, retinal cells, and radial glial cells) and unexpected high proportion of ChP-like cells (Fig. 4-5B). The choroid plexus is responsible for cerebrospinal fluid (CSF) production and serves as a barrier between blood and CSF. The choroid plexus contains a specialized epithelial cell, ependymal cell, which is characterized by the ciliated simple columnar form. ChP cells are different from neurons from morphological, functional aspects, which leads us to suspect the pathological impact of over-produced ChP cells. One predictable outcome of over-populated ChP cells is CSF overproduction, which has been observed in autism patients. Although there is no direct evidence showing patients with mutant AUTS2 to suffer from over-produced CSF, our data provides an insight that during brain development, AUTS2 regulates cell fate commitment by promoting neuron differentiation and repressing ChP cells generation.

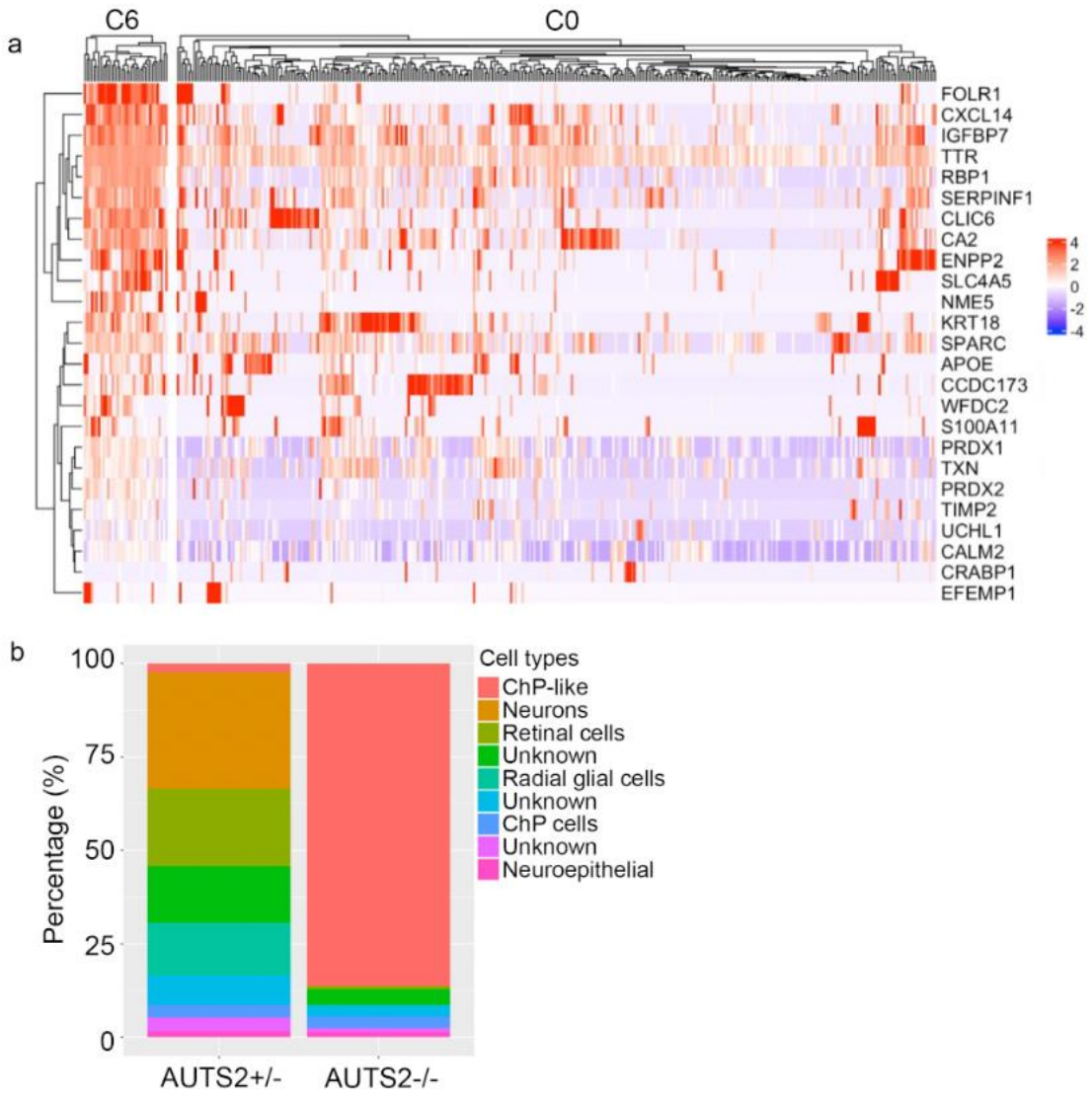

Figure 4-5: Comparison between C0 and C6 and proportion of different cell types in AUTS2+/- and AUTS2-/- organoids.

a, Heatmap of selected ChP epithelial cells markers of the cells in cluster 0 (C0) and cluster 6 (C6). Z-score is used for plotting. **b,** Stacked percentage bar plot shows the proportion of different cell types in *AUTS2+/-* and *AUTS2-/-* organoids.

AUTS2 starts to regulate neuronal differentiation at neuroepithelial cell stage

To achieve successful differentiation, numbers of epigenetic regulatory elements need to function at precise timing. Our previous experiments have proved the importance of AUTS2 at early stage of brain development and hence, our next step is to investigate the effect of AUTS2 on lineage differentiation trajectories of AUTS2 by performing trajectories reconstruction analysis on our scRNA-seq data.

Consistent with clustering results, the pseudo time trajectory analysis showed a clear separation between AUTS2-/- and AUTS2+/- cells (Fig. 4-6A). Based on the previous result in which neuroepithelial cells (C8), ChP (C6) and ChP-like cells (C0) were clustered together, our trajectory analysis showed that forebrain neuron (C1) and radial glial cells (C4) were located on same branch, while retinal cells (C2) were on the different trajectory (Fig. 4-6B). Since neuroepithelial cells are the first cell type that would later differentiate into other cell types, such as radial glial cell, neurons and ChP cells, we set neuroepithelial cells as root for pseudo time analysis. As demonstrated in Fig. 4-6B and C, neuroepithelial cells split into three different branches, leading to neuron, photoreceptor cells and ChP cells, respectively (Fig. 4-6B, C).

The stacked percentage bar plot of each cluster showed that AUTS2-/- and AUTS2+/- organoids showed no obvious difference in cell types at early stages, including neuroepithelial cells (C0) and ChP cells (C6). The differentiation defect of AUTS2-/- organoids happened at later stages, with less than 10% of AUTS2-/- cells in radial glial cells (C4), neurons (C1), and retinal cells (C2) (Fig. 4-6D), suggesting that AUTS2 regulates differentiation at neuroepithelial cell stage. To test this hypothesis, we arranged the cells based on pseudo time and plotted the expression of selected genes across time. The expression of AUTS2 was maintained at a low level

during early stage of brain development (neuroepithelial cells and ChP cells) and then, gradually increased at later stage (neuron and retinal neurons) (Fig. 4-6E). As expected, neuron markers (*MAP2*) and retinal markers (*NEUROD1*) showed a positive correlation with AUTS2 level. In contrast, ChP marker showed a negative correlation with AUTS2 (Fig. 4-6E). These observations suggested that AUTS2 modulates cell fate commitment at neuro-epithelial cell stage and the complete removal of AUTS2 forced the differentiation direction of neuroepithelial cells to choroid plexus cells, rather than towards terminally differentiated neurons.

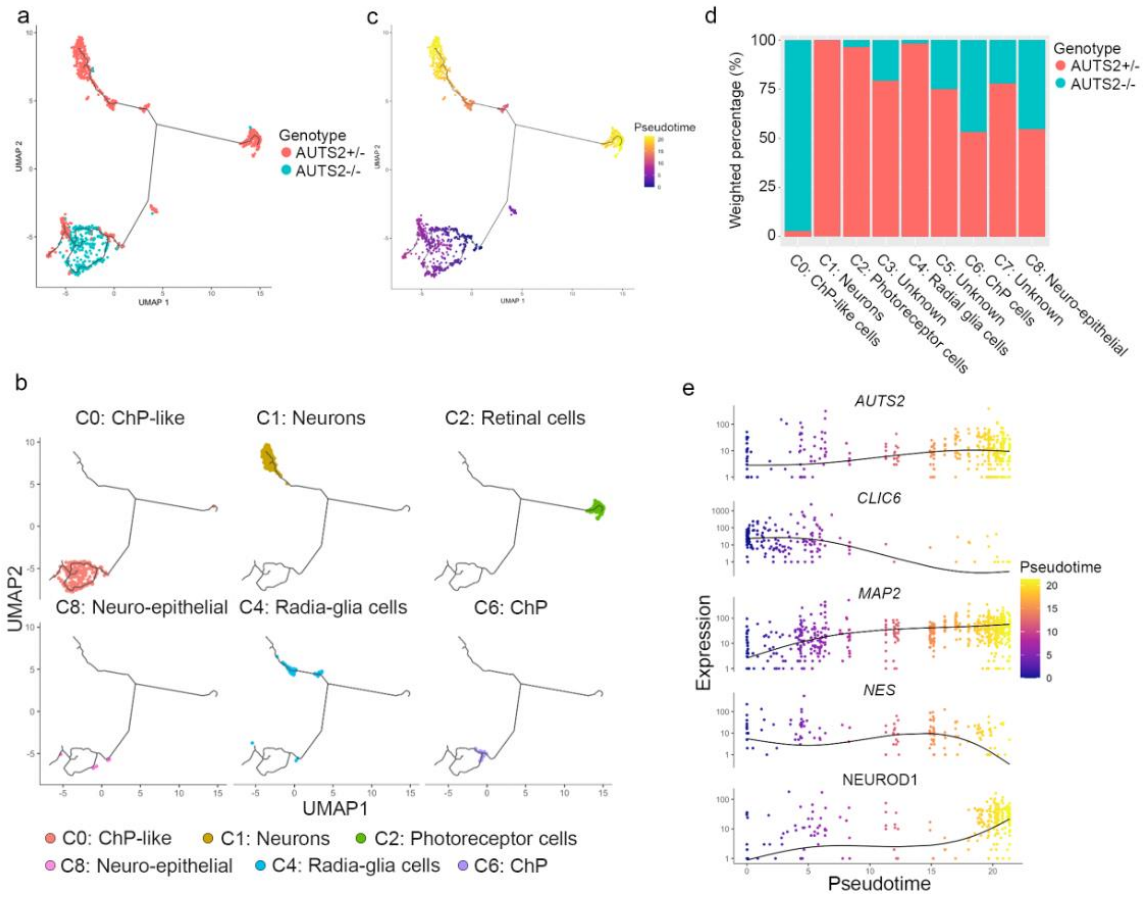

Figure 4-6: Trajectory analysis of scRNA-seq.

a, UMAP plot of 1111 cells from AUTS2+/- and AUTS2-/- organoids at day 70, colored by genotypes. Predicted trajectory is shown as branches. b, UMAP plots of six identified cell types (C0: ChP-like, C1: Neurons, C2: Retinal cells, C8: Neuro-epithelial, C4: Radial glial cells, and C6: ChP) demonstrate the relative position along the trajectory graph. c, Predicted pseudotime of

cells in AUTS2+/- and AUTS2-/- organoids. Based on the trajectory graph and biology of brain development, neuro-epithelial cell (C8) is set as root. d, Stacked percentage plot shows the weighted proportion of AUTS2+/- and AUTS2-/- cells in each cluster. e, Expression pattern of selected genes, AUTS2, ChP marker gene (*CLIC6*), neuronal marker (*MAP2*), neuro-epithelial cell marker (*NES*), and retina cell marker (*NEUROD1*) across the pseudo-time.

AUTS2 represses the transcription of WNT/β-CATENIN signaling pathway during forebrain development

Differentiation and development are tightly regulated by intricate signaling pathways, ensuing the progress from a single cell to a living organism. Disruption of vital signaling pathways could cause pathological consequences. Aware of the importance of AUTS2 in brain development, we tend to understand the molecular mechanism of AUTS2 by exploring the signaling pathways that were potentially affected by AUTS2 removal. First, we performed integrity pathway analysis (IPA) on bulk RNA-seq data of day 78 organoids (Fig. 4-7A). In WT organoids, multiple top activated pathways are tightly related to functionality of neurons and neurogenesis, including “CREB signaling in neurons”, “Neuropathic pain signaling in dorsal horn neurons”, and “Synaptic long-term depression” (Fig. 4-7A), further confirming the importance of AUTS2 in neuronal differentiation. In AUTS2-/- organoids, the only overactivated signaling pathways was WNT/β-Catenin signaling pathway, suggesting the role of AUTS2 as WNT/β-Catenin signaling repressor. Based on the scRNA-seq data, complete removal of AUST2 led to the over-populated ChP cells and decrease in neurons. Coincidentally, Lancaster group has established a organoids differentiation protocol that can induce ChP cells formation by modifying the cerebral organoids protocol we used with 3-day treatment of WNT/β-Catenin and BMP stimulators at early stage (Pellegrini et al. 2020). Their study showed that by activating WNT and BMP signaling pathway, it converts cerebral organoids to choroid plexus organoids could efficiently produce functional ChP cells, which forms fluid-filled cavity and generates CSF. As our scRNA-seq has shown the over-populated ChP-like cells and IPA analysis indicated over-

activated WNT/β-Catenin signaling pathway in AUTS2-/- organoids, we hypothesized that AUTS2 serves as a repressor to WNT/β-Catenin signaling pathway during brain development. To test this hypothesis, first we generated a total AUTS2 knockout 293T cell line using CRIPSR/Cas9 system. Deletion of AUTS2 has been confirmed by PCR and immunoblotting (Fig. 4-7A, B, C). Subsequently, TOPFlash/-FOPFlash luciferase reporter system was employed to measure the influence of AUTS2 on WNT signaling pathway target genes (Fig. 4-7 A). Compared with WT, AUTS2-/- cells showed significantly higher luciferase signal, validating the repressive role of AUTS2 in WNT/β-Catenin signaling pathway.

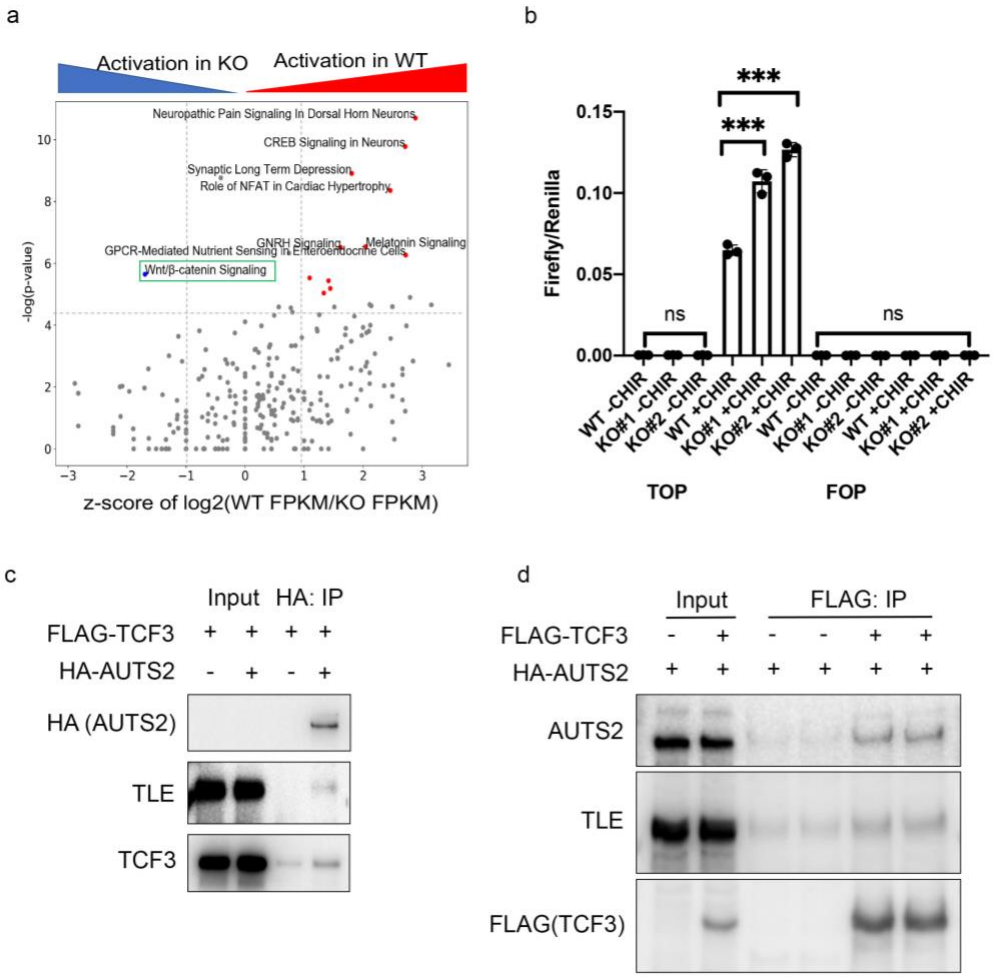

Figure 4-7: AUTS2 represses the transcription of WNT/β-CATENIN signaling pathway during forebrain development.

a, volcano plot of IPA analysis, where x-axis represents the z-score of log2 fold change gene expression between WT and AUTS2-/- organoids and y-axis is log10 p-value. Pathways overactivated in WT are labeled with red and overactivated pathway in AUTS2-/- is labeled with blue. **b,** Bar plot of luciferase assay between WT and AUTS2-/- HEK293 cells. **c,** Immunoblotting of HA-pulldown assay validates the interaction between AUTS2 and TCF3/TLE3. **d,** Immunoblotting of FLAG-pulldown assay confirms that TCF3 interacts with AUTS2 and TLE3.

Since we have proved that the absence of AUTS2 promoted WNT signaling activity, we tend to understand the molecular mechanism of AUTS2 as a WNT/β-Catenin signaling repressor. When we revisit our previous mass spectrometry of AUTS2, we found that TLE3 was shown as potential interacting partner of AUTS2, whose main function is the repressor of the WNT/β-Catenin signaling pathway. TLE family proteins act as transcriptional switch to repress WNT target gene expression through its interaction with TCF3. Given the evidence of the overactivated WNT/β-Catenin signaling pathway in AUTS2-/- organoids, we speculated that AUTS2 inhibits the WNT/β-Catenin signaling pathway by interacting with TCF3/TLE. To test the interaction between AUTS2 and TLE/TCF3, HA-tagged AUTS2 and FLAG-tagged TCF3 were ectopically expressed in 293T cells and co-immunoprecipitation was performed using FLAG-conjugated M2 beads. As expected, TLE co-immunoprecipitated with FLAG-TCF3 and we discovered the interaction between TCF3 and AUTS2 (Fig. 4-7C). To further confirm the association of AUTS2 with TLE and TCF3, we performed a reciprocal co-IP assay against HA-tagged AUTS2 and have successfully validated the formation of AUTS2/TCF3/TLE complex (Fig. 4-7D). Taken together, our results suggested that AUTS2 interacts with TLE and TCF3 to repress the WNT/β-Catenin signaling repressor and its repressive role might be important for proper brain development.

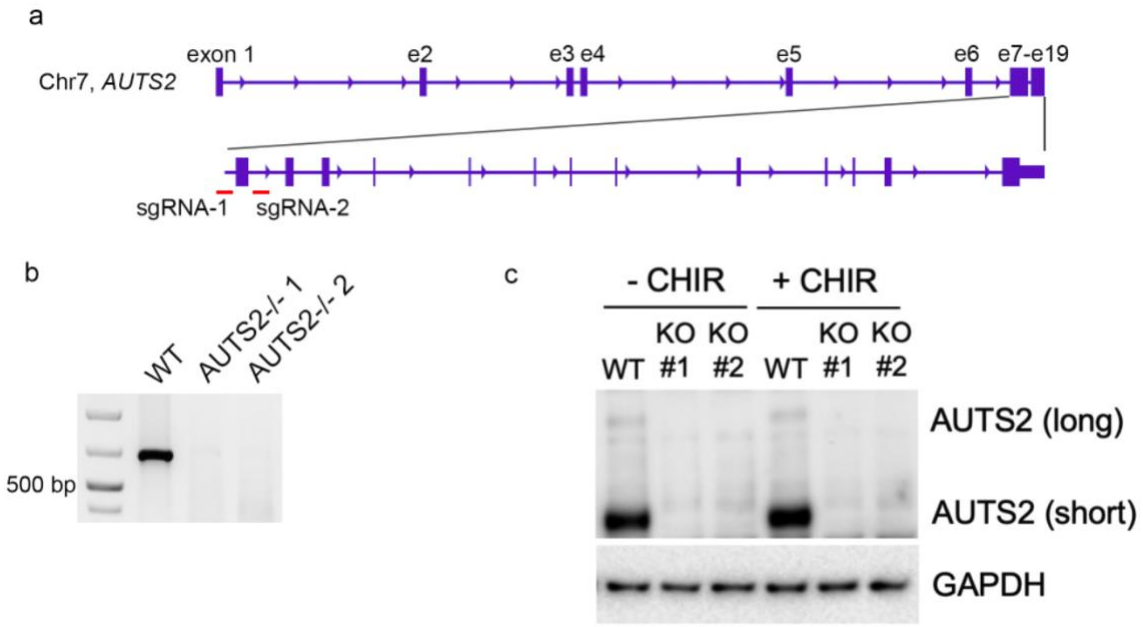

Figure 4-8: The generation of AUTS2-/- HEK 293T stable cell line

a, Schematic of the CRISPR/Cas9 design for AUTS2-/- HEK 293T cell line. sgRNA is designed to target exon 7 of *AUTS2*. **b,** genotyping PCR confirms the successful knockout of AUTS2. **c,** Immunoblotting shows the AUTS2 protein level in of WT and AUTS2-/- cells with/without CHIR treatment.

Neuronal differentiation defect caused by depletion of AUTS2-/- can be partially rescued with WNT inhibitor treatment

To examine if the differentiation abnormities observed in AUTS2-/- cerebral organoids were caused by the overactivation of the WNT/β-Catenin signaling pathway, we performed a rescue experiment by treating AUTS2-/- organoids with XAV939, a well-known WNT inhibitor (Shetti et al. 2019). Since our trajectory analysis showed AUTS2 begins its function at early stage, we decided to treat the AUTS2-/- organoids with XAV939 at expansion stage (day 7 to day 10). When culturing the human brain organoids, across different groups, we observed no morphological difference at early stage, from EB formation to expansion (Fig. 4-9A). However, in the maturation phase, majority of AUTS2-/- organoids had the dark spots, which were hardly

found in WT organoids (Fig. 4-9A). Surprisingly, these dark spots were missing in the AUTS2-/- organoids treated with XAV939. Since our previous experiments suggested that the over-activated WNT signaling pathway led to over-populated ChP cells in AUTS2-/- organoids and the sensitivity of the dark spots towards WNT inhibitor, we suspect these dark spots might be enriched with ChP cells. To delve into the transcriptional characteristics of these dark spots, we isolated these dark spots from AUTS2-/- brain organoids (day 35) by gently pipetting the organoids. The structure of these brain organoids was soft and could be disrupted easily by pipetting. Meanwhile, the dark spots remained intact (Fig. 4-9B). We successfully extracted RNA and performed RNA-seq on the isolated dark spots, with intact AUTS2-/- organoids as control. After calculating the gene abundancy, genes were ranked based on the fold change of its expression between dark spots and AUTS2-/- organoids. Numbers of ChP cell markers, including *FOLR1, TTR, IGFBP7, CLIC6* and *SLC4A5*, were overexpressed in dark spots. Meanwhile, marker genes for neurons and retinal cells, such as *FOXG1, BGN* and *RCVRN*, showed lower expression levels in dark spots, as compared with AUTS2-/- organoids (Fig. 4-9D). The overexpression of ChP marker, TTR was further confirmed using RT-qPCR (Fig. 4-9D, 4-10A). Furthermore, the section of cerebral organoids showed these dark spots were characterized as cavity structures with mono-layer cells on the edge, which mimic ventricles in the brain (Fig. 4-8). Therefore, we identify these dark spots a morphological phenotype caused by AUTS2 removal and was composed of ChP cells.

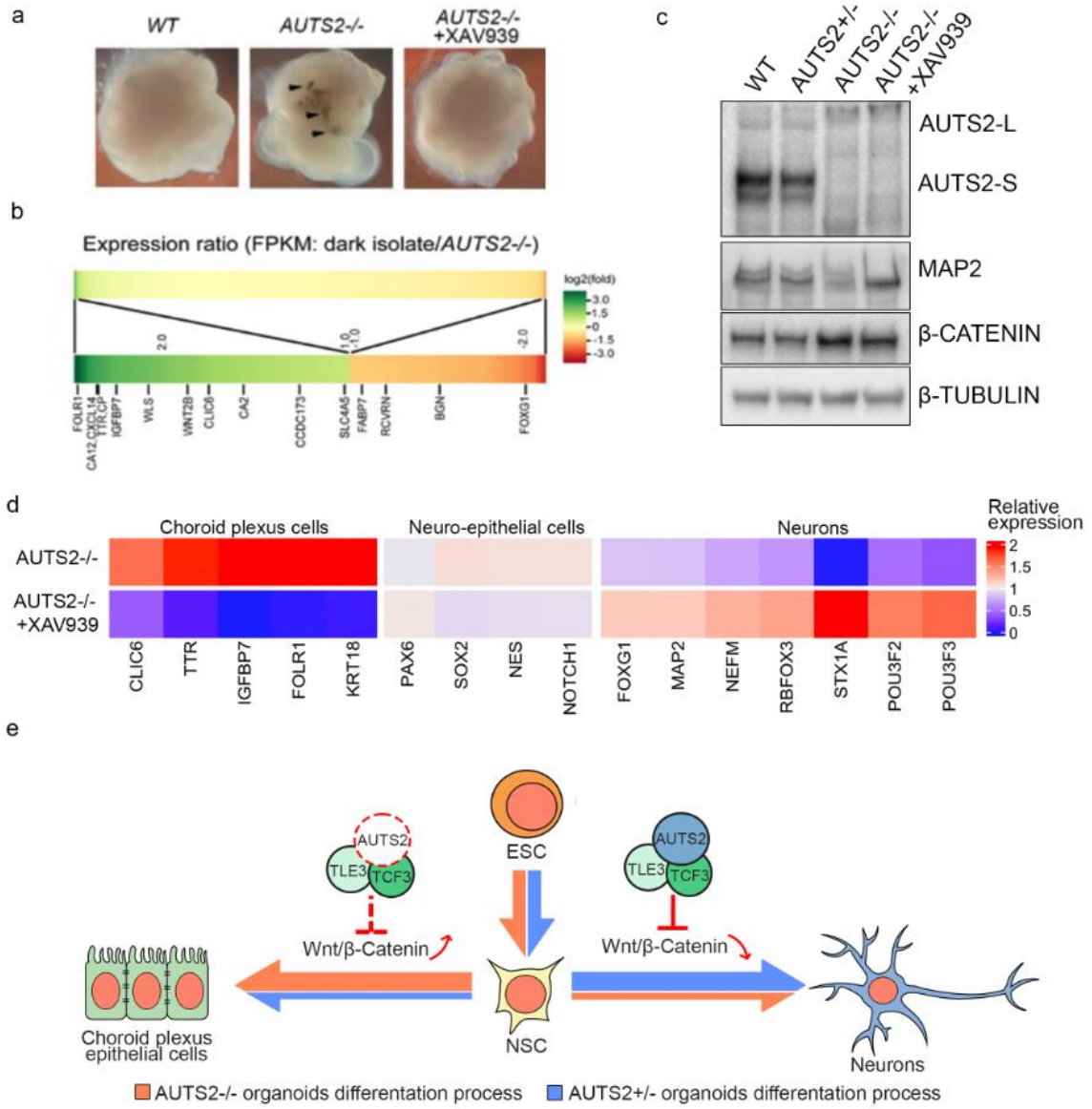

Figure 4-9: Neuronal differentiation defect caused by depletion of AUTS2-/- can be partially rescued with WNT inhibitor treatment.

a, Morphology of WT, AUTS2-/- and AUTS2-/- with XAV939 treatment organoids at day 65. The black spots enriched in AUTS2-/- organoids are labeled with black arrow. **b,** Fold changes of gene expression between AUTS2-/- and AUTS2-/- with XAV939 treatment. Selected neuronal marker genes and ChP epithelial marker genes are annotated. **c,** Immunoblotting shows the protein level of AUTS2, neuronal marker MAP2, WNT signaling pathway component β-CATENIN in WT, AUTS2+/-, AUTS2-/- and AUTS2-/- with XAV939 treatment groups. **d,** Heatmap of the expression level of selected ChP epithelial, neuro-epithelial and neuron markers. **e,** Schematic showed the model of how AUTS2 regulates human brain development.

In addition to the disappearance of dark spots after WNT inhibitor treatment, we also examinate the rescue effect from transcriptional and protein levels. Consistent with our previous experiments suggesting the importance of AUTS2 in neuron differentiation, AUTS2-/- organoids showed a lower MAP2 level using immunoblotting, compared with WT and AUTS2+/- organoids. As we suspected, the neuron differentiation defected in AUTS2-/- organoids was caused by over-activated WNT/β-Catenin signaling pathway, XVA939 treatment rescued the low expression of MAP2 in AUTS2-/- organoid. Agreed with IPA results, immunoblotting results showed that AUTS2-/- organoids had high β-Catenin level, compared with WT and AUTS2+/- organoids. To our surprise, XAV939 could not subside the high β-Catenin level observed in AUTS2-/- organoids, potentially due to the short treatment period. Next, we investigate if the treatment of XVA939 could rescue the phenotype from transcriptional level. Compared with untreated AUTS2-/- organoids, overexpression of ChP cell markers genes, including *TTR, CLIC6, KRT18,* and *SLC4A5* in AUTS2-/- brain organoids was diminished by the WNT inhibitor (Fig. 4-9D). Lower expression of neuron markers, such as *FOXG1, MAP2 and NEFM* in AUTS2-/- were also being reversed by the addition of XVA939. Despite the dramatic changes in the expression of neuron and ChP cell markers, no obvious difference was observed on the neuroepithelial markers. The effect of XVA939 on the ChP (TTR, FOLR1 and SLC4A5) and neuronal (FOXG1, CTIP2 and BRN2) marker genes was then further validated by RT-qPCR (Fig. 4-10A).

To gain a better understanding on the effect of XVA939 on cell fate commitment, we crossed our RNA-seq data with Panglao database, which integrates scRNA-seq from multiple studies, aiming to identify cell markers. ChP cell markers demonstrated a strong tendency to reduce its expression level upon XAV939 treatment (Fig. 4-9C). Marker genes for other cell types, including radial glial cells, immature neurons, neurons, and retinal cells showed no significant difference between XVA939 treated and untreated AUTS2-/- organoids. Besides the repressive function of AUTS2 on WNT/β-Catenin signaling pathway, AUTS2 has multiple other

roles, such as a component in PRC1 complex, which causes the incompetence of XAV939 to fully rescue the phenotype. As we mentioned previously, deletion of AUTS2 caused the imbalance between excitatory and inhibitory neurons; RNA-seq results showed XVA939 treatment exerted no obvious effect on GABAergic neuron (inhibitory neuron) markers but caused an increase in glutamatergic neuron (excitatory neuron) markers (Fig. 4-10C).

In summary, our study has successfully revealed an essential role of AUTS2 in human brain development. AUTS2 initiates its differentiation regulatory function at neuroepithelial stage by promoting neuron generation and repressing the ChP cell differentiation. We have also proved that AUTS2 serves as a repressor of the WNT/β-Catenin signaling pathway, by forming a complex with TCF3 and TLE (Fig. 4-9E).

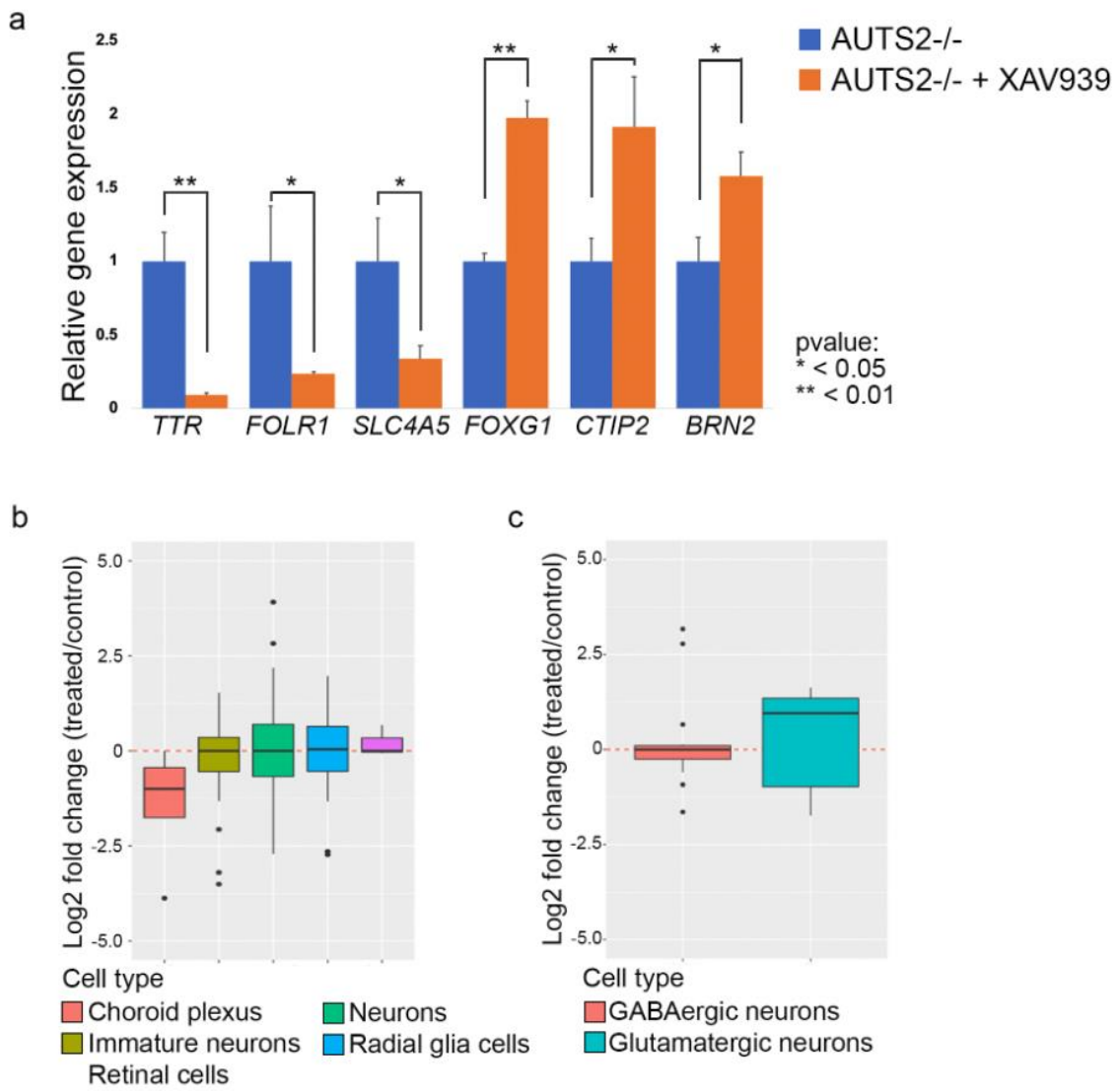

Figure 4-10: WNT inhibitor XAV939 treatment rescues the imbalance between inhibitory and excitatory neurons in AUTS2-/- organoid.

a, RT-qPCR results showed the expression level changes of ChP marker (*TTR, FOLR1* and *SLC4A5*) and neuron markers (*FOXG1, CTIP2* and *BRN2*). **b,** Box plot shows the fold change of selected ChP markers, neuron markers, immature neurons marker, radial glial cell markers and retinal cells markers between AUTS2-/- and AUTS2-/- with XAV939 treatment. **c,** Box plot shows the fold change of selected GABAergic and glutamatergic neuron markers between AUTS2-/- and AUTS2-/- with XAV939 treatment.

Discussion

Delicacy of the human brain has made it difficult to understand the process of brain development using both *in vivo* and *in vitro* model organisms. The human cerebral organoids culturing technique, developed by Lancaster et. al., provides a possibility to study the brain development process *in vitro*, as it can faithfully recapitulate the cell composition and neuron functionality in human developing cerebral cortex (Lancaster et al. 2013). In this study, we took advantage of this system to examine the impact of AUTS2 in human brain development. Our bulk RNA-seq results of 70-day old brain organoids indicated that the complete removal of AUTS2 caused severe neuronal developmental defect by down-regulating neuron development/differentiation-related genes and up-regulating endo/meso-dermal development genes. To our surprise, the transcriptional profile of AUTS2+/- organoids showed no obvious difference from wild type. The possible explanation is that the method we used mimics the early-stage brain development and hence, we fail to detect the difference between WT and AUTS2+/- that might happen at late stage of development. Despite the fact that multiple studies have shown the capability of this 3D cerebral organoid culture to recapitulate the pathological process of certain NDDs, we still cannot rule out the technical limitation of this system. Nevertheless, our results emphasize the importance of AUTS2 during the early-stage human brain development.

In AUTS2 patients, absence of only one copy of AUTS2 is enough to cause phenotype but our transcriptional analysis suggested its similarity with wild type. To better reflect the importance of AUTS2, we decided to compare AUTS2+/- and AUTS2-/- organoids in subsequent experiments. We applied scRNA-seq to dissect the cell composition in AUTS2+/- and AUTS2-/- organoids as the functionality of the brain depends on the orchestration of different cells and abnormality in cell composition can lead to pathological consequences. Removal of AUTS2

promoted the enrichment of ChP cells, a specialized ependymal cell located at ventricles, and reduction of neuron-related cell types, including radial glial cells, neurons, and retinal cells. ChP cell is responsible for CSF production to maintain the wellness of human brain and overpopulated ChP cells may lead to overproduction of CSF, an abnormalities observed in ASD patients (Shen 2018). Our pseudo-time analysis suggested that AUTS2 was involved in differentiation at neuro-epithelial cell stage and hence, removal of AUTS2 in brain organoids caused most of the neuro-epithelial cells to transform into ChP cells instead of neurons. Taken together, our scRNA-seq results proposed a regulatory role of AUTS2 in human brain development by promoting neuronal differentiation and repressing ChP cell differentiation, starting at neuro-epithelial cells stage.

Previous studies have proved that AUTS2 regulates neuronal development in a PRC1-dependent manner (Gao et al. 2014; Monderer-Rothkoff et al. 2021). Studies on the functionality of AUTS2 isoforms suggested that long and short isoforms of AUTS2 contribute to neuronal differentiation, but only the long isoform is capable of interacting with PRC1 complex, which prompted us to explore the PRC1-independent role of AUTS2. Here, our study demonstrated a potential role of AUTS2 in repressing the WNT/β-Catenin signaling pathway by forming a complex with TCF3/TLE3. The WNT/β-Catenin signaling pathway exerts its impact on brain from early patterning stage to adult brains. During neurogenesis, hyperactivation of the WNT/β-Catenin signaling pathway caused the expansion of the neural precursor population (Chenn and Walsh 2002), and inhibition of this signaling pathway induces the premature cell cycle exit of precursor cells and differentiation into neuron (Woodhead et al. 2006). When AUTS2 is absent, our transcriptome analysis showed that the WNT/β-Catenin signaling pathway is overactivated in the brain organoids, which was further confirmed by immunoblotting analysis. We speculated that high WNT activity in the brain organoids altered the cell composition by promoting the generation of ChP cells and inhibiting the production of neurons. Based on our trajectory analysis

and literature, we decided to treat AUTS2-/- organoids with WNT inhibitor from day 7 to day 10, which is a crucial time point for neuroepithelial cells differentiation into other cell types. Treating the organoids with XAV939 for three days reversed the differentiation defect by inducing the neuronal marker expression and repressing ChP cell marker genes. Nonetheless, our immunoblotting analysis showed that the WNT inhibitor treatment failed to rescue the accumulation of β-catenin in AUTS2-/- organoids, suggesting that AUTS2 might have a long-lasting repressive effect on WNT signaling pathway.

Taken together, our study has demonstrated that AUTS2 plays an important role in the maintenance of the neuronal cell identity by exerting its function as a repressor of the WNT/β-Catenin signaling pathway through the interaction with TCF3 and TLE3. Given the strong association between AUTS2 mutations and NDDs, our study may provide a novel therapeutic approach by targeting WNT/β-Catenin signaling pathway.

Chapter 5

Discussion

Overall conclusion

In this dissertation, we explored the function of AUTS2 at different stages of neuronal differentiation. During the differentiation process from embryonic stem cells to neuronal progenitor cells, Auts2 plays an important role in promoting ectodermal cells generation and repressing the production meso/endodermal cells via its repressive role on BMP signaling pathway (**Fig. 5-1**). Our data discovered a novel protein complex, containing AUTS2, WDR68 and SKI, which captures pSMAD1/5/9 in nuclei. AWS complex then promotes poly-ubiquitin protein degradation of pSMAD1/5/9 by recruiting DDB1/CUL4 complex. In the development of human brain, AUTS2 mediates the cell fate commitment at neuro-epithelial cell stage, in which complete removal of AUTS2 hinders the differentiation from neuro-epithelial cells to neuron-related cells and results in the overpopulation of ChP epithelial cells. From molecular level, AUTS2 interacts with TLE3/TCF3 to repress WNT/β-CATENIN signaling pathway (**Fig. 5-1**). Together, our data demonstrates a detailed function of AUTS2, as well as the underlying molecular mechanisms at different stages of brain development. This research provides insights into the regulatory role of AUTS2 in neuronal differentiation, which potentially contributes to the development of new therapeutics.

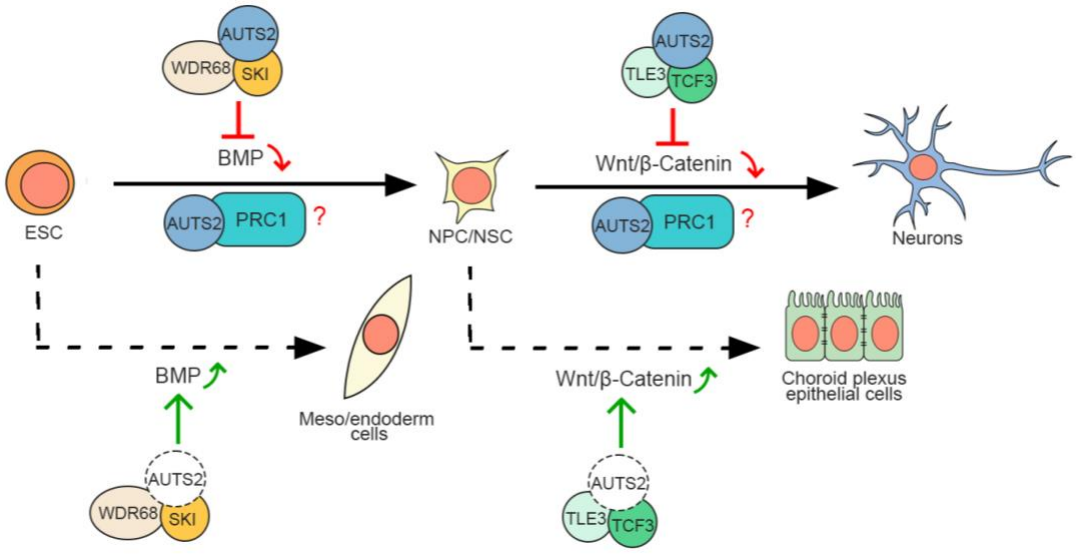

Figure 5-1: Summary of AUTS2 function at different stage of brain development.

In the differentiation process from mESC to NPC, AUTS2 formed a stable complex with WDR68 and SKI (AWS complex), which repressed the activity of BMP pathway. The impairment of this protein complex hindered the differentiation to NPC and promoted the expression of meso/endoderm marker genes. In the process of NPC/NSC to neurons, AUTS2 worked with TCF3/TLE3 to repress WNT/β-Catenin signaling pathway, which facilitated the generation of neuron and suppress the production of ChP-like cells.

The function of AUTS2 on maintaining the transcriptional balance between ectoderm and endo/mesoderm genes

In this study, we used two models, mouse ESC/NPC and human ESC/cerebral organoid, to investigate the impact of AUTS2 on transcription profile regulation. After performing RNA-seq experiment, the GO analysis showed that the removal of *Auts2* in mouse ESC led to the low expression of ectoderm and neuron differentiation-related genes, which highlighted the essential role of AUTS2 in neuronal differentiation. The up-regulated genes in *AUTS2* knockout cells displayed the functional enrichment in meso/endoderm and its derived tissues, including heart, kidney, reproductive and circulation system development (**Fig. 3-5, 4-3**). This observation indicated the role of AUTS2 in maintaining the balance of differentiation between ectoderm and

meso/endoderms. The transcriptional signature observed in *AUTS2* knockout cells has been reported in the cells with dysregulated TGF-β/BMP signaling pathway. Before our study, AUTS2 was known to function in a PRC-dependent manner. One study displayed that from transcription level, the potential role of PRC1.5 in mediating TGF-β/BMP signaling pathway as certain core components in the pathways were targeted by PRC1.5 complex (Yao et al. 2018). Our data demonstrated that from protein level, AWS protein complex repressed BPM signaling pathway by promoting the protein degradation of phosphorylated SMAD1/5/9 (**Fig. 5-1**). Moreover, the phenotype of the imbalanced differentiation in Auts2-/- cells can be rescued by BMP inhibitor treatment, indicating the involvement of BMP pathway in germ layers differentiation. Together, these data displayed a model of Auts2 in neural differentiation, in which Auts2 attenuated BMP signaling pathway via facilitating the protein degradation of pSMAD1/5/9, and in turn, promoted the neuron/ectoderm differentiation and repressed meso/endoderm differentiation.

In the human cerebral organoid study, RNA-seq between WT and AUTS2-/- organoids showed similar gene expression pattern as it in mouse ESC/NPC, characterized by the downregulation of neuron differentiation and neuron functionality-related gene and the upregulation of kidney, blood and urogenital development-related genes. In the differentiation process from human ESC to cerebral organoid, we discovered another PRC1-independent function of AUTS2 in mediating the activity of WNT signaling pathway. Although the treatment of WNT inhibitor can rescue part of phenotypes, such as the overproduction of ChP cells and low yield of neurons, we lack enough data to fully support the validity of AUTS2-WNT-germ layer gene axe. Together, our data revealed the dual function of AUTS2 in regulating the transcription balance of three germ layer genes.

The impact of AUTS2 on cell fate commitment during brain development

In the differentiation from human ESC to cerebral organoid, our scRNA-seq displayed a shift of cell population, from neuron-related cell types to ChP epithelial cells, which was caused by the disruption of AUTS2. ChP cells are located on the lumen of ventricle in brain and the two major biological function of ChP cells includes the production of CSF and maintain the blood-brain barrier. The over-populated ChP cells in AUTS2-/- organoids potentially resulted in the overproduction of CSF, which has been observed in certain autism patients (Shen 2018). Pathway analysis from bulk RNA-seq showed the overactivated WNT signaling pathway in AUTS2-/- organoids. Coincidentally, a recent published paper made adjustment on the cerebral organoid culturing method to generate ChP organoid, by adding WNT and BMP stimulators at early stage (Pellegrini et al. 2020). It fits our finding of AUTS2 in regulating BMP and WNT signaling pathway, while the overactivated WNT signaling was dominant in cerebral organoid, which caused the cell population alteration, which might serve as a potential therapeutic target. Since the phenotypes described previously are mainly in AUTS2 homozygous knockout cells, it may also limit the clinical translational potential of this study to understand the pathological process in AUTS2 mutant patients, as all reported patients are AUTS2 heterozygous mutation. This study provides novel insights into the biological function of AUTS2 in brain development in promoting the generation of neuron-related cells and repressing ChP cells via WNT signaling pathway.

Potential role of AUTS2 in other biological processes

Our findings expand the understanding of AUTS2 in differentiation in regulating two vital signaling pathways, BMP and WNT/β-Catenin pathway. These two pathways are known to be tightly involved in multiple pathways, including differentiation, cell proliferation and

carcinogenesis (M. Katoh and Katoh 2007; Lustig and Behrens 2003; Logan and Nusse 2004; J. Zhang and Li 2005; Bandyopadhyay, Yadav, and Prashar 2013; Z. Li and Chen 2013). Although majority of AUTS2 studies focus on its role in brain development, a few studies also suggested its role in cancer (Denk et al. 2012; Han et al. 2015). Analyzing the public transcriptome data, results showed that patients with AUTS2 mutation potentially affect TGF-β and SHH signaling pathways (Han et al. 2015). Taking advantage of TCGA database, our preliminary analysis showed that certain cancer patients, such as acute leukemia (LAML), can be stratified by AUTS2 expression and the survival rate showed significant difference, further suggesting its potential role in cancers (**Fig. 5-2**). However, to fully understand the impact of AUTS2 in cancers, more efforts are needed. Considering the importance of genetic mutations in the development and process of cancer, further stratifying patients based on their genetic signatures and AUTS2 expression could provide more insights into the impact of AUTS2 on cancer subtypes.

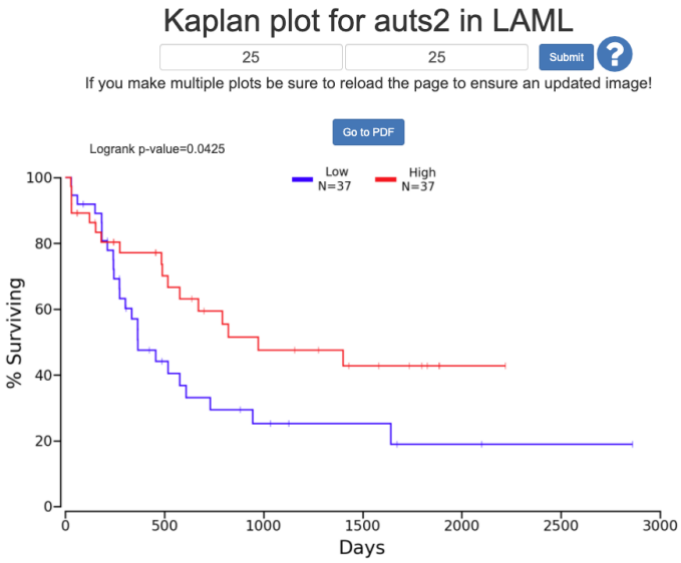

Figure 5-2: Survival rate of patients with high expression and low expression of AUTS2 in acute myeloid leukemia (LAML).

References

Abbas, Tarek, Uma Sivaprasad, Kenta Terai, Virginia Amador, Michele Pagano, and Anindya Dutta. 2008. "PCNA-Dependent Regulation of P21 Ubiquitylation and Degradation via the CRL4Cdt2 Ubiquitin Ligase Complex." *Genes and Development* 22 (18): 2496–2506. https://doi.org/10.1101/gad.1676108.

Abrahams, Brett S., Dan E. Arking, Daniel B. Campbell, Heather C. Mefford, Eric M. Morrow, Lauren A. Weiss, Idan Menashe, Tim Wadkins, Sharmila Banerjee-Basu, and Alan Packer. 2013. "SFARI Gene 2.0: A Community-Driven Knowledgebase for the Autism Spectrum Disorders (ASDs)." *Molecular Autism* 4 (1): 36. https://doi.org/10.1186/2040-2392-4-36.

Ameur, Adam, Ammar Zaghlool, Jonatan Halvardson, Anna Wetterbom, Ulf Gyllensten, Lucia Cavelier, and Lars Feuk. 2011. "Total RNA Sequencing Reveals Nascent Transcription and Widespread Co-Transcriptional Splicing in the Human Brain." *Nature Structural and Molecular Biology* 18 (12): 1435–40. https://doi.org/10.1038/nsmb.2143.

Arlt, Martin F., Alev Cagla Ozdemir, Shanda R. Birkeland, Thomas E. Wilson, and Thomas W. Glover. 2011. "Hydroxyurea Induces de Novo Copy Number Variants in Human Cells." *Proceedings of the National Academy of Sciences of the United States of America* 108 (42): 17360–65. https://doi.org/10.1073/PNAS.1109272108.

Arlt, Martin F., Sountharia Rajendran, Shanda R. Birkeland, Thomas E. Wilson, and Thomas W. Glover. 2012. "De Novo CNV Formation in Mouse Embryonic Stem Cells Occurs in the Absence of Xrcc4-Dependent Nonhomologous End Joining." *PLoS Genetics* 8 (9). https://doi.org/10.1371/JOURNAL.PGEN.1002981.

Ashley-Koch, A., C. M. Wolpert, M. M. Menold, L. Zaeem, S. Basu, S. L. Donnelly, S. A. Ravan, et al. 1999. "Genetic Studies of Autistic Disorder and Chromosome 7." *Genomics* 61

(3): 227–36. https://doi.org/10.1006/geno.1999.5968.

Astorkia, Maider, Herbert M. Lachman, and Deyou Zheng. 2022. “Characterization of Cell-Cell Communication in Autistic Brains with Single-Cell Transcriptomes.” *Journal of Neurodevelopmental Disorders* 14 (1): 1–20. https://doi.org/10.1186/S11689-022-09441-1/FIGURES/7.

Bae, Seung Min, and Ji Yeon Hong. 2018. “The Wnt Signaling Pathway and Related Therapeutic Drugs in Autism Spectrum Disorder.” *Clinical Psychopharmacology and Neuroscience* 16 (2): 129. https://doi.org/10.9758/CPN.2018.16.2.129.

Bakkaloglu, Betul, Brian J. O’Roak, Angeliki Louvi, Abha R. Gupta, Jesse F. Abelson, Thomas M. Morgan, Katarzyna Chawarska, et al. 2008. “Molecular Cytogenetic Analysis and Resequencing of Contactin Associated Protein-Like 2 in Autism Spectrum Disorders.” *American Journal of Human Genetics* 82 (1): 165–73. https://doi.org/10.1016/j.ajhg.2007.09.017.

Bandyopadhyay, Amitabha, Prem Swaroop Yadav, and Paritosh Prashar. 2013. “BMP Signaling in Development and Diseases: A Pharmacological Perspective.” *Biochemical Pharmacology* 85 (7): 857–64. https://doi.org/10.1016/J.BCP.2013.01.004.

Bayés, Mònica, Luis F. Magano, Núria Rivera, Raquel Flores, and Luis A. Pérez Jurado. 2003. “Mutational Mechanisms of Williams-Beuren Syndrome Deletions.” *American Journal of Human Genetics* 73 (1): 131–51. https://doi.org/10.1086/376565.

Bedogni, Francesco, Rebecca D. Hodge, Gina E. Elsen, Branden R. Nelson, Ray A.M. Daza, Richard P. Beyer, Theo K. Bammler, John L.R. Rubenstein, and Robert F. Hevner. 2010. “Tbr1 Regulates Regional and Laminar Identity of Postmitotic Neurons in Developing Neocortex.” *Proceedings of the National Academy of Sciences of the United States of America* 107 (29): 13129–34. https://doi.org/10.1073/PNAS.1002285107.

Bedogni, Francesco, Rebecca D. Hodge, Branden R. Nelson, Erika A. Frederick, Naoko Shiba,

Ray A. Daza, and Robert F. Hevner. 2010. “Autism Susceptibility Candidate 2 (Auts2) Encodes a Nuclear Protein Expressed in Developing Brain Regions Implicated in Autism Neuropathology.” *Gene Expression Patterns : GEP* 10 (1): 9. https://doi.org/10.1016/J.GEP.2009.11.005.

Ben-David, Eyal, Einat Granot-Hershkovitz, Galya Monderer-Rothkoff, Elad Lerer, Shlomit Levi, Maya Yaari, Richard P. Ebstein, Nurit Yirmiya, and Sagiv Shifman. 2011. “Identification of a Functional Rare Variant in Autism Using Genome-Wide Screen for Monoallelic Expression.” *Human Molecular Genetics* 20 (18): 3632–41. https://doi.org/10.1093/hmg/ddr283.

Berk, Michael, Shailesh Y. Desai, Hong Chen Heyman, and Clemencia Colmenares. 1997. “Mice Lacking the Ski Proto-Oncogene Have Defects in Neurulation, Craniofacial Patterning, and Skeletal Muscle Development.” *Genes and Development* 11 (16): 2029–39. https://doi.org/10.1101/gad.11.16.2029.

Beunders, Gea, Jiddeke van de Kamp, Pradeep Vasudevan, Jenny Morton, Katrien Smets, Tjitske Kleefstra, Sonja A. de Munnik, et al. 2016. “A Detailed Clinical Analysis of 13 Patients with AUTS2 Syndrome Further Delineates the Phenotypic Spectrum and Underscores the Behavioural Phenotype.” *Journal of Medical Genetics* 53 (8): 523–32. https://doi.org/10.1136/JMEDGENET-2015-103601.

Beunders, Gea, Els Voorhoeve, Christelle Golzio, Luba M. Pardo, Jill A. Rosenfeld, Michael E. Talkowski, Ingrid Simonic, et al. 2013. “Exonic Deletions in AUTS2 Cause a Syndromic Form of Intellectual Disability and Suggest a Critical Role for the C Terminus.” *American Journal of Human Genetics* 92 (2): 210. https://doi.org/10.1016/J.AJHG.2012.12.011.

Bibel, Miriam, Jens Richter, Katrin Schrenk, Kerry Lee Tucker, Volker Staiger, Martin Korte, Magdalena Goetz, and Yves Alain Barde. 2004. “Differentiation of Mouse Embryonic Stem Cells into a Defined Neuronal Lineage.” *Nature Neuroscience* 7 (9): 1003–9.

https://doi.org/10.1038/NN1301.

Bond, Allison M., Oneil G. Bhalala, and John A. Kessler. 2012. “The Dynamic Role of Bone Morphogenetic Proteins in Neural Stem Cell Fate and Maturation.” *Developmental Neurobiology* 72 (7): 1068. https://doi.org/10.1002/DNEU.22022.

Buchsbaum, Rachel J. 2007. “Rho Activation at a Glance.” *Journal of Cell Science* 120 (7): 1149–52. https://doi.org/10.1242/jcs.03428.

Cao, Ru, Yu Ichi Tsukada, and Yi Zhang. 2005. “Role of Bmi-1 and Ring1A in H2A Ubiquitylation and Hox Gene Silencing.” *Molecular Cell*. https://doi.org/10.1016/j.molcel.2005.12.002.

Castanza, Anthony S., Sanja Ramirez, Prem P. Tripathi, Ray A.M. Daza, Franck K. Kalume, Jan Marino Ramirez, and Robert F. Hevner. 2021. “AUTS2 Regulates RNA Metabolism and Dentate Gyrus Development in Mice.” *Cerebral Cortex* 31 (10): 4808–24. https://doi.org/10.1093/CERCOR/BHAB124.

Chambers, Stuart M., Christopher A. Fasano, Eirini P. Papapetrou, Mark Tomishima, Michel Sadelain, and Lorenz Studer. 2009. “Highly Efficient Neural Conversion of Human ES and IPS Cells by Dual Inhibition of SMAD Signaling.” *Nature Biotechnology* 27 (3): 275–80. https://doi.org/10.1038/nbt.1529.

Chenn, Anjen, and Christopher A. Walsh. 2002. “Regulation of Cerebral Cortical Size by Control of Cell Cycle Exit in Neural Precursors.” *Science* 297 (5580): 365–69. https://doi.org/10.1126/SCIENCE.1074192/SUPPL_FILE/1074192S1_THUMB.GIF.

Cooley, Nicholas P., and Erik S. Wright. 2021. “Accurate Annotation of Protein Coding Sequences with IDTAXA.” *NAR Genomics and Bioinformatics* 3 (3). https://doi.org/10.1093/NARGAB/LQAB080.

Couteur, A. L. Le, I. Gottesman, P. Bolton, E. Simonoff, E. Yuzda, M. Rutter, and A. Bailey. 1995. “Autism as a Strongly Genetic Disorder: Evidence from a British Twin Study.”

Psychological Medicine 25 (1): 63–77. https://doi.org/10.1017/S0033291700028099.

David, Eberhard, Billstedt Eva, and Gillberg Christopher. 2022. "Neurodevelopmental Disorders and Comorbidity in Young Adults Attending a Psychiatric Outpatient Clinic." *Psychiatry Research* 313 (July): 114638. https://doi.org/10.1016/j.psychres.2022.114638.

Denk, Dagmar, Karin Nebral, Jutta Bradtke, Gertrud Pass, Anja Möricke, Andishe Attarbaschi, and Sabine Strehl. 2012. "PAX5-AUTS2: A Recurrent Fusion Gene in Childhood B-Cell Precursor Acute Lymphoblastic Leukemia." *Leukemia Research* 36 (8): e178. https://doi.org/10.1016/J.LEUKRES.2012.04.015.

Dennis, Glynn, Brad T. Sherman, Douglas A. Hosack, Jun Yang, Wei Gao, H. Clifford Lane, and Richard A. Lempicki. 2003. "DAVID: Database for Annotation, Visualization, and Integrated Discovery." *Genome Biology* 4 (5): 1–11. https://doi.org/10.1186/GB-2003-4-9-R60/TABLES/3.

Dennis, Megan K, Angela S Field, Ritwik Burai, Chinnasamy Ramesh, K Whitney, Cristian G Bologa, Tudor I Oprea, et al. 2012. "Sequencing Chromosomal Abnormalities Reveals Neurodevelopmental Loci That Confer Risk across Diagnostic Boundaries." *Cell* 127 (3): 358–66. https://www.sciencedirect.com/science/article/pii/S0092867412004114.

Du, Xiujuan, Xueren Gao, Xin Liu, Lixiao Shen, Kai Wang, Yanjie Fan, Yu Sun, et al. 2018. "Genetic Diagnostic Evaluation of Trio-Based Whole Exome Sequencing Among Children With Diagnosed or Suspected Autism Spectrum Disorder." *Frontiers in Genetics* 9 (November). https://doi.org/10.3389/FGENE.2018.00594.

Elias, Ruben D., Wen Ma, Rodolfo Ghirlando, Charles D. Schwieters, Vijay S. Reddy, and Lalit Deshmukh. 2020. "Proline-Rich Domain of Human ALIX Contains Multiple TSG101-UEV Interaction Sites and Forms Phosphorylation-Mediated Reversible Amyloids." *Proceedings of the National Academy of Sciences of the United States of America* 117 (39): 24274–84. https://doi.org/10.1073/PNAS.2010635117/SUPPL_FILE/PNAS.2010635117.SAPP.PDF.

Eskeland, Ragnhild, Martin Leeb, Graeme R. Grimes, Clémence Kress, Shelagh Boyle, Duncan Sproul, Nick Gilbert, et al. 2010. “Ring1B Compacts Chromatin Structure and Represses Gene Expression Independent of Histone Ubiquitination.” *Molecular Cell* 38 (3): 452–64. https://doi.org/10.1016/j.molcel.2010.02.032.

Eto, Hikaru, Yusuke Kishi, Nayuta Yakushiji-Kaminatsui, Hiroki Sugishita, Shun Utsunomiya, Haruhiko Koseki, and Yukiko Gotoh. 2020. “The Polycomb Group Protein Ring1 Regulates Dorsoventral Patterning of the Mouse Telencephalon.” *Nature Communications* 11 (1). https://doi.org/10.1038/S41467-020-19556-5.

Fair, Summer R, Wesley Schwind, Dominic Julian, Alecia Biel, Gongbo Guo, Ryan Rutherford, Swetha Ramadesikan, et al. 2022a. “Cerebral Organoids Containing an AUTS2 Missense Variant Model Microcephaly.” *Brain : A Journal of Neurology*, July. https://doi.org/10.1093/brain/awac244.

———. 2022b. “Cerebral Organoids Containing an AUTS2 Missense Variant Model Microcephaly.” *Brain*, July. https://doi.org/10.1093/BRAIN/AWAC244.

Fitzgerald, T. W., S. S. Gerety, W. D. Jones, M. Van Kogelenberg, D. A. King, J. McRae, K. I. Morley, et al. 2015. “Large-Scale Discovery of Novel Genetic Causes of Developmental Disorders.” *Nature* 519 (7542): 223–28. https://doi.org/10.1038/NATURE14135.

Flatscher-Bader, T, C. J. Foldi, S. Chong, E. Whitelaw, R. J. Moser, T. H.J. Burne, D. W. Eyles, and J. J. McGrath. 2011. “Increased de Novo Copy Number Variants in the Offspring of Older Males.” *Translational Psychiatry* 1. https://doi.org/10.1038/tp.2011.30.

Francis, Nicole J., Robert E. Kingston, and Christopher L. Woodcock. 2004. “Chromatin Compaction by a Polycomb Group Protein Complex.” *Science*. https://doi.org/10.1126/science.1100576.

Franzén, Oscar, Li Ming Gan, and Johan L.M. Björkegren. 2019. “PanglaoDB: A Web Server for Exploration of Mouse and Human Single-Cell RNA Sequencing Data.” *Database* 2019 (1):

46. https://doi.org/10.1093/DATABASE/BAZ046.

Gámez, Beatriz, Edgardo Rodriguez-Carballo, and Francesc Ventura. 2013. “BMP Signaling in Telencephalic Neural Cell Specification and Maturation.” *Frontiers in Cellular Neuroscience* 7 (MAY). https://doi.org/10.3389/FNCEL.2013.00087.

Gao, Zhonghua, Pedro Lee, James M. Stafford, Melanie Von Schimmelmann, Anne Schaefer, and Danny Reinberg. 2014. “An AUTS2-Polycomb Complex Activates Gene Expression in the CNS.” *Nature* 516 (7531): 349–54. https://doi.org/10.1038/nature13921.

Gao, Zhonghua, Jin Zhang, Roberto Bonasio, Francesco Strino, Ayana Sawai, Fabio Parisi, Yuval Kluger, and Danny Reinberg. 2012. “PCGF Homologs, CBX Proteins, and RYBP Define Functionally Distinct PRC1 Family Complexes.” *Molecular Cell* 45 (3): 344–56. https://doi.org/10.1016/j.molcel.2012.01.002.

Gil, Jesús, and Ana O’Loghlen. 2014. “PRC1 Complex Diversity: Where Is It Taking Us?” *Trends in Cell Biology*. https://doi.org/10.1016/j.tcb.2014.06.005.

Gilissen, Christian, Jayne Y. Hehir-Kwa, Djie Tjwan Thung, Maartje Van De Vorst, Bregje W.M. Van Bon, Marjolein H. Willemsen, Michael Kwint, et al. 2014. “Genome Sequencing Identifies Major Causes of Severe Intellectual Disability.” *Nature 2014 511:7509* 511 (7509): 344–47. https://doi.org/10.1038/nature13394.

Glessner, Joseph T., Kai Wang, Guiqing Cai, Olena Korvatska, Cecilia E. Kim, Shawn Wood, Haitao Zhang, et al. 2009. “Autism Genome-Wide Copy Number Variation Reveals Ubiquitin and Neuronal Genes.” *Nature* 459 (7246): 569–72. https://doi.org/10.1038/NATURE07953.

Graf, Daniel, Zeba Malik, Satoru Hayano, and Yuji Mishina. 2016. “Common Mechanisms in Development and Disease: BMP Signaling in Craniofacial Development.” *Cytokine and Growth Factor Reviews*. NIH Public Access. https://doi.org/10.1016/j.cytogfr.2015.11.004.

Guan, Kaomei, Jürgen Rohwedel, and Anna M Wobus. 1999. “Embryonic Stem Cell

Differentiation Models: Cardiogenesis, Myogenesis, Neurogenesis, Epithelial and Vascular Smooth Muscle Cell Differentiation in Vitro." *Cytotechnology* 30: 211–26.

Han, Yong, Guo Qing Ru, Xiaozhou Mou, Hui ju Wang, Yingyu Ma, Xiang Lei He, Zhilong Yan, and Dongsheng Huang. 2015. "AUTS2 Is a Potential Therapeutic Target for Pancreatic Cancer Patients with Liver Metastases." *Medical Hypotheses* 85 (2): 203–6. https://doi.org/10.1016/J.MEHY.2015.04.029.

Hanafiah, Aflah, Zhuangzhuang Geng, Qiang Wang, and Zhonghua Gao. 2020. "Differentiation and Characterization of Neural Progenitors and Neurons from Mouse Embryonic Stem Cells." *JoVE (Journal of Visualized Experiments)* 2020 (159): e61446. https://doi.org/10.3791/61446.

Higa, Leigh Ann, Min Wu, Tao Ye, Ryuji Kobayashi, Hong Sun, and Hui Zhang. 2006. "CUL4-DDB1 Ubiquitin Ligase Interacts with Multiple WD40-Repeat Proteins and Regulates Histone Methylation." *Nature Cell Biology* 8 (11): 1277–83. https://doi.org/10.1038/ncb1490.

Hodges, Holly, Casey Fealko, and Neelkamal Soares. 2020. "Autism Spectrum Disorder: Definition, Epidemiology, Causes, and Clinical Evaluation." *Translational Pediatrics* 9 (Suppl 1): S55. https://doi.org/10.21037/TP.2019.09.09.

Hori, Kei, and Mikio Hoshino. 2017. "Neuronal Migration and AUTS2 Syndrome." *Brain Sciences* 7 (5). https://doi.org/10.3390/BRAINSCI7050054.

Hori, Kei, Taku Nagai, Wei Shan, Asami Sakamoto, Manabu Abe, Maya Yamazaki, Kenji Sakimura, Kiyofumi Yamada, and Mikio Hoshino. 2015. "Heterozygous Disruption of Autism Susceptibility Candidate 2 Causes Impaired Emotional Control and Cognitive Memory." *PLOS ONE* 10 (12): e0145979. https://doi.org/10.1371/JOURNAL.PONE.0145979.

Hori, Kei, Taku Nagai, Wei Shan, Asami Sakamoto, Shinichiro Taya, Ryoya Hashimoto, Takashi

Hayashi, et al. 2014. “Cytoskeletal Regulation by AUTS2 in Neuronal Migration and Neuritogenesis.” *Cell Reports* 9 (6): 2166–79. https://doi.org/10.1016/J.CELREP.2014.11.045.

Hori, Kei, Kazumi Shimaoka, and Mikio Hoshino. 2022. “AUTS2 Gene: Keys to Understanding the Pathogenesis of Neurodevelopmental Disorders.” *Cells* 11 (1). https://doi.org/10.3390/CELLS11010011.

Hori, Kei, Kunihiko Yamashiro, Taku Nagai, Wei Shan, Saki F. Egusa, Kazumi Shimaoka, Hiroshi Kuniishi, et al. 2020. “AUTS2 Regulation of Synapses for Proper Synaptic Inputs and Social Communication.” *IScience* 23 (6). https://doi.org/10.1016/J.ISCI.2020.101183.

Hsu, Patrick D., David A. Scott, Joshua A. Weinstein, F. Ann Ran, Silvana Konermann, Vineeta Agarwala, Yinqing Li, et al. 2013. “DNA Targeting Specificity of RNA-Guided Cas9 Nucleases.” *Nature Biotechnology 2013 31:9* 31 (9): 827–32. https://doi.org/10.1038/nbt.2647.

Huang, Xin Li, Ying S. Zou, Tom A. Maher, Stephanie Newton, and Jeff M. Milunsky. 2010. “A de Novo Balanced Translocation Breakpoint Truncating the Autism Susceptibility Candidate 2 (AUTS2) Gene in a Patient with Autism.” *American Journal of Medical Genetics Part A* 152A (8): 2112–14. https://doi.org/10.1002/AJMG.A.33497.

Jaenisch, Rudolf, and Richard Young. 2008. “Stem Cells, the Molecular Circuitry of Pluripotency and Nuclear Reprogramming.” *Cell*. Cell Press. https://doi.org/10.1016/j.cell.2008.01.015.

Jin, Jianping, Emily E. Arias, Jing Chen, J. Wade Harper, and Johannes C. Walter. 2006. “A Family of Diverse Cul4-Ddb1-Interacting Proteins Includes Cdt2, Which Is Required for S Phase Destruction of the Replication Factor Cdt1.” *Molecular Cell* 23 (5): 709–21. https://doi.org/10.1016/j.molcel.2006.08.010.

Jolley, Alexandra, Mark Corbett, Lesley Mcgregor, Wendy Waters, Susan Brown, Jillian Nicholl, and Sui Yu. 2013. “De Novo Intragenic Deletion of the Autism Susceptibility Candidate 2

(AUTS2) Gene in a Patient with Developmental Delay: A Case Report and Literature Review." *Pubmed.Ncbi.Nlm.Nih.Gov* 161 (6): 1508–12. https://doi.org/10.1002/ajmg.a.35922.

Kalkman, Hans Otto. 2012. "A Review of the Evidence for the Canonical Wnt Pathway in Autism Spectrum Disorders." *Molecular Autism 2012 3:1* 3 (1): 1–12. https://doi.org/10.1186/2040-2392-3-10.

Kalscheuer, Vera M., David FitzPatrick, Niels Tommerup, Merete Bugge, Erik Niebuhr, Luitgard M. Neumann, Andreas Tzschach, et al. 2007. "Mutations in Autism Susceptibility Candidate 2 (AUTS2) in Patients with Mental Retardation." *Human Genetics 2007 121:3* 121 (3): 501–9. https://doi.org/10.1007/S00439-006-0284-0.

Kalueff, Allan V., Adam Michael Stewart, and Robert Gerlai. 2014. "Zebrafish as an Emerging Model for Studying Complex Brain Disorders." *Trends in Pharmacological Sciences* 35 (2): 63. https://doi.org/10.1016/J.TIPS.2013.12.002.

Kashima, Risa, Sougata Roy, Manuel Ascano, Veronica Martinez-Cerdeno, Jeanelle Ariza-Torres, Sunghwan Kim, Justin Louie, et al. 2016. "Augmented Noncanonical BMP Type II Receptor Signaling Mediates the Synaptic Abnormality of Fragile X Syndrome." *Science Signaling* 9 (431). https://doi.org/10.1126/scisignal.aaf6060.

Katoh, Hironori, and Manabu Negishi. 2003. "RhoG Activates Rac1 by Direct Interaction with the Dock180-Binding Protein Elmo." *Nature* 424 (6947): 461–64. https://doi.org/10.1038/nature01817.

Katoh, Masuko, and Masaru Katoh. 2007. "WNT Signaling Pathway and Stem Cell Signaling Network." *Clinical Cancer Research*. https://doi.org/10.1158/1078-0432.CCR-06-2316.

Kondrychyn, Igor, Lena Robra, and Vatsala Thirumalai. 2017. "Transcriptional Complexity and Distinct Expression Patterns of Auts2 Paralogs in Danio Rerio." *G3: Genes, Genomes, Genetics* 7 (8): 2577–93. https://doi.org/10.1534/G3.117.042622/-/DC1.

Kundu, Sharmistha, Fei Ji, Hongjae Sunwoo, Gaurav Jain, Jeannie T. Lee, Ruslan I. Sadreyev, Job Dekker, and Robert E. Kingston. 2017. "Polycomb Repressive Complex 1 Generates Discrete Compacted Domains That Change during Differentiation." *Molecular Cell* 65 (3): 432-446.e5. https://doi.org/10.1016/j.molcel.2017.01.009.

Lancaster, Madeline A., Magdalena Renner, Carol Anne Martin, Daniel Wenzel, Louise S. Bicknell, Matthew E. Hurles, Tessa Homfray, Josef M. Penninger, Andrew P. Jackson, and Juergen A. Knoblich. 2013. "Cerebral Organoids Model Human Brain Development and Microcephaly." *Nature 2013 501:7467* 501 (7467): 373–79. https://doi.org/10.1038/nature12517.

Lepagnol-Bestel, A. M., G Maussion, B. Boda, A. Cardona, Y. Iwayama, A. L. Delezoide, J. M. Moalic, et al. 2008. "SLC25A12 Expression Is Associated with Neurite Outgrowth and Is Upregulated in the Prefrontal Cortex of Autistic Subjects." *Molecular Psychiatry* 13 (4): 385–97. https://doi.org/10.1038/sj.mp.4002120.

Li, Jing, Marlene Dallmayer, Thomas Kirchner, Julian Musa, and Thomas G.P. Grünewald. 2018. "PRC1: Linking Cytokinesis, Chromosomal Instability, and Cancer Evolution." *Trends in Cancer*. https://doi.org/10.1016/j.trecan.2017.11.002.

Li, Wenhua, Aiyu Yao, Hui Zhi, Kuldeep Kaur, Yong Chuan Zhu, Mingyue Jia, Hui Zhao, et al. 2016. "Angelman Syndrome Protein Ube3a Regulates Synaptic Growth and Endocytosis by Inhibiting BMP Signaling in Drosophila." *PLoS Genetics* 12 (5): e1006062. https://doi.org/10.1371/journal.pgen.1006062.

Li, Zhongwei, and Ye Guang Chen. 2013. "Functions of BMP Signaling in Embryonic Stem Cell Fate Determination." *Experimental Cell Research* 319 (2): 113–19. https://doi.org/10.1016/J.YEXCR.2012.09.016.

Liu, Sanxiong, Kimberly A. Aldinger, Chi Vicky Cheng, Takae Kiyama, Mitali Dave, Hanna K. McNamara, Wukui Zhao, et al. 2021. "NRF1 Association with AUTS2-Polycomb Mediates

Specific Gene Activation in the Brain." *Molecular Cell* 81 (22): 4663-4676.e8. https://doi.org/10.1016/J.MOLCEL.2021.09.020.

Logan, Catriona Y, and Roel Nusse. 2004. "The Wnt Signaling Pathway in Development and Disease." *Annual Review of Cell and Developmental Biology*. https://doi.org/10.1146/annurev.cellbio.20.010403.113126.

Loke, Yuk Jing, Anthony John Hannan, and Jeffrey Mark Craig. 2015. "The Role of Epigenetic Change in Autism Spectrum Disorders." *Frontiers in Neurology* 6 (MAY): 107. https://doi.org/10.3389/FNEUR.2015.00107.

Lowery, Jonathan W., and Vicki Rosen. 2018. "Bone Morphogenetic Protein–Based Therapeutic Approaches." *Cold Spring Harbor Perspectives in Biology* 10 (4): 22327–28. https://doi.org/10.1101/cshperspect.a022327.

Lullo, Elizabeth Di, and Arnold R. Kriegstein. 2017. "The Use of Brain Organoids to Investigate Neural Development and Disease." *Nature Reviews Neuroscience 2017 18:10* 18 (10): 573–84. https://doi.org/10.1038/nrn.2017.107.

Luo, Kunxin, Shannon L. Stroschein, Wei Wang, Dan Chen, Eric Martens, Sharleen Zhou, and Qiang Zhou. 1999. "The Ski Oncoprotein Interacts with the Smad Proteins to Repress TGF̄ Signaling." *Genes and Development* 13 (17): 2196–2206. https://doi.org/10.1101/gad.13.17.2196.

Lustig, B., and J. Behrens. 2003. "The Wnt Signaling Pathway and Its Role in Tumor Development." *Journal of Cancer Research and Clinical Oncology*. Springer. https://doi.org/10.1007/s00432-003-0431-0.

Maenner, Matthew J., Kelly A. Shaw, Jon Baio, Anita Washington, Mary Patrick, Monica DiRienzo, Deborah L. Christensen, et al. 2020. "Prevalence of Autism Spectrum Disorder among Children Aged 8 Years-Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2016." *MMWR Surveillance Summaries* 69 (4): 1–12.

https://doi.org/10.15585/MMWR.SS6904A1.

Margueron, Raphaël, and Danny Reinberg. 2011. “The Polycomb Complex PRC2 and Its Mark in Life.” *Nature*. https://doi.org/10.1038/nature09784.

Mariani, Jessica, Gianfilippo Coppola, Ping Zhang, Alexej Abyzov, Lauren Provini, Livia Tomasini, Mariangela Amenduni, et al. 2015. “FOXG1-Dependent Dysregulation of GABA/Glutamate Neuron in Autism Spectrum Disorders.” *Cell* 162 (2): 375. https://doi.org/10.1016/J.CELL.2015.06.034.

Massagué, Joan. 2012. “TGFβ Signalling in Context.” *Nature Reviews Molecular Cell Biology*. NIH Public Access. https://doi.org/10.1038/nrm3434.

Medina, Matías A., Víctor M. Andrade, Mario O. Caracci, Miguel E. Avila, Daniela A. Verdugo, Macarena F. Vargas, Giorgia D. Ugarte, Ariel E. Reyes, Carlos Opazo, and Giancarlo V. De Ferrari. 2018. “Wnt/β-Catenin Signaling Stimulates the Expression and Synaptic Clustering of the Autism-Associated Neuroligin 3 Gene.” *Translational Psychiatry* 8 (1). https://doi.org/10.1038/S41398-018-0093-Y.

Mellios, N., D. A. Feldman, S. D. Sheridan, J. P.K. Ip, S. Kwok, S. K. Amoah, B. Rosen, et al. 2018. “MeCP2-Regulated MiRNAs Control Early Human Neurogenesis through Differential Effects on ERK and AKT Signaling.” *Molecular Psychiatry* 23 (4): 1051–65. https://doi.org/10.1038/MP.2017.86.

Mervis, Carolyn B, Colleen A Morris, Bonita P Klein-Tasman, Shelley L Velleman, and Lucy R Osborne. 2021. “7q11.23 Duplication Syndrome.” *GeneReviews*®, March. https://www.ncbi.nlm.nih.gov/books/NBK327268/.

Miyata, Yoshihiko, Takeshi Shibata, Masato Aoshima, Takuichi Tsubata, and Eisuke Nishida. 2014. “The Molecular Chaperone TRiC/CCT Binds to the Trp-Asp 40 (WD40) Repeat Protein WDR68 and Promotes Its Folding, Protein Kinase DYRK1A Binding, and Nuclear Accumulation.” *Journal of Biological Chemistry* 289 (48): 33320–32.

https://doi.org/10.1074/jbc.M114.586115.

Mohn, J. L., J. Alexander, A. Pirone, C. D. Palka, S. Y. Lee, L. Mebane, P. G. Haydon, and M. H. Jacob. 2014. “Adenomatous Polyposis Coli Protein Deletion Leads to Cognitive and Autism-like Disabilities.” *Molecular Psychiatry* 19 (10): 1133–42. https://doi.org/10.1038/MP.2014.61.

Monderer-Rothkoff, Galya, Nitzan Tal, Marina Risman, Odem Shani, Malka Nissim-Rafinia, Laura Malki-Feldman, Vera Medvedeva, Matthias Groszer, Eran Meshorer, and Sagiv Shifman. 2021. “AUTS2 Isoforms Control Neuronal Differentiation.” *Molecular Psychiatry* 26 (2): 666–81. https://doi.org/10.1038/s41380-019-0409-1.

Mulligan, Kimberly A., and Benjamin N.R. Cheyette. 2017. “Neurodevelopmental Perspectives on Wnt Signaling in Psychiatry.” *Molecular Neuropsychiatry* 2 (4): 219–46. https://doi.org/10.1159/000453266.

Mullin, A. P., A. Gokhale, A. Moreno-De-Luca, S. Sanyal, J. L. Waddington, and V. Faundez. 2013. “Neurodevelopmental Disorders: Mechanisms and Boundary Definitions from Genomes, Interactomes and Proteomes.” *Translational Psychiatry* 3 (12): e329. https://doi.org/10.1038/TP.2013.108.

Nagamani, Sandesh C.S., Ayelet Erez, Bruria Ben-Zeev, Moshe Frydman, Susan Winter, Robert Zeller, Dima El-Khechen, et al. 2013. “Detection of Copy-Number Variation in AUTS2 Gene by Targeted Exonic Array CGH in Patients with Developmental Delay and Autistic Spectrum Disorders.” *European Journal of Human Genetics : EJHG* 21 (3): 343–46. https://doi.org/10.1038/EJHG.2012.157.

Napoles, Mariana de, Jacqueline E. Mermoud, Rika Wakao, Y. Amy Tang, Mitusuhiro Endoh, Ruth Appanah, Tatyana B. Nesterova, et al. 2004. “Polycomb Group Proteins Ring1A/B Link Ubiquitylation of Histone H2A to Heritable Gene Silencing and X Inactivation.” *Developmental Cell*. https://doi.org/10.1016/j.devcel.2004.10.005.

Nishimura, Takashi, Tomoya Yamaguchi, Katsuhiro Kato, Masato Yoshizawa, Yo Ichi Nabeshima, Shigeo Ohno, Mikio Hoshino, and Kozo Kaibuchi. 2005. “PAR-6-PAR-3 Mediates Cdc42-Induced Rac Activation through the Rac GEFs STEF/Tiam1.” *Nature Cell Biology* 7 (3): 270–77. https://doi.org/10.1038/ncb1227.

O’Roak, Brian J., Laura Vives, Wenqing Fu, Jarrett D. Egertson, Ian B. Stanaway, Ian G. Phelps, Gemma Carvill, et al. 2012. “Multiplex Targeted Sequencing Identifies Recurrently Mutated Genes in Autism Spectrum Disorders.” *Science (New York, N.Y.)* 338 (6114): 1619–22. https://doi.org/10.1126/SCIENCE.1227764.

O ’ Roak, Brian J., Laura Vives, Santhosh Girirajan, Emre Karakoc, Niklas Krumm, Bradley P. Coe, Roie Levy, et al. 2012. “Sporadic Autism Exomes Reveal a Highly Interconnected Protein Network of de Novo Mutations.” *Nature* 485 (7397): 246–50. https://doi.org/10.1038/NATURE10989.

Oksenberg, Nir, and Nadav Ahituv. 2013. “The Role of AUTS2 in Neurodevelopment and Human Evolution.” *Trends in Genetics : TIG* 29 (10): 600–608. https://doi.org/10.1016/J.TIG.2013.08.001.

Oksenberg, Nir, Laurie Stevison, Jeffrey D. Wall, and Nadav Ahituv. 2013a. “Function and Regulation of AUTS2, a Gene Implicated in Autism and Human Evolution.” *PLOS Genetics* 9 (1): e1003221. https://doi.org/10.1371/JOURNAL.PGEN.1003221.

———. 2013b. “Function and Regulation of AUTS2, a Gene Implicated in Autism and Human Evolution.” Edited by James Noonan. *PLoS Genetics* 9 (1): e1003221. https://doi.org/10.1371/journal.pgen.1003221.

Palferman, Sarah, Nicola Matthews, Martha Turner, Janette Moore, Amaia Hervas, Anne Aubin, Simon Wallace, et al. 2001. “Further Characterization of the Autism Susceptibility Locus AUTS1 on Chromosome 7q.” *Human Molecular Genetics* 10 (9): 973–82. https://doi.org/10.1093/hmg/10.9.973.

Parenti, Ilaria, Luis G. Rabaneda, Hanna Schoen, and Gaia Novarino. 2020. "Neurodevelopmental Disorders: From Genetics to Functional Pathways." *Trends in Neurosciences*. Elsevier. https://doi.org/10.1016/j.tins.2020.05.004.

Parker, Matthew O., Alistair J. Brock, Robert T. Walton, and Caroline H. Brennan. 2013. "The Role of Zebrafish (Danio Rerio) in Dissecting the Genetics and Neural Circuits of Executive Function." *Frontiers in Neural Circuits* 7 (MAR). https://doi.org/10.3389/FNCIR.2013.00063.

Pellegrini, Laura, Claudia Bonfio, Jessica Chadwick, Farida Begum, Mark Skehel, and Madeline A. Lancaster. 2020. "Human CNS Barrier-Forming Organoids with Cerebrospinal Fluid Production." *Science* 369 (6500). https://doi.org/10.1126/science.aaz5626.

Platt, Randall J., Yang Zhou, Ian M. Slaymaker, Ashwin S. Shetty, Niels R. Weisbach, Jin Ah Kim, Jitendra Sharma, et al. 2017. "Chd8 Mutation Leads to Autistic-like Behaviors and Impaired Striatal Circuits." *Cell Reports* 19 (2): 335–50. https://doi.org/10.1016/j.celrep.2017.03.052.

Qian, Xuyu, Hongjun Song, and Guo Li Ming. 2019. "Brain Organoids: Advances, Applications and Challenges." *Development (Cambridge)* 146 (8). https://doi.org/10.1242/DEV.166074/19861.

Quadrato, Giorgia, Tuan Nguyen, Evan Z. Macosko, John L. Sherwood, Sung Min Yang, Daniel R. Berger, Natalie Maria, et al. 2017. "Cell Diversity and Network Dynamics in Photosensitive Human Brain Organoids." *Nature 2017 545:7652* 545 (7652): 48–53. https://doi.org/10.1038/nature22047.

Redon, Fiegler, Freeman, Macdonald, Mei, Woodwark, Conrad, and Lee. 2006. "Global Variation in Copy Number in the Human Genome." *Nature* 444 (7118): 444. https://idp.nature.com/authorize/casa?redirect_uri=https://www.nature.com/articles/nature05329&casa_token=atwa3aO_wt4AAAAA:cFdS85LFPBGM8yeTZl2hTk-

p7_AMCmV0IXidfgAfzMTOoMlCb7-8OgonGtxch7ae7M-cwIGsXhxaaxPXzs0.

Risch, Neil, Donna Spiker, Linda Lotspeich, Nassim Nouri, David Hinds, Joachim Hallmayer, Luba Kalaydjieva, et al. 1999. "A Genomic Screen of Autism: Evidence for a Multilocus Etiology." *American Journal of Human Genetics* 65 (2): 493–507. https://doi.org/10.1086/302497.

Ritvo, E. R., B. J. Freeman, A. Mason-Brothers, and A. Mo. 1985. "Concordance for the Syndrome of Autism in 40 Pairs of Afflicted Twins." *The American Journal of Psychiatry* 142 (1): 74–77. https://doi.org/10.1176/AJP.142.1.74.

Rosso, Silvana B., and Nibaldo C. Inestrosa. 2013. "WNT Signaling in Neuronal Maturation and Synaptogenesis." *Frontiers in Cellular Neuroscience* 7 (JUNE). https://doi.org/10.3389/FNCEL.2013.00103.

Russo, Domenico, Floriana Della Ragione, Riccardo Rizzo, Eiji Sugiyama, Francesco Scalabrì, Kei Hori, Serena Capasso, et al. 2018. "Glycosphingolipid Metabolic Reprogramming Drives Neural Differentiation." *The EMBO Journal* 37 (7). https://doi.org/10.15252/embj.201797674.

Rylaarsdam, Lauren, and Alicia Guemez-Gamboa. 2019. "Genetic Causes and Modifiers of Autism Spectrum Disorder." *Frontiers in Cellular Neuroscience* 13 (August): 385. https://doi.org/10.3389/FNCEL.2019.00385/BIBTEX.

Sajan, Samin A., John L.R. Rubenstein, Mark E. Warchol, and Michael Lovett. 2011. "Identification of Direct Downstream Targets of Dlx5 during Early Inner Ear Development." *Human Molecular Genetics* 20 (7): 1262–73. https://doi.org/10.1093/HMG/DDQ567.

Salichs, Eulàlia, Alice Ledda, Loris Mularoni, M. Mar Albà, and Susana De La Luna. 2009. "Genome-Wide Analysis of Histidine Repeats Reveals Their Role in the Localization of Human Proteins to the Nuclear Speckles Compartment." *PLoS Genetics* 5 (3): 1000397.

https://doi.org/10.1371/JOURNAL.PGEN.1000397.

Sanders, Stephan J., A. Gulhan Ercan-Sencicek, Vanessa Hus, Rui Luo, Michael T. Murtha, Daniel Moreno-De-Luca, Su H. Chu, et al. 2011. "Multiple Recurrent de Novo CNVs, Including Duplications of the 7q11.23 Williams Syndrome Region, Are Strongly Associated with Autism." *Neuron* 70 (5): 863–85. https://doi.org/10.1016/J.NEURON.2011.05.002.

Schluth-Bolard, Caroline, Flavie Diguet, Nicolas Chatron, Pierre Antoine Rollat-Farnier, Claire Bardel, Alexandra Afenjar, Florence Amblard, et al. 2019. "Whole Genome Paired-End Sequencing Elucidates Functional and Phenotypic Consequences of Balanced Chromosomal Rearrangement in Patients with Developmental Disorders." *Journal of Medical Genetics* 56 (8): 526–35. https://doi.org/10.1136/jmedgenet-2018-105778.

Schmahmann, Jeremy D., and David Caplan. 2006. "Cognition, Emotion and the Cerebellum." *Brain* 129 (2): 290–92. https://doi.org/10.1093/BRAIN/AWH729.

Shao, Zhaohui, Florian Raible, Ramin Mollaaghababa, Jeffrey R. Guyon, Chao Ting Wu, Welcome Bender, and Robert E. Kingston. 1999. "Stabilization of Chromatin Structure by PRC1, a Polycomb Complex." *Cell* 98 (1): 37–46. https://doi.org/10.1016/S0092-8674(00)80604-2.

Shen, Mark D. 2018. "Cerebrospinal Fluid and the Early Brain Development of Autism." *Journal of Neurodevelopmental Disorders* 10 (1). https://doi.org/10.1186/S11689-018-9256-7.

Shetti, Dattatrya, Bao Zhang, Conghui Fan, Canlong Mo, Bae Hoon Lee, and Kun Wei. 2019. "Low Dose of Paclitaxel Combined with XAV939 Attenuates Metastasis, Angiogenesis and Growth in Breast Cancer by Suppressing Wnt Signaling." *Cells 2019, Vol. 8, Page 892* 8 (8): 892. https://doi.org/10.3390/CELLS8080892.

Simon, Jeffrey A., and Robert E. Kingston. 2009. "Mechanisms of Polycomb Gene Silencing: Knowns and Unknowns." *Nature Reviews Molecular Cell Biology* 10 (10): 697–708. https://doi.org/10.1038/nrm2763.

Sparmann, Anke, and Maarten Van Lohuizen. 2006. “Polycomb Silencers Control Cell Fate, Development and Cancer.” *Nature Reviews Cancer*. https://doi.org/10.1038/nrc1991.

Steffenburg, Suzanne, Christopher Gillberg, Lars Hellgren, Lena Andersson, I. Carina Gillberg, Gun Jakobsson, and Michael Bohman. 1989. “A Twin Study of Autism in Denmark, Finland, Iceland, Norway and Sweden.” *Journal of Child Psychology and Psychiatry, and Allied Disciplines* 30 (3): 405–16. https://doi.org/10.1111/J.1469-7610.1989.TB00254.X.

Stroschein, Shannon L., Wei Wang, Sharleen Zhou, Qiang Zhou, and Kunxin Luo. 1999. “Negative Feedback Regulation of TGF-β Signaling by the SnoN Oncoprotein.” *Science* 286 (5440): 771–74. https://doi.org/10.1126/science.286.5440.771.

Sultana, Razia, Chang En Yu, Jun Yu, Jeffery Munson, Donghui Chen, Wenhui Hua, Annette Estes, et al. 2002. “Identification of a Novel Gene on Chromosome 7q11.2 Interrupted by a Translocation Breakpoint in a Pair of Autistic Twins.” *Genomics* 80 (2): 129–34. https://doi.org/10.1006/GENO.2002.6810.

Sun, Yin, Xuedong Liu, Elinor Ng Eaton, William S. Lane, Harvey F. Lodish, and Robert A. Weinberg. 1999. “Interaction of the Ski Oncoprotein with Smad3 Regulates TGF-β Signaling.” *Molecular Cell* 4 (4): 499–509. https://doi.org/10.1016/S1097-2765(00)80201-4.

Talkowski, Michael E, Jill A Rosenfeld, Ian Blumenthal, Vamsee Pillalamarri, Colby Chiang, Adrian Heilbut, Carl Ernst, et al. 2012. “Sequencing Chromosomal Abnormalities Reveals Neurodevelopmental Loci That Confer Risk across Diagnostic Boundaries.” *Cell* 149: 525–37. https://doi.org/10.1016/j.cell.2012.03.028.

Tang, Shao-Jun. 2014. “Synaptic Activity-Regulated Wnt Signaling in Synaptic Plasticity, Glial Function and Chronic Pain.” *CNS & Neurological Disorders Drug Targets* 13 (5): 737. https://doi.org/10.2174/1871527312666131223114457.

Velmeshev, Dmitry, Lucas Schirmer, Diane Jung, Maximilian Haeussler, Yonatan Perez, Simone

Mayer, Aparna Bhaduri, Nitasha Goyal, David H. Rowitch, and Arnold R. Kriegstein. 2019. "Single-Cell Genomics Identifies Cell Type-Specific Molecular Changes in Autism." *Science (New York, N.Y.)* 364 (6441): 685–89. https://doi.org/10.1126/SCIENCE.AAV8130.

Visel, Axel, Christina Thaller, and Gregor Eichele. 2004. "GenePaint.Org: An Atlas of Gene Expression Patterns in the Mouse Embryo." *Nucleic Acids Research* 32 (Database issue): D552. https://doi.org/10.1093/NAR/GKH029.

Wada, Hironori, and Hitoshi Okamoto. 2009. "Roles of Noncanonical Wnt/PCP Pathway Genes in Neuronal Migration and Neurulation in Zebrafish." *Zebrafish* 6 (1): 3–8. https://doi.org/10.1089/ZEB.2008.0557.

Wagner, Alex H., V. Nikhil Anand, Wan Heng Wang, Jon E. Chatterton, Duo Sun, Allan R. Shepard, Nasreen Jacobson, et al. 2013. "Exon-Level Expression Profiling of Ocular Tissues." *Experimental Eye Research* 111: 105–11. https://doi.org/10.1016/j.exer.2013.03.004.

Wang, Hengbin, Liangjun Wang, Hediye Erdjument-Bromage, Miguel Vidal, Paul Tempst, Richard S. Jones, and Yi Zhang. 2004. "Role of Histone H2A Ubiquitination in Polycomb Silencing." *Nature* 431 (7010): 873–78. https://doi.org/10.1038/nature02985.

Wang, Qiang, Zhuangzhuang Geng, Yi Gong, Kaitlyn Warren, Haiyan Zheng, Yuka Imamura, and Zhonghua Gao. 2018. "WDR68 Is Essential for the Transcriptional Activation of the PRC1-AUTS2 Complex and Neuronal Differentiation of Mouse Embryonic Stem Cells." *Stem Cell Research* 33 (December): 206–14. https://doi.org/10.1016/j.scr.2018.10.023.

Wang, Richard N., Jordan Green, Zhongliang Wang, Youlin Deng, Min Qiao, Michael Peabody, Qian Zhang, et al. 2014. "Bone Morphogenetic Protein (BMP) Signaling in Development and Human Diseases." *Genes & Diseases* 1 (1): 87–105. https://doi.org/10.1016/J.GENDIS.2014.07.005.

Watabe, Tetsuro, and Kohei Miyazono. 2009. "Roles of TGF-β Family Signaling in Stem Cell

Renewal and Differentiation." *Cell Research*. Cell Res. https://doi.org/10.1038/cr.2008.323.

Wodarz, Andreas, and Roel Nusse. 1998. "Mechanisms of Wnt Signaling in Development." *Annual Review of Cell and Developmental Biology* 14: 59–88. https://doi.org/10.1146/ANNUREV.CELLBIO.14.1.59.

Wong, C. C.Y., E. L. Meaburn, A. Ronald, T. S. Price, A. R. Jeffries, L. C. Schalkwyk, R. Plomin, and J. Mill. 2014. "Methylomic Analysis of Monozygotic Twins Discordant for Autism Spectrum Disorder and Related Behavioural Traits." *Molecular Psychiatry* 19 (4): 495. https://doi.org/10.1038/MP.2013.41.

Woodhead, Gregory J., Christopher A. Mutch, Eric C. Olson, and Anjen Chenn. 2006. "Cell-Autonomous β-Catenin Signaling Regulates Cortical Precursor Proliferation." *Journal of Neuroscience* 26 (48): 12620–30. https://doi.org/10.1523/JNEUROSCI.3180-06.2006.

Yamashiro, Kunihiko, Kei Hori, Esther S.K. Lai, Ryo Aoki, Kazumi Shimaoka, Nariko Arimura, Saki F. Egusa, et al. 2020. "AUTS2 Governs Cerebellar Development, Purkinje Cell Maturation, Motor Function and Social Communication." *IScience* 23 (12). https://doi.org/10.1016/J.ISCI.2020.101820.

Yao, Mingze, Xueke Zhou, Jiajian Zhou, Shixin Gong, Gongcheng Hu, Jiao Li, Kaimeng Huang, et al. 2018. "PCGF5 Is Required for Neural Differentiation of Embryonic Stem Cells." *Nature Communications* 9 (1): 1–12. https://doi.org/10.1038/s41467-018-03781-0.

Yoshizawa, Masato, Takeshi Kawauchi, Masaki Sone, Yoshiaki V Nishimura, Mami Terao, Kaori Chihama, Yo-Ichi Nabeshima, and Mikio Hoshino. 2005. "Involvement of a Rac Activator, P-Rex1, in Neurotrophin-Derived Signaling and Neuronal Migration." *Soc Neuroscience*. https://doi.org/10.1523/JNEUROSCI.4955-04.2005.

Zhang, Jiwang, and Linheng Li. 2005. "BMP Signaling and Stem Cell Regulation." *Developmental Biology* 284 (1): 1–11. https://doi.org/10.1016/J.YDBIO.2005.05.009.

Zhang, Xinwang, Menglong Rui, Guangmin Gan, Cong Huang, Jukang Yi, Huihui Lv, Wei Xie,

and Roger J. Colbran. 2017. “Neuroligin 4 Regulates Synaptic Growth via the Bone Morphogenetic Protein (BMP) Signaling Pathway at the Drosophila Neuromuscular Junction.” *The Journal of Biological Chemistry* 292 (44): 17991–5. https://doi.org/10.1074/JBC.M117.810242.

Zhao, Tianyu, Qini Gan, Arjun Stokes, Rhonda N.T. Lassiter, Yongping Wang, Jason Chan, Jane X. Han, David E. Pleasure, Jonathan A. Epstein, and Chengji J. Zhou. 2014. “β-Catenin Regulates Pax3 and Cdx2 for Caudal Neural Tube Closure and Elongation.” *Development (Cambridge, England)* 141 (1): 148–57. https://doi.org/10.1242/DEV.101550.

Zhao, Wukui, Yikai Huang, Jingzi Zhang, Mengjie Liu, Haijing Ji, Congcong Wang, Ning Cao, et al. 2017. “Polycomb Group RING Finger Proteins 3/5 Activate Transcription via an Interaction with the Pluripotency Factor Tex10 in Embryonic Stem Cells.” *Journal of Biological Chemistry* 292 (52): 21527–37. https://doi.org/10.1074/jbc.M117.804054.

Zi, Zhike, Douglas A. Chapnick, and Xuedong Liu. 2012. “Dynamics of TGF-β/Smad Signaling.” *FEBS Letters*. NIH Public Access. https://doi.org/10.1016/j.febslet.2012.03.063.

VITA

Zhuangzhuang Geng

EDUCATION

M.S. in Pharmacogenomics 2014 – 2016
Temple University, Philadelphia, PA

B.S. in Pharmacology 2010 - 2014
China Pharmaceutical University, Nanjing, China

PUBLICATIONS

1) **Geng, Z**., Yen-Teng, T., Hobbs, R., DeGraff, D., Wang, Y., Stafford J., Gao, Z. (2022) AUTS2 regulate cell fate commitment through Wnt signaling pathway during human brain development. (manuscript in preparation)

2) **Geng, Z**., Wang, Q., Miao, W., Wolf, T., Chavez J., Giddings, E., Hobbs, R., DeGraff, D., Wang, Y., Stafford J., Gao, Z. (2022) AUTS2 controls neuronal lineage choice through a novel PRC1-independent complex and BMP inhibition. Stem Cell Reports

3) **Geng, Z**., & Gao, Z. (2020). Mammalian PRC1 Complexes: Compositional Complexity and Diverse Molecular Mechanisms. International Journal of Molecular Sciences, 21(22), 8594.

4) Hanafiah, A., **Geng, Z**., Wang, Q., & Gao, Z. (2020). Differentiation and Characterization of Neural Progenitors and Neurons from Mouse Embryonic Stem Cells. JoVE (Journal of Visualized Experiments), (159), e61446.

5) Chen, S., Wang, Q., Yu, H., Capitano, M. L., Vemula, S., Nabinger, S. C., Gao, R., Yao, C., Kobayashi, M., **Geng, Z**., ... & Liu, Y. (2019). Mutant p53 drives clonal hematopoiesis through modulating epigenetic pathway. Nature communications, 10(1), 1-14.

6) Wang, Q., **Geng, Z**., Gong, Y., Warren, K., Zheng, H., Imamura, Y., & Gao, Z. (2018). WDR68 is essential for the transcriptional activation of the PRC1-AUTS2 complex and neuronal differentiation of mouse embryonic stem cells. Stem cell research, 33, 206-214.