ZNF804A Transcriptional Networks in Differentiating Neurons Derived from Induced Pluripotent Stem Cells of Human Origin

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Abstract

ZNF804A (Zinc Finger Protein 804A) has been identified as a candidate gene for schizophrenia (SZ), autism spectrum disorders (ASD), and bipolar disorder (BD) in replicated genome wide association studies (GWAS) and by copy number variation (CNV) analysis. Although its function has not been well-characterized, ZNF804A contains a C2H2-type zinc-finger domain, suggesting that it has DNA binding properties, and consequently, a role in regulating gene expression. To further explore the role of ZNF804A on gene expression and its downstream targets, we used a gene knockdown (KD) approach to reduce its expression in neural progenitor cells (NPCs) derived from induced pluripotent stem cells (iPSCs). KD was accomplished by RNA interference (RNAi) using lentiviral particles containing shRNAs that target ZNF804A mRNA. Stable transduced NPC lines were generated after puromycin selection. A control cell line expressing a random (scrambled) shRNA was also generated. Neuronal differentiation was induced, RNA was harvested after 14 days and transcriptome analysis was carried out using RNA-seq. 1815 genes were found to be differentially expressed at a nominally significant level ($p < 0.05$); 809 decreased in expression in the KD samples, while 1106 increased. Of these, 370 achieved genome wide significance (FDR < 0.05); 125 were lower in the KD samples, 245 were higher. Pathway analysis showed that genes involved in interferon-signaling were enriched among those that were down-regulated in the KD samples. Correspondingly, ZNF804A KD was found to affect interferon-alpha 2 (IFNA2)-mediated gene expression. The findings suggest that ZNF804A may affect a differentiating neuron’s response to inflammatory cytokines, which is consistent with models of SZ and ASD that support a role for infectious disease, and/or autoimmunity in a subgroup of patients.
**Introduction**

ZNF804A has been implicated in SZ and BD in replicated genetic and molecular studies [1–2]. In addition, rare copy gain and copy loss CNVs, and an inversion affecting the gene have been found in patients with ASD, psychosis, developmental disabilities, and anxiety disorder [3–6]. ZNF804A codes for a protein containing a C2H2-type zinc-finger domain, suggesting that it has DNA binding properties and can act as a transcription factor. This is supported by Girgenti et al. who showed that the protein localized to the nucleus, and more specifically to the promoters of several SZ candidate genes in E13 rat embryo cortical neural progenitors [7]. In addition, Hill et al. knocked down ZNF804A expression in NPCs derived from human cortical neuroepithelium using siRNAs and identified 152 differentially expressed genes by microarray analysis, including genes involved in cell adhesion, neural migration, neurite outgrowth and synaptogenesis [8].

ZNF804A is one of many genes coding for transcription factors, splicing regulators and chromatin remodeling proteins that have been implicated in SZ, BD, and ASD, including POU3F2, A2BP1, MYT1L, JARID2, TCF4, ARNT2, CHD8, SMARCA2, and β-catenin (which is regulated by the lithium and DISC1 target GSK3β) [9–24]. Analyzing the effect of these genes on downstream targets is a reasonable strategy for identifying gene networks—both unique and shared—involved in neuropsychiatric disorders.

To further enhance our understanding of the role of ZNF804A on downstream targets, and to expand on the findings reported by Hill et al., we have knocked down the gene in NPCs derived from iPSCs with shRNA vectors. Using iPSCs as a source of human neural progenitor cells (NPCs) for genetic manipulation has some advantages over others, such as nasal neuroepithelium and cortical neuroepithelium, one of which is the capacity to generate patient-specific neurons [25–27]. In addition, since iPSCs are self-renewing, permanent, genetically manipulated cell lines can be generated. In this study, we report on a transcriptome analysis carried out on early differentiating human neurons derived from iPSC-generated NPCs in which ZNF804A has been knocked down using shRNAs.

**Methods and Materials**

**Development of iPSC lines**

The study was approved by the Albert Einstein College of Medicine Committee Institutional Review Board (IRB). All participants signed an informed consent that was approved by the Einstein IRB. For this study, ZNF804A knockdown (KD) was carried out on a line from a healthy male control. iPSCs were generated using non-integrating plasmids containing OCT4, SOX2, KLF4, L-MYC, LIN28, and a p53 shRNA vector, introduced by nucleofection [28–31]. The iPSCline used in this experiment has a normal karyotype, expresses pluripotency markers, and is capable of differentiating into all 3 germ layers. (S1 Fig and S1 Text).

**Generating NPCs and inducing neuronal differentiation**

The protocol for generating NPCs and inducing neuronal differentiation has been previously described [32–34]. Details can be found in the S1 Text.

**Lentiviral particle transduction**

NPCs were transduced with a mix of three shRNAs targeting ZNF804A (Santa Cruz Biotechnology sc-94548-V). In addition, a scrambled shRNA vector was used as a control (Santa Cruz Biotechnology, sc-108080). NPCs were grown in NBF medium (DMEM/F12, 0.5 x N2, 0.5 x B27 and1% P/S) containing FGF2 (20ng/ml) in 6-well plates coated with poly-L-ornithine.
hydrobromide and laminin. Cells at ~50% confluence were fed with fresh medium and 5ug/ml Polybrene (Santa Cruz Biotechnology, sc-134220) and transduced with shRNA lentiviral particles. Cells were incubated for 24 hours, after which medium with virus was removed and replaced with complete NBF medium without Polybrene. After 24–48 hours, 5 µg/ml puromycin was added to select for transduced cells. Medium with puromycin was changed every other day for 5–7 days. Transduced cells were expanded and induced to differentiate into neurons once ~50% confluence was reached, which occurred approximately 5–10 days after expansion). Cells were harvested for RNA after 14 days of differentiation. Two separate NPC preps from the same KD experiment were induced to differentiate into neurons (scrambled 1, 2; KD 1, 2). In addition, a separate KD was carried out using different shRNA vectors (scrambled 3, KD 3) supplied by the Albert Einstein College of Medicine shRNA Core Facility (S1 Text). The shRNAs contained the following ZNF804A target sense sequences: Albert Einstein College of Medicine shRNA core facility (AGAAGCUUAGUUCUUCAAA); Santa Cruz, sc-94548-VA (CAAGGAUGUAUCUACAGAA), sc-94548-VB (GAAGCAGAGAAUAGUUACA), sc-94548-VC (CAACCACCAUUACAUUCA).

RNA-seq
Paired-end RNA-seq was carried out on an Illumina HiSeq 2000. We obtained 101-bp mate-paired reads from DNA fragments with an average size of 250-bp (standard deviation for the distribution of inner distances between mate pairs is approximately 100 bp). RNA-seq reads were aligned to the human genome (GRCh37/hg19) using the software TopHat (version 2.0.8) [35]. The category of transcripts used for our expression analysis is described at http://vega.sanger.ac.uk/info/about/gene_and_transcript_types.html. We counted the number of RNA-seq fragments mapped to each gene annotated in the GENCODE database (version 18) [36]. The count was divided by effective gene length (derived from regions covered by reads) to obtain expression level in Transcripts Per Million (TPM), which is calculated by multiplying the estimated fraction of transcripts made up by a given gene by 10^6 [37]. The measure is independent of the mean expressed transcript length and is thus more comparable across samples; thus it is favored over another popular transcript measure, FPKM, based on previous studies [38]. We used DESeq (an R package developed by Anders and Huber) to determine differential expression from the count data [39]. Afterwards, only genes with average TPMs greater than 1 across samples (n = 15,330) were considered for differential expression. A multiple comparison correction was applied to adjust the p-values for false discovery rate (FDR) [40]. Sequencing data have been deposited in the Gene Expression Omnibus (GEO) repository (accession number GSE54112).

Quantitative real-time PCR (qPCR)
qPCR was carried out on reverse transcribed PCR using the 2^ΔΔCt method as previously described [30,34]. A detailed description and the primers used for this analysis can be found in S1 Text.

Proliferation assay
Cell proliferation was assayed using the Vybrant MTT cell proliferation assay kit (invitrogen) according to the protocol manual. Briefly, similar amounts of NPCs (50,000 cells in triplicate) were seeded on 96 well plates coated with poly-L-ornithine hydrobromide and laminin. Cells were assayed daily for 5 days. At the time of the assay, 100ul of medium was removed from the well and replaced with an equal volume of fresh medium without FGF2, along with 10ul of the 12mM MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) stock solution.
The cells were incubated at 37°C for 4 hours. 85 ul of medium was removed and 50 ul of DMSO was added, followed by a 10 minute incubation at 37°C. The samples were mixed well, transferred to a microplate, and the absorbance at 540 nm was determined.

**Interferon inducible gene expression**

NPC cultures were prepared from the parent iPSC line (untreated control), a ZNF804A KD and a scrambled shRNA control (KD2 and scrambled 2). The NPCs were previously frozen in aliquots of 10⁶ cells. After thawing, the cells were cultured for several days until 50% confluence was reached, after which they were treated with interferon-alpha 2 (IFNA2; 1mg/ml) or vehicle for 4 hours. IFNA2 was chosen because IPA analysis showed that this was the most significant upstream regulator among the down-regulated genes. RNA was harvested and analyzed by qPCR. Duplicate cultures were treated and analyzed in triplicate for qPCR. We analyzed several IFN-inducible genes, including IFITM2, IFITM3, IFI6, STAT1 and STAT3. However, neither IFITM2 nor IFITM3 were significantly affected by IFNA2 treatment under our experimental conditions (not shown). Consequently, the analysis of IFN-inducible genes was restricted to IFI6, STAT1 and STAT3. Relative expression means were then normalized to a control, vehicle-treated sample, which was arbitrarily set at 1.0. Fold differences were calculated and statistical significance was determined using a Student’s t-test.

**Results**

**RNA-seq**

A total of 1,815 genes were found to be differentially expressed at a nominally statistically significant level (p<0.05); 809 decreased in expression in the KD samples, while 1,106 increased (S1 Table for complete dataset). Of these, 370 reached genome wide significance after correction for multiple testing (FDR<0.05); 125 were lower in the KD samples, 245 were higher. This included 10 long non-coding RNAs that decreased in expression and seven that increased. Reduced expression of ZNF804 in the KD was confirmed by the RNA-seq findings, which showed a 69% decrease (p = 9.4e-08; FDR = 6.7e-05), as well as by qPCR (S1 Table, Fig 1). The most significantly down-regulated genes based on FDR were TIMP1, LINCO0645, IFITM3, IFITM2, KAL1, RTL1, and PCDH7 (Table 1). TIMP1 is metallopeptidase inhibitor that has an effect on cellular proliferation, especially in response to cytokines, and acts as an inflammatory mediator in the brain [41]; a connection to immune activation is a recurrent theme in this study, as described below. LINCO0645 is expressed throughout the brain and maps to a large, coding gene poor region on chromosome 14. The closest is NOVA1, which is ~1Mb upstream and codes for a splicing co-factor that mediates the response to cellular and behavioral stress, and inflammatory cytokines [42,43]. KAL1 is expressed throughout the brain and involved in neural migration [44]. RTL1 is a retrotransposon-derived, paternally expressed gene that maps to a well-established imprinted locus on 14q32; it is expressed in the brain and has been found to be up-regulated by Neurogenin 2 during dorsal telencephalon development [45,46]. PCDH7 is a non-clustered protocadherin expressed in the brain that was also found to be differentially expressed by Hill et al., one of 17 genes that overlapped between our respective studies, the others being CRYAB, CCL2, SEZ6, STAC, MCM3, CDCA4, ARHGAP19, NCAPG2, CDKN1A, MUTYH, EIF4A2, ANTXR1, ATP1B1, ACTG2, EIF4G1, and PPP4R1 [8]. The overlap of the differentially expressed genes is statistically significant (p = 4.6e-9, hypergeometric test).

Among the four genes affected by ZNF804A in the Girgenti study, which was carried out in rat NPCs, one overlapped with our findings; the SZ and BD candidate PDE4B (increased in
expression by overexpressing ZNF804A in rat NPCs, while it decreased following KD in our study—see S1 Table) [7].

Finally, two of the more interesting down-regulated genes are IFITM2 and IFITM3, which decreased several fold (Table 1); they code for interferon-induced transmembrane proteins that play a role in interferon-signaling and the innate defense against influenza and other viruses [47–49]. This is particularly interesting from a SZ pathogenesis perspective, considering the clinical and epidemiological evidence pointing towards prenatal influenza as a risk factor in SZ, which will be described in more detail in the discussion [50–59].

One concern about the dramatic decrease in IFITM2 and IFITM3 expression in the KD lines is that it’s an artifact of lentiviral transduction; that is, could the reduced expression in the

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Fig 1. Validation of selected genes by qPCR. qPCR was carried out as described in methods. Each assay was carried out in triplicate, and the mean fold change was calculated. This was repeated 3–6 times for each sample. Asterisk indicates significance at p<0.05 calculated using a Student’s t-test. Actual p-values are: ZNF804A (0.003); IFITM2 (0.040); IFITM3 (0.001); EZH2 (0.016); APOE (0.018); CRYAB (0.015); SLC6A1 (0.0004); GRIA2 (0.011); TCF4 (0.710); IGF2 (0.0004); BEGAIN (0.010); CDK1 (0.015).

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ZNF804A KD lines compared with the scrambled controls be due to differences in the expression of lentiviral sequences? However, in an analysis of *IFITM2* and *IFITM3* expression in this experiment and in 17 other KD and scrambled lines we have generated in NPCs, no correlation was observed between viral gene expression (inferred from RNA-seq reads mapped to vectors) and *IFITM2* and *IFITM3* RNA levels (correlation coefficients, -0.014 and 0.280, respectively).

In addition to these two interferon-related genes, a number of others that code for various proteins involved in cytokine signaling are significantly lower in the KD samples, including *IFIT3*, *IL17RC*, *IL17D*, *CCL2*, *CXCL16*, *IFNAR1*, *IL1RAP*, *IL6ST*, *C1QTNF5*, *TNFAIP3*, *TNFRSF21*, *TNFAIP8*, *TNFAIP8L1*, *TNFAIP2*, *C1QTNF6* and *TNFSF12* (S1 Table).

The most significantly up-regulated genes in the KD samples were *KIRREL2*, *ZIC3*, *MTFP1*, and *KIF4A*. *KIRREL2* (Kin of IRRE-like 2), is a cell adhesion molecule that regulates activity-dependent axonal projections in the olfactory system of mice [60]. *ZIC3* is a member...
of a family of zinc-finger transcription factors that plays a role in regulating the proliferation and differentiation of neuronal progenitors in the medial forebrain and the cerebellum, retinoic acid signaling in the hindbrain, and the generation of mouse iPSCs [61–63]. Two other members of the ZIC family, ZIC1 and ZIC4, are also significantly up-regulated in the KD cells (S1 Table). ZIC1 was also found to be differentially expressed in the Hill et al ZNF804A KD study, as noted above. MTFP1 (mitochondrial fission process 1) is a nuclear gene that is a downstream target of the phosphatidylinositol 3-kinase signaling pathway, PALM3 is a member of a multigene family involved in membrane dynamics, and is expressed in axons and dendritic spines; it is also up-regulated by LPS and may be involved in IL-1 signaling [64,65].

We validated the changes in gene expression for 12 genes including ZNF804A; all but one (TCF4) was confirmed (Fig 1). We chose to validate genes of interest in neuropsychiatric disorders, as well as several that overlapped with a study we carried out on neuronal aggregates derived from iPSCs exposed to heat shock [66]. TCF4 expression decreased, as expected, but statistical significance was not achieved because of a large standard deviation.

Pathway Analysis

Based on the function of the top differentially expressed genes, ZNF804A appears to have an effect on genes involved in immune responses. In order to evaluate this possibility more systematically, we assessed the 1815 differentially expressed genes that were nominally significant (p < 0.05) using Ingenuity Pathway Analysis (IPA). For genes that decreased in the ZNF804A KDs, the top disease pathways and biological functions were neuromuscular disease, dyskinesia, movement disorders and Huntington’s Disease. Genes implicated in SZ and BD were the 5th and 9th most significant (Table 2; S2 Table for complete IPA analysis).

Inspection of the list of down-regulated genes showed several others that are of interest in neuropsychiatric disorders, but were not called by IPA, including TIMELESS, DISCI, PTEN, RANBP1, TCF4, NPS3, PCDH9, NLGN4X, and CDH7 [73–84].

Consistent with the marked decrease we detected in IFITM2 and IFITM3, IPA showed a significant enrichment for genes involved in inflammation and response to cytokines among the KD down-regulated genes. In the IPA “upstream regulators” analysis, in which IPA assesses the observed gene expression changes for transcriptional activators, the top 2, and 4 of the top 10 upstream regulators were members of the interferon family: IFNA2 (interferon alpha 2), IFN1 (interferon lambda 1), ISG15 (interferon stimulated gene 15), a ubiquitin-like modifier that is induced by type I interferons, and IFNG (interferon gamma) (Table 3). Similar results were obtained when the data were analyzed by Gene Ontology (GO): among the top GO terms were 3 out of 11 involved in interferon signaling (Table 4; S3 Table).

The IPA findings suggest that interferon signaling will be affected by ZNF804A. This is supported by an analysis of IFNA2-mediated gene expression carried out in NPCs from an untreated control, a scrambled control, and a ZNF804A KD, as described in the methods section. Following treatment with IFNA2, IFI6 and STAT1 gene expression significantly increased, while STAT3 decreased significantly (~2–10 fold changes in expression compared with controls; all p-values < 0.05, not shown). As seen in Fig 2, there was a statistically significant increase in the fold induction of IFI6 in the KD samples compared with both the untreated control NPC line used for the shRNA experiment, and the scrambled control KD (~2-fold more robust induction with INFRA2; p = 0.01 and 0.03, respectively, two-tailed Student’s t-test). By contrast, no significant differences were found when the untreated control and the scrambled control were compared. Significant differences were also found in the ZNF804A KD for STAT1 and STAT3 in comparison with the scrambled control (p = 0.004; 0.05, respectively). However, for these two genes, the induction (STAT1) or decrease (STAT3) that occurs
Table 2. Diseases and biological functions for ZNF804A down-regulated genes.

| Diseases/Functions          | p-value  | Molecules                                                                 |
|-----------------------------|----------|---------------------------------------------------------------------------|
| neuromuscular disease       | 1.56E-08 | ADRA2A, AEBP1, AK5, ALDH6A1, APOE, ATP2B2, BRINP1, CCL2, CDS1, CHRM3, CNR1, CPNE5, CRYAB, CRYM, DGKB, ESRRG, FAS, FLRT2, GABRA2, GRIK2, HAP1, HCG22, IFNAR1, ITPKB, KCNA5, KCNA1, KCNA2, KCNIP1, MX1, NR4A2, PCDH7, PDE4B, PPARA, PPP3CA, PRNP, PSMB8, RFXG, SCARB2, SCN2A, SCN4B, SEZ6, SH3GL2, SLC1A1, SLC24A3, SPOCK3, ST8SIA4, TM2D1, TOMM20, TDPS2 |
| dyskinesia                  | 1.31E-07 | ADRA2A, AEBP1, ALDH6A1, APOE, ATP2B2, CDS1, CHRM3, CNR1, CPNE5, CRYAB, CRYM, DGKB, ESRRG, FLRT2, GABRA2, GRIK2, HAP1, ITPKB, KCN5, KCNA1, KCNIP1, PCDH7, PPARA, PPP3CA, PSMB8, RGS2, RFXG, SCARB2, SCN2A, SCN4B, SEZ6, SH3GL2, SLC1A1, SPOCK3, ST8SIA4, TM2D1, TOMM20, TDPS2 |
| Movement Disorders          | 2.46E-07 | ADRA2A, AEBP1, ALDH6A1, APOE, ATP2B2, BEAN1, CASB, CDS1, CHRM3, CNR1, CPNE5, CRYAB, CRYM, DGKB, ESRRG, FAS, FLRT2, GABRA2, GRIK1, GRIK2, HAP1, ITPKB, KCN5, KCNA1, KCN3, KCNIP1, KLHL1, NR4A2, PCDH7, PDE4B, PPARA, PPP3CA, PSMB8, RGS2, RFXG, SCARB2, SCN2A, SCN4B, SEZ6, SH3GL2, SLC1A1, SPOCK3, ST8SIA4, TM2D1, TOMM20, TDPS2 |
| Huntington's Disease        | 3.01E-07 | AEBP1, ALDH6A1, APOE, ATP2B2, CALY, CCK, CHRM3, CNR1, CPNE5, CRYAB, CRYM, DGKB, ESRRG, FLRT2, GABRA2, GRIK2, HAP1, ITPKB, KCN5, KCNA1, KCNIP1, PCDH7, PPARA, PPP3CA, PSMB8, RFXG, SCARB2, SCN2A, SCN4B, SEZ6, SH3GL2, SLC1A1, SPOCK3, ST8SIA4, TM2D1, TOMM20, TDPS2 |
| Schizophrenia               | 5.13E-07 | ADRA2A, ALDH3B1, ALDH5A1, APOE, ATP2B2, CALY, CCK, CHRM3, CNR1, CPNE5, CRYAB, CRYM, DGKB, ESRRG, FLRT2, GABRA2, GRIK1, GRIK2, HAP1, ITPKB, KCN5, KCNA1, KCNIP1, KLHL1, NR4A2, PCDH7, PDE4B, PDE8B, PPARA, PPP3CA, PSMB8, RGS2, RFXG, SCARB2, SCN2A, SCN4B, SEZ6, SH3GL2, SLC1A1, SPOCK3, ST8SIA4, TM2D1, TOMM20, TDPS2 |
| disorder of basal ganglia   | 8.33E-07 | ADRA2A, AEBP1, ALDH6A1, APOE, ATP2B2, CDS1, CHRM3, CNR1, CPNE5, CRYAB, CRYM, DGKB, ESRRG, FLRT2, GABRA2, GRIK2, HAP1, ITPKB, KCN5, KCNA1, KCNIP1, NR4A2, PCDH7, PDE4B, PDE8B, PPARA, PPP3CA, PSMB8, RGS2, RFXG, SCARB2, SCN2A, SCN4B, SEZ6, SH3GL2, SLC1A1, SPOCK3, ST8SIA4, TM2D1, TOMM20, TDPS2 |
| progressive motor neuropathy| 3.73E-06 | ADRA2A, AK5, ALDH5A1, APOE, BRINP1, CCL2, CDKN1A, CHRM3, CNR1, CPNE5, CRYAB, CRYM, DGKB, ESRRG, FLRT2, GABRA2, GRIK1, GRIK2, HAP1, ITPKB, KCN5, KCNA1, KCN2D, MX1, NR4A2, PDE4B, PLFA2G4C, PRNP, SCN2A, SCN4B, SH3GL2, SLC1A1, SLC24A3, SPARC, ST8SIA4, SUSD1, TIMP1 |
| epilepsy                    | 1.12E-05 | ALDH5A1, ARRDC3, ASAH1, CASB, CDKN1A, CRYM, ERRFI1, GABRA2, GRIK1, KAL1, LINC00152, NR4A3, PRIKCLEL1, PRNP, RGS2, SCARB2, SCN2A, SCN4B, SLC6A1, TRIM9 |
| bipolar disorder            | 3.32E-05 | ADRA2A, ALDH5A1, APOE, CASB, CCL2, CHRM3, GABRA2, GRIK2, GRID1, GRIK1, GRID2, GRIK4, GRM5, MCHR1, PPP2R2C, PPP3CA, RABGAP1L, SCN2A, SCN4B, SLC1A1, TCF4 |
| inflammatory demyelinating disease | 6.41E-05 | ADRA2A, AK5, BRINP1, CCL2, CHRM3, CNR1, CRYAB, FAS, HCG22, IFNAR1, KCN2D, MX1, SLC24A3, TIMP1 |

This included several kainate ionotropic receptors, AMPA and metabotropic glutamate receptor subtypes, and the GABA-A receptor subtype, GABRA2 [67]. In addition, a decrease in cholecystokinin expression was detected in the KD neurons. CCK codes for a neuropeptide that’s expressed in a subset of GABAergic neurons; it has a role in mediating anxiety traits and panic disorder, and is differentially expressed in the entorhinal cortex in SZ [68–72].

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following INFA2 treatment was attenuated compared with the scrambled control. These findings support that idea that INFA2 signaling is affected by ZNF804A.

For the up-regulated genes, the top disease pathways and biological functions were all related to the mitotic cell cycle, cellular assembly, DNA replication, and cancer with very low p-values ranging from E-19 to E-31 (S4 Table). Similar results were obtained when the enriched GO terms were determined (Table 5 and S5 Table for complete list). The findings suggest that knocking down ZNF804A affects proliferation. To test this idea, a proliferation assay was carried out in NPCs. After a three day lag period, the ZNF804A KD line showed a 30–37% increase in proliferative capacity compared with a scrambled control on days 4 and 5. However, differences were not statistically significant (Fig 3). The ZNF801A KD line also showed an increase in proliferation compared with the untreated control (wild type line; parent line, but not transduced with an shRNA vector), which almost reached statistical significance (Student’s t-test, two tailed, p = 0.052 and 0.056 for days 4 and 5, respectively).

The marginal, albeit statistical insignificant increase in proliferation could suggest that some of the changes in the expression of genes implicated in neuropsychiatric disorders and in cytokine-signaling found in day 14 neurons are secondary to an effect on proliferation and maturation from NPCs into neurons. However, there was no consistent difference in expression of NPC markers; SOX2, VIM (vimentin) and PAX6 expression levels did not differ between the KD and scrambled control, although NES (nestin) expression increased by ~40% (S1 Table). It should be noted, however, that IFITM2 and IFITM3, are not differentially expressed during the transition from NPCs to neurons [30]. A decrease in IFITM2 and IFITM3 expression independent of differentiation status was confirmed by qPCR carried out on KD and scrambled control NPCs (p = 0.04 and 0.001, respectively; not shown). Thus, the differentially expressed genes we detected in the KD experiment in day 14 neurons do not appear to be an artifact of differential proliferation or differentiation status.

**Table 3. Upstream Regulators for ZNF804A KD down-regulated genes.**

| Upstream Regulator | p-value | Target molecules in dataset |
|--------------------|---------|-----------------------------|
| IFNA2              | 1.11E-08| C19orf66,FAS,HERC6,IFI35,IFI44L,IFI6,IFIT3,IFITM3,IRF9,ISG20, LGALS3BP,MX1,PARP12 |
| IFNL1              | 2.44E-08| C19orf66,HERC6,IFI35,IFI44L,IFI6,IFIT3,IFITM3,IRF9,ISG20, LGALS3BP,MX1 |
| MAPK1              | 4.07E-06| CDKN1A,DEPTOR,IFI16,IFIT3,IFITM3,IRF9,ISG20,LGALS3BP, MVP,PARP12,PSMB8,SPOCK1 |
| USP18              | 1.02E-05| IFI6,IFITM3,IRF9,MX1         |
| ISG15              | 3.09E-05| IFI6,IFITM3,MX1              |
| EIF2AK2            | 8.26E-05| IFI35,IFI6,IFITM2,ISG20, LGALS3BP,PARP12,PARP9 |
| IFNG               | 3.16E-04| CCL2,CDKN1A,CXCL16,FAS,HERC6,HRK,IFI44L,IFI6,IFIT3,IGFBP4,IRF9, ISG20,MCHR1,MX1,PSMB8,TNFSF12 |
| PKC(s)             | 3.39E-04| CCL2,IGF2,NR4A3,Ppara,Rgs2   |
| Smad1/5/8          | 3.96E-04| CDKN1A,CDKN2A                |
| LIN9               | 3.96E-04| CDKN1A,CDKN2A                |

Table 3. Upstream Regulators for ZNF804A KD down-regulated genes.

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Discussion

SZ, ASD and BD, and other neuropsychiatric disorders are genetically heterogeneous complex genetic traits that will make it difficult to translate genetic findings into novel therapeutics. One way to address heterogeneity is to find common pathways onto which different candidate genes converge. This is particularly feasible for genes that code for transcription factors or chromatin remodeling proteins that could potentially have an impact on shared target genes or pathways. A convergence of target genes is suggested by our finding that the expression of a number of SZ and BD candidates was affected by ZNF804A KD, as noted above.

The utility of identifying common molecular and genetic targets could also be applied when considering the role of environmental factors in the development of SZ and ASD. Although these disorders are highly heritable, environmental factors play a role as well; maternal immune activation (MIA) caused by infectious diseases or autoimmune phenomena, maternal nutritional deprivation, obstetrical complications, oxidative stress, anoxic stress, paternal age and environmental toxins have all been implicated [85–95].

One major finding in this study—altered expression of genes involved in cytokine signaling following ZNF804A KD—is consistent with these observations, and suggests that allelic variants in the gene interact with cytokine signaling pathways in some manner leading to an increased risk of disease. One of the more interesting cytokine-related findings, a decrease in IFITM2 and IFITM3 gene expression, suggests another possibility; an impact on viral

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Table 4. Gene Ontology (GO) for genes down-regulated following ZNF804A KD.

| GO term                          | Biological process                          | # DEGs | p-value     | FDR      |
|----------------------------------|---------------------------------------------|--------|-------------|----------|
| GO:0003008 system process        | 72                                           | 1.43E-09 | 5.03E-06    |          |
| GO:0044707 single-multicellular organism process | 169                                           | 7.41E-09 | 1.31E-05    |          |
| GO:0007268 synaptic transmission | 41                                           | 1.53E-08 | 1.80E-05    |          |
| GO:0032501 multicellular organismal process | 171                                           | 2.40E-08 | 2.12E-05    |          |
| GO:0050877 neurological system process | 56                                           | 4.61E-08 | 3.25E-05    |          |
| GO:0035637 multicellular organismal signaling | 44                                           | 6.50E-08 | 3.82E-05    |          |
| GO:0019226 transmission of nerve impulse | 43                                           | 8.32E-08 | 4.20E-05    |          |
| GO:0048731 system development    | 119                                         | 3.70E-07 | 1.63E-04    |          |
| GO:0071357 cellular response to type I interferon | 10                                           | 4.87E-07 | 1.72E-04    |          |
| GO:0060337 type I interferon signaling pathway | 10                                           | 4.87E-07 | 1.72E-04    |          |
| GO:0034340 response to type I interferon | 10                                           | 6.13E-07 | 1.97E-04    |          |
| GO:0007267 cell-cell signaling   | 48                                          | 6.94E-07 | 2.04E-04    |          |
| GO:0023052 signaling            | 150                                         | 9.25E-07 | 2.33E-04    |          |
| GO:0044700 single organism signaling | 150                                         | 9.25E-07 | 2.33E-04    |          |
| GO:0007154 cell communication    | 152                                         | 1.46E-06 | 3.43E-04    |          |
| GO:0051239 regulation of multicellular organismal process | 69                                           | 7.57E-06 | 0.0017      |          |
| GO:0007610 behavior             | 29                                          | 9.39E-06 | 0.002       |          |

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**Fig 2. Induction of interferon-responsive genes.** NPCs were treated with IFNA2 (1mg/ml) for 4 hours, after which RNA was isolated and analyzed by qPCR. IFI6 and STAT1 increased with treatment, while STAT3 decreased. Fold changes were calculated as described in the methods section. Significant differences in the fold change were found in the comparison between the scrambled control and the ZNF804A KD line for all three genes using a Student’s t-test (all results two-tailed): IFI6, p = 0.03; STAT1, p = 0.004; STAT3, p = 0.05. No other significant differences were found for the other comparison, except for untreated control vs ZNF804A KD (p = 0.01). The bar graph represents the mean of 6 determinations (biological duplicates analyzed in triplicate) +/- standard error.

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**Table 5. Gene Ontology (GO) for ZNF804A KD up-regulated genes.**

| GO_term         | Biological process                      | # of DEGs | p-value    | FDR     |
|-----------------|-----------------------------------------|-----------|------------|---------|
| GO:0000278      | mitotic cell cycle                      | 162       | 5.07E-65   | 2.05E-61|
| GO:0007049      | cell cycle                              | 203       | 2.29E-59   | 4.63E-56|
| GO:0022402      | cell cycle process                      | 176       | 3.14E-58   | 4.24E-55|
| GO:0000280      | nuclear division                        | 96        | 2.19E-50   | 1.78E-47|
| GO:0007067      | mitosis                                 | 96        | 2.19E-50   | 1.78E-47|
| GO:0048285      | organelle fission                       | 98        | 5.92E-49   | 4.00E-46|
| GO:0051301      | cell division                           | 115       | 1.88E-46   | 1.09E-43|
| GO:1902589      | single-organism organelle organization  | 175       | 1.17E-37   | 5.92E-35|
| GO:0006259      | DNA metabolic process                   | 121       | 1.15E-34   | 5.19E-32|
| GO:0007059      | chromosome segregation                  | 51        | 3.12E-32   | 1.26E-29|

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pathogens that have cytopathic effects on neurons. IFITM2 and IFITM3 code for interferon-induced transmembrane proteins, which are viral restriction factors that play a role in protecting cells against the entry of influenza and other viruses, as well as other aspects of interferon-signaling [47–49]. This is of interest because of previous studies suggesting that maternal exposure to influenza and other viruses increases SZ risk in offspring [50–56]. Thus, a decrease in IFITM2 and IFITM3 expression, for example due to reduced ZNF804A expression or function, could increase the cytopathogenic effects of some viruses. Investigators have reported an alteration in IFITM2 and IFITM3 gene expression in various brain regions in SZ and ASD autopsy samples; however, expression is increased [57,103–108]. The opposite effects on expression in our ZNF804A KD neurons and the autopsy studies could be due to differences related to neuronal maturation or stage of brain development, as well the obvious differences in the choice of tissue: live cells vs autopsy samples. In addition, IFITM2 and IFITM3 are many fold more
highly expressed in non-neuronal cells in the brain, such as endothelium and microglia, so differential expression in the brain vs pure neuronal cultures in could be due opposite effects of these genes in neurons compared with other cell types [109].

In addition to IFITM2 and IFITM3, a number of other genes involved in interferon signaling were differentially expressed, as described in the results section. This too is consistent with a number of findings in humans and animal models. For example, an increase in INF-γ levels was found in the CSF of patients with SZ and in the plasma of patients with first episode psychosis [110–111]. And, an alteration in the ratio of IFN-γ to various interleukins was found to be significantly decreased in SZ, consistent with a Th2 shift [112]. Finally, an increase in serum IFN-γ was found in patients with 22q11.2 del who experienced ASD-like behaviors [113].

As for other cytokines, several genes involved in interleukin and TNF signaling were also found to be differentially expressed in the ZNF804A KD samples, as noted above. These could be meaningful considering that fact that IL-1β, IL-6 and TNF-α mediate the effects of MIA on brain development and behavior in animal models, and altered expression has been found in the brain and peripheral blood of patients with SZ and ASD [114–117]. And interestingly, differences in IL-17 expression have been found in SZ and ASD compared with controls in first episode, medication naïve SZ patients, and animal models [118–124].

Two other down-regulated genes related to immune function that we detected were CCL2 and CXCL16; CCL2 was also significantly reduced in the Hill et al. study, as noted in the results section [8]. CCL2 codes for a chemokine expressed in neurons, microglia, and astrocytes that is induced by viral infection, head trauma, and cytokines [40,125–130]. A number of studies show that plasma CCL2 levels differ in SZ, ASD, BD and major depression compared to controls [120,131–136]. Although chemokines are primarily known for their role in innate immunity, in particular chemotaxis, they are increasingly being recognized for their non-immune effects in the brain (neurogenesis, neuroprotection, dopamine transmission, migration of NPCs, and controlling blood–brain barrier permeability) [137–141].

CXCL16 codes for a chemokine that is primarily expressed in lymphoid tissue, but also appears to have a protective effect on excitotoxic cell death in the brain and is induced in a mouse model of multiple sclerosis [142,143]. In addition, SNPs in the gene were found to be associated with SZ in a combined GWAS/pathway analysis [144].

Another differentially expressed gene worth noting in the general context of inflammatory responses is CRYAB, which decreased in the KD cells, similar to the Hill et al. study [8]. CRYAB codes for heat shock inducible chaperone that suppresses apoptosis and neuroinflammation [145,146]. It was one of the top genes induced by heat shock in human fetal telencephalon-like structures we differentiated from iPSCs [66]. This suggests that reduced expression caused by a decrease in ZNF804A could make differentiating neurons more vulnerable to infectious disease, autoimmune attack or cellular stress.

Overall, our results suggest that ZNF804A modulates the expression of genes that regulate cytokine and chemokine signaling pathways in differentiating neurons, perhaps affecting their response to immune activation and/or viral infection. This hypothesis is also supported by immunological studies and replicated GWAS findings, in which association signals in the MHC locus have been found, suggesting an infectious disease and/or autoimmune process in subgroups of SZ and ASD patients [147–158]. However, non-immune effects of MHC antigens on brain development and neuronal function is a possible explanation for the association [159–161].

Finally, it should be noted that an effect of ZNF804A on cytokine signaling could also help explain the development of metabolic syndrome, which occurs as a polymorphic trait in many patients treated with psychotrophic medications (and some untreated patients as well); metabolic syndrome is mediated by immune cytokines [133,162–167]. It should also be noted that
there are four binding sites for EGR (early growth response) transcription factors in the ZNF804A promoter region. EGR transcription factors play a role in synaptic plasticity and behavior, and are activated by pro-inflammatory cytokines, providing another potential connection between immune activation and ZNF804A expression, and suggesting the possibility of feedback interactions [168–172].

These findings, while interesting, must be viewed cautiously, however. Although we did not detect a correlation between expression of lentiviral sequences and IFITM2 and IFITM3 expression, it is still possible that the changes we detected in cytokine signaling genes are an artifact of viral transduction that cannot be explained simply by analyzing vector transcripts. Consequently, validation using a gene knockout approach with CRISPR or zinc finger nucleases, rather than shRNA mediated gene KD will be extremely valuable to confirm our findings, as well as performing these analyses using additional iPSC lines to account for genetic background differences.

Supporting Information

S1 Fig. Germ line markers are expressed in the iPSC line used in this experiment: AFP (alpha-fetoprotein; endoderm), TUJ1 (TUBB3; Neuron-specific class III beta-tubulin) (ectoderm), Desmin (mesoderm). In addition, the iPSC line expresses the pluripotency markers Tra 1–60 and Tra-81, and has a normal karyotype. (JPG)

S1 Table. Entire list of RNA-seq data from 3 scrambled controls and 3 ZNF804A KDs arranged by lowest FDR. All genes showing genome-wide significance (FDR; q-value <0.05) are highlighted in bold type. (XLSX)

S2 Table. Ingenuity Pathway Analysis (IPA) for ZNF804A down-regulated genes. (XLS)

S3 Table. Gene Ontology (GO) for ZNF804A down-regulated genes. (XLSX)

S4 Table. Ingenuity Pathway Analysis (IPA) for ZNF804A up-regulated genes. (XLS)

S5 Table. Gene Ontology (GO) for ZNF804A up-regulated genes. (XLSX)

S1 Text. Comprehensive description of methods, PCR primers and antibodies. (DOCX)

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Author Contributions

Conceived and designed the experiments: HML DZ. Performed the experiments: JC AH EP JD SJ. Analyzed the data: ML DZ HML. Contributed reagents/materials/analysis tools: ML DZ. Wrote the paper: HML DZ ML.
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