Transcription factor POU3F2 regulates TRIM8 expression contributing to cellular functions implicated in schizophrenia

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Abstract
Schizophrenia (SCZ) is a neuropsychiatric disorder with aberrant expression of multiple genes. However, identifying its exact causal genes remains a considerable challenge. The brain-specific transcription factor POU3F2 (POU domain, class 3, transcription factor 2) has been recognized as a risk factor for SCZ, but our understanding of its target genes and pathogenic mechanisms are still limited. Here we report that POU3F2 regulates 42 SCZ-related genes in knockdown and RNA sequencing experiments of human neural progenitor cells (NPCs). Among those SCZ-related genes, TRIM8 (Tripartite motif containing 8) is located in SCZ-associated genetic locus and is aberrantly expressed in patients with SCZ. Luciferase reporter and electrophoretic mobility shift assays (EMSA) showed that POU3F2 induces TRIM8 expression by binding to the SCZ-associated SNP (single nucleotide polymorphism) rs5011218, which affects POU3F2 binding efficiency at the promoter region of TRIM8. We investigated the cellular functions of POU3F2 and TRIM8 as they co-regulate several pathways related to neural development and synaptic function. Knocking down either POU3F2 or TRIM8 promoted the proliferation of NPCs, inhibited their neuronal differentiation, and impaired the excitatory synaptic transmission of NPC-derived neurons. These results indicate that POU3F2 regulates TRIM8 expression through the SCZ-associated SNP rs5011218, and both genes may be involved in the etiology of SCZ by regulating neural development and synaptic function.

Introduction

Schizophrenia (SCZ) is a highly heritable psychiatric disorder with complex pathogenic mechanisms that are not fully understood\(^1\). Several leading hypotheses have been aimed at its etiology. Two of these are based upon the disruption of neural development and synaptic function, proposing that abnormal neural structure, function, and connectivity are at fault\(^2\)–\(^3\). Genetic studies also support such theories, associating SCZ with widespread gene dysregulations. Indeed, large scale genome-wide association studies (GWAS) have identified hundreds of genes linked to SCZ\(^4\). Dozens of these SCZ-related genes, such as GLT8D1, MiR-137, and CACNA1C, affect neural development and synaptic functions\(^5\)–\(^7\). However, for most of these risk genes, little is known about their functional relationship to SCZ and even less about their coordinated function.

SCZ is likely caused by many genes with small effects, but their collective interactions form a functional regulatory network that magnifies their effects considerably\(^8\). Several regulatory genes act as hub genes regulating their targets within the network, such as transcription factors, microRNAs, and long non-coding RNAs\(^6\),\(^9\),\(^10\). In a previous study, we constructed a gene coexpression module underlying SCZ risk using 394 brain samples from patients with SCZ and healthy controls\(^9\). We found brain-specific transcription factor POU3F2 to be one of the key regulators co-expressed with many genes within the SCZ-related module. POU3F2 is expressed primarily in the central nervous system and has an essential role in brain development\(^11\). However, the target genes of POU3F2 and their relationship with SCZ remain largely unknown.

Findings from several studies support the role of POU3F2 in SCZ etiology. POU3F2 involves neuronal functions that favor the neurodevelopmental hypothesis of SCZ. For example, POU3F2 regulates layer production and cortical neuron migration in the
developing neocortex\textsuperscript{11, 12}, while \textit{POU3F2}-deficient mice exhibit impaired hippocampal neurogenesis\textsuperscript{13}. Further, GWAS and brain imaging analyses have identified SCZ-related brain activity affected by \textit{POU3F2}\textsuperscript{14}. Additionally, \textit{POU3F2} expression is found to be aberrantly increased in patients with SCZ in three independent RNA-seq datasets\textsuperscript{15}. While such convergent evidence supports \textit{POU3F2} as a contributor to the risk for SCZ, knowledge of the pathogenic mechanism of \textit{POU3F2} is meager. Identifying \textit{POU3F2} targets and associated cellular functions may further clarify its pathogenic mechanism within SCZ.

We hypothesize that \textit{POU3F2} affects neurodevelopment and synaptic functions by regulating its co-expressed genes. To test this hypothesis, we first identified the co-expressed genes of \textit{POU3F2} for those with expression affected by \textit{POU3F2} knockdown in NPCs. Among these \textit{POU3F2} targets, we selected \textit{TRIM8} as a promising SCZ-related gene for downstream studies. \textit{TRIM8} is an E3 ubiquitin ligase and best known for its role in cell proliferation and immune response\textsuperscript{16, 17}. To interpret the regulatory mechanisms of \textit{POU3F2}-regulated \textit{TRIM8} expression, we investigated SCZ GWAS SNPs located in the \textit{TRIM8} promoter region. Lastly, to study the impact of \textit{POU3F2} and \textit{TRIM8} on neural development and synaptic function, we studied several cellular phenotypes associated with the expression changes of \textit{POU3F2} and \textit{TRIM8} in neural cells.

\section*{Results}

\subsection*{SCZ-related target genes of \textit{POU3F2} identified in NPCs}

From a gene coexpression module constructed in our previous study\textsuperscript{9}, we identified genes whose expression levels were affected by \textit{POU3F2}. The module contained 545 protein-coding genes (Table S1). We wanted to determine if any of these genes were being regulated by \textit{POU3F2} using a cellular model. We first characterized the human induced pluripotent stem cells (iPSCs) and differentiated them into NPCs (Supplementary Fig. 1). Next, we performed \textit{POU3F2} knockdown in the NPCs through lentiviral transduction and observed that 3,421 genes were changed significantly by RNA-seq analysis (false discovery rate (FDR) \(q < 0.05\), Table S2), with 1,961 of them were upregulated and 1,460 were downregulated (Fig. 1A). Of the 3,421 genes with expression changes, 60 overlapped with the 545 module genes and had a consistent direction of alteration in the \textit{POU3F2} knockdown and coexpression analyses (enrichment \(P = 9.1\text{e}^{-4}\); Fig. 1B, Table S3).

To explore whether these 60 \textit{POU3F2}-regulated genes are also risk genes for SCZ, we examined three published lists of SCZ candidate genes (Table S4) and performed gene enrichment analysis. The first gene list, obtained from the SZDB (schizophrenia database), contained 2,752 genes assembled from the Psychiatric Genomics Consortium (PGC) GWAS study, the sherlock integrative analysis, and a study of genes affected by copy number variations\textsuperscript{18}. The second list consisted of 106 genes with SCZ-associated rare variants that were selected from our published study\textsuperscript{9}. The third list had 4,096 differentially expressed protein-coding genes from PsychENCODE data (FDR \(q < 0.05\); SCZ \(n = 559\); Control \(n = 936\))\textsuperscript{19}. A total of 6,235 SCZ candidate genes resulted from combining these three gene lists. Enrichment analysis identified 42 out of the 60 \textit{POU3F2}-regulated genes from the 6,235 SCZ-related genes (enrichment \(P = 2.1\text{e}^{-5}\); Fig. 1C, Table S5). Functional enrichment

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analysis showed that the 42 genes were significantly enriched in the development of neural cells and the central nervous system (FDR q ≤0.05; Fig. 1D).

**TRIM8 stood out as an SCZ candidate gene among the 42 SCZ-related genes**

We prioritized the genes that have been identified as associated with an increased risk for SCZ among the 42 target genes using GWAS and gene expression data. PGC identified TRIM8, NCAN, and C1orf54 as SCZ candidate genes in a GWAS study using a cohort of 36,989 cases and 113,075 controls. The other 39 genes were identified in studies of rare variants and differential expression from smaller study samples. TRIM8, NCAN, and C1orf54 were selected for further analysis as they were identified in a well-powered study. We found that TRIM8 was located in the most significant GWAS locus (ranking the third locus, GWAS p = 6.198e-19). NCAN and C1orf54 followed far behind ranking as 47th and 48th loci, respectively (GWAS p = 3.634e-10; p = 4.487e-10). Furthermore, PsychENCODE data showed that TRIM8 expression increased in the brain tissues of patients with SCZ compared with controls (SCZ n = 559; control n = 936; differential expression p = 0.016), whereas NCAN and C1orf54 expression were not significantly altered. Therefore, we selected TRIM8 for further study.

**Three SCZ SNPs may mediate POU3F2-regulated TRIM8 expression by disrupting POU3F2 binding**

Although RNA-seq results showed that TRIM8 expression decreased after POU3F2 knockdown, POU3F2-specific mechanisms for regulating TRIM8 expression and its association with SCZ remain unknown. SCZ-related genetic variants are frequently located in non-coding regions, such as in promoter and enhancer regions. These variants regulate gene expression and generate gene expression quantitative trait loci (eQTL). Furthermore, SCZ-related variants can affect gene expression by disrupting transcription factor binding. Therefore, we hypothesized that SCZ-related variants in the regulatory region of TRIM8 influence its expression by affecting POU3F2 binding.

To identify the POU3F2 binding-disrupting SNPs associated with TRIM8 expression, we first identified POU3F2 binding sequences within the 6kb upstream region of TRIM8 (Table S6), referencing the JASPAR database. We then identified brain eQTL SNPs of TRIM8 within this 6kb genomic region using Brain eQTL Almanac (BRAINEAC) data from the UK Brain Expression Consortium (Table S7). Combining these results, we identified two eQTL SNPs (rs5011218 and rs11191359) that were co-localized with the predicted POU3F2 binding sites, and another eQTL SNP (rs4146428) which was near the predicted site. Data from the Lieber Institute for Brain Development (LIBD) also confirmed rs5011218 and rs4146428 to be TRIM8 eQTL signals in the dorsolateral prefrontal cortex (FDR q = 0.0027 for both SNPs, Fig. 2B).

To study the regulatory activity of these three SNPs (rs5011218, rs11191359, and rs4146428), we annotated them using the Encyclopedia of DNA Elements (ENCODE) data as summarized in the University of California Santa Cruz (UCSC) Genome Browser website. Graphical results showed that these three SNPs were covered by H3K27AC and DNaseI hypersensitivity signals, indicating regional high transcriptional activity (Fig. 2C).
We then tested the direct interaction between POU3F2 and the three SNPs. ChIP-seq data from the ENCODE project revealed that POU2F2 binds to the site surrounding rs4146428 in B-lymphocyte cells (Fig. 2C). POU3F2 and POU2F2 have a similar core binding motif within the POU domain. Therefore, POU3F2 could also bind to this site. Considering that POU3F2 is a brain-specific transcription factor and that SCZ-related SNPs confer their effects mainly in the brain, we downloaded POU3F2 ChIP-seq data from NPCs. The analysis revealed ChIP-seq peak signals overlapping these three SNPs sites (Fig. 2C), indicating their direct interaction with the POU3F2 protein.

Finally, we found that the three SNPs described above are associated with SCZ. These three SNPs were included in the PGC-reported set of SCZ GWAS SNPs having a 99% probability of containing SCZ causal variants (rs5011218 GWAS $P = 3.7e^{-9}$, rs11191359 GWAS $P = 2.5e^{-8}$, rs4146428 GWAS $P = 2.8e^{-8}$, Fig. 2A). All three SNPs were in linkage disequilibrium (non-random association of alleles at multiple loci, $r^2 > 0.6$) with the SCZ index SNP rs7907645 (GWAS $P = 1.3e^{-11}$, Fig. 2A). Data from BRAINEAC showed that the index SNP rs7907645 was also an eQTL SNP of TRIM8 in the occipital cortex ($P = 6.2e^{-3}$, Fig. 2A).

**POU3F2 promoted TRIM8 expression, and SNP rs5011218 modulated this regulation by altering POU3F2 binding efficiency**

To verify that POU3F2 positively regulates TRIM8 expression, we performed transient transfection assays to alter POU3F2 expression using POU3F2 overexpression plasmid or small interfering RNA (siRNA). POU3F2 overexpression in NPCs significantly increased mRNA expression of TRIM8 ($P < 0.01$, Fig. 3A) and TRIM8 protein expression ($P < 0.01$, Fig. 3C). Conversely, POU3F2 knockdown significantly decreased TRIM8 mRNA expression ($P < 0.001$, Fig. 3B) and lowered TRIM8 protein expression ($P < 0.001$, Fig. 3D). These results were consistent with the RNA-seq analysis, confirming that POU3F2 is a positive regulator of TRIM8 expression in NPCs.

Next, we tested what regulatory effects the different alleles of the three SNPs (rs5011218 A>C, rs11191359 A>T and rs4146428 G>A) have on TRIM8 expression using the dual-luciferase reporter assay. We first cloned the regulatory element of TRIM8 containing the reference alleles of three SNPs (rs5011218 A, rs11191359 A, rs4146428 G, Table S8) into luciferase reporter vectors. To see the effects of POU3F2 protein on the activity of the constructed vector, we then co-transfected the reporter vectors and the POU3F2 overexpression vector into NPCs. The results suggested that POU3F2 significantly increased the luciferase activity of the reporter vector through the regulatory element containing the reference alleles ($P < 0.001$, Fig. 3E). We then mutated the three reference alleles to test the regulatory effects of the alternative alleles. We found that the C allele of rs5011218, the T allele of rs11191359, and the A allele of rs4146428 each significantly inhibited the POU3F2-induced upregulation respectively ($P < 0.001$, Fig. 3E).

Finally, we studied the direct interaction between POU3F2 protein and the SNPs using EMSA. While the three SNPs all showed allelic effects in luciferase reporter assays, rs4146428 was not located within the predicted POU3F2 binding sites (Fig. 2A). Therefore, we studied the impact of rs5011218 and rs11191359 on POU3F2 binding by designing
several different probes for EMSA assays (table S9). We observed two shifted bands for rs5011218, suggesting that POU3F2 bound to the designed probes containing either the A or C allele (Fig. 3F). Moreover, POU3F2 protein had higher affinity for the A allele than the C allele. This observation explains the luciferase reporter results for rs5011218, in which the vector carrying allele A showed increased reporter activity. For rs11191359, no direct interaction between POU3F2 protein and the corresponding probes was detected (Supplementary Fig. 2).

**POU3F2 and TRIM8 expressions peaked in the prefrontal cortex during early developmental stages**

POU3F2 and TRIM8 expression patterns were assessed in human tissues using GTEx (genotype-tissue expression) data27, and we found that both POU3F2 and TRIM8 are widely expressed in various brain regions (Supplementary Fig. 3A and Supplementary Fig. 3B). In addition, spatiotemporal expression data from SZDB (schizophrenia database) showed that the mRNA expression levels of POU3F2 and TRIM8 gradually increased and then peaked during the early developmental stages of the prefrontal cortex (fetal and infant stages; Supplementary Fig. 3C). These data suggest that POU3F2 and TRIM8 may have a role in brain development.

**POU3F2 and TRIM8 co-regulated several signaling pathways associated with neural development and synaptic functions**

To further understand the functional roles of POU3F2 and TRIM8 implicated in SCZ, we performed pathway enrichment analysis of their co-regulated genes. A total of 1040 genes were significantly changed after TRIM8 knockdown in NPCs (FDR q < 0.05, Table S10). Genes totaling 600 were upregulated, and 440 downregulated (Supplementary Fig. 4A). When comparing RNA-seq data from TRIM8 and POU3F2 knockdown NPCs (Table S10 and Table S2), we found an overlap of 548 genes with consistent alteration direction in the two lists (Supplementary Fig. 4B, Table S11). Compared with the 6235 SCZ-related genes we collected (Table S4), 202 out of the 548 common genes were significantly enriched in the 6,235 SCZ genes (enriched P < 0.001, Table S12). The 548 POU3F2 and TRIM8 co-regulated genes were used in pathway enrichment analysis by Ingenuity Pathway Analysis (IPA) software. We identified many significantly enriched pathways related to neural development and synaptic functions, such as synaptic long-term depression, Wnt/β-catenin signaling, and axonal guidance signaling (enrichment P < 0.05; Supplementary Fig. 4C, Table S13).

**POU3F2 and TRIM8 regulated NPC proliferation by affecting the cell cycle**

Based on gene expression pattern and pathway enrichment analysis, we investigated the functional effects of POU3F2 and TRIM8 knockdown on cell proliferation. To examine the specificity of our POU3F2 or TRIM8 shRNAs, we used three different shRNAs to transfect the NPCs and obtained RNA-seq data from each (Fig. 1A and Supplementary Fig. 4A). We then compared the gene expression signatures produced by these shRNAs, according to the previous study28. Pearson correlation analysis showed that the expression levels of each gene from various RNA-seq data were highly correlated (Pearson Correlation Coefficient (PCC) > 0.98, P < 0.001; Supplementary Fig. 5). It means that these shRNAs produce the
same gene expression signatures, so the probability of an off-target effect was fairly low since off-target effects are typically random and are not reproducible. We then selected one of the shRNAs to knock down POU3F2 and TRIM8 expression, independently, in NPCs. Lentivirus encoding shRNA against POU3F2 (shPOU3F2-C) significantly decreased endogenous POU3F2 expression, and TRIM8 expression was also significantly decreased (respectively by 51%, \( P < 0.001 \) and by 43%, \( P < 0.001 \); Fig. 4A and 4B). However, TRIM8 shRNA (shTRIM8-C) decreased TRIM8 expression by 59% (\( P < 0.001 \); Fig. 4B) but had no effect on POU3F2 expression (\( P > 0.05 \); Fig. 4A). We also significantly upregulated TRIM8 expression in the POU3F2 knockdown cell line to determine if we could functionally rescue the phenotype changes in POU3F2 knockdown cells. (TRIM8 increased by 51%, \( P < 0.001 \); Fig. 4B).

We then studied the effects of expression changes of POU3F2 and TRIM8 on cell proliferation using EdU (5-ethynyl-2'-deoxyuridine) staining and CCK-8 (cell counting kit-8). With EdU staining, POU3F2 and TRIM8 knockdown significantly increased the ratio of EdU-positive cells (dividing cells) compared to control, respectively (increased by 39.8% and 36%, respectively, \( P < 0.001 \) for both; Fig. 4C and 4D). We also observed that TRIM8 overexpression reversed the increased ratio, induced by POU3F2 knockdown (decreased by 31.5%, \( P < 0.001 \); Fig. 4C and 4D). With the CCK-8 assay, we observed that silencing POU3F2 or TRIM8 increased the number of cells significantly at day 5 (\( P < 0.001 \); Fig. 4E), and overexpressing TRIM8 produced the opposite effect in POU3F2 knockdown NPCs (\( P < 0.01 \); Fig. 4E). Here we also performed the CCK-8 assay in another NPC line from StemCell Technologies. The result of this clone was the same. POU3F2 and TRIM8 knockdown significantly increased the number of cells at day 5, and TRIM8 overexpression reversed the proliferation rate in POU3F2 knockdown NPCs (\( P < 0.001 \); Supplementary Fig. 6).

To study how POU3F2 and TRIM8 knockdown promote cell proliferation, we performed cell cycle analyses. Flow cytometric analysis showed that POU3F2 or TRIM8 knockdown significantly decreased the proportion of NPCs in G1 phase respectively, compared with the control (\( P < 0.001 \) for both, Fig. 4F and 4G). In contrast, the proportion of cells in S and G2 phases significantly increased after reducing POU3F2 or TRIM8 expression in NPCs (\( P < 0.001 \) for both, Fig. 4F and 4G). These suggest that POU3F2 and TRIM8 knockdown promote cell transition from the G1 phase to the S and G2 phases. Finally, we found that TRIM8 overexpression in POU3F2 knockdown cells significantly reversed the cell proportion of each phase (\( P < 0.001 \), Fig. 4F and 4G).

To exclude the possibility of off-target effects on NPC proliferation for the shRNA lentivirus (shPOU3F2-C and shTRIM8-C), we used additional lentivirus shPOU3F2-B and shTRIM8-B to study the functional effects of POU3F2 and TRIM8 on cell proliferation. The results for shPOU3F2-B and shTRIM8-B lentivirus were consistent with the original lentivirus. The shPOU3F2-B and shTRIM8-B lentivirus significantly reduced the expressions of POU3F2 and TRIM8 compared with controls (decreased by 54% and 49%, respectively, \( P < 0.001 \) for both; Supplementary Fig. 7A and 7B). With EdU staining, EdU-positive cells were significantly increased in POU3F2 and TRIM8 knockdown NPCs compared with controls (increased by 41% and 33%, respectively, \( P < 0.01 \) for both; Supplementary Fig. 7C and 7D). With the CCK-8 assay, silencing POU3F2 or TRIM8 increased the number of cells

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significantly at day 5 ($P < 0.001$; Supplementary Fig. 7E). Cell cycle analysis showed that
*POU3F2* and *TRIM8* knockdown both significantly decreased the proportion of NPCs in
G1 phase, compared with the control ($P < 0.001$, Supplementary Fig. 7F and 7G). The
proportion of cells in S and G2 phases significantly increased after reducing *POU3F2* or
*TRIM8* expression in NPCs ($P < 0.01$ for both, Supplementary Fig. 7F and 7G). Finally, we
found that *TRIM8* overexpression in *POU3F2* knockdown NPCs significantly reversed each
cellular phenotype ($P < 0.01$, Supplementary Fig. 7).

**POU3F2** and **TRIM8** regulated the neuronal differentiation and excitatory synaptic
transmission

We tested the effects of *POU3F2* and *TRIM8* on the differentiation of NPCs to neurons
and astrocytes. Compared to controls cells, *POU3F2* or *TRIM8* knockdown in NPCs
significantly reduced the ratio of Tuj1 (Neuron specific class III beta-tubulin, immature
neurons marker)-positive neurons (for *POU3F2*, $P < 0.01$; for *TRIM8*, $P < 0.001$; Fig. 5A
and 5B). *TRIM8* overexpression rescued the differentiation defect of *POU3F2* knockdown
NPCs, as confirmed by significantly increased Tuj1-positive neurons ($P < 0.01$; Fig. 5A and
5B). Unexpectedly, *POU3F2* and *TRIM8* knockdown did not affect the differentiation of
NPCs into astrocytes (Supplementary Fig. 8A and 8B). We quantified the number and length
of neuronal dendrites after differentiating NPCs into mature neurons. Dendrite length and
number were significantly decreased in neurons with lower *POU3F2* or *TRIM8* expression
(for *POU3F2*, $P < 0.001$ for length and number; for *TRIM8*, $P < 0.01$ for length and $P <$
0.001 for number; Fig. 5C, 5D and 5E). Dendrite length and number in *POU3F2* knockdown
neurons were rescued by *TRIM8* overexpression (Fig. 5C, 5D and 5E). We also used
another NPC line to study the effects of *POU3F2* and *TRIM8* on neuronal differentiation.
This clone also showed that *POU3F2* or *TRIM8* knockdown significantly reduced the ratio
of Tuj1-positive neurons, and *TRIM8* overexpression reversed the ratio of Tuj1-positive
neurons in *POU3F2* knockdown cells (Supplementary Fig. 8C and 8D).

In addition to neurodevelopmental defects, aberrant expression of SCZ susceptibility genes
often causes the dysfunctions of excitatory synaptic transmission of neurons$^{29, 30}$. To
examine whether *TRIM8* and *POU3F2* cause this dysfunction, we first differentiated wild
type NPCs into mature neurons and then altered the *TRIM8* and *POU3F2* expression in
the neurons. We then measured the miniature excitatory postsynaptic currents (mEPSCs),
a key indicator of excitatory synaptic transmission. Lentivirus encoding shRNA against
*POU3F2* (sh*POU3F2*-C) significantly decreased endogenous *POU3F2* expression, and
*TRIM8* expression was also significantly decreased in the mature neurons (respectively by
55%, $P < 0.001$ and by 48%, $P < 0.001$; Supplementary Fig. 9). However, *TRIM8* shRNA
(sh*TRIM8*-C) decreased *TRIM8* expression by 58% ($P < 0.001$; Supplementary Fig. 9C)
but had no effect on *POU3F2* expression (Supplementary Fig. 9B). We also significantly
upregulated *TRIM8* expression in the *POU3F2* knockdown neurons to determine if we
could functionally rescue the phenotype changes. (*TRIM8* increased by 42%, $P < 0.001$;
Supplementary Fig. 9C). The mEPSCs recorded from *POU3F2* or *TRIM8* knockdown
neurons showed a significant decrease in frequency (for *POU3F2*, $P < 0.01$; for *TRIM8*, $P$
< 0.001; Fig. 5F and 5G), but no change in amplitude (Fig. 5F, 5H and 5J), compared to
controls. The decreased frequency in cells with *POU3F2* and *TRIM8* knockdown is also
revealed with the rightward shift of the cumulative probability curves (Fig. 5I). As expected, TRIM8 overexpression in POU3F2 knockdown neurons significantly restored the frequency of mEPSCs (P < 0.01; Fig. 5F, and 5G).

To exclude the possibility of off-target effects on neuronal differentiation and synaptic transmission of shPOU3F2-C and shTRIM8-C lentivirus, we used shPOU3F2-B and shTRIM8-B lentivirus to replicate the functional study. The results of shPOU3F2-B and shTRIM8-B lentivirus were consistent with the results of shPOU3F2-C and shTRIM8-C. POU3F2 or TRIM8 knockdown significantly reduced the ratio of Tuj1-positive neurons (P < 0.01 for both; Supplementary Fig. 10A and 10B). Dendritic length and number were significantly decreased in mature neurons with reduced POU3F2 or TRIM8 expression (P < 0.01; Supplementary Fig. 10C, 10D and 10E). In addition, mEPSCs recorded from POU3F2 or TRIM8 knockdown neurons showed a significant decrease in frequency (P < 0.05; Supplementary Fig. 10F, 10G, and 10I), without any change in amplitude (Supplementary Fig. 10F, 10H, and 10J), compared with controls. Finally, TRIM8 overexpression significantly restored each cellular phenotype in the POU3F2 knockdown cells (P < 0.05; Supplementary Fig. 10).

Discussion

Our functional study suggests that POU3F2 and TRIM8 are involved in the pathophysiology of SCZ, which is characterized as a disorder of neural development and synaptic function. Focusing on the regulatory mechanisms of POU3F2-mediated TRIM8 expression, we found that two alleles of SCZ-related SNP rs5011218, A and C, showed different binding affinities to POU3F2. The A allele had greater affinity and promoted TRIM8 expression more effectively than the C allele. PGC reported that patients with SCZ have a significantly higher frequency of the A allele of rs5011218 than healthy controls (P = 9.65e-9; SCZ n = 33,426, Control n = 32,541). Moreover, studies showed that POU3F2 and TRIM8 have an abnormally high level of expression in the brain tissue of patients with SCZ. Based on these data, we speculate that both increased expression of POU3F2 and the rs5011218 A allele aberrantly stimulate high expression of TRIM8, which in turn heightens SCZ risk. These results exemplify how non-coding variants can contribute to SCZ risk by affecting gene expression.

POU3F2, TRIM8, and their co-regulated genes appear to form a complex regulatory network contributing to SCZ. We observed that POU3F2 and TRIM8 co-regulated genes enriched in many established neurodevelopmental and synaptic pathways implicated in SCZ. These pathways include the Wnt/β-catenin, long-term depression (LTD), axonal guidance, and ephrin receptor signaling pathways (Table S13). The Wnt/β-catenin pathway is critical in the formation of neuronal circuits by regulating neuronal differentiation, dendritic development, and synaptic function. SCZ has previously been characterized by dysfunction of Wnt/β-catenin signaling pathway. The LTD pathway is associated with neuronal connectivity by modulating synaptic plasticity. Research shows that patients with SCZ display reduced LTD-like plasticity. The axonal guidance pathway ensures the accurate positioning of axons during synaptic connection and neural circuitry formation.
Ephrin receptor signaling is also involved in many neuronal functions, including axonal directional guidance, synaptic plasticity, and cell migration. To elucidate the pathogenic mechanisms of POU3F2 and TRIM8, we studied the cellular functions of the genes using iPSC-derived NPCs and neurons. iPSCs technology and the following neural differentiation methods are useful approaches for the in vitro modeling of SCZ. Many studies show that iPSC-derived NPCs and neurons carrying SCZ-related genetic changes exhibit cellular phenotypes similar to those observed in human postmortem studies and animal models. By altering POU3F2 and TRIM8 expression, we determined that both regulate NPC proliferation by impacting the cell cycle and that both regulate neuronal differentiation. Abnormalities in cell differentiation and proliferation have been reported in patients with SCZ. Since bipolar disorder is also a neurodevelopmental disorder, and POU3F2 is located in its genome-wide significant risk locus, POU3F2-regulated neural functions might also be related to the etiology of bipolar disorder. Moreover, POU3F2 or TRIM8 knockout reduces excitatory synaptic transmission. This similar defect of synaptic function has been observed in postmortem and in vivo studies of patients with SCZ. In this way, our study highlights the possible cellular and molecular mechanisms for these phenotypes.

Other published findings also associate POU3F2 and TRIM8 with neural development and implicate them in terms of SCZ risk. Children with a mutation in POU3F2 possess varying degrees of developmental delay and intellectual disability. According to two other studies, children with TRIM8 mutations also exhibit developmental delays, limited language development, and abnormalities in brain magnetic resonance imaging. POU3F2 has shown influence over neurogenesis with the regulation of neurodevelopmental genes, such as Delta1, Hes5, and Tbr2. Fibroblasts can be directly differentiated into neurons by POU3F2 overexpression with a combination of other transcription factors. Santina et al. identified several TRIM8-regulated neurodevelopmental and synaptic pathways in mouse neural stem cells, such as axonal guidance, ephrin receptor, and synaptic long-term depression pathways. TRIM8 is an E3 ubiquitin-protein ligase, and this type of ligase plays a key role in the developmental pathogenesis of schizophrenia by regulating synapse plasticity and function. For example, the E3 ligase TRAF6 can affect synaptic transmission by regulating PSD-95 (postsynaptic density protein 95) ubiquitination. These findings support our data showing that POU3F2 and TRIM8 regulate neural development and synaptic function. Moreover, TRIM8 is involved in immune responses by regulating TNFα and IL-1β, and these neurotoxic proinflammatory factors may contribute to brain volume loss in SCZ.

There are many interesting genes from the 42 POU3F2 targeted gene list yet to be studied, including PAX6 (paired box 6), NTRK2 (neurotrophic receptor tyrosine kinase 2), NDRG2 (N-myc downstream-regulated gene 2 protein), and NCAN (neurocan core protein). The transcription factor PAX6 has essential functions in early forebrain development. The NTRK2 protein was shown to be a receptor for BDNF (brain derived neurotrophic factor) and regulates aspects of neural development, including neurite outgrowth, neuron migration, and synaptic plasticity. NDRG2 expressed in different brain regions and involved in neurite outgrowth and astrocyte differentiation. Gene NCAN is expressed mainly in brain.
nervous system tissue and has effects on cell migration similar to those of \textit{POU3F2}. Therefore, \textit{POU3F2}-regulated target genes may contribute to neurodevelopment in various ways.

Meanwhile, our study bears some limitations. Our validation found only 60 of the 545 genes of the previously constructed coexpression module to be regulated by \textit{POU3F2}. The remainder of \textit{POU3F2} target genes may be missing due to a difference in the cell types and developmental stages used in the two studies. In our previous work, the gene expression data for predicting the \textit{POU3F2} regulatory network was derived from adult brain tissues containing various neural cell types\textsuperscript{9}, whereas our current study used the early neurodevelopmental cells (NPCs) to validate \textit{POU3F2} regulation. The iPSCs-derived 3D organoids contain multiple neural cell types and can be used to recapitulate complex features of the brain\textsuperscript{66}. Therefore, we could select a 3D organoid model to further study gene regulatory relationships and developmental functions. On the other hand, \textit{POU3F2} is only one of the transcription factors in the gene coexpression module, other transcription factors could regulate remaining target genes.

In summary, our study suggests that \textit{POU3F2} and \textit{TRIM8} as risk genes associated with SCZ etiology and SCZ risk variant rs5011218 mediates \textit{POU3F2}-regulated \textit{TRIM8} expression.

\section*{Materials and methods}

\subsection*{Generation of NPCs and differentiation into neurons and astrocytes}

The normal human-induced pluripotent stem cells (iPSCs) were obtained from the ATCC (#ACS-1011) and cultured in mTeSR1 medium (StemCell Technologies, #05850). Human iPSCs were differentiated into NPCs using a neural induction medium (StemCell Technologies, #05835), and NPCs were maintained in Neural Progenitor Medium (StemCell Technologies, #05833). An additional NPC line (StemCell Technologies, #70901) was ordered for cell proliferation and neuronal differentiation assays. Neuronal differentiation was induced by a Neuron Differentiation and Maturation Kit (StemCell Technologies, #08500, #08510). For astrocyte differentiation, NPCs were plated on Matrigel-coated culture dish, then incubated in Astrocyte Differentiation and Maturation media (StemCell Technologies, #08540, #08550).

\subsection*{Karyotyping}

The karyotyping was performed as previously described\textsuperscript{67}. Cells were treated with colcemid (final concentration 0.1 \text{ug/mL}) for 4 hours (h) in 37°C incubator. The cells were harvested using Accutase (StemCell Technologies, #07920) and then incubated with 0.075 \text{M KCl} for 10 minutes (min) at 37°C. Cells were then treated with Carnoy fixative. metaphase spreads were prepared from cells using the air-drying method, followed by the protocol for G binding using Giemsa stain. The number and structure of chromosomes were examined in twenty cells.
Lentivirus infection

ShRNA lentiviruses targeting POU3F2 and TRIM8 were purchased from Origene (# TL310273V, # TL300821V). The POU3F2 shRNA lentiviral particles (# TL310273V) contain four specific shRNAs (shPOU3F2-A, shPOU3F2-B, shPOU3F2-C, and shPOU3F2-D) and one scrambled shRNA. The TRIM8 shRNA lentiviral particles (# TL300821V) also contain four specific shRNAs (shTRIM8-A, shTRIM8-B, shTRIM8-C, and shTRIM8-D) and one scrambled shRNA. For RNA-seq analysis, we selected three shRNAs to silence POU3F2 or TRIM8 expression in NPCs and used the scrambled shRNA as a negative control. NPCs were infected according to the manufacturer’s protocol (MOI=20). After 72 h viral infection, cells were treated with puromycin (1µg/ml) to generate mixed NPCs expressing specific shRNA sequence. The shRNA sequences were as following: shPOU3F2-A, 5′-CCTGTATGGCAACGTGTTCTCGCAGACCA-3′; shPOU3F2-B, 5′-GGCTCTGGAGAGCCATTTCCTCAAATGCC-3′; shPOU3F2-C, 5′-ATGGCGACCGCAGCGTCTAACCACTACAG-3′; shTRIM8-A, 5′-AACCTGAACAGATCCTGGAGGCTGACCA-3′; shTRIM8-B, 5′-TAAGATCGGCCACCTGAACTCCAAGCTGCTC-3′; shTRIM8-C, 5′-GGCAAGATTCTCGTCTGTTCTGTGGACAA-3′; scrambled shRNA: 5′-GCACTACCAGAGCTAACTCAGATAGTACT-3′. We selected shPOU3F2-C, shPOU3F2-B, shTRIM8-C, shTRIM8-B, and the scrambled shRNA to study the functional effects of POU3F2 and TRIM8. Lentiviral ORF (open reading frame) particle of TRIM8 (Origene, # RC205812L2V) and control lentivirus (Origene, # PS100071V) were used for TRIM8 overexpression in functional rescue assay.

RNA-seq library preparation and sequencing

Total RNA was extracted from cells using the miRNeasy Mini Kit (217004, Qiagen) and treated with DNAase. The RNA quality was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies). All samples were of high quality having a RIN index of 10. 450 ng RNA per sample was used for library preparation with Illumina TruSeq Stranded Total RNA with Ribo-Zero Gold Kit following manufacturer protocols. Sequencing was performed using an Illumina NextSeq500 sequencer.

RNA-seq data and differential expression analyses

An average of 46M paired-end 75bp reads per sample were generated from sequencing. Raw data in FASTQ format were transferred from the SUNYMAC core facility server. Sequencing quality was accessed by FastQC v0.11.7 program, low-quality bases and reads were filtered from raw reads by Trimmomatic v0.36 with a Phred score cutoff of 30. The trimmed reads were mapped to Gencode GRCh38.p12 Release 29 human reference genome using STAR aligner v2.6.0c. Reads mapped to genes were summarized by featureCounts program in subread v1.6.1. Genes were filtered by CPM (count-per-million) ≥ 1 in at least two samples, and data were normalized to effective library size by edgeR v3.22.3 program. Differential gene expression analyses were performed using edgeR. These RNA-seq data (GSE143692) has been submitted to the GEO (Gene Expression Omnibus) database.
Transient transfection of plasmid and small interfering RNA (siRNA)

The human POU3F2 overexpression plasmid (pYr-CMV-Kan2-POU3F2-Flag) was purchased from Changsha Yingrun Biotechnology (#HO005604). This vector was engineered to express the complete POU3F2 ORF with an expression flag tag. The corresponding control plasmid (pYr-CMV-Kan2-Flag) was also purchased from Changsha Yingrun Biotechnology (VPY0900). POU3F2 siRNA was synthesized by Genepharma, with the following sequence: 5′-GGCGGAUCAAACUGGGAUU-3′. The control siRNA sequence: 5′-UUCUCCGAACGUUCAGUUG-3′. For plasmid transfection, cells at 80% confluency in 6-well plates were transfected with 2.5 ug plasmid using Lipofectamine LTX & PLUS Reagent (Invitrogen). For siRNA transfection, cells at 40% confluency in 6-well plates were transfected with siRNA at a final concentration of 50 nmol/L using Lipofectamine RNAiMAX Reagent (Invitrogen). After 36 h, we extracted RNA or protein for expression analyses.

Quantitative Real-time PCR

Total RNA was extracted from cells using the miRNeasy Mini Kit (217004, Qiagen). cDNA was synthesized using the HiScriptII Q Select RT SuperMix (R223–01, Vazyme), after which ChamQ SYBR qPCR Master Mix (Q311–01, Vazyme) was used to quantitate PCR according to the manual. Endogenous housekeeping genes (β-actin or GAPDH) were used as internal standards. The cycling protocol was completed as follows: 30 seconds at 98°C, followed by 35 cycles of 98°C for 15 seconds and 60°C for 30 seconds. The primer sequences are listed in supplementary table S14.

Western Blot

Protein isolation was performed with cell lysis buffer (Beyotime, #P0013). Protein concentration was detected using a Detergent Compatible Bradford Protein Assay Kit (Beyotime). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and blocked by 5% milk for 1.5 h. Membranes were incubated with primary antibodies overnight at 4°C. The following antibodies were used: POU3F2 (1:1000, Cell Signaling Technology 12137), TRIM8 (1:1000, Abcam ab155674), and GAPDH (1:10000, Sigma G9295). Corresponding secondary antibodies (1:5000, Jackson ImmunoResearch 111–035-144) were used to bind with primary antibodies at room temperature for 2 h. Protein bands were detected by chemiluminescence using the BeyoECL Plus (Beyotime, #P0018S). Relative intensities of the protein bands were quantified by software ImageJ (https://imagej.nih.gov/ij/).

Immunocytochemistry

After washing twice with PBS, cells were fixed with 4% formaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. After washing thrice with PBS, cells were blocked in PBS containing 5% BSA for 30 min, followed by incubation with primary antibodies for 1.5 h at room temperature. Primary antibodies were as follows: Pax6 (1:100, Santa Cruz sc-32766), Nestin (1:100, Santa Cruz sc-23927), Nanog (1:200, Cell Signaling Technology 4903), OCT4 (1:200, Cell Signaling Technology 2750), Tuji(1:200, Cell Signaling Technology 5568), MAP2 (1:200, Cell Signaling Technology...
8707), and GFAP (1:200, Cell Signaling Technology 3670). After washing twice with PBS, cells were incubated with fluorescence-labeled secondary antibodies (1:200, Jackson ImmunoResearch 115–165-003 and 115–545-003) for 1 h at room temperature. Finally, 1 ug/ml DAPI was used for staining the nuclei.

**Prediction of POU3F2 binding sites and regulatory SNPs**

To identify POU3F2 binding sites and regulatory SNPs located within the regulatory region of TRIM8, we downloaded the 6kb upstream regulatory sequences of TRIM8 from the UCSC website. After this, we predicted putative POU3F2 binding sites from these data using the JASPAR database. The relative profile score threshold was 60%. For regulatory SNPs prediction, we collected all SNPs within the 6kb upstream region of TRIM8 using the UCSC website. Among these SNPs, eQTL SNPs of TRIM8 were selected for further analysis using the BRAINEAC database.

**Dual-luciferase reporter assay**

The upstream regulatory region containing the reference alleles of three SNPs (rs5011218, rs11191359, and rs4146428) was cloned into the PGL3-Basic vector using a One Step Cloning Kit (Vazyme, C112). This region is 1003 bp and contains the A alleles of rs5011218 and rs11191359 and G allele of rs4146428. The sequence and SNPs information are listed in table S8. The Fast Mutagenesis Kit V2 (Vazyme, C214) was used for SNP mutation. NPCs were co-transfected with 500 ng reconstructed vector, 10 ng pRL-TK, and 500 ng POU3F2 overexpression vectors (or empty vectors) in 24-well plates. After 48 h, the activities of firefly luciferase and renilla luciferase were measured using the dual-luciferase assay kit (Promega, E1910).

**Electrophoretic mobility shift assay**

After transient transfection with the POU3F2 overexpression vector for 48 h, nuclear proteins were extracted from NPCs using NE-PER nuclear reagents (Thermo scientific, 78833). Oligonucleotide probes were designed with corresponding SNPs, flanked by 15 bp, in both a cold and 5’ biotinylated form (supplementary table S9). Oligonucleotides were annealed by heating to 95°C for 5 min with subsequent cooling to room temperature (5°C/min). POU3F2 antibody (Santa Cruz, sc-393324X) was used to achieve gel supershift. EMSA was performed with Light Shift Chemiluminescent EMSA Kit (Thermo scientific, 20148). The binding reaction was carried out at 25°C for 30 min with 6 ng of nuclear extract and 20 fmol of labeled probes. The samples were electrophoresed in a 6% polyacrylamide gel at 100 V until the bromophenol migrated approximately 3/4 down the length of the gel. The transfer was completed 200mA for 1.5 h. When the transfer was completed, the transferred DNA was crosslinked to a nylon membrane. Finally, biotin-labeled DNA was detected by chemiluminescence.

**NPCs proliferation assay**

Cell proliferation was assessed using cell counting kit-8 (Beyotime, C0038) and BeyoClick™ EdU cell proliferation kit (Beyotime, C0075L). For the cell counting kit-8 assay, 2x10^3 NPCs were seeded into 96-well plates, initially. At various later time points,
20 μl WST-8 was added into each well, which contained 200 μl of media. The plates were then incubated at 37°C for 4 h before the optical density was measured at 450 nm in a microplate reader (BioTek). For the EdU staining assay, 1 × 10^6 NPCs were plated on 25 mm coverslips and cultured overnight. Next, we removed half of the medium volume and added an equal volume of the EdU working solution (20 μM) to make a final concentration of 10 μM. After incubating for 5 h, we used 4% formaldehyde for cell fixation and 0.3% TritonX-100 for permeabilization. Finally, we performed the EdU detection and DNA staining using click additive solution and Hoechst 33342.

Cell cycle analysis

We used the cell cycle analysis kit (Beyotime, C1052) for cell staining. Cells were briefly washed twice in 1X PBS before being fixed overnight in 70% ethanol. On the following day, cells were stained with a solution of propidium iodide (PI) at 37°C for 30 min after being washed three times with 1X PBS. Finally, the cells were analyzed using FACScalibur (BD Biosciences).

Quantification of dendritic length and number

Neurons were stained with MAP2 for dendritic length and number measurements. We used the NeuronJ program to trace dendrites following the instructions. Dendrites were assigned to clusters, with each cluster comprised of a primary dendrite and all its associated branches. After tracing was completed and clusters were assigned, a text file containing the dendritic length and number information was generated. A total of 64 neurons were measured for each group.

Electrophysiological recordings

We seeded NPCs onto glass coverslips and differentiated them into neurons for 50 days using a Neuron Differentiation and Maturation Kit (StemCell Technologies, #08500, #08510) for electrophysiological recordings. We then used the purchased lentivirus (shPOU3F2-C, shTRIM8-C, and scrambled shRNA, MOI=20) to alter the gene expression in the neurons, after which we cultured the neurons for 5 additional days before whole-cell patch-clamp recordings as previously described. At room temperature, mEPSCs were recorded at −60 mV. The pipettes were filled with solutions containing (in mM): 130 K-glucoritate, 8 NaCl, 10 HEPES, 0.4 EGTA, 2 Mg-ATP, and 0.25 GTP-Tris, pH 7.25. During the recording, neurons were maintained in the artificial cerebrospinal fluid (ACSF) bubbled with a mixture of CO2 (5%) and O2 (95%). The ACSF contained (in mM): 121 NaCl, 4.2 KCl, 1.1 CaCl2, 1 MgSO4, 29 NaHCO3, 0.45 NaH2PO4-H2O, 0.5 Na2HPO4, and 20 glucose. Picrotoxin (PTX, 100 μM) and tetrodotoxin (TTX, 1 μM) were added to the ACSF to block GABAA receptor-mediated inhibitory responses and action potential-evoked synaptic responses, respectively. The Multiclamp 200B and pCLAMP software (Molecular Devices) were used for signal recording. Data were analyzed by Clampfit 10.7 and Mini Analysis softwares.
Statistical Analysis

Data are expressed as mean ± standard deviation (SD) with statistical comparisons performed using Student’s t-test. Results were considered statistically significant at p<0.05. P values were adjusted for multiple testing using the Benjamini-Hochberg method. We used Fisher’s exact test or Chi-square test to assess the significance of gene enrichment analysis, and image fields were randomly selected under 20X or 40X magnification.

URLs

For JASPAR database22, http://jaspar.genereg.net/; For BRAINEAC data23, http://www.braineac.org/; For LIBD database24, http://eqtl.brainseq.org/phase1/eqt/l; For UCSC Genome Browser25, https://genome.ucsc.edu; For GTEx27, https://gtexportal.org/; For SZDB18, http://www.szdb.org/index.html;

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Walker E, Kestler L, Bollini A, Hochman KM. Schizophrenia: etiology and course. Annu Rev Psychol 2004; 55: 401–430. [PubMed: 14744221]
2. Frankle WG, Lerma J, Laruelle M. The Synaptic Hypothesis of Schizophrenia. Neuron 2003; 39(2): 205–216. [PubMed: 12873379]
3. Birnbaum R, Weinberger DR. Genetic insights into the neurodevelopmental origins of schizophrenia. Nat Rev Neurosci 2017; 18(12): 727–740. [PubMed: 29070826]
4. Schizophrenia Working Group of the Psychiatric Genomics C. Biological insights from 108 schizophrenia-associated genetic loci. Nature 2014; 511(7510): 421–427. [PubMed: 25056061]
5. Yang CP, Li X, Wu Y, Shen Q, Zeng Y, Xiong Q et al. Comprehensive integrative analyses identify GLT8D1 and CSNK2B as schizophrenia risk genes. Nat Commun 2018; 9(1): 838. [PubMed: 29483533]
6. Yin J, Lin J, Luo X, Chen Y, Li Z, Ma G et al. miR-137: a new player in schizophrenia. Int J Mol Sci 2014; 15(2): 3262–3271. [PubMed: 24566148]
7. Simms BA, Zamponi GW. Neuronal voltage-gated calcium channels: structure, function, and dysfunction. Neuron 2014; 82(1): 24–45. [PubMed: 24698266]
8. Fromer M, Roussos P, Sieberts SK, Johnson JS, Kavanagh DH, Perumal TM et al. Gene expression elucidates functional impact of polygenic risk for schizophrenia. Nat Neurosci 2016; 19(11): 1442–1453. [PubMed: 27668389]
9. Chen C, Meng Q, Xia Y, Ding C, Wang L, Dai R et al. The transcription factor POU3F2 regulates a gene coexpression network in brain tissue from patients with psychiatric disorders. Sci Transl Med 2018; 10(472): eaat8178. [PubMed: 30545964]
10. Ren Y, Cui Y, Li X, Wang B, Na L, Shi J et al. A co-expression network analysis reveals lncRNA abnormalities in peripheral blood in early-onset schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 2015; 63: 1–5. [PubMed: 25967042]

11. He X, Treacy M, Simmons D, Ingraham H, Swanson L, Rosenfeld M. Expression of a large family of POU-domain regulatory genes in mammalian brain development. Nature 1989; 340(6228): 35–41. [PubMed: 2739723]

12. McEvilly R, de DM, Schonemann M, Hooshmand F, Rosenfeld M. Transcriptional regulation of cortical neuron migration by POU domain factors. Science 2002; 295(5559): 1528–1532. [PubMed: 11859196]

13. Hashizume K, Yamanaka M, Ueda S. POU3F2 participates in cognitive function and adult hippocampal neurogenesis via mammalian-characteristic amino acid repeats. Genes Brain Behav 2018; 17(2): 118–125. [PubMed: 28782255]

14. Potkin SG, Turner JA, Guffanti G, Lakatos A, Fallon JH, Nguyen DD et al. A genome-wide association study of schizophrenia using brain activation as a quantitative phenotype. Schizophr Bull 2009; 35(1): 96–108. [PubMed: 19023125]

15. Pearl JR, Colantuoni C, Bergey DE, Funk CC, Shannon P, Basu B et al. Genome-Scale Transcriptional Regulatory Network Models of Psychiatric and Neurodegenerative Disorders. Cell Syst 2019; 8(2): 122–135 e127. [PubMed: 30772379]

16. Li Q, Yan J, Mao AP, Li C, Ran Y, Shu HB et al. Tripartite motif 8 (TRIM8) modulates TNAfalpha- and IL-1beta-triggered NF-kappaB activation by targeting TAK1 for K63-linked polyubiquitination. Proc Natl Acad Sci U S A 2011; 108(48): 19341–19346. [PubMed: 22084099]

17. Caratozzolo MF, Micale L, Turturo MG, Cornacchia S, Fusco C, Marzano F et al. TRIM8 modulates p53 activity to dictate cell cycle arrest. Cell Cycle 2012; 11(3): 511–523. [PubMed: 22262183]

18. Wu Y, Yao YG, Luo XJ. SZDB: A Database for Schizophrenia Genetic Research. Schizophr Bull 2017; 43(2): 459–471. [PubMed: 27451428]

19. Gandal MJ, Zhang P, Hadjimichael E, Walker RL, Chen C, Liu S et al. Transcriptome-wide isoform-level dysregulation in ASD, schizophrenia, and bipolar disorder. Science 2018; 362(6420): eaat8127. [PubMed: 30545856]

20. Roussos P, Mitchell AC, Voloudakis G, Fullard JF, Pothula VM, Tsang J et al. A role for noncoding variation in schizophrenia. Cell Rep 2014; 9(4): 1417–1429. [PubMed: 25453756]

21. Huo Y, Li S, Liu J, Li X, Luo XJ. Functional genomics reveal gene regulatory mechanisms underlying schizophrenia risk. Nat Commun 2019; 10(1): 670. [PubMed: 30737407]

22. Wasserman WW, Sandelin A. Applied bioinformatics for the identification of regulatory elements. Nat Rev Genet 2004; 5(4): 276–287. [PubMed: 15131651]

23. Glass D, Vinuela A, Davies MN, Ramasamy A, Parts L, Knowles D et al. Gene expression changes with age in skin, adipose tissue, blood and brain. Genome Biol 2013; 14(7): R75. [PubMed: 23889843]

24. Jaffe AE, Straub RE, Shin JH, Tao R, Gao Y, Collado-Torres L et al. Developmental and genetic regulation of the human cortex transcriptome illuminate schizophrenia pathogenesis. Nat Neurosci 2018; 21(8): 1117–1125. [PubMed: 30050107]

25. Rosenbloom KR, Sloan CA, Malladi VS, Dresser TR, Learned K, Kirkup VM et al. ENCODE data in the UCSC Genome Browser: year 5 update. Nucleic Acids Res 2013; 41(Database issue): D56–63. [PubMed: 23193274]

26. Xue Y, Qian H, Hu J, Zhou B, Zhou Y, Hu X et al. Sequential regulatory loops as key gatekeepers for neuronal reprogramming in human cells. Nat Neurosci 2016; 19(6): 807–815. [PubMed: 27110916]

27. Consortium GT. The Genotype-Tissue Expression (GTEx) project. Nat Genet 2013; 45(6): 580–585. [PubMed: 23715323]

28. Semizarov D, Frost L, Sarthy A, Kroeger P, Halbert DN, Fesik SW. Specificity of short interfering RNA determined through gene expression signatures. Proc Natl Acad Sci U S A 2003; 100(11): 6347–6352. [PubMed: 12746500]
29. Cui L, Sun W, Yu M, Li N, Guo L, Gu H et al. Disrupted-in-schizophrenia1 (DISC1) L100P mutation alters synaptic transmission and plasticity in the hippocampus and causes recognition memory deficits. Mol Brain 2016; 9(1): 89. [PubMed: 27729083]

30. Mao W, Watanabe T, Cho S, Frost JL, Truong T, Zhao X et al. Shank1 regulates excitatory synaptic transmission in mouse hippocampal parvalbumin-expressing inhibitory interneurons. Eur J Neurosci 2015; 41(8): 1025–1035. [PubMed: 25816842]

31. Ruderfer DM, Ripke S, McQuillan A, Boocock J, Stahl EA, Pavlides JMW et al. Genomic dissection of bipolar disorder and schizophrenia, including 28 subphenotypes. Cell. 2018;173: 1705–15 [PubMed: 29906448]

32. Rosso SB, Inestrosa NC. WNT signaling in neuronal maturation and synaptogenesis. Front Cell Neurosci 2013; 7: 103. [PubMed: 23847469]

33. Isabella P, Flavia N, Alberto MF, Giorgio D K, Antonio DC, Chiara R et al. Neurodevelopment in Schizophrenia: The Role of the Wnt Pathways. Current Neuropharmacology 2013; 11: 535–558. [PubMed: 24403877]

34. Massey PV, Bashir ZI. Long-term depression: multiple forms and implications for brain function. Trends Neurosci 2007; 30(4): 176–184. [PubMed: 17335914]

35. Hasan A, Nitsche MA, Herrmann M, Schneider-Axmann T, Marshall L, Gruber O et al. Impaired long-term depression in schizophrenia: a cathodal tDCS pilot study. Brain Stimul 2012; 5(4): 475–483. [PubMed: 21945231]

36. Russell SA, Bashaw GJ. Axon guidance pathways and the control of gene expression. Dev Dyn 2018; 247(4): 571–580. [PubMed: 29226467]

37. Klein R, Kania A. Ephrin signalling in the developing nervous system. Curr Opin Neurobiol 2014; 27: 16–24. [PubMed: 24608162]

38. Liu YN, Lu SY, Yao J. Application of induced pluripotent stem cells to understand neurobiological basis of bipolar disorder and schizophrenia. Psychiatry Clin Neurosci 2017; 71(9): 579–599. [PubMed: 28393474]

39. Brennand KJ, Simone A, Jou J, Gelboin-Burkhart C, Tran N, Sanga S et al. Modelling schizophrenia using human induced pluripotent stem cells. Nature 2011; 473(7346): 221–225. [PubMed: 21490598]

40. Yoon K-J, Nguyen Ha N, Ursini G, Zhang F, Kim N-S, Wen Z et al. Modeling a Genetic Risk for Schizophrenia in iPSCs and Mice Reveals Neural Stem Cell Deficits Associated with Adherens Junctions and Polarity. Cell Stem Cell 2014; 15(1): 79–91. [PubMed: 24996170]

41. Habela CW, Song H, Ming GL. Modeling synaptogenesis in schizophrenia and autism using human iPSC derived neurons. Mol Cell Neurosci 2016; 73: 52–62. [PubMed: 26655799]

42. Wang L, Lockstone HE, Guest PC, Levin Y, Palotás A, Pietsch S et al. Expression Profiling of Fibroblasts Identifies Cell Cycle Abnormalities in Schizophrenia. J Proteome Res 2010; 9(1): 521–527. [PubMed: 19916557]

43. Allen KM, Fung SJ, Weickert CS. Cell proliferation is reduced in the hippocampus in schizophrenia. Aust N Z J Psychiatry 2016; 50(5): 473–480. [PubMed: 26137345]

44. Sanches M, Keshavan MS, Brambilla P, Soares JC. Neurodevelopmental basis of bipolar disorder: a critical appraisal. Prog Neuropsychopharmacol Biol Psychiatry 2008; 32(7): 1617–1627. [PubMed: 18538910]

45. Stahl EA, Breen G, Forstner AJ, McQuillan A, Ripke S, Trubetskov Y et al. Genome-wide association study identifies 30 loci associated with bipolar disorder. Nat Genet 2019; 51(5): 793–803. [PubMed: 31043756]

46. Stephan KE, Friston KJ, Frith CD. Dysconnection in schizophrenia: from abnormal synaptic plasticity to failures of self-monitoring. Schizophr Bull 2009; 35(3): 509–527. [PubMed: 19153455]

47. Wong AHC, Van Tol HHM. Schizophrenia: from phenomenology to neurobiology. Neuroscience & Biobehavioral Reviews 2003; 27(3): 269–306. [PubMed: 12788337]

48. Karlsgodt KH, Sun D, Cannon TD. Structural and Functional Brain Abnormalities in Schizophrenia. Curr Dir Psychol Sci 2010; 19(4): 226–231. [PubMed: 25414548]
49. Westphal DS, Riedhammer KM, Kovacs-Nagy R, Meitinger T, Hoefele J, Wagner M. A De Novo Missense Variant in POU3F2 Identified in a Child with Global Developmental Delay. Neuropediatrics 2018; 49(6): 401–404. [PubMed: 30199896]

50. Kasher PR, Schertz KE, Thomas M, Jackson A, Annunziata S, Ballesta-Martinez MJ et al. Small 6q16.1 Deletions Encompassing POU3F2 Cause Susceptibility to Obesity and Variable Developmental Delay with Intellectual Disability. Am J Hum Genet 2016; 98(2): 363–372. [PubMed: 26833329]

51. Assoum M, Lines MA, Elpeleg O, Darmency V, Whiting S, Edvardson S et al. Further delineation of the clinical spectrum of de novo TRIM8 truncating mutations. Am J Med Genet A 2018; 176(11): 235–240. [PubMed: 27346735]

52. Sakai Y, Fukai R, Matsushita Y, Miyake N, Saitsu H, Akamine S et al. De Novo Truncating Mutation of TRIM8 Causes Early-Onset Epileptic Encephalopathy. Ann Hum Genet 2016; 80(4): 235–240. [PubMed: 27346735]

53. Dominguez MH, Ayoub AE, Rakic P. POU-III transcription factors (Brn1, Brn2, and Oct6) influence neurogenesis, molecular identity, and migratory destination of upper-layer cells of the cerebral cortex. Cereb Cortex 2013; 23(11): 2632–2643. [PubMed: 22892427]

54. Castro DS, Skowronska-Krawczyk D, Armant O, Donaldson IJ, Parras C, Hunt C et al. Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. Dev Cell 2006; 11(6): 831–844. [PubMed: 17141158]

55. Pfisterer U, Kirkeby A, Torper O, Wood J, Nelander J, Dufour A et al. Direct conversion of human fibroblasts to dopaminergic neurons. Proc Natl Acad Sci U S A 2011; 108(25): 10343–10348. [PubMed: 21646515]

56. Venuto S, Castellana S, Monti M, Appolloni I, Fusilli C, Fusco C et al. TRIM8-driven transcriptional profile of neural stem cells identified gliala-related nodal genes and pathways. Biochem Biophys Acta Gen Subj 2019; 1863(2): 491–501. [PubMed: 30528352]

57. Kohlbrenner EA, Shaskan N, Pietersen CY, Sonntag KC, Woo TW. Gene expression profile associated with postnatal development of pyramidal neurons in the human prefrontal cortex implicates ubiquitin ligase E3 in the pathophysiology of schizophrenia onset. J Psychiatr Res 2018; 102: 110–117. [PubMed: 29635114]

58. Ma Q, Ruan H, Peng L, Zhang M, Gack MU, Yao WD. Proteasome-independent polyubiquitin linkage regulates synapse scaffolding, efficacy, and plasticity. Proc Natl Acad Sci U S A 2017; 114(41): E8760–E8769. [PubMed: 28973854]

59. van Kesteren CF, Gremmels H, de Witte LD, Hol EM, Van Gool AR, Falkai PG et al. Immune involvement in the pathogenesis of schizophrenia: a meta-analysis on postmortem brain studies. Transl Psychiatry 2017; 7(3): e1075. [PubMed: 28350400]

60. Georganos PA, Carr CB, Price DJ. The role of Pax6 in forebrain development. Dev Neurobiol 2011; 71(8): 690–709. [PubMed: 21538293]

61. Zamani A, Xiao J, Turnley AM, Murray SS. Tropomyosin-Related Kinase B (TrkB) Regulates Neurite Outgrowth via a Novel Interaction with Suppressor of Cytokine Signalling 2 (SOCS2). Mol Neurobiol 2019; 56(2): 1262–1275. [PubMed: 29881947]

62. Huang EJ, Reichardt LF. Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 2003; 72: 609–642. [PubMed: 12676795]

63. Takahashi K, Yamada M, Ohata H, Honda K, Yamada M. Ndrg2 promotes neurite outgrowth of NGF-differentiated PC12 cells. Neurosci Lett 2005; 388(3): 157–162. [PubMed: 16039777]

64. Lin K, Yin A, Yao L, Li Y. N-myc downstream-regulated gene 2 in the nervous system: from expression pattern to function. Acta Biochim Biophys Sin (Shanghai) 2015; 47(10): 761–766. [PubMed: 26341979]

65. Christa K P, Len A P, Kimberly L, Wufang F, Gregory G L. Characterization of the human neurocan gene, CSPG3. Gene 1998; 221: 199–205. [PubMed: 9795216]

66. Logan S, Arzua T, Canfield SG, Seminary ER, Sison SL, Ebert AD et al. Studying Human Neurological Disorders Using Induced Pluripotent Stem Cells: From 2D Monolayer to 3D Organoid and Blood Brain Barrier Models. Compr Physiol 2019; 9(2): 565–611. [PubMed: 30873582]
67. Hu Z, Zhou M, Wu Y, Li Z, Liu X, Wu L et al. ssODN-mediated in-frame deletion with CRISPR/Cas9 to restore the FVIII function in hemophilia A patient-derived iPSCs and ECs. Mol Ther Nucleic Acids 2019; 17: 198–209. [PubMed: 31261034]

68. Andrews S FastQC: a quality control tool for high throughput sequence data. Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom 2010.

69. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014; 30(15): 2114–2120. [PubMed: 24695404]

70. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013; 29(1): 15–21. [PubMed: 23104886]

71. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 2013; 30(7): 923–930. [PubMed: 24227677]

72. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010; 26(1): 139–140. [PubMed: 19910308]

73. Popko J, Fernandes A, Brites D, Lanier LM. Automated analysis of NeuronJ tracing data. Cytometry A 2009; 75(4): 371–376. [PubMed: 18937344]

74. Wang J, Vasaikar S, Shi Z, Greer M, Zhang B. WebGestalt 2017: a more comprehensive, powerful, flexible and interactive gene set enrichment analysis toolkit. Nucleic Acids Res 2017; 45(W1): W130–W137. [PubMed: 28472511]
Figure 1. **POU3F2 regulates SCZ-related genes involved in neural development.**

(A) Volcano plot of 3,421 differentially expressed genes. NS, not significant; Down, significantly downregulated; Up, significantly upregulated. A total of six samples were analyzed (three POU3F2 shRNA: shPOU3F2-A, shPOU3F2-B and shPOU3F2-C; three controls). (B) 60 coexpression module genes overlapped with the POU3F2 knockdown gene list. (C) 42 out of 60 POU3F2-regulated genes overlapped with SCZ-related genes. (D) Results of functional enrichment analysis of POU3F2-regulated SCZ genes using WebGestalt Toolkit\textsuperscript{74}. 

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Figure 2. *POU3F2* may affect *TRIM8* expression through three SCZ-related SNPs.

(A) Reference alleles of three SNPs (rs5011218, rs11191359, and rs4146428) are located in the regulatory region of *TRIM8*. The *TRIM8* transcription start site is indicated as +1. Each SNP is marked with an eQTL P value and a SCZ GWAS P value. OCTX, occipital cortex. (B) LIBD database showed that rs5011218 and rs4146428 are significantly associated with *TRIM8* expression in the dorsolateral prefrontal cortex (FDR q = 0.0027 for both SNPs). These eQTLs were identified using 237 samples. (C) The SNPs were located in upstream regions of *TRIM8* that contains H3K27AC Histone modification, DNase I hypersensitivity and ChIP-seq signals.
Figure 3. *POU3F2* promoted *TRIM8* expression through the regulatory SNP rs5011218.

(A, B) mRNA expression analyses of *TRIM8* by RT-qPCR after *POU3F2* overexpression (A) and knockdown (B). Control (A), transfection of control plasmid; *POU3F2* OE, *POU3F2* overexpression, transfection of *POU3F2* overexpression plasmid; Control (B), transfection of control siRNA; *POU3F2* KD, *POU3F2* knockdown, transfection of *POU3F2* siRNA.

(C, D) Protein expression analyses of *TRIM8* by Western blotting after *POU3F2* overexpression (C) and knockdown (D).

(E) Luciferase reporter assay of the constructed vectors. FL, Firefly luciferase; RL, Renilla luciferase; PGL-AAG, luciferase reporter vector containing the reference alleles of three SNPs; NC, negative control, transfection of control plasmid; PO, *POU3F2* overexpression, transfection of *POU3F2* overexpression plasmid; PGL-CAG, the A allele of rs5011218 was mutated into the C allele; PGL-ATG, the A allele of rs11191359 was mutated into the T allele; PGL-AAA, the G allele of rs4146428 was mutated into the A allele. N=3 independent biological experiments. For each biological replicate, we designed three technical replicates. Values represent mean±sd, significance was determined using Student’s t-test. **P < 0.01, ***P < 0.001.

(F) EMSA results of Ding et al. Page 23 Mol Psychiatry. Author manuscript; available in PMC 2021 October 13.
rs5011218. Lane 1: reaction as the negative control; lane 2: biotin-labeled probes (A allele) plus nuclear proteins; lane 3: excess unlabeled probes displaced binding of biotin-labeled probes; lane 4: anti-POU3F2 antibody combined with protein/DNA complex; lane 5: POU3F2 binding efficiency decreased after the A allele was mutated into C allele; lane 6: binding reaction was totally inhibited after mutating POU3F2 core binding sequences.
Figure 4. Silencing POU3F2 and TRIM8 promote NPCs proliferation through advancing cell cycle progression.

(A) POU3F2 mRNA expression level in four different cell lines. NC, negative control, infection of scrambled shRNA lentivirus; PO KD, POU3F2 knockdown, infection of shPOU3F2-C lentivirus; T8 KD, TRIM8 knockdown, infection of shTRIM8-C lentivirus; PO KD + T8 OE, POU3F2 knockdown plus TRIM8 overexpression, infection of shPOU3F2-C and TRIM8 overexpression lentivirus; NS, not significant. (B) The mRNA expression level of TRIM8 in four different cell lines. (C) EdU staining was used to assess the proliferation ability of NPCs after POU3F2 and TRIM8 knockdown. (D) Data
quantification for (C). (E) Cell proliferation was evaluated by CCK-8 assay in *POU3F2* and *TRIM8* knockdown cells. (F) Silencing of *POU3F2* and *TRIM8* promoted the cell cycle progression. (G) Data quantification for (F). **P < 0.01, ***P < 0.001. Two-tailed Student’s t-test. Values represent the mean±sd from three independent biological replicates. For each biological replicate, we designed three technical replicates.
Figure 5. Suppressing *POU3F2* and *TRIM8* expression inhibited neuronal differentiation and excitatory synaptic transmission.

(A) The proportion of cells expressing Tuj1 in relation to total number of cells. Tuj1, a marker for staining immature neurons. NC, negative control, infection of scrambled shRNA lentivirus; PO KD, *POU3F2* knockdown, infection of sh*POU3F2*-C lentivirus; T8 KD, *TRIM8* knockdown, infection of sh*TRIM8*-C lentivirus; PO KD + T8 OE, *POU3F2* knockdown plus *TRIM8* overexpression, infection of sh*POU3F2*-C and *TRIM8* overexpression lentivirus; (B) Data quantification for (A). (C) *POU3F2* and *TRIM8* knockdown affected dendrite development. MAP2 (microtubule associated protein 2), a
marker for staining neuronal dendrites. DAPI (4',6-diamidino-2-phenylindole), a standard DNA staining. (D) Data quantification for dendrite length. (E) Data quantification for dendrite number. All data are mean ± sd from three independent biological replicates. For each biological replicate, we designed three technical replicates. **P < 0.01, ***P < 0.001, two-tailed Student’s t-test. (F) Representative traces of miniature excitatory postsynaptic currents. (G and H) POU3F2 and TRIM8 knockdown reduced mEPSC frequency (G) without affecting mEPSC amplitude (H). 16 neurons were recorded for NC and T8 KD groups, 17 and 19 neurons were recorded for PO KD and PO KD + T8 OE groups, respectively. N = 3 independent biological experiments. (I and J) Cumulative probability plot of the inter-event interval (I) and amplitude (J).