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BASIC SCIENCE ARTICLE

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In the developing cerebral cortex: axonogenesis, synapse formation, and synaptic plasticity are regulated by *SATB2* target genes

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BACKGROUND: Special AT-rich sequence-binding protein 2 is essential for the development of cerebral cortex and key molecular node for the establishment of proper neural circuitry and function. Mutations in the *SATB2* gene lead to SATB2-associated syndrome, which is characterized by abnormal development of skeleton and central nervous systems.
METHODS: We generated *Satb2* knockout mouse model through CRISPR-Cas9 technology and performed RNA-seq and ChIP-seq of embryonic cerebral cortex. We conducted RT-qPCR, western blot, immunofluorescence staining, luciferase reporter assay and behavioral analysis for experimental verification.
RESULTS: We identified 1363 downstream effector genes of *Satb2* and correlation analysis of *Satb2*-targeted genes and neurological disease genes showed that *Satb2* contribute to cognitive and mental disorders from the early developmental stage. We found that *Satb2* directly regulate the expression of *Ntng1*, *Cdh13*, *Kitl*, genes important for axon guidance, synaptic formation, neuron migration, and *Satb2* directly activates the expression of *Mef2c*. We also showed that *Satb2* heterozygous knockout mice showed impaired spatial learning and memory.
CONCLUSIONS: Taken together, our study supportsroles of *Satb2* in the regulation of axonogenesis and synaptic formation at the early developmental stage and provides new insights into the complicated regulatory mechanism of *Satb2* and new evidence to elucidate the pathogen of SATB2-associated syndrome.

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IMPACT:

- 1363 downstream effector genes of Satb2 were classified into 5 clusters with different temporal expression patterns.
- We identified *Plxnd1*, *Ntng1*, *Efnb2*, *Ephb1*, *Plxna2*, *Epha3*, *Plxna4*, *Unc5c*, and *Flrt2* as axon guidance molecules to regulate axonogenesis.
- 168 targeted genes of Satb2 were found to regulate synaptic formation in the early development of the cerebral cortex.
- Transcription factor *Mef2c* is positively regulated by *Satb2*, and 28 *Mef2c*-targeted genes can be directly regulated by *Satb2*.
- In the Morris water maze test, $Satb2^{+/-}$ mice showed impaired spatial learning and memory, further strengthening that *Satb2* can regulate synaptic functions.

INTRODUCTION

Special AT-rich sequence-binding protein 2(SATB2) is a transcription factor that plays essential roles in chromatin remodeling and regulation of gene expression in a nuclear-matrix-attachment regions (MARs) dependent manner.[1] *Satb2* has been demonstrated to function in multiple biological processes, including craniofacial patterning, bone formation, cortical regionalization, development of corpus callosum and neuron projection in the neocortex.[2–4] *Satb2* is also important as a regulator of synaptic plasticity in the hippocampus that underlies memory functions.[5] In humans, microdeletions or mutations of the *SATB2* gene lead to SATB2-associated syndromes (SAS) or Glass syndrome (OMIM 612,313), which are clinically manifested as craniofacial malformation, teeth anomalies, developmental delay, poor speech development, hyperactivity, intellectual disability, seizures, and symptoms of autism.[6,7]

In developing mouse brain, *Satb2* expressing neurons extend axons across the corpus callosum. While in *Satb2* knockout mice, axons cannot pass through the corpus callosum instead descend along the corticospinal tract and neurons failed to reach their

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targeted regions.[3] *Satb2* was reported to regulate the differentiation of both callosal and subcerebral projection neurons in the developing cerebral cortex.[8] Generation and outgrowth of axons, termed as axonogenesis, are critical for establishing and maintaining the polarized structure of the neurons.[9] Proper axon guidance is essential in neuron migration and projection to their targets.[10,11] *Satb2* was known to regulate genes involved in axonogenesis, such as *Ctip2*, *Unc5c*, and *Epha7*.[12–14] The cerebral cortex plays a central role in high-level cognitive functions such as learning, memory, thinking and decision-making.[15] Synaptic transmission between neurons, commonly termed synaptic plasticity, are key for primarily learning and memory.[16] Many researches demonstrate functions of *Satb2* in neuronal projection, axonogenesis and synaptic functions in the cerebral cortex, but molecular mechanisms of *Satb2* underlining these functions are still not fully understood.

In this study, to further understand molecular mechanisms of *Satb2* in the early development of cerebral cortex, we generated *Satb2* knockout mice, and performed RNA-seq and Chromatin Immunoprecipitation sequence (ChIP-seq) and functional study to understand functions of *Satb2* in the development of the cortical cortex.

METHODS

Experimental animals

All animal research was approved by the Children's Hospital of Fudan University (2014-025). All experimental mice were fed in a specific pathogen-free room with automatically controlled temperature (16 °C), humidity (40%), ventilation and light conditions. *Satb2* knock out model was generated through CRISPR-Cas9 technology by Biocytogen Pharmaceuticals (Beijing) Co., Ltd. Genotypes were determined by polymerase chain reaction (PCR) and agarose gel electrophoresis with two pairs of primers near the deleted sequence (Table S1).

RNA-seq analyses

RNA-seq data were processed through the Nextflow nf-core/rnaseq pipeline (v3.4).[17] Differential expression analysis was performed for replicates using DESeq2 (v1.30)[18] and genes with a false discovery rate (FDR) < 0.1 were considered as differentially expressed genes, to be consistent with FDR of 0.1 for cutoff in P0 RNA-seq analysis. P0 cortex data from McKenna et al. (GSE68911)[19] and adult cortex data from Cera et al. (GSE123992)[20] were re-analyzed using the same pipeline for E17.5 cortex and the same threshold cutoffs for differential expression were applied.

ChIP-seq

ChIP reactions were used 22 µg of embryonic mouse brain tissue chromatin and 4 µg of anti-SATB2 antibody (Novus, cat# NBP176912). The library of $Satb2^{+/+}$ ChIP DNA fraction was generated according to the manufacture protocol (Active Motif). The 75nt single-end sequence reads were obtained by Illumina sequencing NextSeq500. Reads were aligned to the mouse genome (mm10) by BWA (v 0.7.17),[21] and after removal of duplicate and non-uniquely mapped reads by Picard (v2.23.1) (http://broadinstitute.github.io/picard/). A signal map capturing fragment densities along the genome was generated and visualized in the Integrated Genome Browser (IGB). MACS2 (v2.2.7)[22] was used to call narrow peaks with FDR < 0.001. Peaks were annotated using the R package ChIPseeker (1.31.3).[23]

Gene function and disease enrichment analyses

Gene ontology (GO), kyoto encyclopedia of genes and genomes (KEGG) and human phenotype ontology (HPO) enrichment analysis was performed using the R package clusterProfiler (v4.3.4).[24] Sets with less than 10 (5 for HPO) or more than 500 genes were omitted. *p*-values were corrected using the Benjamini–Hochberg (BH) method. Gene sets with FDR < 0.05 (FDR < 0.2 for KEGG) were considered to be significant and the top ten sets were plotted. Ontology diagrams were visualized using ontologyPlot (v1.6). KEGG pathways were plotted by the R package pathview (v1.31.3).[25] HPO annotations for mouse genes were acquired from Molecular Signatures Database (MSigDB) through the R package msigdbr (v7.4.1). 707 neurological disease genes were downloaded from OMIM website (https://www.omim.org/) using "Head & neck, CS, Neurologic" as the clinical synopsis search quires. Disease genes were mapped to the orthologous mouse genes through annotations from the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org/). The list of genes encoding mouse transcription factors was downloaded from the AnimalTFDB database.[26] The *p*-values for the overlapping gene sets were calculated by the Fisher exact test.

RESULTS

Generation of *Satb2* knockout mouse model

Satb2 knockout mouse model was generated through CRISPR-Cas9 technology (Fig. 1a). Real-time quantification PCR(RT-qPCR) showed significantly decreased mRNA level of *Satb2* in $Satb2^{-/-}$ ($p < 0.001$) and $Satb2^{+/-}$ ($p < 0.001$) mice when compared with $Satb2^{+/+}$ mice (Fig. 1b). Western blot analysis of cerebral cortex lysates showed a lower expression of SATB2 protein in $Satb2^{+/-}$ mice and nearly no expression in $Satb2^{-/-}$ mice (Fig. 1c). Relative quantification of bands also verified knockout effects (Fig. 1d). We performed immunofluorescence staining of frozen sections of mouse brain to further test the knockout efficiency of SATB2 in situ in the cerebral cortex. Consistent with previous reports, most SATB2-positive cells reside in layers 2/3/4 and a few numbers of SATB2-positive cells are present in layer 5 and even fewer in deep layer 6 in wild-type mice (Fig. 1e). In contrast, SATB2 was not obviously expressed in neocortex region of $Satb2^{-/-}$ mice brain. *Satb2* was reported to promote the development of callosal neurons through repressing expression of CTIP2, which is expressed in Layer 5/6 neurons and expanded into layer 2/3/4 in the absence of SATB2.[3,12] Taken together, we successfully generated a *Satb2* knockout mouse model for further study.

Identification of dysregulated *Satb2*-targeted genes in the developing cerebral cortex

To decipher mechanisms of *Satb2* in callosal projection and neuronal development, we performed RNA-seq of cerebral cortex samples from wild-type and homozygous mutant mice at E17.5. Compared with $Satb2^{+/+}$ mice, 144 genes were significantly upregulated and 168 genes were downregulated in $Satb2^{-/-}$ mice (Fig. S1A and Table S5). GO of these differentially expressed genes mainly enriched on axonogenesis, axon guidance, neuron differentiation, neuron projection extension, synapse function, dendrite development, cell adhesion and cognition (Fig. S1B). *Satb2* has been reported to promote the development of callosal and subcerebral neurons in a cell context-dependent manner. We analyzed the expression of genes important for the development of callosal projection neurons(CPN) and subcerebral projection neurons(ScPN) (Fig. S1C, D). Most CPN marker genes, such as *Cux1/2* and *Pou3f2/3*, were significantly decreased, while most ScPN marker genes, such as *Ctip2* and *Sox5*, were significantly increased, which means the transcriptome characteristics of cerebral cortex were transformed from CPN to ScPN in $Satb2^{-/-}$ mouse.

ChIP-seq of fresh cortices dissected from E17.5 embryos helped us identify 8719 genomic regions (also referred to as peaks) enriched in SATB2-precipitated DNA, which mapped to a total of 4834 gene loci in the $Satb2^{+/+}$ group (Fig. S2A). All peak regions distributed ±3 kb upstream of transcription start sites (TSS) (Fig. S2B). The detailed location of peaks relative to genomic annotations was presented in a pie chart (Fig. S2C).

To identify Satb2-targeted genes in the 4834 candidate genes, we integrated our E17.5 RNA-seq data and P0 RNA-seq data published by Mckenna et al.[19] Re-analysis of P0 RNA-seq data with identical bioinformatics procedures applied in the above E17.5 RNA-seq dataset helped us identify 4209 differentially expressed genes (DEGs) in either E17.5 or P0 *Satb2*-mutant cortices. 1363 genes overlapped between DEGs and ChIP peaks associated genes, suggesting these genes could be *Satb2*-targeted genes (Fig. 2a and Table S6). According to the GO and KEGG pathway

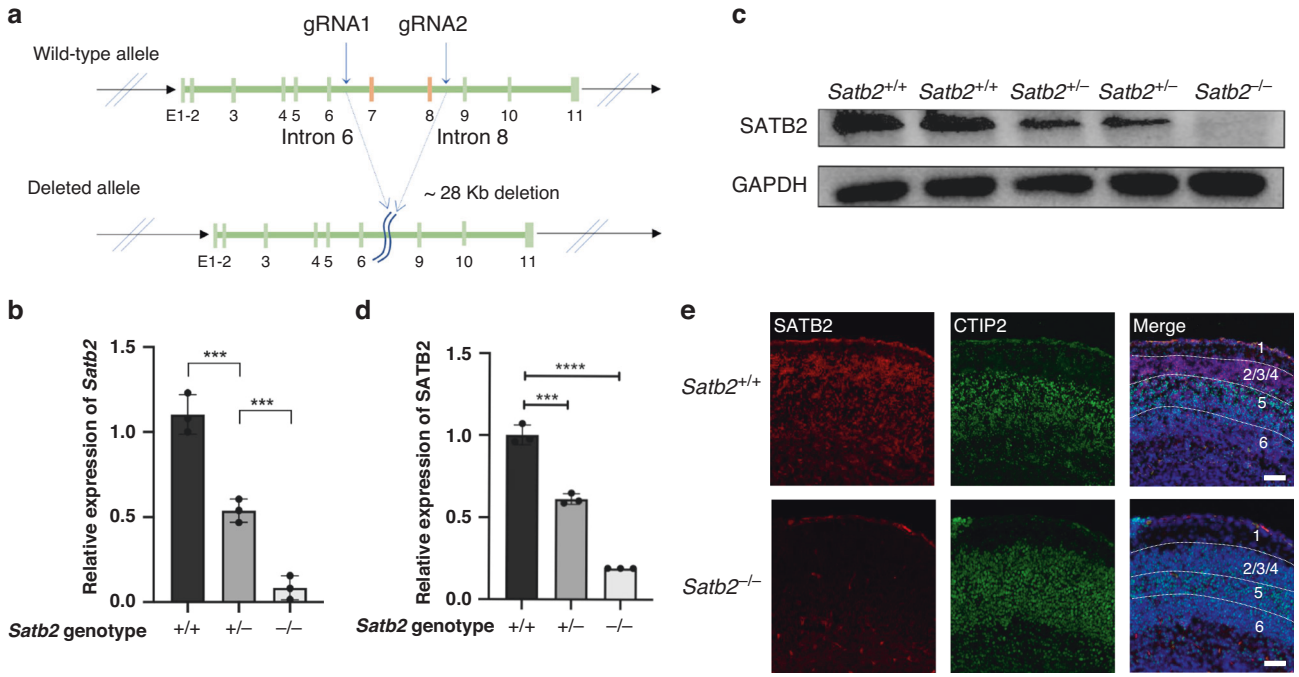

Fig. 1 Generation of *Satb2* knockout mouse model. a Knockout strategy through targeting intron 6 and intron 8 of *Satb2* gene by CRISPR-Cas9 technology. **b** qRT-PCR of *Satb2* in the cerebral cortex of E17.5 $Satb2^{+/+}$, $Satb2^{+/-}$, and $Satb2^{-/-}$ mice. Unpaired *t*-test, $***p < 0.001$, $n = 3$, error bars represent standard error of mean. **c** Representative western blot images of SATB2 protein in the cerebral cortex of E17.5 $Satb2^{+/+}$, $Satb2^{+/-}$, and $Satb2^{-/-}$ mice. **d** Relative quantitative analysis of western blot bands. Unpaired *t*-test, $***p < 0.001$, $****p < 0.0001$, $n = 3$. **e** Representative images of SATB2 and CTIP2 immunostaining in E17.5 $Satb2^{+/+}$ and $Satb2^{-/-}$ cortex. Scale bar, 100 μm.

analysis, there is a significant enrichment for genes with roles in synapse organization, axonogenesis, cell junction assembly, cognition, learning or memory and cell adhesion, indicating that *Satb2* regulates various aspects of axongenesis and synaptic function in cerebral cortex (Fig. 2b, c). Next, we want to know whether these *Satb2*-targeted genes contribute to SAS, so we downloaded neurological disease genes from OMIM website, and performed correlation analysis with *Satb2*-targeted genes. We found *Satb2* targets were enriched for 178 transcription factor ($p = 1.269e\text{-}12$) and 73 neurological disease genes ($p = 0.0007568$) (Fig. 2d).

Developmental expression pattern of *Satb2*-targeted genes

To explore the developmental functions of the Satb2-targeted genes, we investigated their developmental expression trajectories in the wild-type cerebral cortex based on previous published RNA-seq data.[27] The expression profile of the 1363 gene showd noticeable temporal changes and can be grouped into five clusters using soft-clustering method[28] (Fig. 3a, b). The five cluster genes regulated by Satb2 were further classified into three groups with different biological functions that were C1 and C2 genes that regulate neurodevelopment, C3 genes that regulate cell adhesion, C4 and C5 genes that regulate synaptic related functions. Functional differences in the three groups are consistent with the role of *Satb2* as a cell fate and neuron projection determinant at neonatal stage and regulator of synaptic plasticity/physiology at the adult stage.[20] GO analysis of each cluster is consistent with differences in biological processes of *Satb2* in different developmental stages (Fig. 3c).

Kitl encodes KITL (stem cell factor, SCF), a ligand growth factor for c-kit, and functions in neuronal migration and survival, so we choose *Kitl* as a representative of C3 genes to verify whether they are directly regulated by *Satb2*.[29,30] Both RNA-seq data and RT-qPCR results showed significantly increased expression of *Kitl* in $Satb2^{-/-}$ mouse cerebral cortex (Fig. 3d, *p*-value < 0.01). ChIP-seq analysis identified a strong and specific binding of SATB2 to intron 1 of *Kitl* gene (Fig. 3e). To test whether SATB2 can directly regulate the expression of *Kitl*, we performed luciferase reporter assay. When co-transfected with SATB2, luciferase activity significantly decreased in *Kitl*-S-pEZX group compared with the empty pEZX group (Fig. 3f). We found a de novo SATB2 c.715 C > T (p.R239X) mutation in a 3-year-old boy who was diagnosed with SAS. The mutation had been reported in literatures and was thought of as a hot spot mutation. Individuals with the R239X mutation usually exhibited craniofacial dysmorphism, generalized osteoporosis, profound mental retardation and epilepsy.[31] When co-transfected mutant SATB2 R239X with *Kitl*-S-pEZX, the activity of luciferase increased compared with SATB2 and *Kitl*-S-pEZX group, suggesting that mutant SATB2 R239X weakened the repression activity of SATB2 for *Kitl*. The results indicated that SATB2 could negatively regulate the expression of *Kitl* in the developing cerebral cortex through a conserved regulatory element in intron 1 of *Kitl*.

Satb2-targeted genes may be involved in axonogenesis in the developing cerebral cortex

We used the R package "ontologyPlot" to generate the ontology diagram of the enriched descendant GO terms to "axonogenesis". *Satb2*-targeted axonogenesis genes mainly enriched on axon extension and axon guidance processes (Fig. 4a). We detected several axon guidance molecules dysregulated in $Satb2^{-/-}$ mice compared with $Satb2^{+/+}$ group (Fig. 4b) and mRNA level changes of these axon guidance molecules were verified by RT-qPCR (Fig. 4c). Expression of *Plxnd1*, *Ntng1*, *Efnb2*, and *Ephb1* were significantly increased, while expression of *Plxna2*, *Epha3*, *Plxna4*, *Unc5c*, and *Flrt2* were significantly decreased. Proper axon guidance is essential in neuron migration and projection to their targets. Disrupted axon guidance cues might lead to attractive or repulsive environmental disorganized, which growth cone of axons can sense, thus causing misprojection of neurons, so we inferred that *Satb2*-targeted axonogenesis genes may have influences on axon growth.

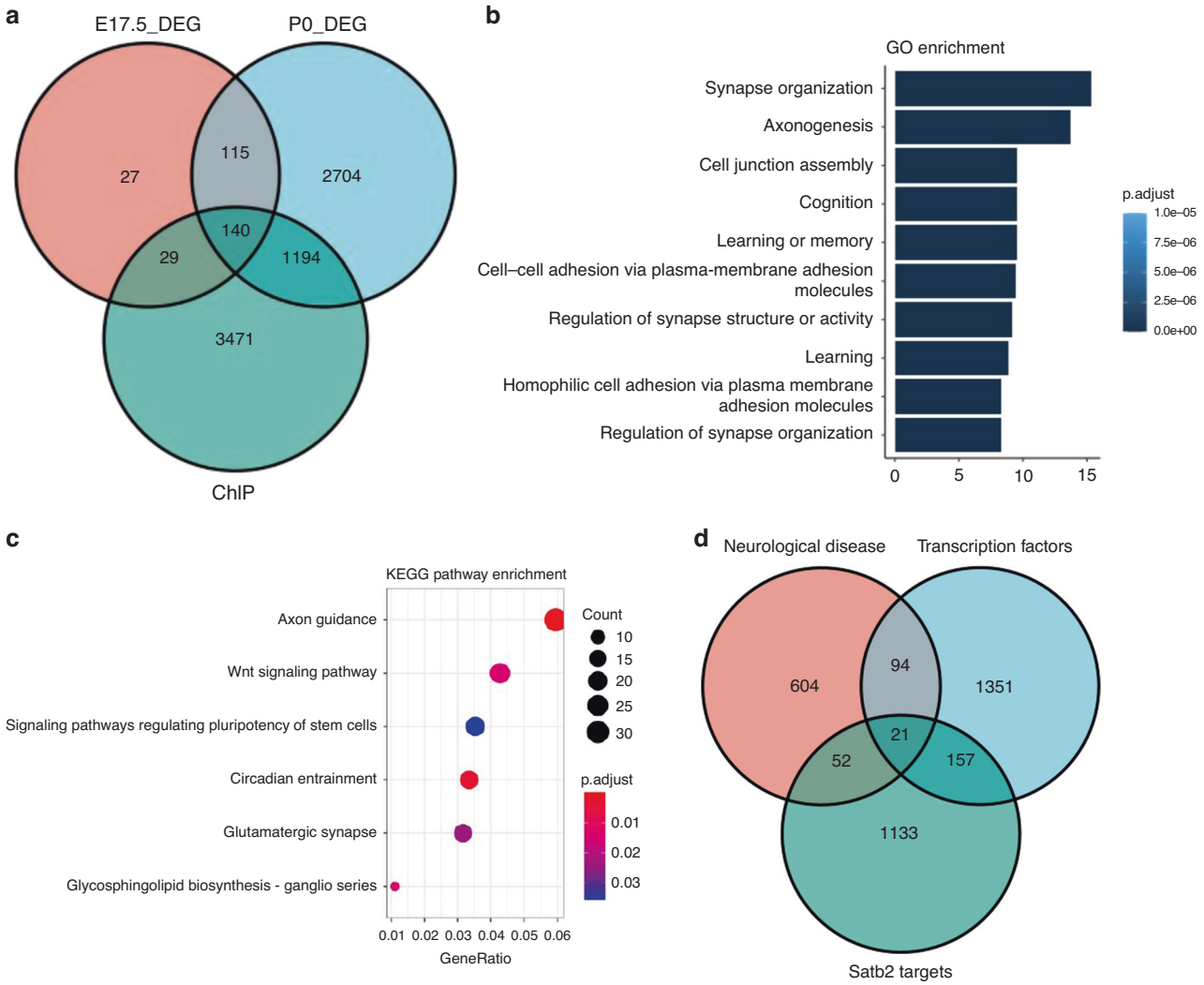

Fig. 2 Identification of *Satb2*-targeted genes in the mouse developing cerebral cortex. a Venn diagram shows the 1363 overlapping genes between SATB2-binding genes and E17.5 DEGs or P0 DEGs. **b** GO enrichment of *Satb2*-targeted genes (adjusted *p*-value < 0.05). **c** KEGG pathway enrichment of *Satb2*-targeted genes (adjusted *p*-value < 0.05). **d** Venn diagram shows relations between *Satb2*-targeted genes, transcription factors and neurological disease genes. Satb2 targets are enriched for transcription factors (178 genes, $p = 1.269e\text{-}12$) and neurological disease genes (73 genes, $p = 0.0007568$).

Among the upregulated axon guidance genes, we identified a new *Satb2* target, *Ntng1*, encoding Netrin-G1 (Fig. 4d, *p*-value < 0.01). Netrin-G1 is a vertebrate-specific guidance molecule, mediating several aspects of neural-circuit formation, such as neurite elongation and laminar organization of dendrites in mice.[32] ChIP-seq analysis identified a strong and specific binding of SATB2 to highly conserved intron 4 of the *Ntng1* gene (Fig. 4e). Luciferase activity significantly decreased when co-transfected SATB2 with *Ntng1*-S-pEZX (Fig. 4f). When co-transfected mutant SATB2 R239X with *Ntng1*-S-pEZX, the activity of luciferase increased compared with wild-type SATB2 group. The result indicates that *Satb2* can directly and negatively regulate the expression of *Ntng1* in the developing cerebral cortex through a conserved regulatory element in intron 1 of *Ntng1*.

Satb2-targeted genes may be involved in synapse formation in the developing cerebral cortex

To further understand synaptic functions of *Satb2* in cerebral development, we next focus on synaptic genes from SynaptomeDB.[33] Comparing DEGs at E17.5 and P0 stages with ChIP-targeted genes, we found 168 targeted synaptic genes of *Satb2* (Fig. 5a and Table S7). GO analysis showed the 168 synaptic genes were enriched in synapse organization, regulation of synaptic structure or activity or membrane, synaptic vesicle and synaptic transmission (Fig. 5b). Over-represented KEGG pathways included glutamatergic synapse, synaptic vesicle cycle and arginine biosynthesis.

To find out whether *Satb2* regulates different groups of synaptic genes during developmental stages, we re-analyzed RNA-seq data of *Satb2*-mutant cortices from adult mice published previously.[20] Comparing of DEGs detected from E17.5, P0 and adult samples revealed that only 72 synaptic genes overlapped between the early developmental stage and the adult stage while most differential expressed synaptic genes were stage specific (Fig. S3). Most of the synaptic genes were differentially expressed in P0 stage, and only a small number of genes were simultaneously upregulated or downregulated in *Satb2* knockout mouse at E17.5, P0 and adult stages (genes linked by pink or blue lines in different developmental stages) (Fig. 5c). The patterns were similar among presynaptic, postsynaptic, preactivezone and vesicles genes. When considering *Satb2* ChIP-targeted synaptic genes, we found 25 are consistently regulated among different stages and 143 genes are E17.5/P0 specific (Fig. 5d), including *Cdh10, Cdh13, Cacna2d1, Cntnap2, Epha4* and *Gabra1* (Labeled in Fig. 3b). E17.5/

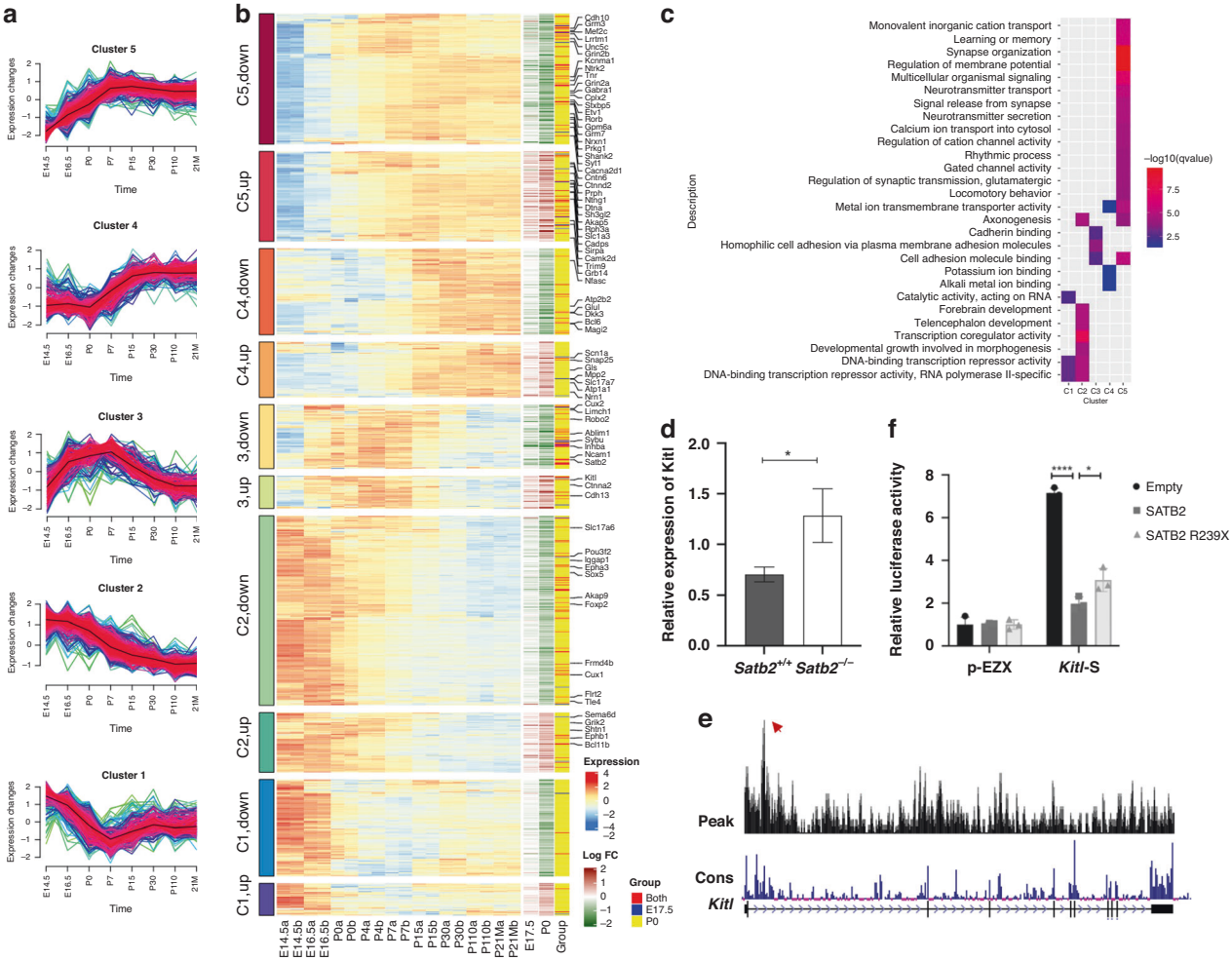

Fig. 3 Developmental expression pattern of *Satb2*-targeted gene. a Five clusters of *Satb2*-targeted genes with different temporal expression patterns. **b** Expression heatmap of *Satb2*-targeted genes. Bottom, developmental stage of RNA-seq samples; Left, gene cluster with up- or downregulated features; Right, DEG *Satb2*-targeted genes in E17.5 and P0. **c** Biological process, molecular function, and cellular component in GO analysis of each cluster. **d** Increased expression of *Kitl* in *Satb2*$^{-/-}$ mouse cortex. **e** SATB2 binds to conserved intronic sequences of *Kitl* gene. Red arrows indicate a binding peak. **f** Relative luciferase activity changes when co-transfection of SATB2 or SATB2 R239X and *Kitl*-S-pEZX. $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 3$.

P0-specific *Satb2*-targeted synaptic genes mainly enriched on calmodulin binding, cell adhesion molecule binding and actin filament binding (Fig. 5e), which were essential for initiation of synaptic formation.[34] Adult-specific synaptic genes mainly enriched on enzyme activator and postsynaptic neurotransmitter receptor activity and these processes were important for synapse specification and synaptic plasticity. These results demonstrated that *Satb2* can also regulate synaptic formation at early developmental stage by targeting different sets of genes compared to adult stage.

Cadherin-13 (CDH13) is critical in the regulation of cell migration,[35] neurite outgrowth and synapse formation in neurons with monoaminergic or GABAergic specification.[36–38] *Cdh13* was an E17.5/P0-specific *Satb2*-targeted synaptic gene and showed increased expression in *Satb2*$^{-/-}$ mouse cerebral cortex compared with *Satb2*$^{+/+}$ group from RNA-seq and RT-qPCR (Fig. 5f, *p*-value < 0.01). ChIP-seq analysis identified a strong and specific binding of SATB2 to the highly conserved promoter region of the *Cdh13* gene (Fig. 5g). Luciferase activity significantly decreased when co-transfected SATB2 with *Cdh13*-S-pEZX (Fig. 5h). However, when co-transfected mutant SATB2 R239X with *Cdh13*-S-pEZX, activity of luciferase increased compared with SATB2 and *Cdh13*-S-pEZX group. These results showed that *Satb2* can directly and negatively regulate expression of *Cdh13* in the developing cerebral cortex through the conserved element in *Cdh13* promoter region.

Satb2 positively regulates *Mef2c* expression

To further investigate mechanisms of *Satb2* in the regulation of synaptic function in the early developmental stage, we analyzed transcription factors among E17.5 DEG genes, Satb2-targeted genes and neurological disease genes. We found 4 transcription factors overlapping among the three dataset, that is *Satb2*, *Mef2c*, *Zbtb18* and *Zeb2* (labeled by red color in Fig. S1A). *Mef2c* has been demonstrated to be essential for synaptic function and regulate excitatory/inhibitory synapse density predominantly as a cell-autonomous, transcriptional repressor.[39] We identified decreased expression of *Mef2c* in *Satb2*$^{-/-}$ mouse cerebral cortex from RNA-seq data and verified by RT-qPCR (Fig. 6a, *p*-value < 0.01). Western blot and relative quantification of bands also help us verified decreased expression of MEF2C at protein level in *Satb2*$^{-/-}$ mouse cerebral cortex (Fig. 6b, c). Immunofluorescence staining of SATB2 and MEF2C showed that MEF2C had a high expression in the cerebral cortex especially in layer 2/3/4 in wild-type mouse brain and expression level of MEF2C was reduced significantly in the cerebral cortex of *Satb2*$^{-/-}$ mouse (Fig. 6d). Co-staining of SATB2

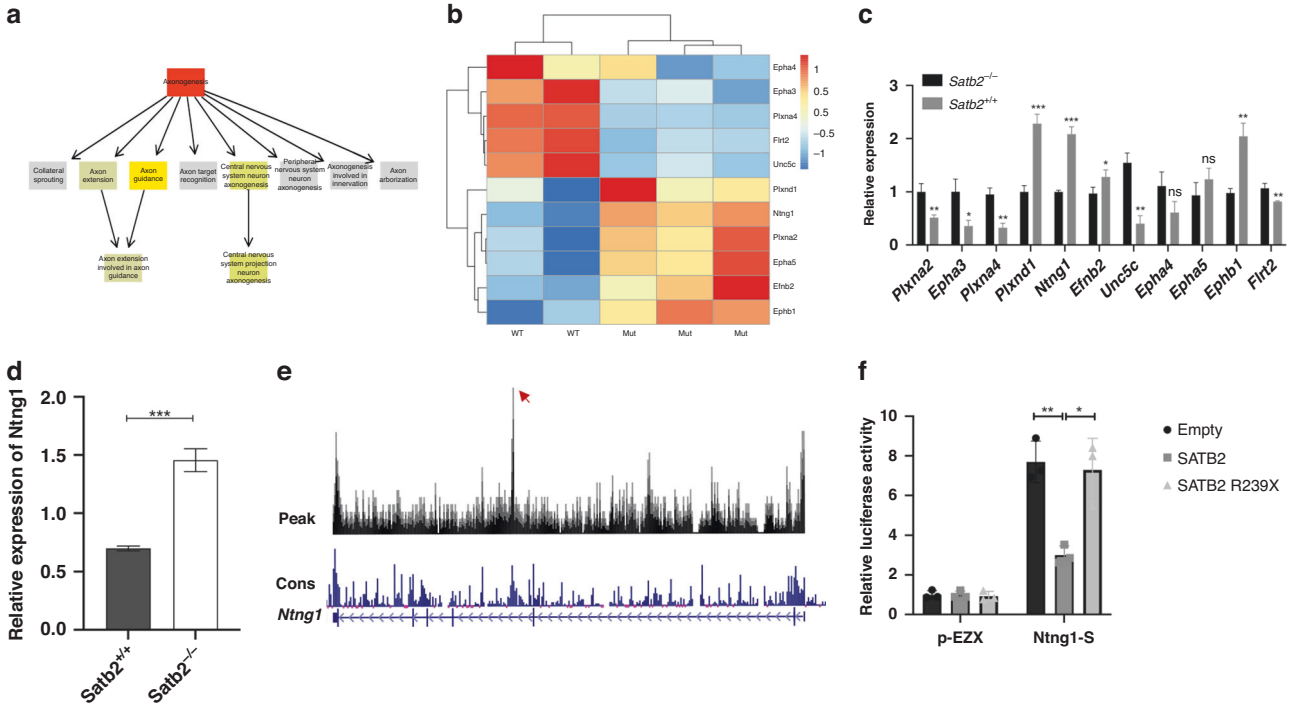

Fig. 4 ***Satb2* regulate axonogenesis in the developing cerebral cortex. a** *Satb2*-targeted axonogenesis genes mainly enriched on axon extension and axon guidance included in axonogenesis. The darker yellow color represents the lower *p*-value in the GO enrichment analysis. **b** Differentially expressed axon guidance molecules in *Satb2*$^{-/-}$ mouse cortex. Unpaired *t*-test, $**p < 0.05$, $n = 3$, error bars represent standard error of mean. **c** RT-qPCR verification of genes in **b** in the cerebral cortex of E17.5 *Satb2*$^{+/+}$ and *Satb2*$^{-/-}$ mice. **d** Increased expression of *Ntng1* in *Satb2*$^{-/-}$ mouse cortex. **e** SATB2 binds to conserved intronic sequences of *Ntng1* gene. Red arrows indicate a binding peak. **f** Luciferase reporter assay showed regulation of *Ntng1*-S-pEZX by SATB2. $**p < 0.01$, $***p < 0.001$, $n = 3$.

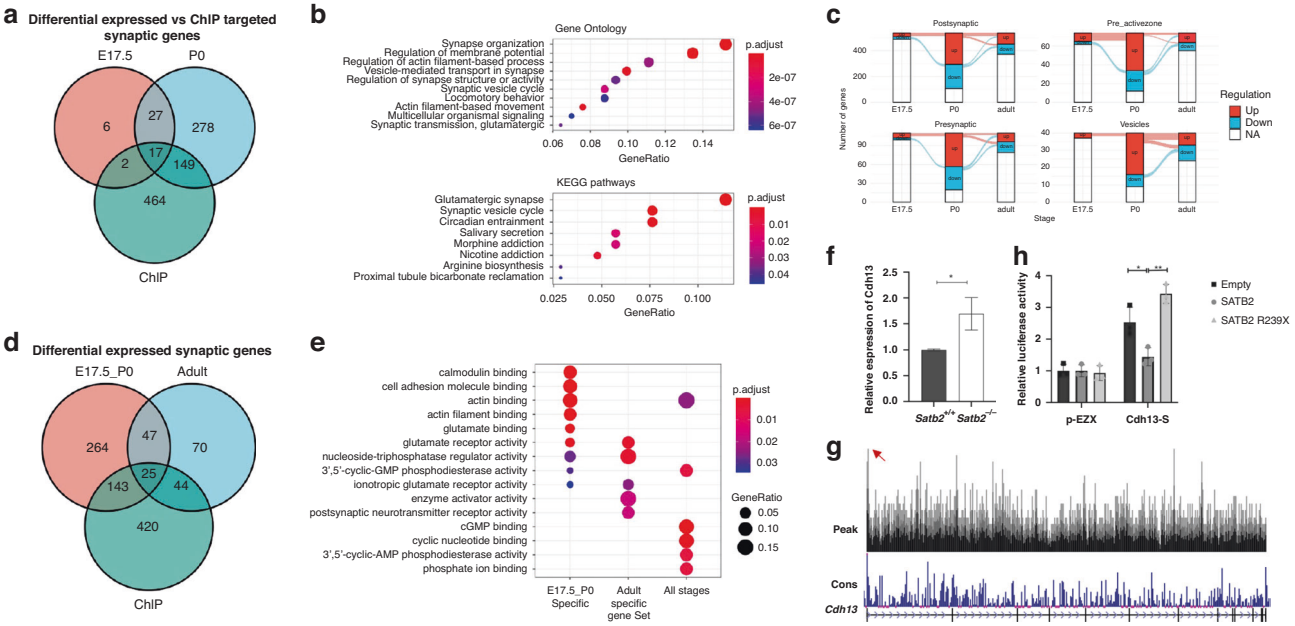

Fig. 5 ***Satb2* regulate synaptic formation at early developmental stage. a** Venn diagram shows 168 genes overlapping between *Satb2*-binding synaptic genes and E17.5_DEG synaptic genes or P0_DEG synaptic genes. **b** Gene Ontology or KEGG pathway analysis of *Satb2*-targeted synaptic genes. **c** Localization of *Satb2*-targeted synaptic genes in the synapses and expression changes in different stages. Genes linked by pink or blue lines are simultaneously upregulated or downregulated in *Satb2* knockout mice at E17.5, P0 and adult stages. **d** Venn diagram shows gene overlap between *Satb2*-binding synaptic genes and E17.5_P0 DEG synaptic genes or adult_DEG synaptic genes. **e** Gene ontology enrichment of genes in **d**. **f** RT-qPCR of *Cdh13* in the cerebral cortex of E17.5 *Satb2*$^{+/+}$ and *Satb2*$^{-/-}$ mice. Unpaired *t*-test, $**p < 0.05$, $n = 3$. **g** SATB2 binds to conserved promoter sequences of *Cdh13* gene. Red arrows indicate binding peak. **h** Luciferase reporter assay showed regulation of *Cdh13*-S-pEZX by SATB2. $**p < 0.01$, $***p < 0.001$, $n = 3$.

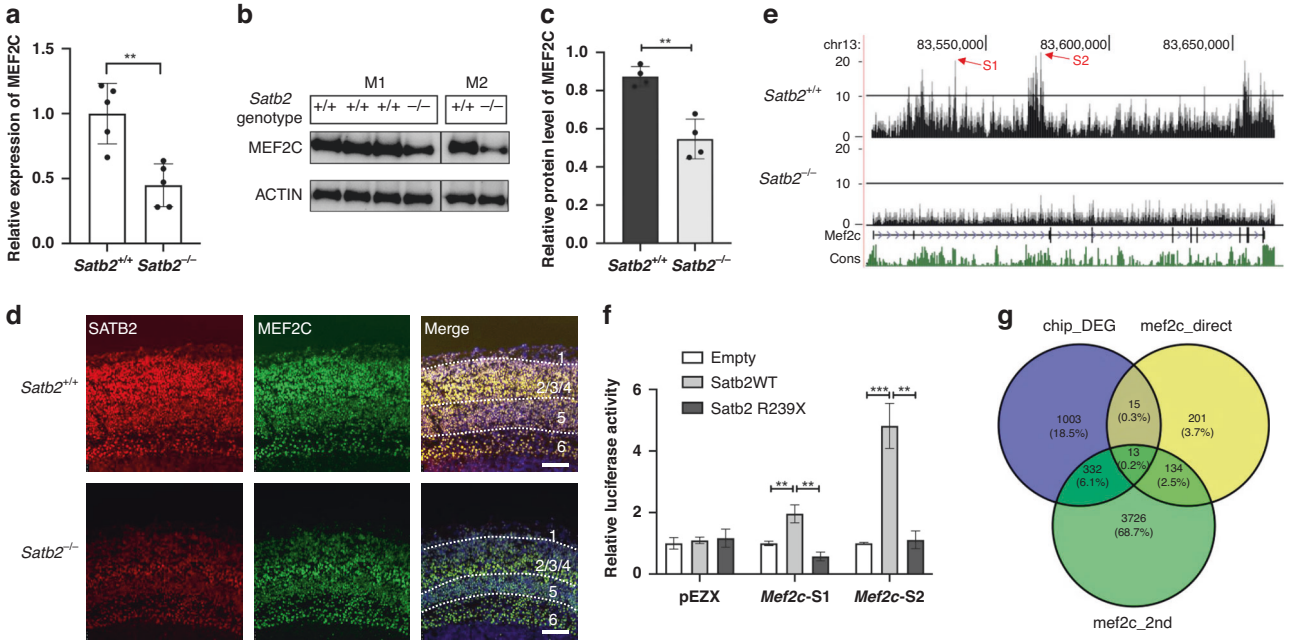

Fig. 6 *Satb2* positively regulates the expression of *Mef2c*. **a** RT-qPCR of *Mef2c* in the cerebral cortex of E17.5 $Satb2^{+/+}$ and $Satb2^{-/-}$ mice. Unpaired *t*-test, $**p < 0.05$, $n = 3$, error bars represent standard error of mean. **b** Representative western blot images of MEF2C protein in the cerebral cortex of E17.5 $Satb2^{+/+}$ and $Satb2^{-/-}$ mice. **c** Relative quantitative analysis of western blot bands. Unpaired *t*-test, $**p < 0.05$, $n = 4$. **d** Representative images of SATB2 and MEF2C immunostaining in E17.5 $Satb2^{+/+}$ and $Satb2^{-/-}$ cortex. **e** SATB2 binds to conserved intronic sequences of *Mef2C* gene. Red arrows indicate two binding peaks. **f** Luciferase reporter assay showed regulation of *Mef2c*-S1-pEZX or *Mef2c*-S2-pEZX by SATB2. $**p < 0.01$, $***p < 0.001$, $n = 3$. **g** Venn diagram shows that 28 *Satb2*-targeted genes overlap with the *Mef2c* direct targeted genes and 345 Satb2-targeted genes overlap with the *Mef2c* secondary targeted genes.

and MEF2C also showed both SATB2 and MEF2C localized in nucleus, and there were cells showed co-localization of SATB2 and MEF2C in layer 2/3 in E17.5 $Satb2^{+/+}$ cerebral cortex (Fig. S4). These results showed that knockout of *Satb2* resulted in reduced expression of MEF2C.

ChIP-seq analysis identified strong and specific bindings of SATB2 to highly conserved intron 2 regions of the *Mef2c* gene. Two sequences (*Mef2c*-S1 and *Mef2c*-S2) were inserted into the luciferase reporter plasmid to test whether *Satb2* directly regulates the detected regions of *Mef2c* (Fig. 6e). Co-transfection of SATB2 or SATB2 R239X with empty pEZX-FR01 plasmids slightly increased luciferase activity. However, luciferase activity significantly increased when co-transfection SATB2 with *Mef2c*-S1-pEZX or *Mef2c*-S2-pEZX (Fig. 6f). Moreover, the activity of luciferase didn't increase when co-transfection mutant SATB2 R239X with *Mef2c*-S1-pEZX or *Mef2c*-S2-pEZX. The result indicated that mutant SATB2 R239X lead to a weakened regulatory activity. We also observed similar pattern of luciferase activity in *Mef2c*-S2- pEZX group (Fig. 6f). These results suggested that *Satb2* can regulate the conserved intron regions of *Mef2c* and *Satb2* likely directly regulate the expression of *Mef2c* in the developing cerebral cortex.

To find out whether *Satb2* can regulate the expression of synaptic function-related genes through *Mef2c*, we compared *Satb2*-targeted genes and *Mef2c*-targeted genes from the study of Allaway et al.[40] We found 28 genes overlap between *Satb2*-targeted genes and *Mef2c*_direct genes in the early developmental stage, indicating that expression of the 28 genes can be regulated by *Satb2* directly or by *Mef2c* which is regulated by *Satb2* (Fig. 6g). Among the 28 genes, *Arhgef28*, *Dtna*, *Cdh7*, *Cacna2d1* and *Caln1* have been reported to be associated with synaptic functions.[41–45] Additionally, we found 332 genes overlapping between *Satb2*-targeted genes and *Mef2c*_2nd genes. We inferred that these genes were regulated by *Satb2* directly or by other transcription factors that *Mef2c* regulates. By integrating of *Satb2* ChIP-seq data and RNA-seq data of adult *Satb2* and *Mef2c* knockout cortical cortex,[20,39] we identified 206 genes were directly regulated by *Satb2* or *Mef2c* in the adult stage (Fig. S5A). The 206 genes are enriched mainly on synapse organization, synapse assembly, forebrain development and cell junction assembly (Fig. S5B). These results suggested that the numbers of synaptic genes regulated by *Satb2* or *Mef2c* increased when synapse and cognitive function getting more important and complex from early developmental stage to adult stage.

Impaired spatial learning and memory in $Satb2^{+/-}$ mice

Since $Satb2^{-/-}$ mice die immediately after birth,[2] we performed behavioral tests with $Satb2^{+/+}$ and $Satb2^{+/-}$ mice. We recorded the weights of $Satb2^{+/+}$ and $Satb2^{+/-}$ mice after birth and found $Satb2^{+/-}$ mice were significantly lighter than $Satb2^{+/+}$ mice since D12 (Fig. S6A). Open field test, passive avoidance test, elevated plus-maze test and novel object recognition test showed no differences between $Satb2^{+/+}$ and $Satb2^{+/-}$ mice (data not shown). In the Morris water maze test (MWM), $Satb2^{+/-}$ mice exhibited a longer latency in finding the platform than $Satb2^{+/+}$ mice during the learning phase (Fig. S6B). The result indicated that the $Satb2^{+/-}$ mice may have impaired learning capacity during the consecutive 4-days training phase of the MWM task than $Satb2^{+/+}$ mice. During the memory test, the number of platform crossings was lower in the $Satb2^{+/-}$ mice compared to $Satb2^{+/+}$ mice (Fig. S6C). The distance or time to the platform showed no obvious difference between $Satb2^{+/+}$ and $Satb2^{+/-}$ mice (Fig. S6D, E). Thus, we thought $Satb2^{+/-}$ mice showed impaired spatial learning and memory, further strengthening that *Satb2* regulates functions of synapse.

DISCUSSION

Here, we identified downstream targeted genes of *Satb2* and showed that *Satb2* directly regulated genes important in axonogenesis, synaptic organization and cell adhesion during the early development of the cerebral cortex.

Satb2 has been demonstrated to regulate many biological processes as a transcription factor. Integration of RNA-seq and ChIP-seq data helped us identified 1363 targeted genes of *Satb2* with different temporal expression patterns (Fig. 2). Interestingly, 73 *Satb2*-targeted genes are associated with neurological disease, which could explain the mental abnormalities in SAS patients.[6] The growth of axons towards their targets is critical during the establishment of neuronal connections under the regulation of axon guidance ligands and receptors.[46] *Satb2* and *Ctip2* directly repress the expression of two Netrin1 receptors- DCC and Unc5C to control the interhemispherical projection in a subset of early born, deep layer callosal neurons.[13] *Satb2* also has been reported to regulate EphA7 to control soma spacing and self-avoidance of cortical pyramidal neurons.[14] In this study, we found eight additional downstream axon guidance genes regulated by *Satb2*, that is *Plxna2*, *Plxna4*, *Plxnd1*, *Ntng1*, *Epha3, Ephb1, Efnb2*, and *Flrt2* (Fig. 4c). *Plxna2*, *Plxna4* and *Plxnd1* code cell surface receptors for class 3 semaphorins (SEMA3A/3C/3E), SEMA4A and SEMA6A, and have been demonstrated to regulate neuronal migration or ensure specificity of synapse formation.[47–49] *PLXNA2* has recently been shown as a candidate gene in autism spectrum disorder and intellectual disability.[50,51] *Epha3, Ephb1, Efnb2* code ligand or receptors of the ephrins family, which inhibit axon growth by mediating growth cone collapse through regulation of Ras and Rho GTPases.[52] *Epha3* also promotes axon growth through RhoA signaling and NMIIA in developing neurons.[53] We provided experimental evidence of *Ntng1* to be a new axon guidance molecule regulated by *Satb2* (Fig. 4d–f). Therefore, these evidences provided new insights into the complexity of *Satb2* in regulating axonogenesis.

During neuronal development, axons and dendrites establish initial synaptic contacts mostly during development and the early postnatal period, although synapse formation continues throughout life at a lower rate.[34] It has been revealed that *Satb2* has different roles as a cell fate and neuron projection determinant at the neonatal stage while a regulator of synaptic plasticity/physiology at the adult stage.[20] Our study identified 168 *Satb2*-targeted synaptic genes and 143 early-stage-specific *Satb2*-targeted synaptic genes, suggesting that *Satb2* can also regulate synaptic formation at the early developmental stage (Fig. 5). 168 synaptic genes enriched on synapse organization, regulation of synaptic structure or activity or membrane, synaptic vesicle and synaptic transmission. *Cdh13*, one of the 143 early-stage-specific *Satb2*-targeted synaptic genes, had an increased expression in the absence of *Satb2*. Several studies revealed *Cdh13* variants associated with memory impairment[54] and hyperactivity/impulsivity, specifically during childhood and adolescents.[55] Luciferase reporter assay provides evidence that *Satb2* can directly regulate the expression of *Cdh13* (Fig. 5h). *Satb2* was thought as a cell fate and neuron projection determinant at neonatal stage and regulator of synaptic plasticity/physiology at the adult stage. Our study firstly demonstrated the function of *Satb2* in synaptic formation at the embryonic stage; our findings enriched the functions of *Satb2* in the early development stage.

Myocyte enhancer factor 2 C (MEF2C) is crucial for programming early neuronal differentiation and regulation of dendritic spine density, synapse development, synapse number, and AMPA-mediated postsynaptic strength.[39,56,57] We provided experimental evidence that *Mef2c* was downregulated in *Satb2*$^{-/-}$ mice (Fig. 6a–d). Our study firstly suggests *Mef2c* as a downstream effector of *Satb2* to regulate synaptic transmission in the developing cerebral cortex (Fig. 6e, f). Compared analysis of targeted genes of *Satb2* and *Mef2c* showed that several targets of *Mef2c* were regulated directly or indirectly by *Satb2* (Fig. 6f), indicating a complex regulatory network of *Satb2*.

Satb2 cKO mice showed hyperactivity, increased impulsivity, abnormal social novelty, and impaired spatial learning and memory.[58] MWM test of global *Satb2*$^{+/-}$ mice showed impaired spatial learning and memory (Fig. S6), further strengthening that *Satb2* regulates synapse functions. Considering *SATB2* heterozygous variations in humans cause disease and SATB2 expression in mice and human has similar patterns during prenatal cortex development, our results showed that *Satb2* heterozygous knockout mice had similar phenotypes and were more consistent with human disease model.

CONCLUSION

Our study has increased the knowledge of the potential target genes of *Satb2* in the developing cerebral cortex and these target genes may be involved in axonogenesis, synapse formation and synaptic plasticity. We also explored the direct regulation of *Satb2* in 4 downstream effectors (*Kitl*, *Ntng1*, *Cdh13*, and *Mef2c*) although these still need further investigation. Our study demonstrates complicated molecular regulatory mechanism of *Satb2* in axonogenesis and synaptic function in the early developmental stage and provides new insights to elucidate the pathogen of SAS.

DATA AVAILABILITY

RNAseq and ChIP-seq data that support the findings of this study have been deposited to GEO database with the accession number GSE201562.

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AUTHOR CONTRIBUTIONS

H.W. designed the study and revised the manuscript; Q.G. performed experiment, collected and interpreted the data, drafted the initial manuscript; Y.W. analyzed the RNA-seq and ChIP-seq data; Y.Q., X.C. and X.L. involved experiment, X.D. and H.C. involved data analysis, Q.W., Y.J., S.Y., J.Z., and S.S. involved animal experiment; B.W. and W.Z. revised the manuscript; and all authors read and approved the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

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