Supplemental Information:

Supplemental Figure 1: ZCCHC17 is expressed in neurons. Immunohistochemistry was performed for ZCCHC17. At 1:100, anti-ZCCHC17 antibody stains nuclei strongly and cytoplasm weakly in grey matter (A), and nuclei weakly in white matter (B). At 1:400, anti-ZCCHC17 selectively stains the nucleoli of large neurons in grey matter, as shown in C (low magnification) and D (high magnification).
Supplemental Figure 2: In this figure, we display all of the ZCCHC17 and Beta-actin blots used to generate Figure 2A. Three controls and two severe AD cases are each repeated as technical replicates; all of these cases have similar optical density between the two technical replicates when normalized by beta-actin, and in these cases the two technical replicates are averaged into one value for purposes of making Figure 2A. These cases are (all lanes counting from leftmost sample as lane 1); lane 5 from gel 1 and lane 10 from gel 2; lane 2 from gel 1 and lane 6 from gel 3; lane 11 from gel 2 and lane 3 from gel 3; lane 14 from gel 1 and lane 4 from gel 3, and lane 3 from gel 2 and lane 1 from gel 3. The blue arrow points to the ZCCHC17 band. Of note, we occasionally see higher molecular weight bands reacting with ZCCHC17 antibody in AD cases. We are currently unclear as to the nature of these bands, although they are largely absent from control cases.
Supplemental Figure 3: In this figure, we display all of the NeuN and Beta-actin blots used to generate Figure 2B.
Supplemental Figure 4: In this figure, we display all of the GFAP and Beta-actin blots used to generate Figure 2C.

** ladder for GFAP blots seen best on beta-actin channel
Supplemental Figure 5: ZCCHC17 nuclear staining decreases after knock-down. Rat cortical cultures were incubated with control siRNA (A-D) or ZCCHC17 siRNA (E-H). ZCCHC17 staining was primarily nuclear (A and E) and overlapped with the DAPI signal (B and F). Beta-tubulin staining was performed to highlight the cytoplasm (C and G; panels D and H are composite). ZCCHC17 signal intensity was analyzed using Image J, and ZCCHC17 siRNA incubation leads to an approximate 50% reduction in signal (I). In (I), 3 confocal slides were analyzed per group, and 10 images were taken per slide. p-value = 2*10^-5, one-sided t-test. Error bars are standard error.
Supplemental Figure 6: ZCCHC17 has a conserved function across species. (A) Our human interactome work predicts the relationship of genes with ZCCHC17 in our rat data. Every gene that is linked to ZCCHC17 through our interactome analysis (which uses human data) and that also has a rat homologue that significantly correlates with ZCCHC17 in our rat data is represented by a point. For each gene, the x-axis displays the human interactome-derived Spearman’s rank correlation of a given gene with ZCCHC17. The y-axis displays the Spearman’s rank correlation of a given gene with ZCCH17 as derived from our rat data. Genes that with strong positive correlations or strong negative correlations in both humans and rats are colored blue; genes with discordant correlations (positive in one species, negative in another) are colored black. Note that for genes that positively correlate with ZCCHC17 in our human data (right half), more reach positive significant correlations with ZCCHC17 in our rat data (169 genes; blue dots upper right quadrant) than reach negative significant correlations in our rat data (37 genes; black dots lower right quadrant). The same relationship holds for the minority of genes predicted to negatively correlate with ZCCHC17 in our human data (i.e. there are more blue dots in lower left quadrant (20 genes) than black dots in the upper left quadrant (7 genes)). Overall, 81% of the genes that reach significance in the rat data have the same sign of correlation in the human data (p-value < 2.2 * 10^{-16}, binomial test). (B) ZCCHC17 knock-down is
characterized by impaired expression of ontology groups related to synaptic function. Shown here are the top 10 most significant categories, which are all characterized by a net decrease in gene expression (see negative Z values). Six of these categories (shaded in grey) relate to synaptic function. Note that this table was generated by first screening out ontology groups with over 200 genes in order to eliminate broad ontology groups that contain less specific information (see Supplemental Methods for details).
Supplemental Table 1: Ontology analysis was performed for all genes in the ZCCHC17 regulon. Shown are the top ten categories when ranked by FDR corrected p-value (using Benjamini–Hochberg procedure; original p-values are generated using the hypergeometric test, see Supplemental Methods for details). Although synaptic categories are well represented (four out of the top ten, see shaded categories), other non-synaptic ontology groups are also highly represented in the ZCCHC17 regulon.

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Supplemental Table 2: Shown is a list of all of the synaptic genes that 1) Correlate with ZCCHC17 in our interactome, and 2) Show statistically significant changes in expression after
ZCCHC17 knock-down in our rat cortical cultures. The column second from right is the p-values adjusted for multiple hypothesis testing with a Benjamini-Hochberg procedure, and the column on the far right is the Spearman’s rank correlation of these genes with ZCCHC17 in the interactome. Note that most of these genes are positively correlated with ZCCHC17 in the interactome, and subsequently decrease in expression (see log2FoldChange values) when ZCCHC17 is knocked-down.

The following Supplemental tables are provided as separate files

Supplemental Table 3: Listed are all of the genes from the ZCCHC17 human interactome that have a rat homologue that appears in our rat RNA-seq data. For each of these genes, we have listed 1) The name of the human gene, 2) The human gene Entrez number, 3) The Spearman’s rank correlation of this gene with ZCCHC17 in our human data 4) The rat homologue, 5) The rat homologue Entrez number, 6) The Spearman’s rank correlation with ZCCHC17 in our rat data, followed by 7) The p-value and 8) The Benjamini–Hochberg adjusted p-value for this correlation in our rat data. See main text for analysis of these results.

Supplemental Table 4: Listed are the full RNA-seq results after ZCCHC17 knock-down in rat cortical cultures.

Supplemental Methods:

Computational Analysis Identifying ZCCHC17

In this study, we use the initial work from Aubry et al. (2015) as a starting point to identify master regulators that drive synaptic dysfunction in AD. Here, we will first briefly describe the work in Aubry et al. that is relevant to the computational work in this study, and then describe in more detail our analysis. In Aubry et al., the authors used computational tools to interrogate RNA expression profiles to determine whether there are factors (“master regulators”) that may be driving a large percentage of the changes in gene expression seen in a disease state. These
tools consist of two main algorithms. The first algorithm is ARACNe (Algorithm for the Reconstruction of Accurate Cellular Networks) (Basso, et al., 2005; Margolin, et al., 2006; Palomero, et al., 2006). In short, ARACNe determines whether mRNA levels of a given transcriptional regulator correlate with mRNA levels of a given gene. A list is then assembled of every gene whose mRNA levels correlate with the mRNA levels of a given transcriptional regulator (mutual information is used to fully determine this relationship (Basso, et al., 2005; Margolin, et al., 2006; Palomero, et al., 2006)). This analysis is performed for every transcriptional regulator in the genome. For our purposes, we broadly define a “transcriptional regulator” as any gene whose protein product is known or predicted to bind DNA and/or RNA. We define “transcriptional regulator” in this way because any protein with a direct role in gene expression could potentially cause large scale changes in gene expression in AD. Thus, our definition of “transcriptional regulators” includes transcription factors, cofactors, and other proteins involved in RNA synthesis and processing. We call the resulting data set the “interactome” for the given tissue/cell type that we are analyzing. mRNA levels of a transcriptional regulator will only loosely be correlated with mRNA levels of its down-stream targets, so the power of this technique is sensitive to sample size. The data set used by Aubry et al. consists of a previously published set of Affymetrix U133 Plus 2.0 Array expression data from laser-capture micro-dissected neurons from various regions of AD brain tissue (Liang, et al., 2008; Liang, et al., 2010; Liang, et al., 2007). The total data set represents 14 controls, 10 non-demented individuals with AD-type changes in their brains at autopsy, and 34 demented individuals with the histopathological confirmation of AD. To assemble the interactome, Aubry et al. used the full set of 193 gene expression profiles representing the entorhinal cortex, hippocampus, middle temporal gyrus, posterior cingulate, superior frontal gyrus, and visual cortex of these individuals. This number of samples is within the range of sample sizes used in previous implementations of this algorithm (Basso, et al., 2005; Margolin, et al., 2006; Palomero, et al., 2006). Note that ARACNe is designed to detect only direct interactions
between a transcriptional regulator and a target gene. This is accomplished by eliminating
correlations between ZCCHC17 and a secondary effect gene using the data processing
inequality (Basso, et al., 2005; Margolin, et al., 2006; Palomero, et al., 2006).

The interactome is species and cell-type specific, and tells us what transcriptional regulators
likely have a direct effect on the expression level of genes that are expressed in the specific
species and cell-type that formed the sample pool. The human neuronal interactome derived
from ARACNe was subsequently queried by Aubry et al. using a second algorithm: MARINa
(Master Regulator INference algorithm) (Carro, et al., 2010; Lefebvre, et al., 2010). MARINa
outputs a list of transcriptional regulators whose regulons are significantly over-represented
amongst genes that showed significant changes in expression for a given phenotype (in this
case AD) when compared to control (this ranking of regulons is accomplished through
generating an enrichment score using Gene Set Enrichment Analysis (Subramanian, et al.,
2005), see [(Carro, et al., 2010; Lefebvre, et al., 2010)] for details on how this is used in
MARINa). Transcriptional regulators that have passed this threshold are called master
regulators (MRs).

Aubry et al. (2015) identified a set of MRs that may play a role in overall changes in gene
expression in AD neurons. In this paper, we used this same approach to identify MRs that drive
synaptic dysfunction in AD. To do this, we screened the MARINa output for MRs that 1) Have
regulons with a large number of synaptic target genes, and 2) Have synaptic target genes that
are decreasing in expression in AD. We used a pre-defined list of “synaptic” genes from the
Ingenuity database (Kramer, et al., 2014) for this purpose. We curated this list using a broad
definition that tagged any gene that was related to synaptic function (according to Ingenuity’s
filter), even if its protein product is not technically localized to the synapse. This relatively broad
definition fit our purpose, as our goal was to identify master regulators that drive synaptic and
neurophysiologic dysfunction. Although a narrower focus limited to proteins actually localized to the synapse would be interesting, it was not the goal of this project.

Specifically, we assembled a list of every MR that had been identified in every brain region analyzed in Aubry et al. These regions include entorhinal cortex, hippocampus, middle temporal gyrus, posterior cingulate, superior frontal gyrus, and visual cortex. Aubry et al. identified MRs by region of the brain, and each region had a separate list of MRs that drive gene expression in AD brain tissue in that region. As might be expected, the lists have a high degree of overlap across regions, although there are also some differences (i.e. some MRs were found exclusively in one region, and not any other). Not surprisingly, MARINa predicts the fewest number of MRs in visual cortex (Aubry, et al., 2015), which is consistent with the known regional sparing of visual cortex in AD (Braak, et al., 2006). With this initial list from Aubry et al., we first assembled a list of MRs that were predicted to have differential activity in three or more of these six regions, as we are interested in finding MRs whose dysfunction may be generalizable to neurons throughout the brain in AD. For each MR that passed this threshold, we then calculated the average change in gene expression for each “synaptic” gene across all regions where this MR is predicted to have differential activity. We then calculated the synaptic score for a given MR ($S_{MR}$) with the following equation:

$$S_{MR} = \sum_{i=1}^{n} r_i * d_i$$

where $n$ is the number of synaptic genes that correlate with a given MR, $r_i$ is the Spearman’s rank correlation between the MR and $i^{th}$ synaptic gene, and $d_i$ is the difference in the expression values of the $i^{th}$ synaptic gene. The $d_i$ value is calculated by first taking the average expression value for the $i^{th}$ synaptic gene from control and AD – this average is calculated from the original Affymetrix Human Genome U133 Plus 2.0 data which was previously normalized (Aubry, et al., 2015) with gcrma (Wu, et al., 2004) to adjust for background intensities in Affymetrix array data.
We then took the difference between these two values and eliminated differences that were not statistically significant using an FDR of 0.05. We then normalized remaining synaptic genes showing a significant difference in expression by the control value for each synaptic gene in order to control for genes with large absolute values but small relative changes. The resulting $d_i$ value is:

$$d_i = (a_i - c_i)/c_i$$

where $a_i$ is the average expression value of gene $i$ in the AD samples and $c_i$ is the average expression value of gene $i$ in the control samples. MRs were subsequently ranked based on their $S_{MR}$ values; the MR with the most negative $S_{MR}$ value is ZCCHC17 (Figure 1). Note that $S_{MR}$ is essentially a sum of the change in a MR's synaptic targets, weighted by the correlations between the MR and each target. $S_{MR}$ values will therefore be large for MRs that have high correlations to synaptic target genes that show large changes in value in AD. MRs with large negative $S_{MR}$ values will either A) Be positively correlated with synaptic genes with lower expression in AD, or B) Be negatively correlated with synaptic genes with higher expression in AD. Although both scenarios are mathematically possible, the lower expression level of synaptic genes in our AD dataset meant that scenario (A) was the only scenario that actually occurred. All of the above analysis was performed in Matlab. Ontology analysis for the ZCCHC17 regulon (Supplemental table 1) was performed using gene ontology (GO) categories curated by the ConsensusPathDB database (Kamburov, et al., 2013). We submitted the genes in the ZCCHC17 regulon to this online resource, which provides a p-value for each category using the hypergeometric test as well as FDR correction using the Benjamini–Hochberg procedure. We performed our ontology analysis with GO categories in the most specific level of biological process (“Level 5”) in order to focus on biological categories that would be most informative for characterizing the ZCCHC17 regulon (i.e. we eliminated broader categories that contain less specific information).
**Human tissue**

All human tissue was de-identified and was obtained from the Columbia University Alzheimer’s Disease Research Center/New York Brain Bank, and as such, is IRB exempt under NIH IRB exemption four (E4). For western blot, frozen autopsy tissue from superior temporal gyrus was used from subjects with different stages of AD pathology. Samples matched for age and post-mortem interval were obtained from control subjects, subjects with moderate AD and subjects with severe AD. Control subjects had no history of cognitive impairment and had no pathology that met the criteria for AD or any other age-related neurodegenerative disease. Subjects with moderate and severe AD had a history of cognitive impairment and Alzheimer’s pathology. Cortical samples were classified as “moderate” and “severe” based on a local assessment of the density of tau pathology. Specifically, for each frozen section of superior temporal gyrus, an adjacent formalin fixed/paraffin embedded section was stained for tau (AT8). This stained section was then assessed by a neuropathologist at 100x magnification and the field with the highest density of tau-positive neurons was located. Sections were classified as “moderate” AD if this highest-density field had 6 or fewer tau-positive neurons. “Severe” cases had more than 6 tau-positive neurons in the highest density field. This methodology was employed because the Braak staging system is a global assessment of disease burden (Braak, et al., 2006), and we wished to have a more local assessment of disease burden in a section of tissue immediately adjacent to our frozen samples in order to better correlate the relationship of ZCCHC17 to Alzheimer’s pathology. For immunohistochemistry, formalin-fixed, paraffin-embedded surgical brain tissue was used from adult patients.

**Immunohistochemistry**

Immunohistochemistry was performed with a primary antibody against ZCCHC17 from AbCam (abcam ab80454); the primary antibody was used at 1:100 or 1:400 (see Results section). All
slides were counterstained with hematoxylin. Immunostaining was performed in the Ventana automated slide stainer without manual antigen retrieval and was detected using the Ventana ultraView universal DAB detection kit (Tucson, AZ) as recommended by the manufacturer.

**Western Blot**

Frozen fragments of human cortex were homogenized in 3% LDS buffer (3% LDS, 10 mM EDTA, 50 mM Tris-HCL) and rat cortical cultures were homogenized in RIPA buffer (Thermo Scientific); in both cases we added protease inhibitor (Thermo Scientific) at 4°C, followed by centrifugation at 10,000 rpm for 30 min. Supernatant protein was quantified using BCA protein assay reagent (Pierce), and 22 mg of supernatant protein per sample was electrophoresed on Novex Wedgewell 4–20% Tris-Glycine gels (Invitrogen) according to the manufacturer’s specification. Gels were transferred to nitrocellulose membranes in Tris-Glycine transfer buffer (Invitrogen) using Mini Trans-Blot Cells (Bio-Rad) overnight at 4°C. Membranes were then blocked for 24 h in Seablock (Thermo Scientific), followed by overnight incubation in primary antibody in 50% TBST (tris buffered saline + tween; Fisher Scientific) + 50% Seablock. Blots for ZCCHC17 were first incubated over night with anti-ZCCHC17 (Abcam: ab80454, 1:1000), developed, and then incubated overnight with anti-Beta-actin (Cell Signaling: 8H10D10, 1:10 000). NeuN blots were first incubated overnight with anti-NeuN (Abcam ab104224, 1:2000), developed, and then incubated overnight with anti-Beta-actin (Cell Signaling: 4970T, 1:2500). GFAP blots were first incubated overnight with anti-GFAP (Cell Signaling 3670, 1:2000), developed, and then incubated overnight with anti-Beta-actin (Cell Signaling: 4970T, 1:2500). After incubation with primary antibody, blots were washed in TBST, and incubation in fluorescently labeled secondary antibody (Thermo scientific #SA5-10044 and Invitrogen 35568) at 1:10,000 for 1 h. Blot images were taken using Odyssey imaging system (LI-COR).
RNA was extracted from rat cortical culture using Quick-RNA Mini Prep Plus kit (Zymo Research). Residual DNA was removed using the DNA free kit from Ambion (AM 1906), and RNA was quantified using a nano-drop spectrophotometer. cDNA was made from 400ng of RNA using the SuperScript VILO (Invitrogen). Quantitative RT-PCR was performed on ABsystems 7500 FAST using SYBR green (Invitrogen). Melting curve was used to confirm primer specificity.

Primers used: ZCCHC17 forward: 5'-CTGAACACGACCTGCAAGAA-3', reverse: 5'-CCTCTTCAGGGACCAGAGAATA-3', SYT1 forward: 5'-AAAGGAAGAGCCCAAGGAAG-3', reverse: 5'-GACGTATGGATCGGATGTACC-3', Sv2B forward 5'-TTGTCATCGTCTGTGCTCTG-3', reverse 5'-CCTTAGCTCTCATGGTGGTGC -3', SYN2 forward: 5'-GCCCTCACCCAAAACCTATG-3', reverse: 5'-AGTGTTTGTCTTCCAGTCTCCC-3', GAPDH forward: 5'-CCATCAACGACCCCTTCATT-3', reverse: 5'-GACCAGCTTCCCATTCTCAG-3'. Values were normalized to RPL13 mRNA forward: 5'-CATCACGGAAGAGGAAGAAC-3', reverse: 5'-TCCAGCCGCGCATTTTCTTTTATTT-3'.

Cell Culture

Primary cortical cultures were prepared from E18 OFA Sprague Dawley rat embryos (Charles River, Wilmington, MA, USA) as previously described (Izzo, et al., 2014). Rats were handled in accordance with NIH Guidelines for Humane Care and Use of Laboratory Animals. Cortices were dissected in cold HBSS (Invitrogen), followed by digestion in a 0.05% solution of Trypsin-EDTA (Life Technologies) at 37° C for 15 min. Tissue was then mechanically dissociated in Dulbecco's Modified Eagle Medium (Life Technologies). Cells were counted and plated at 600 thousand cells per ml in Neurobasal media supplemented with 2% B27, 2mM Glutamax (all from Life Technologies). Wells were coated with poly-D-Lysine (Sigma-Aldrich, Saint-Louis, MO,
USA) the day before. Cells were fed and treated after 7 days in vitro (DIV) with Accell siRNA 1 uM (Dharmacon) and harvested at DIV 14 for corresponding experiment. siRNA consisted of a pool of four ZCCHC17 sequences or four control siRNA sequences.

**Generation and analysis of RNA-seq data from rat cultures**

Rat cortical cultures were incubated with siRNA (control or ZCCHC17) at one week of age, and then one week later RNA was extracted as described above. RNA-seq was performed on 6 biological replicates with ZCCHC17 siRNA and 6 biological replicates with control siRNA. RNA-seq was performed at the Columbia University Sulzberger Genome Center core facility. RNA was prepared for sequencing using the Illumina TruSeq Stranded mRNA Library Prep Kit, and RNA-seq was performed on Illumina HiSeq2000 sequencers. FASTQ files were aligned using the STAR 2.5 aligner (Dobin, et al., 2013) and count data was generated using HTseq 0.6.1p1 (Anders, et al., 2015). Differential expression was calculated using DESeq2 (Love, et al., 2014). All subsequent analysis of the differential count data was performed using scripts written in R that analyzed the data as described in the Results section. Correlations between ZCCHC17 and other mRNA transcripts were calculated using the \texttt{rcorr} function in the Hmisc package in Bioconductor (Frank E. Harrell, 2016). For correlation analysis, we curated a list of rat homologue genes that corresponded to the genes in the human ZCCHC17 regulon using the bioDBnet database (Mudunuri, et al., 2009). We did this by first assigning our human ZCCHC17 regulon genes Entrez gene IDs, and then using the bioDBnet database to convert these IDs into rat homologue Entrez IDs. ZCCHC17 human regulon genes with no rat homologue or multiple possible rat homologues were discarded. Genes with a single unambiguous rat homologue Entrez ID were then used in our correlation analysis with ZCCHC17 in the rat data, and the Spearman’s rank correlation value of the rat homologue with rat ZCCHC17 was compared to the Spearman’s rank correlation value between ZCCHC17 and the corresponding human gene in our interactome analysis (see Supplemental Table 3).
Ontology analysis was performed using ontology groups as defined by the Reactome project (Croft, et al., 2014; Fabregat, et al., 2016). For each ontology group, we performed a 2-sided t-test to determine whether the log2 fold change values of the genes in that ontology group showed a significant change in expression as compared to the rest of the gene expression profile. After performing this with every ontology group in the Reactome dataset, we then corrected for multiple hypothesis testing with a Benjamini-Hochberg procedure and set the FDR of 0.05. Supplemental Figure 6B was generated after screening out ontology groups with over 200 genes in order to eliminate broad ontology groups that contain less specific information.

**Immunocytochemistry and confocal microscopy**

Cells that were plated on poly-L-lysine-coated 12mm coverslips were incubated with ZCCHC17 or control siRNA at DIV7 and then fixed at DIV14 using a solution of 4% paraformaldehyde and 4% sucrose in Phosphate Buffered Saline (PBS) (Invitrogen) for 20 min. Cells were then washed three times in PBS. After that the cells were permeabilized with a solution of 0.02% Triton-X (Fisher scientific) in PBS for 10 min. The blocking step involved incubating coverslips in Blocking buffer (1% Bovine Serum Albumin (BSA) (Fisher Scientific) in PBS) for 30 minutes. Cells were subsequently incubated for 1 h with primary antibody anti-Zcchc17 (Abcam: ab80454) 1:100 and anti-Beta-tubulin (Biolegend: 801201) 1:100 in Blocking buffer followed by the washing step three times with Blocking buffer. Secondary antibody (goat anti-rabbit conjugated to Alexa 466 diluted in Blocking buffer 1:400) was added for 1h. Coverslips were mounted in Vectashield Mounting Medium for Fluorescence with DAPI (Vetcor laboratories). Z-stacks of neurons were acquired on a AX10/LSM800 confocal microscope (Carl Zeiss). Fluorescence was collected with a × 63 plan apochromat immersion oil (Zeiss) objective.
(numerical aperture (NA) 1.4). All the experiments were performed at room temperature. Acquired images were analyzed using ImageJ Ver. 2.00-rc-49.

References:


