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ORIGINAL ARTICLE

Genome-wide distribution of Auts2 binding localizes with active neurodevelopmental genes

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The autism susceptibility candidate 2 gene (*AUTS2*) has been associated with multiple neurological diseases including autism spectrum disorders (ASDs). Previous studies showed that *AUTS2* has an important neurodevelopmental function and is a suspected master regulator of genes implicated in ASD-related pathways. However, the regulatory role and targets of Auts2 are not well known. Here, by using ChIP-seq (chromatin immunoprecipitation followed by deep sequencing) and RNA-seq on mouse embryonic day 16.5 forebrains, we elucidated the gene regulatory networks of Auts2. We find that the majority of promoters bound by Auts2 belong to genes highly expressed in the developing forebrain, suggesting that Auts2 is involved in transcriptional activation. Auts2 non-promoter-bound regions significantly overlap developing brain-associated enhancer marks and are located near genes involved in neurodevelopment. Auts2-marked sequences are enriched for binding site motifs of neurodevelopmental transcription factors, including Pitx3 and TCF3. In addition, we characterized two functional brain enhancers marked by Auts2 near *NRXN1* and *ATP2B2*, both ASD-implicated genes. Our results implicate Auts2 as an active regulator of important neurodevelopmental genes and pathways and identify novel genomic regions that could be associated with ASD and other neurodevelopmental diseases.

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INTRODUCTION

Autism spectrum disorders (ASDs) are common (1/88 in the United States), 1 highly heritable 2 pervasive developmental disorders characterized by variable deficits in social communication, language and restrictive and repetitive behaviors.³ AUTS2 has been increasingly implicated as an ASD candidate gene with over 50 unrelated individuals with ASD or ASD-related phenotypes, such as intellectual disability or developmental delay, having structural variants disrupting the AUTS2 region.4-17 AUTS2 structural variants have also been associated with other neurological phenotypes including epilepsy, 18 schizoaffective disorder, 1 bipolar disorder, 20,21 attention deficit-hyperactivity disorder, 22 differential processing speed,²³ suicidal tendencies under the influence of alcohol²⁴ and dyslexia.¹⁰ In addition, a non-coding single-nucleotide polymorphism within AUTS2 (rs6943555) is significantly associated with alcohol consumption.²⁵ AUTS2 has also been implicated in human-specific evolution. $^{26-28}$

AUTS2 is a nuclear protein that is expressed in numerous neuronal cell types including glutamatergic neurons (in the cortex, olfactory bulb and hippocampus), GABAergic neurons (Purkinje cells) and tyrosine hydroxylase-positive dopaminergic neurons (substantia nigra and ventral tegmental area).²⁹ In mice, *Auts2* is expressed in the developing olfactory bulb, cerebral cortex and cerebellum, and is located in the nuclei of neurons and some neuronal progenitors.²⁹ At embryonic day (E) 16, Auts2 shows strong expression in the cerebral cortex with a gradient of high rostral to low caudal expression.²⁹

The precise function of *AUTS2* is not well known; however, zebrafish knockdowns have shown *auts2* to be critical in neurodevelopment. *auts2* morphant fish display microcephaly with a decrease in neuronal cells in the brain, ^{13,30} which may be caused by failure of cells to differentiate into mature neurons. ³⁰ *auts2* knockdown also leads to craniofacial abnormalities in zebrafish ¹³ and reduced movement, possibly caused by fewer motor and/or sensory neurons. ³⁰ Sequence analysis of AUTS2 identified a predicted PY motif (PPPY) at amino acids 515–519, ³¹ a potential WW-domain-binding region involved in protein–protein interactions. This motif is thought to be involved in the activation of transcription factors, suggesting that AUTS2 may be involved in transcriptional regulation. ⁵

Several proteins are suggested to affect the expression of AUTS2. T-box, brain, 1 (Tbr1), a transcription factor that has been implicated in ASD,³² regulates regional and laminar identity in postmitotic neurons²⁹ and is critical for proper neocortex development,³³ interacts with the *Auts2* promoter in the developing neocortex.³³ *Tbr1*-deficient mice have decreased levels of reelin,³³ a protein necessary for proper neuronal migration in developing brains that can be expressed at decreased levels in individuals with ASD.³⁴ *Auts2* expression is reduced in SATB homeobox 2 (*Satb2*) null mice, with *Satb2* being a known regulator of Tbr1 expression in callosal projection neurons.³⁵ *Auts2* has been reported to have a 1.33-fold change in cerebellar gene expression in methyl CpG binding protein 2 (*Mecp2*) null mice, a gene implicated in neurodevelopmental disorders including Rett syndrome and autism.³⁶ *AUTS2* is a potential target of GTF2I

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repeat domain-containing 1 (*GTF2IRD1*), one of 26 genes deleted in neurodevelopmental disorder Williams–Beuren syndrome. ^{37,38} Zinc-finger matrin-type 3 (*Zmat3*, also known as *wig-1*) is a transcription factor regulated by p53 and has an important role in RNA protection and stabilization. wig-1 downregulation leads to a significant reduction in *Auts2* mRNA levels in the brains of BACHD mice, a Huntington's disease mouse model. ³⁹

AUTS2 is also thought to interact with other genes and pathways including the notch and ERK signaling pathways, *PRC1*, and *SEMA5A*. Notch signaling has been shown to be involved in neuronal migration through its interaction with Reelin^{42,43} and AUTS2 expression was found to oscillate in phase with other notch pathway genes. 44,45 Polycomb-group repressive complex 1 (PRC1), a polycomb-group gene often involved in transcriptional repression physically interacts with AUTS2, implicating a role for AUTS2 in developmental transcriptional regulation.⁴⁶ The regulatory pathway of the autism candidate gene semaphorin 5 A (SEMA5A) contains multiple ASD-associated genes, including AUTS2, that overlap rare copy number variations that have been associated with ASD. Twelve regulators of SEMA5Aregulated genes were identified, including AUTS2, suggesting that AUTS2 is a master regulator in ASD-related pathways.⁴⁷ Drosophila melanogaster Tay bridge gene, which has a region of homology with AUTS2 (30% identity in the amino acids 1764--2019 with human AUTS2 amino acids 486-782) in the C-terminal region, is a component of the EGFR/Erk signaling pathway that antagonizes EGFR signaling and interacts with Erk. 41 This suggests that AUTS2's normal function in humans may be involved in Erk signaling, having a role in the differentiation and survival of cells during development.41 Given the limited homology between the proteins, it is difficult to deduce any functional conservation.⁴⁸ However, expression of human AUTS2 in the wing disc was shown to interfere with EGFR signaling, albeit in an opposite manner to Tay. The authors conclude that the effects of AUTS2 on drosophila EGFR signaling are consistent with a role in the regulation of Erk in humans.41 Combined, these interactions implicate AUTS2 in neurodevelopment pathways, including processes important for cell differentiation and ASD. 40 However, the actual downstream regulatory targets of AUTS2 still remain largely unknown.

Given *AUTS2*'s suspected role in neurodevelopment and ASD, and that *AUTS2* may be a master regulator in ASD-related pathways,⁴⁷ we explored the genomic targets of *Auts2* in E16.5 mouse forebrains using chromatin immunoprecipitation followed by deep sequencing (ChIP-seq), RNA-seq and zebrafish enhancer assays. We found multiple lines of evidence that Auts2 is associated with promoters and distal enhancers of genes that are active during neurodevelopment. In addition, motif analysis of Auts2-marked sites found enrichment for known motifs involved in neurodevelopment including paired-like homeodomain 3 (Pitx3), transcription factor 3 (TCF3) and forkhead box O3 (FOXO3). Finally, we identified two novel brain enhancers marked by Auts2, which are located near neurexin 1 (*NRXN1*) and ATPase Ca++ transporting plasma membrane 2 (*ATP2B2*), two genes implicated in ASD.

MATERIALS AND METHODS

ChIP-seq

Mouse embryos were harvested from timed pregnant CD-1 females (Charles River, Wilmington, MA, USA) at E16.5. The forebrains were dissected in cold phosphate-buffered saline and batches of eight forebrains each were collected in a tube and washed twice with phosphate-buffered saline. Forebrains were cut to <1 mm size and crosslinked with 1% formaldehyde for 10 min. Chromatin from forebrain tissue was isolated and sheared using a Bioruptor (Diagenode, Denville, NJ, USA) and immunoprecipitation was performed using 5 mg of anti-AUTS2 antibody (HPA000390, Sigma Aldrich (St Louis, MO, USA), lot no. A33089; previously verified to be specific for the Auts2 protein).²⁹ The antibody is

polyclonal, which can be prone to batch variability and can contain multiple epitopes leading to nonspecific background. We confirmed Auts2 antibody specificity through immunoblot and immunoprecipitation assays. To further validate our results, we performed ChIP-seq with three additional polyclonal Auts2 antibodies: Everest (Upper Heyford, England; catalog no. EB09003), Santa Cruz (Dallas, TX, USA; catalog no. sc-163717) and Abcam (Cambridge, England; catalog no. ab96326). We obtained peaks using the Everest (139 peaks) and Santa Cruz (51 peaks) antibodies but no unique peaks were obtained with the Abcam antibody. Analysis of the overlap of those peaks with the Sigma antibody ChIP-seg peaks found 137 (98.5%) of the Everest peaks and all 51 (100%) of the Santa Cruz peaks to overlap the Sigma peaks (Supplementary Table S1). Furthermore, we performed quantitative PCR to validate the ChIP-seq results and showed specific enrichment for four Everest peaks that overlap Sigma peaks, and no enrichment for four negative controls (regions lacking Auts2 association; data not shown). Chromatin from the same sample was processed for the input control. Illumina libraries were constructed from ChIP and input DNA by the UC Davis Genomics core and sequenced on a HiSeq2500 (Illumina). Auts2 ChIP-seq data from this study are available in SRA (http://www.ncbi.nlm.nih.gov/sra; SRA experiment SRR1292304 (Sigma), SRR1292309 (input), SRR1365079 (Everest), SRR1365081 (Santa Cruz)). ChIP-seg reads were demultiplexed and aligned to the mouse genome (mm9) using Bowtie⁴⁹ allowing one mismatch per read and retaining only reads with a single reportable alignment (example command: bowtie -p 12 -m 1 -v 1 -S). Resulting SAM files were converted to BAM format for peak calling with MACS2 (version 2.0.10).⁵⁰ For peak calling, MACS2 extended the reads to 300 bp and kept only peaks where FDR ≤ 0.01 after normalizing relative to input DNA (example command: macs2 callpeak -f BAM -g mm --keep-dup 1 --nomodel --extsize 300 - q 0.01). A total of 49 364 210 reads were generated with the Sigma Auts2 input and 49 289 630 reads were generated from the input control. For both Auts2 and input, the read length was 50 bp.

RNA-seq

Total RNA was extracted from two replicates of E16.5 mouse forebrain tissue and purified using the RNeasy Maxi Kit (Qiagen, Venlo, Limburg, The Netherlands) and sent to Otogenetics for ribosomal RNA depletion, cDNA production using random primers, library preparation, and pair end sequencing (~100 bp) using Illumina HiSeq2000. Resulting reads were demultiplexed and aligned to the mouse genome (mm9) using TopHat v2.0. 7. 49.51.52 The two replicates were merged and read counts mapping to each transcript were obtained using HTseq. 53 Expression of each transcript was quantified as fragments per kilobase of transcript per million fragments mapped (FPKM) by dividing the total number of reads mapping to each transcript by transcript length, and the total number of reads aligned to the genome divided by one million. The Wilcoxon test from the statistical toolkit R was used to calculate differences in gene expression between genes whose promoter contains an Auts2-marked site and all other genes. RNA-seq data from this study are available in SRA (http://www.ncbi.nlm.nih.gov/sra; SRR experiment SRR1298758 (replicate 1) and SRR1298760 (replicate 2)).

Analyzing the genomic context near Auts2 peaks

Distance from Auts2-marked sites to nearest transcription start site (TSS) was assessed using genomic coordinates downloaded from the UCSC Genome Browser's mm9 RefSeq Genes track. Histone modification ChIP-seq data from mouse E14.5 whole brain⁵⁴ were downloaded from UCSC Genome Browser (H3K4me3, H3K27ac and H3K27me3). All data sets were downloaded in October 2013. BedTools^{,55} intersectBed command was used to identify overlap between this study's Auts2-marked sites and histone modification peaks from E14.5 mouse whole brain. A single base pair overlap was sufficient to consider two regions overlapping. We observed a minimum of 3 bp, maximum of 1475 bp and a median of 349 bp overlap. Significance was calculated using a permutation p-test (1000 permutations). To determine how many Auts2-marked sites reside within each gene in the mouse genome, genes were defined by transcription start and end sites in mm9 RefSeq. BedTools' windowBed command (with 1 bp window) was used to count the number of Auts2-marked sites within each gene.

Gene ontology, pathway and motif analysis

Pathway analysis was performed using ingenuity pathway analysis (IPA; Ingenuity Systems, www.ingenuity.com) by inputting a list of genes near Auts2-marked regions (one gene per Auts2-marked site, based on closest

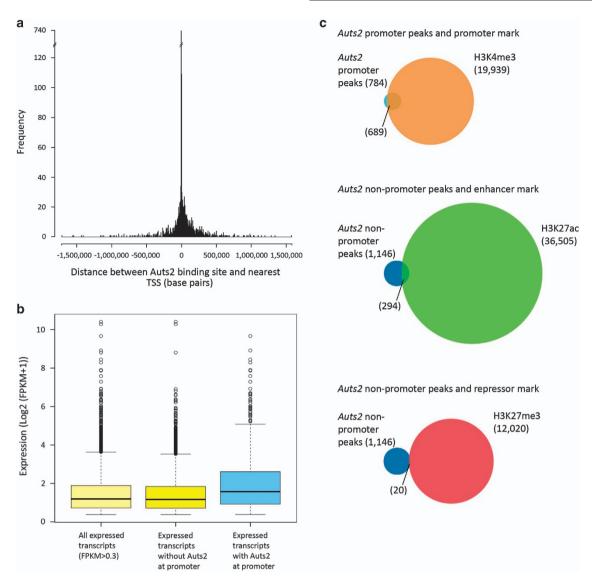


Figure 1. Analysis of Auts2 ChIP-seq peaks. (a) Distance distribution of the 1930 Auts2-marked sites to the nearest transcription start site (TSS) shows preferential binding near TSSs. Histogram displays bins of 5 kb. (b) FPKM transcript expression scores (FPKM>0.3) for genes whose promoters localized with Auts2 display significantly higher expression than those that do not ($P < 2.2e^{-16}$; Wilcoxon test). (c) Overlaps of Auts2-marked sites with histone modifications show significant localization of Auts2 at promoters (H3K4me3) and active enhancers (H3K27ac; P < 0.001; permutation test) but not repressed regions (H3K27me3; P-value = 0.081; permutation test). Histone data were acquired from previously reported ChIP-seq for mouse E14.5 whole brain. P-value = 0.081; permutation test).

distance to TSS, listed in Supplementary Table S2). IPA performs multiple hypothesis test correction using the Benjamini-Hochberg method. Gene ontology analysis was performed using Genomic Regions Enrichment of Annotations Tool (GREAT).⁵⁶ The *Q*-value, calculated by GREAT, applies a false discovery rate correction to the binomial raw P-value to correct for multiple hypothesis testing.⁵⁶ To associate genomic regions with genes in GREAT, we used the 'basal plus extension' setting, except when examining promoter marked sites, in which case 'single nearest gene' was chosen. Background regions were defined using GREAT's 'whole genome' setting except when examining promoter marked sites, where we defined a promoter-specific background containing all promoter regions (2500 bp upstream and 500 bp downstream of a TSS) plus a window the size of the largest Auts2 ChIP-seq peak (1457 bp). MEME and ChIPmunk^{57,58} were used to search for de novo motifs within Auts2-marked sites. MEME-ChIP and GOMO^{59,60} were used to identify experimentally characterized motifs and their gene ontologies. MEME-ChIP and GOMO analyses were performed on 26 March 2014. For MEME-Chip, transcription factorbinding motif input came from JASPAR vertebrates and UniPROBE mouse databases. The expected motif site distribution was set to zero or one occurrence per sequence and motif width was set between 6 and 30. For GOMO, the supported database category was set to multiple species and

the database was set to Mus Musculus. The signal threshold was set to $q \le 0.05$.

Transgenic enhancer assays

Enhancer candidate sequences were selected from Auts2-marked sites based on proximity to the *SFARI* genes that had a gene score of 1–3 (corresponding to high confidence genes, strong candidates and genes with suggestive evidence),⁶¹ evolutionary conservation (sequences showing ≥70% identity for at least 100 bp) and/or overlap with enhancer-associated histone marks (H3K27ac and H3K4me1), but not promoters (H3K4me3) from whole-brain E14.5 ChIP-seq data sets.⁵⁴ PCR was carried out on human genomic DNA (Roche) and products were cloned into the E1b-GFP (green-fluorescent protein)-Tol2 enhancer assay vector containing an E1b minimal promoter followed by GFP⁶² and verified by sequencing. Constructs were injected following standard procedures^{63,64} into at least 100 zebrafish embryos along with Tol2 mRNA⁶⁵ to facilitate genomic integration. GFP expression was observed and annotated up to 48 h post fertilization (hpf). An enhancer was considered positive if at least 15% of all fish surviving to 48 hpf showed a consistent expression pattern after subtracting out percentages of tissue expression in fish injected with the



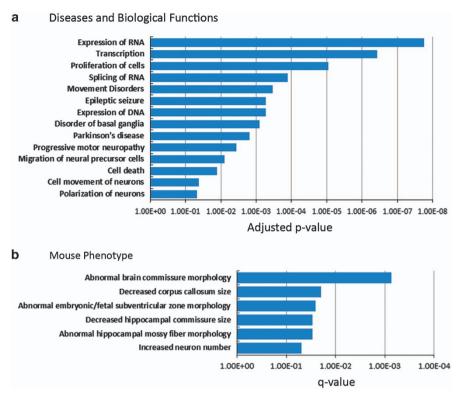


Figure 2. Pathway analysis and gene ontology of Auts2-marked sites. (**a**) ingenuity pathway analysis (IPA) pathway analysis of genes whose promoters contain an Auts2-marked site; the figure shows selected significant (after Benjamini–Hochberg correction) neurological, gene expression and cell cycle-related disease and biological functions. (**b**) GREAT⁵⁶ gene ontology analysis of non-promoter Auts2-marked sites; the figure shows all significant (after false discovery rate correction) neurological-related mouse phenotypes.

empty enhancer vector. For each construct, at least 50 fish were analyzed for GFP expression at 48 hpf. All animal work was approved by the UCSF Institutional Animal Care and Use Committee (protocol number AN100466).

RESULTS

We performed RNA-seq and ChIP-seq using an Auts2 antibody on E16.5 mouse forebrains. E16.5 was chosen because of the reported strong Auts2 expression in the forebrain²⁹ and the established neurogenesis for many relevant brain structures at this time point.⁶⁶ Through RNA-seq, we identified 8897 transcripts expressed at this time point (quantified as FPKM>0.3). Our Auts2 ChIP-seq found 1930 marked sites, the majority of which (1146=59%) do not overlap gene promoters (2500 bp upstream and 500 bp downstream of a TSS). Nonetheless, the 784 Auts2-marked promoters we detected are significantly more than expected by chance (P < 0.001; permutation test), and most promoter peaks (602=31% of all peaks) directly overlap the TSS (Figure 1a).

Promoters of actively transcribed neurodevelopmental genes are marked by Auts2

We initially focused on the 784 Auts2-marked sites that reside within promoter regions. These promoter peaks correspond to 776 genes, as a few genes have multiple Auts2-marked sites within their promoter region. Our RNA-seq analysis showed that these genes display significantly higher expression levels at E16.5 than transcripts that were not marked by Auts2 ($P < 2.2e^{-16}$; Wilcoxon test; Figure 1b). Consistent with their association with highly expressed genes, 88% of Auts2 marks at promoters (689/784) are also marked by the active promoter histone modification H3K4me3 in previously published ChIP-seq data generated from E14.5 whole brain 54 (P < 0.001; permutation test; Figure 1c). These

results indicate that the presence of Auts2 at promoters correlates with transcriptional activation.

Using IPA, we comprehensively analyzed pathways and networks of the 776 genes whose promoters overlap Auts2-marked sites, and therefore may be directly regulated by the Auts2 protein. We found that these genes are significantly enriched for diseases and biological functions related to neurodevelopment, including epileptic seizures, disorders of the basal ganglia, migration of neural precursor cells, cell movement of neurons, polarization of neurons and more (Figure 2a, Supplementary Table S2). Interestingly, these genes are also enriched for processes involved in gene expression and cell cycle, including expression of RNA, transcription, proliferation of cells, splicing of RNA, expression of DNA and cell death (Figure 2a).

Non-promoter Auts2-marked regions function as enhancers

We next analyzed the 1146 Auts2 ChIP-seg marked sites that did not overlap promoters. Supporting the hypothesis that these distal peaks are functionally important genomic elements, 74% are evolutionarily conserved (phastcons 30-way mammal conserved elements),⁶⁷ significantly more than expected by chance (P < 0.001; permutation test). Further suggesting that Auts2 is primarily an activator, 26% (294/1146) of Auts2 distal peaks overlap previously reported mouse E14.5 whole-brain H3K27ac ChIP-seq peaks,⁵⁴ an active enhancer mark, which is significantly more than expected by chance (P < 0.001; permutation test). In contrast, only 2% (20/1146) overlap E14.5 mouse forebrain H3K27me3 peaks, 54 a repressive mark (P = 0.081; permutation test; Figure 1c). Five Auts2-marked sites were identified within the Auts2 gene itself, all of which overlap mouse E14.5 forebrain H3K4me1 marks,⁵⁴ and two that overlap H3K27ac marks,⁵⁴ suggesting an autoregulatory active role for Auts2. None of the Auts2-marked sites overlap the TSS of any of the transcripts

(Supplementary Figure S1). Combined, these results imply that many of the non-promoter Auts2-marked sites likely function as activate regulatory elements.

We investigated the regulatory functions of the 1146 non-promoter Auts2-marked sites using the gene ontology tool GREAT. These Auts2-marked non-promoter sites reside near genes involved in mouse brain development (Figure 2b) including corpus callosum size and neuron number, both of which have been implicated in ASD. ^{68–70} Taken together, these data support a neurodevelopmental and gene expression role for genes associated with Auts2-marked regulatory regions.

Given our observed correlation between Auts2-marked sites and enhancer marks, we next tested whether these sequences function as enhancers *in vivo*. Ten Auts2-marked enhancer candidates (AMECs; Supplementary Table S3) were tested for enhancer activity using a zebrafish transgenic enhancer assay. Candidates were selected based on proximity to ASD-related genes, conservation and overlap with enhancer-associated histone modifications.⁵⁴ Four of the ten candidates were positive enhancers at 24 or 48 hpf (Supplementary Figure S2). AMEC1 lies in an intron of *NRXN1* and showed positive enhancer activity in the heart and forebrain (olfactory epithelium) at 48 hpf (Figure 3a, Supplementary Figure S2a). AMEC2, which lies ~ 56 kb upstream of

contactin 4 (*CNTN4*), displayed enhancer activity in the somitic muscles at 48 hpf (Supplementary Figure S2b). AMEC5, which lies \sim 571 kb upstream of the RNA-binding protein fox-1 homolog (*RBFOX1*), had enhancer activity in the notochord at 48 hpf (Supplementary Figure S2c). AMEC8, which lies in an intron of *ATP2B2*, showed enhancer activity in the midbrain and hindbrain (potentially in the trigeminal sensory neuron) and the spinal cord at 24 hpf, and in somitic muscles at 48 hpf (Figure 3b, Supplementary Figure S2d). These results show that Auts2 occupies functionally active enhancers, some of which drive expression in the developing brain.

Additional genome-wide pathway analyses confirm neurodevelopmental function

We examined the function of nearby genes for all 1930 Auts2-marked sites and the subset of 784 promoter marked sites using GREAT (Supplementary Table S2) and observed an enrichment for gene expression in the mouse cerebral cortex (promoter sites) and in the lower jaw (all marked sites; Supplementary Table S2), which fits with previously reported human phenotypes ¹³ as well as *auts2* morpholino knockdown phenotypes of smaller heads ^{13,30} and undersized and reduced jaws. ¹³ GREAT disease ontology

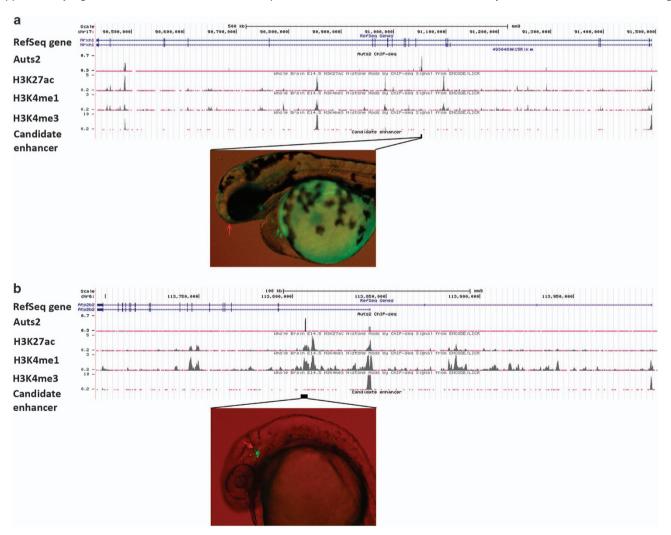


Figure 3. Auts2-marked enhancers. (**a**) A UCSC Genome Browser snapshot of the *Nrxn1* locus in mm9, including tracks for the RefSeq gene, Auts2 ChIP-seq, whole-brain E14.5 H3K27ac/H3k4me1/H3kme3 ChIP-seq⁵⁴ and the Auts2-marked enhancer candidate (AMEC) 1. A representative picture of AMEC1 shows positive enhancer activity in the zebrafish heart and forebrain (olfactory epithelium (red arrow)) at 48 h post fertilization (hpf) is shown below. (**b**) UCSC browser snapshot of the *Atp2b2* region including RefSeq and ChIP-seq tracks. Below, a representative 24 hpf embryo showing enhancer activity of AMEC8 in the midbrain (red arrow) and hindbrain (trigeminal sensory neurons).



enrichment also identified motor neuron disease and hereditary degenerative disease of central nervous system (promoter marked sites; Supplementary Table S2), which also matches the *auts2* knockdown phenotype of fewer motor neuron cell bodies in the spinal cord along with improperly angled weaker projections.³⁰

We also performed IPA analysis on all Auts2-marked sites and the subset of 1146 non-promoter Auts2-marked sites using the gene whose TSS was closest to the ChIP-seq peak (Supplementary Table S2). For both data sets, we found significant enrichment of many diseases and functions including multiple neurodevelopmental related categories (for example, migration of neurons, differentiation of neurons, development of the cerebral cortex, disorders of the basal ganglia, Parkinson's disease, schizophrenia, and so on; Supplementary Table S2). In addition, we observed enrichment for genes involved in the axonal guidance signaling canonical pathway ($P=4.6e^{-9}$; right-tailed Fisher exact test; for all Auts2-marked sites; Supplementary Figure S3, Supplementary Table S2) and ERK/MAPK signaling canonical pathway ($P=6.79e^{-7}$; right-tailed Fisher exact test; for all Auts2-marked sites; Supplementary Figure S4, Supplementary Table S2).

Motif analysis identifies transcription factors involved in neuronal development

To identify known motifs present in Auts2-marked sites, we compared the sequences of those sites with position weight matrices of several hundreds of experimentally determined transcription factor-binding sites using MEME-ChIP. 59 We analyzed gene ontology terms linked to identified motifs with the gene ontology tool GOMO.60 In Auts2-marked promoter regions, we identified motifs involved in many functions including olfactory receptor activity, translation, transcription, neuron fate commitment and structural constituent of ribosomes (Supplementary Table S4). Among the neuro-associated enriched motifs is a binding motif for Pitx3, a transcriptional regulator involved in the differentiation and maintenance of dopaminergic neurons during development.⁷¹ A paralog of PITX3 with a similar binding motif is PITX1, which has been implicated in autism.⁷² In Auts2-marked non-promoter regions, we identified motifs involved in many functions including calcium ion binding, translation, structural constituent of ribosomes and olfactory receptor activity (Supplementary Table S4). Among the known motifs enriched in non-promoter marked regions is the T-cell acute lymphocyte leukemia/TCF3 heterodimer (TAL1::TCF3). Tcf3 is a transcriptional regulator expressed in the developing cerebral cortex involved in the regulation of cell growth, differentiation and commitment of multiple cell lineages including neurons.^{73–75} TAL1 and TCF3 are also associated with acute lymphoblastic leukemia (ALL), 76,77 which has been associated with AUTS2.⁴⁰ In addition enriched is FOXO3, a transcriptional activator involved in neuronal cell death⁷⁸ (Supplementary Table S4). Using MEME and ChlPmunk, two *de novo* motif algorithms,^{57,58} we were only able to identify motifs comprising simple repeats, for instance [CA]ACA[CA]ACA [CA]ACA.

DISCUSSION

Our mouse E16.5 forebrain RNA-seq and ChIP-seq analyses lend further support to a neurodevelopmental role for Auts2. We showed that Auts2 is frequently and significantly localized near promoters of active genes, suggesting that Auts2 is involved in the activation or maintenance of gene expression. IPA and GREAT analyses identified significant associations with neurodevelopmental genes and pathways near Auts2 ChIP-seq peaks. Both GREAT and IPA found highly significant enrichment for genes involved in gene expression and ribosome-related proteins. Interestingly, dynamic regulation of individual ribosomal proteins control gene expression and mammalian development within

vertebrate embryos.⁷⁹ Future experiments are needed to determine whether and how *AUTS2* and ribosomes work together to affect neurodevelopment. IPA analysis also identified axonal guidance signaling as a significantly enriched canonical pathway, which includes the *SEMA5A* gene and supports *AUTS2's* role in *SEMA5A*-related pathways including neurodevelopment and ASD progression.⁴⁷

Our analyses identified several interesting genes that Auts2 may target. Glycerophosphodiester phosphodiesterase domain containing 1 (Gdpd1) contains an Auts2 mark at its promoter and is highly expressed in our RNA-seg data set (FPKM = 2.58). GDPD1 encodes a glycerophosphodiester phosphodiesterase and could be a candidate region linking AUTS2 to alcoholism, given that other classes of phosphodiesterases are involved in alcohol seeking and consumption behaviors.80 GABA B receptor 1 (Gabbr1), which also contains an Auts2-marked site at its promoter and is highly expressed in our data set (FPKM = 11.18), is a key component of GABAergic signaling important for synaptic regulation and is implicated in autism, epilepsy and alcohol and cocaine addiction. 81–83 Ubiquitin carboxyl-terminal esterase L1 (Uchl1) is very highly expressed in our data set (FPKM = 43.36) and displays Auts2 localization at its promoter. Uchl1 is expressed specifically in neurons and is a strong Parkinson's disease susceptibility gene,84 providing insight on a potential new role for AUTS2 in the progression of Parkinson's disease. Another very highly expressed gene (FPKM = 28.08) that has a promoter marked with Auts2 is neruocan (Ncan), a gene thought to be involved in axon guidance⁸⁵ that is implicated in bipolar disorder and schizophrenia.^{86,87} These genes represent possible connections between AUTS2 and a wide range of neurological disorders and provide a list of candidate interactions for further functional studies of the role of AUTS2 in neurodevelopment and disease.

In addition to neurodevelopment, AUTS2 may have a role in cancer. Previous studies have linked AUTS2 with ALL, and matching our finding of enrichment of the TCF3 motif in Auts2-marked sites, as TCF3 is associated with ALL. In addition, our GREAT results identified the MSigDB perturbation term 'genes whose DNA methylation differ between primary ALL cells and peripheral blood samples' as significant (p=7.82e⁻⁶; Binomial test; non-promoter marks).

De novo motif analysis of Auts2-marked regions did not find any non-repetitive novel motifs, and the distribution of known motifs was not centrally enriched within the Auts2-marked regions, supporting the theory that Auts2 acts as a cofactor rather than by binding directly to DNA. AUTS2 lacks identified DNA-binding motifs but contains several predicted protein–protein interaction domains including an SH2, a PY and 13 SH3 domains.^{29,31,40} More research needs to be performed to determine Auts2-binding partners and how they converge to bind DNA.

Auts2-binding analysis revealed that non-promoter Auts2marked sites overlap the enhancer mark H3K27ac significantly more than expected by chance, but not with the repressive H3K27me3 mark, suggesting that Auts2 binds to active enhancer regions throughout the genome. Taken together, this suggests that Auts2 also has an activating role in distant gene regulatory elements. We functionally characterized 10 Auts2-marked sequences near genes implicated in ASD using a transgenic zebrafish enhancer assay. Four AMECs showed positive zebrafish enhancer expression, two of which were positive in the brain. Given our small sample size, testing only 10 AMECs and finding 4 to be positive, we cannot definitively conclude that Auts2 can be used as an enhancer mark or that it is directing the activity of these enhancers. AMEC1, which showed positive zebrafish expression in the olfactory epithelium, is in the intron of NRXN1, a gene involved in synapse formation and signaling that has been implicated in ASD and other neurological disorders.^{88,89} Nrxn1 is also expressed in the olfactory epithelium at E14.5,90 suggesting this enhancer could regulate Nrxn1. Several other lines of

evidence, including our motif analysis, known expression patterns of Auts2 in mouse and zebrafish, ^{29,30} and our IPA and GREAT analyses, further support an olfactory role for Auts2. AMEC8 lies in an intron of *ATP2B2*, which is implicated in ASD, likely because of altered Ca²⁺ signaling when *ATP2B2* is defective. ^{91,92} This sequence showed positive zebrafish enhancer activity in trigeminal sensory neurons matching *Atp2b2*"s expression in the mouse trigeminal ganglion. ⁹⁰ AMEC4, which is within *AUTS2* (Supplementary Figure S1), did not display enhancer activity. It is possible that this region is not an enhancer, or may be active at time points outside the annotated 24–48 hpf.

Using ChIP-seq and RNA-seq on mouse E16.5 forebrains we identified potential Auts2-regulated regions and found that they mark active regulatory elements. We located 1930 targets of Auts2, ~40% of which lie in promoter regions. Transcripts with Auts2-bound promoters had significantly higher expression levels than ones without Auts2 binding. Both the genes and regulatory sequences that are bound by Auts2 provide distinctive candidate regions to investigate nucleotide variation associated with neurodevelopmental disorders. AUTS2 is emerging as a critical regulator of active neurodevelopmental genes, and future studies such as mouse knockouts could confirm and increase our understanding of the neurodevelopmental function of this gene.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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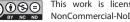


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