

## ORIGINAL ARTICLE

Autism-associated *Dyrk1a* truncation mutants impair neuronal dendritic and spine growth and interfere with postnatal cortical developmentT Dang<sup>1</sup>, WY Duan<sup>2,5</sup>, B Yu<sup>3</sup>, DL Tong<sup>3</sup>, C Cheng<sup>3</sup>, YF Zhang<sup>3</sup>, W Wu<sup>2,6</sup>, K Ye<sup>2,7</sup>, WX Zhang<sup>2,8</sup>, M Wu<sup>1,2</sup>, BB Wu<sup>1,2</sup>, Y An<sup>1,2</sup>, ZL Qiu<sup>3,9</sup> and BL Wu<sup>1,4,9</sup>

Autism is a prevailing neurodevelopmental disorder with a large genetic/genomic component. Recently, the dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1 A (*DYRK1A*) gene was implicated as a risk factor for autism spectrum disorder (ASD). We identified five *DYRK1A* variants in ASD patients and found that the dose of *DYRK1A* protein has a crucial role in various aspects of postnatal neural development. *Dyrk1a* loss of function and gain of function led to defects in dendritic growth, dendritic spine development and radial migration during cortical development. Importantly, two autism-associated truncations, R205X and E239X, were shown to be *Dyrk1a* loss-of-function mutants. Studies of the truncated *Dyrk1a* mutants may provide new insights into the role of *Dyrk1a* in brain development, as well as the role of *Dyrk1a* loss of function in the pathophysiology of autism.

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## INTRODUCTION

Autism spectrum disorder (ASD) is a complex, heterogeneous neurodevelopmental disorder<sup>1</sup> with multiple different etiologies. The heritability of ASD has been estimated to be as high as 90%.<sup>2</sup> ASD is usually diagnosed by clinical assessment and the onset of three core disturbances before 3 years of age: impaired social interaction, unusual patterns of highly restricted interests and repetitive behaviors, and language abnormalities.<sup>2</sup> The risk of recurrence for ASD is approximately 2–3%.<sup>3</sup>

Autism is thought to occur when an array of potential genetic vulnerabilities, possibly in concert with epigenetic factors and/or gene–environment interactions, affect neural connectivity and neurodevelopment.<sup>4</sup> To date, we have learned that the symptoms of autism may arise when some specific region of the genome appears to be abnormal; for instance, multiple genetic/genomic factors have been reported to be associated with autism, including chromosomal abnormalities, genomic copy number variants and genetic variants of specific genes.<sup>5–8</sup>

Dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1A (*DYRK1A*), which is encoded by a gene on human chromosome 21, is involved in many of the biological processes of neural development and performs critical functions by phosphorylating various downstream substrates, including transcription factors, splice factors and synaptic proteins.<sup>9</sup> For example, *DYRK1A*-mediated phosphorylation of the P53 protein at Ser15 inhibits cell

proliferation and is therefore associated with cell growth and differentiation.<sup>10</sup> The tau protein and the N-WASP protein affect the outgrowth of microscopic fibers and actin assembly when they are phosphorylated by *DYRK1A* and thereby contribute to the development of neuronal dendrites and dendritic spines.<sup>11–13</sup> Considering the critical contribution of *DYRK1A* to Down syndrome (DS),<sup>14</sup> numerous studies have focused on the *Dyrk1a* gain-of-function model, in which transgenic mice that overexpress *Dyrk1a* by 1.5-fold show severe learning and memory defects.<sup>15–17</sup> The dosage imbalance of *Dyrk1a* led to delayed neural development.<sup>18–20</sup> Previous studies have identified the molecular mechanisms by which *DYRK1A* affects the occurrence of DS and mental retardation.<sup>9,15,21–23</sup> In several recent exome sequencing studies of ASD patients, some *DYRK1A* variants were found to be associated with autism, suggesting that *DYRK1A* is another risk factor for ASD.<sup>6,24–26</sup>

In an exome sequencing pilot study, we identified several *DYRK1A* mutations in ASD patients with intellectual disability (ID) and developmental delay (DD) (short name ASD/ID/DD or ASD), consistent with the findings of other independent studies.<sup>27</sup> Furthermore, we screened 892 patients with ASD/ID/DD and/or epilepsy/seizure for the *DYRK1A* gene and found additional variants of *DYRK1A* (Supplementary Table S1). Using this sequencing approach, a total of 11 novel variants and 1 known mutation in the coding region were detected, of which 9 variants were

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predicted to alter protein function according to bioinformatics prediction (Table 1), whereas the other 3 variants were benign. Given the locations of the *DYRK1A* variants in the specific protein domains, we postulate that those variants may affect *DYRK1A* function in three ways: nuclear localization, kinase activity, and abnormal translation termination that results in truncated *DYRK1A* proteins (Table 1 and Figure 1c). Previous reports have suggested that the R205X mutant acts in the third way.<sup>28,29</sup> Owing to the critical role of *Dyrk1a* in neural development, we decided to

investigate the role of *Dyrk1a* in neural development, as well as whether these *Dyrk1a* variants have *loss-* or *gain-of-function* effects.

## MATERIALS AND METHODS

Selected *DYRK1A* variants for the neuronal function study  
Previously, we collaboratively screened 892 patients at Boston Children's Hospital (437 samples of the western population) and the Children's

**Table 1.** The genomic and bioinformatics analysis of *DYRK1A* variants that may be associated with ASD/ID/DD

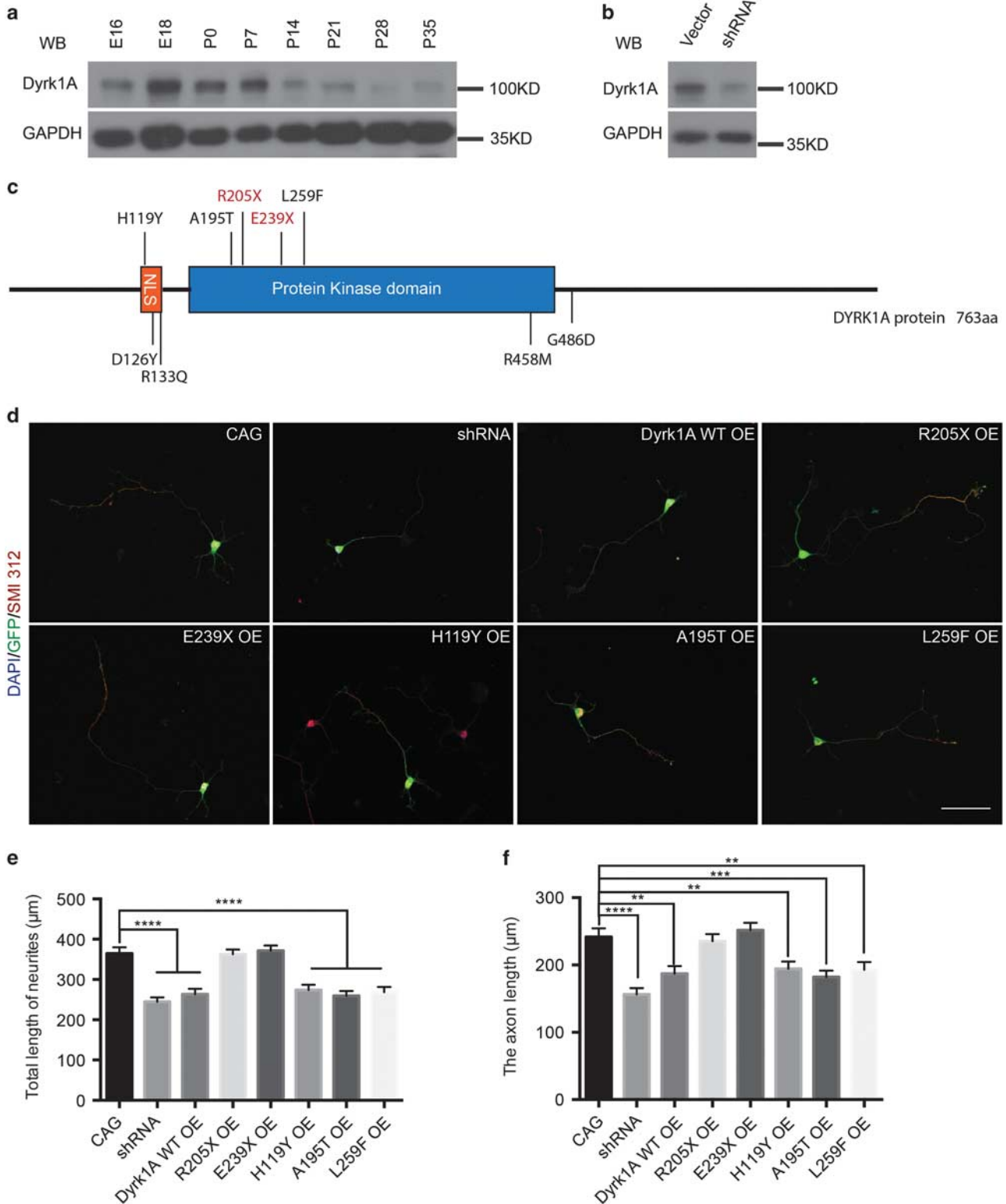
Exon	Nucleotide change	Genome position	Protein change	Type	Function prediction by bioinformatics tools	MAF in ASD patients	MAF in controls
<b>6</b>	<b>c.355C&gt;T</b>	<b>Chr21:38852967</b>	<b>p.H119Y</b>	<b>Missense</b>	<b>SIFT: possibly damaging</b> <b>Polyphen: tolerated</b> <b>MutationTaster: disease causing</b> <b>PhyloP: highly conserved</b>	<b>0.00151</b>	<b>0</b>
6	c.376G>T	Chr21:38852988	p.D126Y	Missense	SIFT: possibly damaging Polyphen: deleterious MutationTaster: disease causing PhyloP: highly conserved	0.00151	0
6	c.398G>A	Chr21:38853010	p.R133Q	Missense	SIFT: possibly damaging Polyphen: deleterious MutationTaster: disease causing PhyloP: moderately conserved	0.00151	0
<b>7</b>	<b>c.583G&gt;A</b>	<b>Chr21:38858835</b>	<b>p.A195T</b>	<b>Missense</b>	<b>SIFT: possibly damaging</b> <b>Polyphen: tolerated</b> <b>MutationTaster: disease causing</b> <b>PhyloP: weekly conserved</b>	<b>0.00146</b>	<b>0</b>
<b>7</b>	<b>c.613C&gt;T</b>	<b>Chr21:38858865</b>	<b>p.R205X</b>	<b>Nonsense</b>	<b>SIFT: possibly damaging</b> <b>Polyphen: deleterious</b> <b>MutationTaster: disease causing</b> <b>PhyloP: NA</b>	<b>0.00158</b>	<b>0</b>
<b>8</b>	<b>c.715G&gt;T</b>	<b>Chr21:38862527</b>	<b>p.E239X</b>	<b>Nonsense</b>	<b>SIFT: possibly damaging</b> <b>Polyphen: deleterious</b> <b>MutationTaster: disease causing</b> <b>PhyloP: NA</b>	<b>0.00158</b>	<b>0</b>
<b>8</b>	<b>c.777G&gt;T</b>	<b>Chr21:38862589</b>	<b>p.L259F</b>	<b>Missense</b>	<b>SIFT: possibly damaging</b> <b>Polyphen: deleterious</b> <b>MutationTaster: disease causing</b> <b>PhyloP: highly conserved</b>	<b>0.00158</b>	<b>0</b>
11	c.1373G>T	Chr21:38877719	p.R458M	Missense	SIFT: possibly damaging Polyphen: deleterious MutationTaster: disease causing PhyloP: highly conserved	0.00138	0
11	c.1457G>GA	Chr21:38877803	p.G486D	Missense	SIFT: possibly damaging Polyphen: deleterious MutationTaster: disease causing PhyloP: highly conserved	0.00138	0

Abbreviations: ASD, autism spectrum disorder; DD, developmental delay; ID, intellectual disability; MAF, minor allele frequency; NA, not available. Note: (1) The inheritance pattern of the variants in this table could not be determined because they were de-identified proband samples and no parental samples were available for this study, which were indicated in the section of Materials and Methods. (2) The 5 *DYRK1A* variants in bold in this table (p.R205X, p.E239X, p.H119Y, p.A195T and p.L259F) were selected to investigate whether these variants may exhibit *loss-* or *gain-of-function* effects. (3) The prediction value by Bioinformatics tools are listed in Supplementary Table S1: *DYRK1A* variants detected in 892 ASD/ID/DD samples.

**Figure 1.** Wild-type (WT) *Dyrk1a* (dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1 A) overexpression reduced total neurite and axon length of neurons in primary cultured mouse cortical neurons. **(a)** The *Dyrk1a* expression pattern in the cerebral cortex during the embryonic and postnatal period. **(b)** Specific knockdown of endogenous *Dyrk1a* in the mouse cortical neurons. Immunoblotting of the knock-down using specific short hairpin RNA (shRNA) against endogenous *DYRK1A* protein. The protein marker ladders are shown on the right. **(c)** Representation of the specific locations of missense variants related to autism in the *DYRK1A* protein structure, with the two truncations highlighted in red. The five variants located above the protein structure were included in the additional functional study. The smaller orange bar shows the NLS (bipartite nuclear localization signal) motif, and the larger bar (blue) represents the protein kinase domain. The black thread shows the other amino acids making up *DYRK1A*. **(d)** The specific cellular morphology of neurons expressing the CAG vector (as a control), *Dyrk1a* RNAi, *Dyrk1a* WT overexpression, the two truncations (R205X and E239X) and the three missense mutations (H119Y, A195T and L259F). All neurons were co-labeled with 4,6-diamidino-2-phenylindole (DAPI; to identify nuclei), green fluorescent protein (GFP; to identify overall neuronal morphology) and SMI 312 (an axonal marker). Scale bar 50  $\mu$ m. **(e, f)** The statistical results for the total neurites **(e)** and axon length **(f)** between specific cell types. The bar graph shows the mean value  $\pm$  s.e.m.; approximately 70 cells from three independent experiments were counted during the statistical analysis. Unpaired *t*-test statistical method was used. \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001.

Hospital of Fudan University (455 samples of Han Chinese) with ASD/ID/DD for *DYRK1A*. All samples were de-identified prior to collection under Institutional Review Board protocols for non-identifiable biological specimens, which were deemed 'leftover' from patient samples after clinical tests. Of these, we selected samples with an ASD/ID/DD and/or epilepsy/seizure diagnosis according to Diagnostic and Statistical Manual of Mental

Disorders, Fourth Edition. This study was approved by each institute. The sequencing experiments were also performed at each institute. For the bioinformatics analysis of the *DYRK1A* variants identified in the screen, the longest of *DYRK1A* isoform was used as a reference sequence (NM\_001396.3, NP\_001387.2). Considering the high homology between human and mouse *Dyrk1a*, we introduced the mouse *Dyrk1a* cDNA



(NM\_007890.2, NP\_031916.1) into our study, and the *DYRK1A* variants in the coding region that were identified in individuals with ASD corresponded to *Dyrk1a* (Supplementary Table S2). Variants were classified according to *DYRK1A* protein domains listed in UniProt (<http://www.uniprot.org/>).

Supplementary Table S1 lists the variants detected in these patients. Based on the classification of the 11 variants, we selected 5 representative variants (three missense: H119Y, A195T and L259F; and two nonsense: R205X and E239X), which covered all three kinds of variants described previously, to investigate the role of *DYRK1A* in neurodevelopment, as well as the effects of these variants on the *DYRK1A* protein (Figure 1c). Considering that *DYRK1A* is highly conserved between human and mouse (approximately 99% according to protein sequence alignment using the MegAlign software; Supplementary Figure S1), we used the cDNA of mouse *Dyrk1a* rather than of human *DYRK1A*. The five variants were located in the mouse *Dyrk1a* cDNA, which corresponded to *DYRK1A* (Supplementary Table S2).

#### Clinical information on patients harboring *DYRK1A* mutations

The patient with R205X was diagnosed with ID and global DD. She exhibited microcephaly, chronic static encephalopathy and aphasia, oral motor apraxia, seizure dysmorphism and anxiety. All test results for karyotyping, fragile X and chromosomal microarray analysis were negative. The patient with E239X was diagnosed with ASD and global DD. He had a history of autism, anxiety and developmental coordination disorder, as well as abnormal electroencephalography but no seizures. Tests results for karyotyping, fragile X and chromosomal microarray analysis were negative. The patient with H119Y was diagnosed with PDD-NOS (pervasive developmental disorder, not otherwise specified). She had a history of global DD that was most marked for speech delay, motor delay and motor coordination disorder, seizure and mild physical dysmorphism. Tests results for karyotyping, fragile X and chromosomal microarray analysis were negative. Magnetic resonance imaging and magnetic resonance spectroscopy were normal. The patient with A195T was diagnosed with ASD/ID. He had a history of infantile spasms and global DD, with particular impairments in language, social interaction and repetitive behaviors, mild ID, complex partial epilepsy with epileptic encephalopathy and anxiety. Tests results for karyotyping, fragile X and chromosomal microarray analysis were negative. The patient with L259F was diagnosed with PDD-NOS and then ASD/ID. He demonstrated extremely low cognitive abilities and adaptive functioning, consistent with the mild range of ID. He had anxiety, perseveration and additional upsets and aggressive behavior. Because the parents of the patients were not available for testing, no other genetic information was available.

#### Plasmids

The full-length *Dyrk1a* ORF was amplified from the total cDNA of mouse cortical neurons. *Dyrk1a* was then inserted into pRK5-Myc, which already contained a 1xMyc epitope tag (5'-EQKLISEEDL-3') at the 5' end. For immunofluorescence experiments, we cloned *Myc-Dyrk1a* into pCAGGS-IRES-green fluorescent protein (GFP). The mutants, including both missense mutations and the two truncations, were generated using KOD-Plus, and specific primers were designed (Supplementary Table S3) according to the manufacturer's instructions (#132000; Toyobo, Osaka, Japan). The mouse *Dyrk1a* shRNA was directed against the following sequence, 5'-AGAAGACGATGCATACAGG-3', which was introduced into the pSUPER vector.

Lentivirus was provided by BrainVTA (Wuhan, China).

#### Antibodies

The antibodies used in this study included the following: *DYRK1A* (no. 2771; Cell Signaling Technology, Danvers, MA, USA); GAPDH (no. ab8245; Abcam, Cambridge, UK); Myc epitope tag (no. M20002; Abmart, Berkeley Heights, NJ, USA); GFP (no. G10362; Invitrogen, Carlsbad, CA, USA), and SMI 312 (no. SMI-312R; COVANCE, Princeton, NJ, USA).

#### Cell culture

Embryonic day 15 (E15)–E16 mouse cortical neurons were cultured and transfected via electroporation with an Amaxa Nucleofector (Amaxa, Cologne, Germany) at 0 days *in vitro* (DIV) on cover glasses that had been coated with a poly-D-lysine solution (1 mg ml<sup>-1</sup> in dd water) according to

the manufacturer's instructions. After DIV 3, the cells were fixed for further analysis.

For neurite and axon outgrowth rescue experiments, we co-transfected each group with two plasmids, using a 1:1 ratio of wild-type (WT) or mutant forms of *Dyrk1a* and short hairpin RNAs (shRNAs), via electroporation as described above. Cells were collected for immunofluorescence analysis after 72 h of *in vitro* culturing.

#### Immunofluorescence

Cells were washed with 1× phosphate-buffered saline (PBS) for 5 min, fixed in 4% paraformaldehyde (PFA) for 20 min and blocked in 1× PBS buffer with 3% bovine serum albumin and 0.1% Triton-X-100 for 60 min at room temperature (RT). The cells were incubated in primary antibodies overnight at 4 °C, washed three times in 1× PBS and then incubated in secondary antibodies at RT for 90–120 min. The signals were observed via fluorescence microscopy.

#### Immunoblotting

For *Dyrk1a*-specific expression in the mouse cortex, cortical tissue homogenates were prepared from the mouse brains at different developmental stages, immersed in RIPA buffer, boiled in 1× sodium dodecyl sulfate loading buffer and then resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All of the protein on the gel was transferred to polyvinylidene fluoride membranes (Amersham Biosciences, Uppsala, Sweden), and the membranes were blocked with 5% non-fat milk in Tris-buffered saline and Tween 20 (TBST) buffer for 1 h. The membranes were then incubated in primary antibodies overnight at 4 °C, washed three times in TBST and incubated in secondary antibodies for 1 h at RT. The signals were revealed by horseradish peroxidase reaction using SuperSignal Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

#### Hippocampal slice culture

Postnatal day 5 (P5) Sprague Dawley rats were included in the spine density study. Hippocampal tissue slices (400 μm) were cultured on Millicell (0.4 μm; Millipore, Boston, MA, USA) in six-well plates with 1 ml of slice culture medium as described previously.<sup>30,31</sup> The cultured slices were incubated in 5% CO<sub>2</sub> at 37 °C and transfected using a Helios Gene Gun (Bio-Rad, Hercules, CA, USA) at DIV 2. Bullets were prepared containing 50 μg of plasmid DNA coated on 25 mg of 1.0-μm gold particles (Bio-Rad). The medium was changed every 2 days. The slices were fixed at DIV 7 in ice-cold 4% PFA. GFP immunostaining was then conducted using a GFP antibody and an Alexa 488-conjugated secondary antibody (1:1000). DAPI (4,6-diamidino-2-phenylindole; 1:500) was also used to visualize cell nuclei.

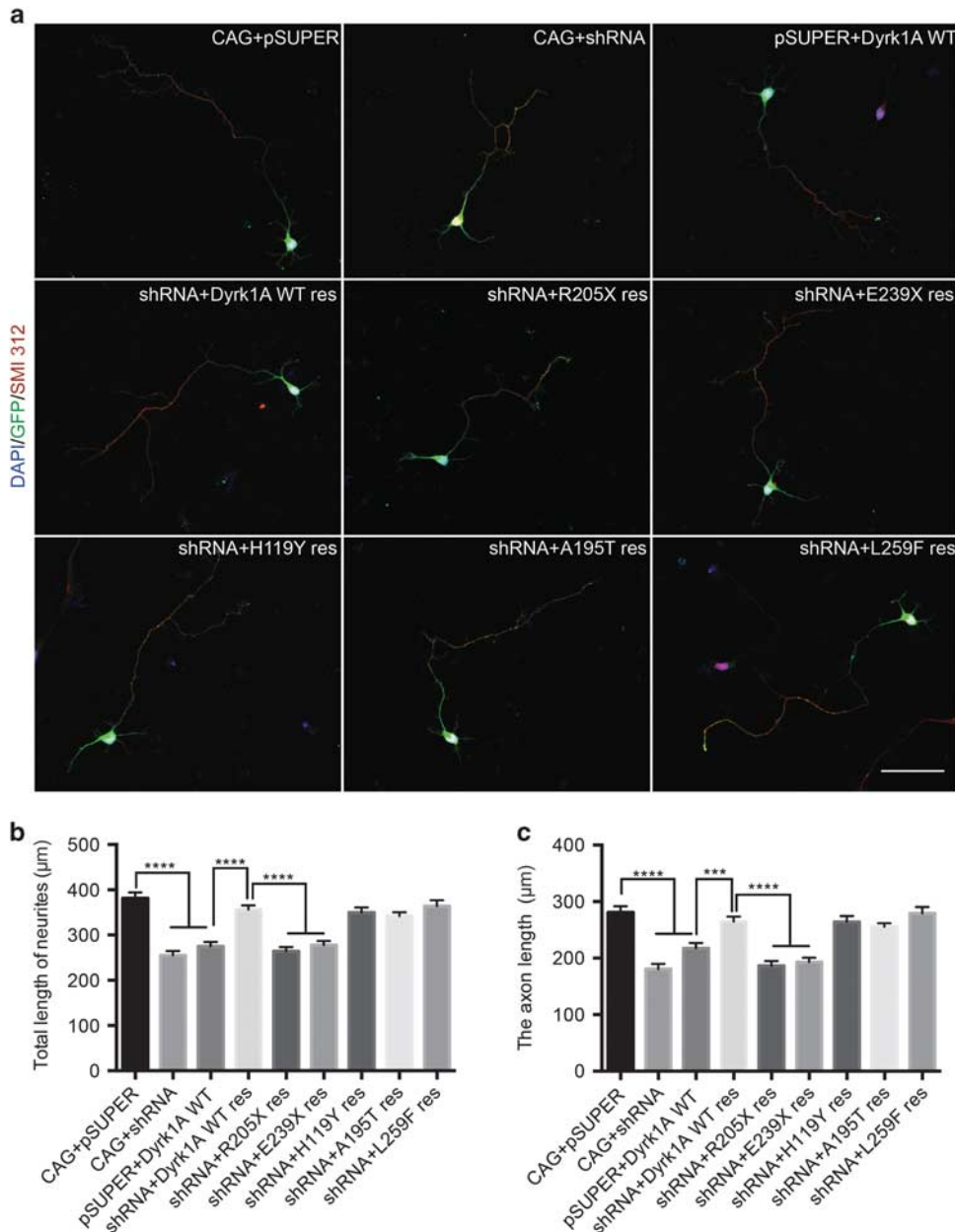
#### *In utero* electroporation

Plasmids encoding WT *Dyrk1a*, R205X and the empty vector were prepared in a final volume of 15 μl (2 μg μl<sup>-1</sup> plasmid DNA, 0.01% Fast Green and 0.5 μg μl<sup>-1</sup> enhanced GFP) for each pregnant mouse. The plasmids were then injected into the ventricles of E14.5 mouse brains. Electroporation (50-ms square pulses at 1000-ms intervals) was performed using an Electro Square Porator (ECM 830, San Diego, CA, USA) at 30 V.

Mouse pups were harvested at P0, and whole brains were fixed in 4% PFA for 24 h and sequentially dehydrated in 15% and 30% sucrose solutions (in 1× PBS) for >48 h. Longitudinal brain sections (30 μm) were obtained via frozen sectioning for later immunofluorescence experiments in which GFP immunostaining was performed using a GFP antibody and an Alexa 488-conjugated secondary antibody (1:1000) to recognize GFP-positive neurons.

#### Data analysis

Images were obtained using a Nikon E80i fluorescence microscope (Nikon, Tokyo, Japan) equipped with a ×20 objective (for neurite development, axonal polarity formation and subcellular localization in cultured mouse cortical neurons), a Nikon E80i equipped with a ×10 objective (for cortical migration and *in utero* electroporation (IUE)), a Nikon A1 confocal microscope equipped with a ×20 objective (for neurite outgrowth in rat hippocampal slice cultures and the IUE examples) and a Nikon A1 confocal microscope equipped with a ×60 oil immersion lens (for spine density).



**Figure 2.** Wild-type (WT) *Dyrk1a* (dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1 A) rescues the phenotype of decreased neurites and axon length in cultured mouse cortical neurons following *Dyrk1a* short hairpin RNA (shRNA) knockdown. **(a)** The specific morphology of neurons expressing the vectors (CAG and pSUPER), shRNA, WT *Dyrk1a* and WT or mutant forms of *Dyrk1a* along with the shRNA. All neurons were co-labeled with 4,6-diamidino-2-phenylindole (DAPI), green fluorescent protein (GFP) and SMI 312 (to distinguish axons from total neurites). Scale bar 50 μm. **(b, c)** The statistical results for the total neurites **(b)** and axon length **(c)** between specific cell types. The bar graph shows the mean value ± s.e.m.; approximately 60 cells from three independent experiments were counted, and unpaired *t*-tests were used for the statistical analysis. \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001.

To evaluate neurite length in cultured primary cortical neurons, GFP-positive cells were randomly selected from each condition, and the total neurite length was traced with the Fiji software (<http://imagej.net/Fiji/Downloads>). At least three independent experiments were performed, and approximately 70 neurons were analyzed.

To evaluate spine density in rat hippocampal slice cultures, images were obtained as a Z-stack of approximately 15–20 sections (0.2 μm per section). The average spine density of specific neurons was analyzed statistically and compared among the four treatments. The average spine density was calculated as the total number of spines per 10 μm of dendritic length of each neuron. Approximately 45 neurons from 3 independent experiments

were evaluated. The specific dendritic length was traced with the Fiji software (<http://imagej.net/Fiji/Downloads>).

To analyze total neurite outgrowth in slice cultures, images were obtained as a Z-stack of approximately 10–15 sections (1 μm per section). The total neurite length of each neuron was then traced with the Fiji software, as described previously.

For IUE, GFP-positive cells were counted using the ImageJ software (<https://imagej.nih.gov/ij/download.html>). The ratio of GFP-positive cells in the cortical plate included the total number of GFP-positive cells in the intermediate zone, the subventricular zone and the ventricular zone and was analyzed for each condition.

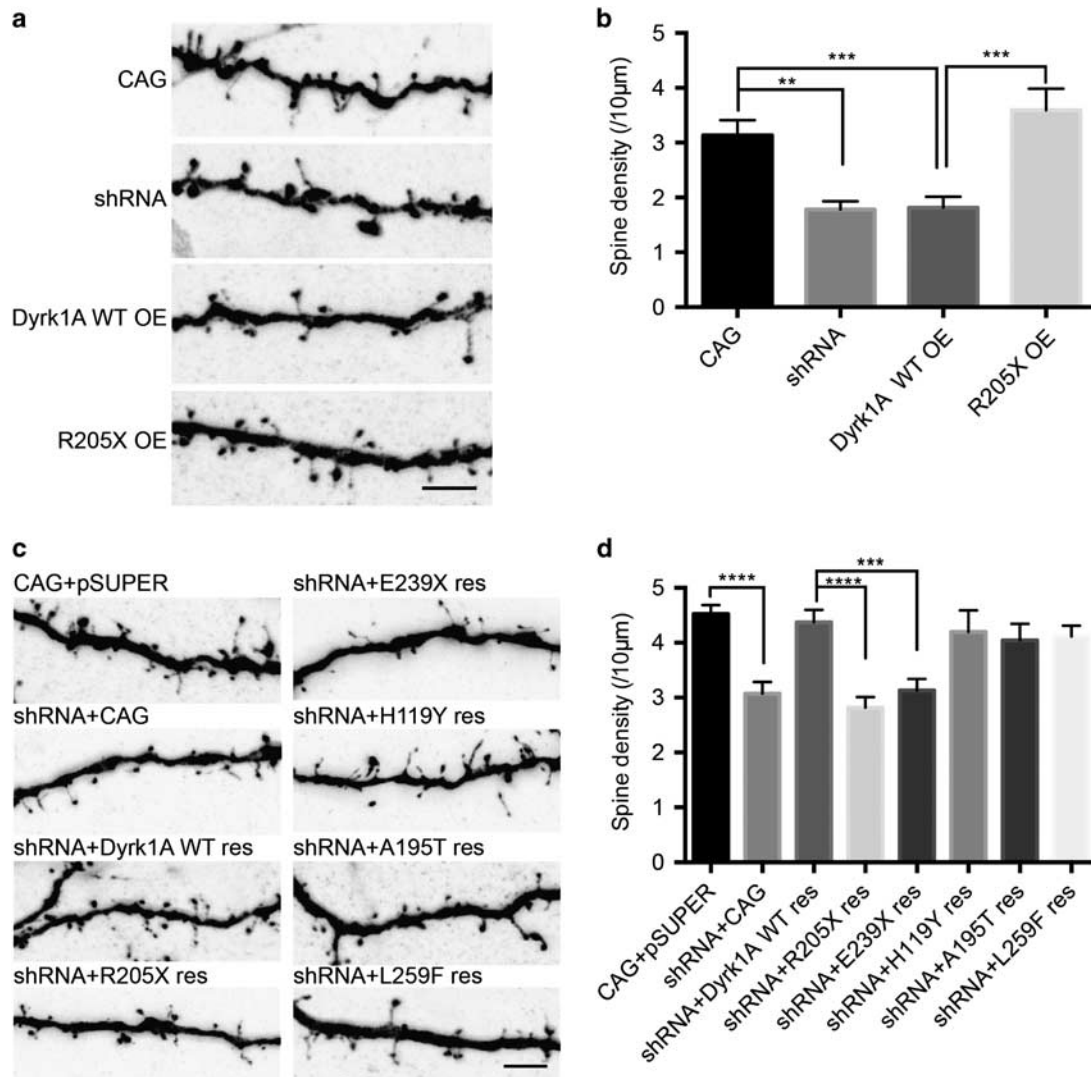
All statistical analyses were conducted using unpaired *t*-tests, with  $P < 0.05$  considered significant.

## RESULTS

### *Dyrk1a* expression profiling during brain development

*Dyrk1a* is widely expressed in the developing nervous system,<sup>10,32</sup> suggesting that it has a crucial role in brain development. To

examine DYRK1A protein levels in the embryonic and postnatal cortex, we collected protein lysates from embryonic and early postnatal mouse brains and examined DYRK1A protein levels via western blotting. We found that *Dyrk1a* was highly expressed during the embryonic stage, from E16 to P0, and this high expression level persisted until P7 (Figure 1a). In contrast, DYRK1A protein expression gradually decreased from P7 and remained at a relatively low level thereafter (Figure 1a). This

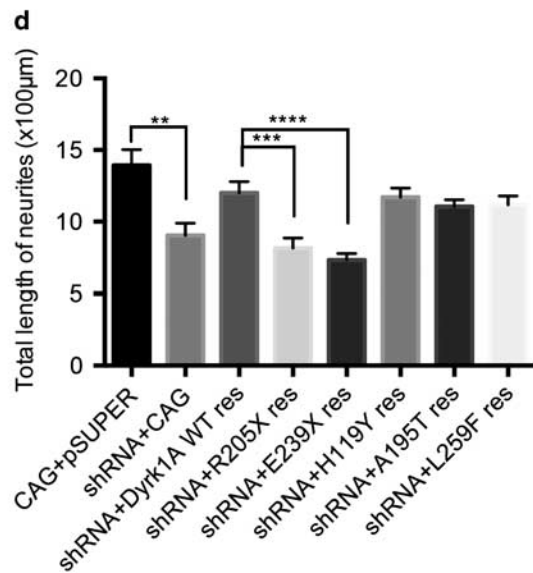
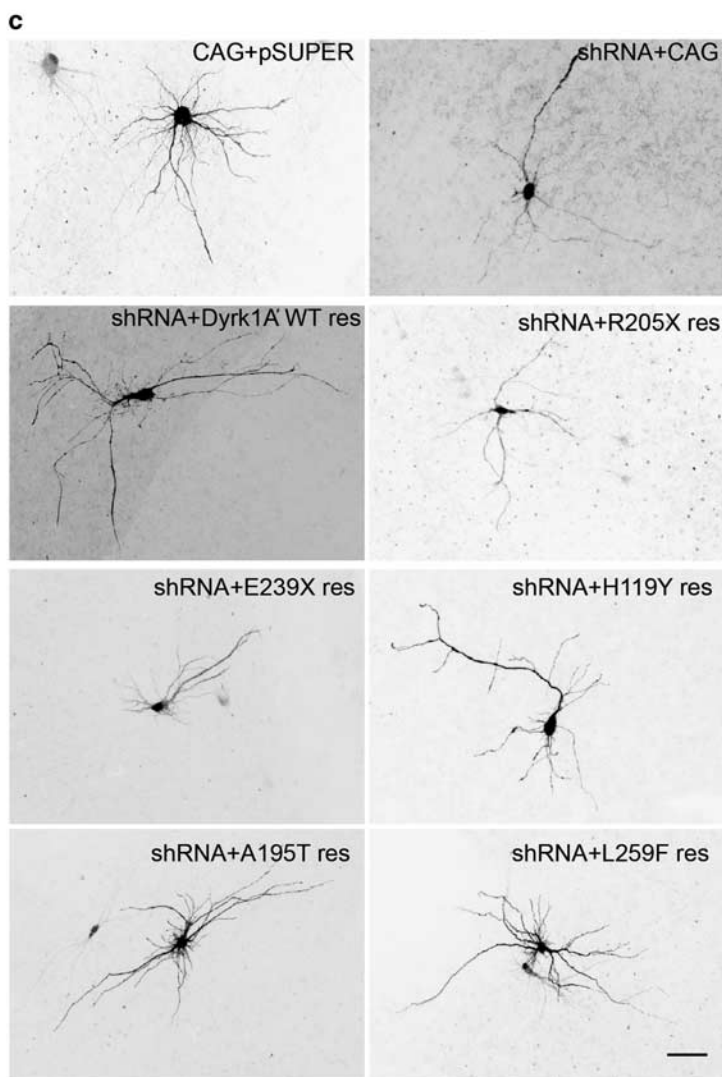
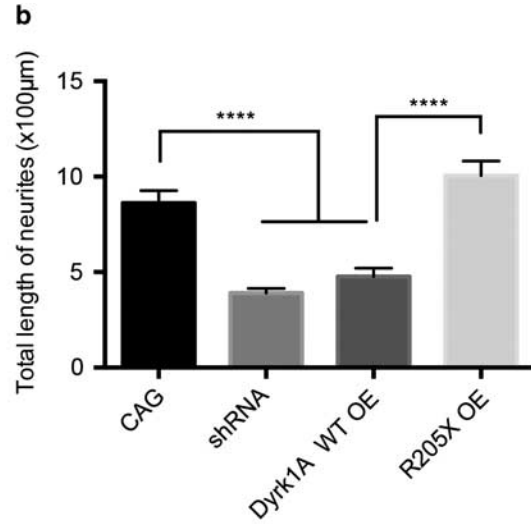
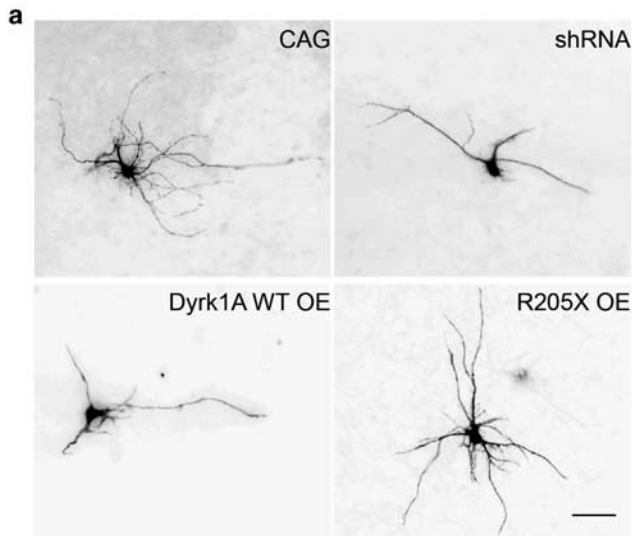


**Figure 3.** *Dyrk1a* (dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1 A) dosage imbalance reduced dendritic spine density in rat hippocampal slice culture, and wild-type (WT) *Dyrk1a* rescued the phenotype caused by *Dyrk1a* knockdown. (a, c) Morphology of the spine density under specific conditions with the expression of different plasmids, including CAG vector, *Dyrk1a* short hairpin RNA (shRNA), *Dyrk1a* WT, R205X (a) and shRNA rescue using WT or mutant *Dyrk1a* (c). Scale bar 5 μm. (b, d) Statistical analysis of the spine density (per 10 μm) in specific types of neurons. Forty-five neurons from three independent experiments were counted, and the individual neuronal spine density was determined. Unpaired *t*-tests were performed for the statistical analysis to compare each condition.  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ ,  $^{****}P < 0.0001$ .

**Figure 4.** *Dyrk1a* (dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1 A) inhibited total neurite outgrowth in rat hippocampal neurons, and short hairpin RNA (shRNA)-mediated loss of function was rescued by wild-type *Dyrk1a*. (a, c) The morphology of neurons in each condition expressing the indicated plasmids. (a) Neurons expressed CAG vector, *Dyrk1a* short hairpin RNA (shRNA), *Dyrk1a* wild-type (WT) and R205X. (c) Neurons expressed specific plasmids for the rescue conditions, including WT and the five mutant forms of *Dyrk1a* plus *Dyrk1a* shRNA. (b, d) Statistical analysis of total neurite length in overexpression (b) and rescue conditions (d). Scale bar 50 μm. Approximately 50 neurons from three independent experiments were counted, and the total neurite length of each cell was determined. Unpaired *t*-tests were performed for the statistical analysis.  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ ,  $^{****}P < 0.0001$ .

expression pattern suggests that *Dyrk1a* has a critical role in the developing brain. To study the role of *Dyrk1a* in neural development, we designed a series of shRNAs against the mouse

*Dyrk1a* gene and identified one set of effective shRNAs that significantly decreased DYRK1A protein levels in mouse primary cortical neurons (Figure 1b).



The dosage of *Dyrk1a* is crucial for dendritic growth in cultured neurons

Dendrites have a vital role in the biological functions of neurons, such as the receipt of information from other neurons. Previous studies have observed dendritic abnormalities in the brains of DS patients; in these patients, the *DYRK1A* gene is overexpressed owing to the excess of chromosome 21-associated genes.<sup>15,18</sup> Furthermore, Lepagnol-Bestel *et al.*<sup>21</sup> showed that overexpressing *Dyrk1a* in mouse cortical neurons significantly decreased neurite length. Thus we decided to investigate whether autism-associated *Dyrk1a* mutations are involved in neurite development.

During our analysis of *DYRK1A* variants, we classified the nine variants into three groups according to their specific localization in the functional domains of *DYRK1A* (Figure 1c), as described above, and decided to select five representative mutants (R205X, E239X, H119Y, A195T and L259F) for further analysis.

In this study, we transfected mouse primary cortical neurons with plasmids expressing the empty CAG vector (as a control), *Dyrk1a* shRNA, WT *Dyrk1a* and the five *Dyrk1a* mutants, and we measured total neuritic and axonal length after 72 h. First, we confirmed a previous finding that overexpression of WT *Dyrk1a* severely reduced the total neurite length in cultured mouse cortical neurons (Figures 1d–f). Importantly, when endogenous *Dyrk1a* was specifically knocked down by RNA interference (RNAi), neurite length was also significantly decreased compared with control neurons expressing GFP alone. This finding was similar to the observation that overexpressing WT *Dyrk1a* decreased neurite growth (Figures 1d–f). These data indicate that the precise dosage of *DYRK1A* protein is critical for proper neurite development.

We further examined whether mutations in *Dyrk1a* affected neurite growth. We found that overexpressing either of the two *Dyrk1a* truncation mutants (R205X and E239X) did not inhibit total neurite growth; neurite growth in cells that were transfected with these mutants resembled that of controls, indicating that these two mutants lacked the normal function of WT *Dyrk1a* (Figures 1d–e). However, all three *Dyrk1a* missense mutants (H119Y, A195T and L259F) retained the ability to repress neurite outgrowth when overexpressed (Figures 1d–e), similar to WT *Dyrk1a*, indicating that non-haploinsufficient mutations may affect different aspects of brain development.

We next investigated whether *Dyrk1a* might affect axonal growth in neurons. During the neurite development assay, we measured axon length via immunohistochemistry using an antibody against the axonal marker SIM 312 and found that axonal growth was indeed affected by the *Dyrk1a* dosage and autism-associated mutations (Figures 1d and f). From these results, we found that shRNA-mediated *Dyrk1a* knockdown, as well as the overexpression of the WT form and the three missense mutants, decreased axon length compared with the control. In contrast, R205X- and E239X-expressing neurons exhibited the same phenotype as CAG vector-expressing neurons (Figures 1d and f).

Because WT *Dyrk1a* overexpression led to a similar phenotype to that of shRNA-mediated *Dyrk1a* knockdown, we performed a rescue experiment to confirm the specificity of the shRNA-mediated *Dyrk1a* knockdown and acquire a better understanding of the critical impact of the *Dyrk1a* dosage on neurite and axonal development. WT *Dyrk1a* rescued the inhibition of both neurite and axon length caused by *Dyrk1a* RNAi (Figures 2a–c). Neurons co-expressing WT *Dyrk1a* and shRNA exhibited similar outgrowth as control neurons expressing GFP alone, confirming the specificity of the *Dyrk1a* shRNA in mouse cortical neurons.

Moreover, we previously showed that overexpression of nonsense mutations did not mimic the phenotype of WT *Dyrk1a* overexpression on neurite and axonal growth, revealing the important effect of these two *Dyrk1a* truncations. For further insight into the function of these mutants, we also conducted a

rescue experiment using the five mutant forms of *Dyrk1a* and found that only the three missense mutations could rescue the knockdown phenotype (Figures 2a–c). The two truncations, R205X and E239X, did not restore neurite and axon lengths to control levels. In fact, staining revealed decreased outgrowth, similar to that observed with *Dyrk1a* RNAi (Figures 2a–c). Therefore, we surmise that these two *Dyrk1a* truncations act as *Dyrk1a* loss-of-function mutants.

We conclude that the *Dyrk1a* dosage is critical for proper neurite and axonal outgrowth and that the two nonsense mutations, R205X and E239X, are loss-of-function mutants.

*Dyrk1a* is crucial for the growth of neurites and spines in hippocampal neurons in slices

Next we wanted to examine the role of *Dyrk1a* in spine formation in hippocampal neurons. Dendritic spines are small protrusions along dendrites and represent the typical postsynaptic structure of excitatory synapses. Studies have shown that dendritic spine abnormalities are strongly associated with various neurological and neuropsychiatric disorders.<sup>33</sup> *Dyrk1a* is reported to phosphorylate and inactivate the N-WASP protein, suggesting that *Dyrk1a* has a role in spine formation.<sup>12</sup>

We investigated the role of *Dyrk1a* in spine formation using a hippocampal slice culture system. We used gene-gun-mediated delivery to introduce plasmids encoding GFP along with WT *Dyrk1a*, *Dyrk1a* R205X and *Dyrk1a* shRNA into hippocampal neurons in postnatal hippocampal slices. Interestingly, we found that either knocking down *Dyrk1a* or overexpressing the WT form strongly repressed dendritic spine formation in hippocampal neurons, further indicating that a precise amount of *DYRK1A* protein is required for proper spine development (Figures 3a and b). However, overexpression of the R205X mutant did not similarly inhibit dendritic growth (Figures 3a and b).

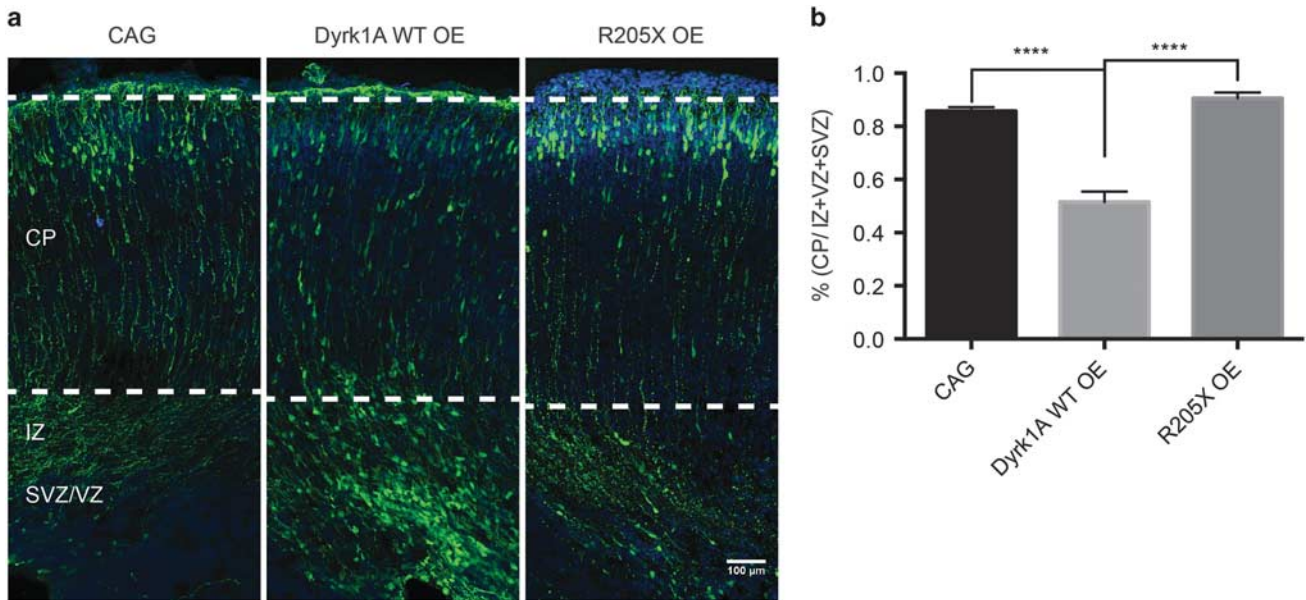
Furthermore, we conducted rescue assays to confirm the critical role of *Dyrk1a* in spine formation. WT *Dyrk1a* successfully rescued the loss of function, and neurons that received this treatment exhibited phenotypes similar to those of neurons that only expressed the CAG vector (Figures 3c and d). Therefore, we concluded that the shRNA against *Dyrk1a* was specific in rat hippocampal neuron. The three missense mutations also rescued the decreased spine density caused by *Dyrk1a* RNAi, whereas the two truncation mutants, R205X and E239X, did not rescue the effects on spine formation (Figures 3c and d), consistent with the rescue phenotype that was observed in mouse cortical neurons.

We also measured neurite outgrowth in hippocampal neurons that received the aforementioned manipulations. Consistently, either RNAi or WT *Dyrk1a* overexpression strongly inhibited neurite growth in hippocampal neurons in brain slices (Figures 4a and b). However, overexpression of the *Dyrk1a* R205X mutant did not affect neurite growth compared with WT *Dyrk1a* (Figures 4a and b). We next re-expressed WT and different *Dyrk1a* mutants along with RNAi in hippocampal slice cultures to assess whether these defects could be rescued. Only WT *Dyrk1a* and the missense mutants, but not R205X and E239X, were able to rescue the defects caused by RNAi (Figures 4c and d). The rescue results further confirmed the role of *Dyrk1a* in neurite development in hippocampal slices and that the truncations resulted in *Dyrk1a* loss of function. This evidence again indicates that the autism-related *Dyrk1a* truncations (R205X and E239X) are loss-of-function mutations.

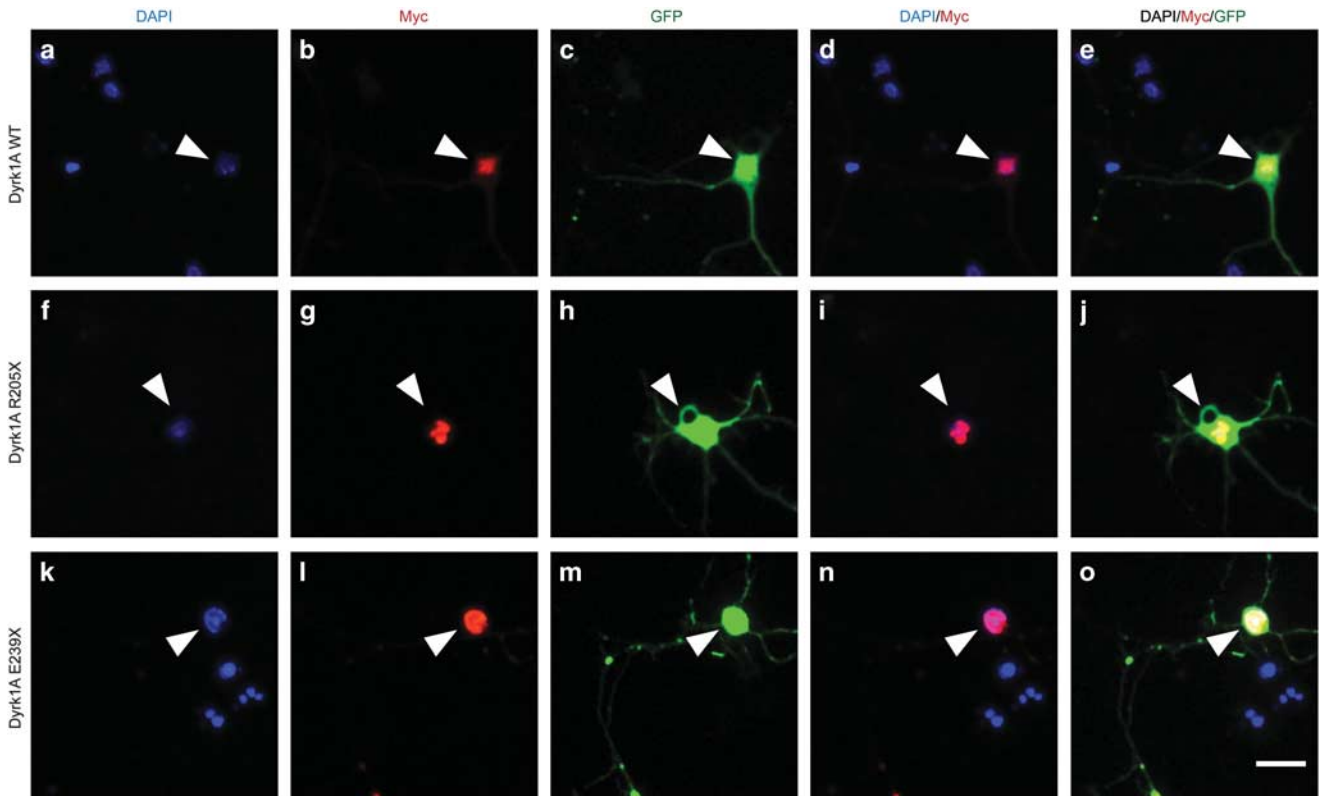
*Dyrk1a* is critical for cortical migration during early postnatal development

To further understand the role of *Dyrk1a* in embryonic and postnatal cortical development in the mouse brain, we examined cortical migration by manipulating *Dyrk1a* via IUE in mice.





**Figure 5.** *Dyrk1a* (dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1 A) wild-type (WT) overexpression delayed neuronal migration in mouse embryonic cortical development. (a) Cortical migration in each condition, including CAG (left), *Dyrk1a* WT overexpression (middle) and R205X (right). (b) Quantification of panel (a). The green fluorescent protein (GFP)-positive cells in the cortical plate (CP), intermediate zone (IZ), subventricular zone (SVZ) and ventricular zone (VZ) were counted, and the ratio of GFP-positive cells in the CP/(IZ and VZ/SVZ) was analyzed statistically using unpaired *t*-tests. P0 mice were collected in three independent experiments, including CAG ( $n = 7$ ), *Dyrk1A* WT ( $n = 6$ ) and R205X ( $n = 5$ ) mice. \*\*\*\* $P < 0.0001$ . Scale bar 100  $\mu\text{m}$ .



**Figure 6.** The two *Dyrk1a* (dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1 A) truncations did not disturb *Dyrk1a* subcellular localization. (a–e) Neurons expressing *Dyrk1a* wild-type (WT) were labeled with 4,6-diamidino-2-phenylindole (DAPI; to identify nuclei), a Myc epitope tag (to identify the precise location of the expressed protein) and green fluorescent protein (GFP; to identify overall neuronal morphology). Merge 1 shows DAPI and Myc co-labeling, and merge 2 shows DAPI, Myc and GFP labeling. Panels (f–j) and (k–o) depict the two nonsense mutants, respectively. Both R205X and E239X, as well as *Dyrk1a* WT, localized to the nucleus. The images were merged using the ImageJ software. Scale bar 20  $\mu\text{m}$ .

Plasmids encoding GFP plus either the WT *Dyrk1a* or the R205X mutant were electroporated into the ventricular zone of E14.5 mouse brains, and the migration status of the GFP-labeled neurons was observed in neonatal pups on P0. Overexpression of WT *Dyrk1a* significantly delayed cortical migration compared with GFP expression alone (Figures 5a and b). In contrast, expressing the *Dyrk1a* R205X truncation in migrating neurons did not similarly affect cortical migration, again suggesting that the R205X truncation has a *loss-of-function* effect (Figures 5a and b). This evidence again indicates that the R205X truncation may be a *loss-of-function* mutant of *Dyrk1a*, which affects various aspects of postnatal neural development, including dendrite and spine formation and cortical migration.

#### Autism-related *Dyrk1a* truncations do not affect subcellular localization

The histidine-repeat domain near the C-terminus of DYRK1A is critical for its localization in nuclear speckles.<sup>34</sup> Therefore, we wanted to examine whether the R205X or E239X truncation affected the subcellular localization of *Dyrk1a* in neurons. We transfected cultured mouse primary cortical neurons with plasmids encoding myc-tagged WT *Dyrk1a* or the two truncated mutants. By performing immunocytochemistry with an anti-myc antibody, we first confirmed that WT *Dyrk1a* was expressed exclusively in the nucleus (Figure 6). Importantly, the R205X and E239X *Dyrk1a* truncations were also located in the nucleus, suggesting that these mutations did not affect *Dyrk1a* localization (Figure 6).

## DISCUSSION

*DYRK1A* is a critical gene located on human chromosome 21, encoding a proline-directed serine/threonine kinase that has been reported to phosphorylate several substrates related to gene transcription, neuronal degeneration and synapse function.<sup>9</sup> *Dyrk1a* transgenic mice (*TgDyrk1A*), a mouse model of DS that overexpresses *Dyrk1a* by 1.5-fold,<sup>19</sup> show defects in learning and memory, as well as hyperactivity and altered motor learning.<sup>22</sup> Duplications of *Dyrk1a* result in abnormal neural development, such as compromised dendrite outgrowth and cell differentiation. At early embryonic stages, *Dyrk1a* overexpression reduces the proliferation of neuronal progenitor cells,<sup>35–40</sup> whereas increasing neuronal differentiation and enhancing GABAergic differentiation, which were observed in neuronal stem cells isolated from *TgDyrk1A* mice.

For decades, different types of genetic/genomic defects have been linked to ASD, including chromosomal abnormalities, genomic copy number variants and genetic variants of hundreds of genes, highlighting the etiological heterogeneity underlying the genetic and phenotypic variation in ASD. Recently, *DYRK1A* was proposed as a candidate gene for syndromic ASD and ID or DD, and approximately 100 alterations have thus far been identified. Of these, the majority are *de novo* heterozygous mutations (nonsense, frame-shift, splicing alteration) and large chromosomal rearrangements (micro-deletion/duplication, insertion and translocation). The majority were haploinsufficiency alterations, which are relevant for many ASD-associated genes. The eight novel and one recurrent *DYRK1A* mutations detected in our patients with ASD/ID/DD are consistent with these findings.<sup>6,8,24–26,41–44</sup>

In the present study, the phenotypes of the two patients with *DYRK1A* truncations partially overlapped with the findings in a recent report, including microcephaly, seizures and impaired speech, supporting the hypothesis that *DYRK1A* truncations define a syndromic form of ASD and ID.<sup>26</sup> Interestingly, the higher incidence of truncations identified in our patients (0.22%; 2 truncations in 892 patients) is similar to work by van Bon *et al.*<sup>26</sup> (0.21%; 15 truncations in 7162 patients), further supporting the

observation that *DYRK1A* truncations may be a relatively common type of mutation associated with syndromic ASD and ID or DD.

Fewer than 10 missense mutations have been reported in the literature, and most are predicted to be pathogenic; however, no functional studies have been described. In the present study, we found that the three missense mutations behaved similarly to WT *Dyrk1a* when overexpressed, with all three repressing neurite and axon outgrowth and affecting spine development; however, all could be rescued, which indicated that they were not *loss-of-function* mutations but may affect other *Dyrk1a* functions that were not assessed in this study. These findings also suggested that non-haploinsufficient missense mutations may affect brain development in different ways than haploinsufficient truncated mutations. Further studies to explore the pathogenesis of such missense mutations are needed. In addition, this study was limited to detecting only *DYRK1A* mutations; therefore, these missense mutations may in fact function with other unknown mutations or may not contribute to the risk of autism.

In this study, we show that *Dyrk1a* has an important role in neural development. The imbalance of the *Dyrk1a* dosage affected dendrite development and axon length. Consistent results were found in primary cultured mouse cortical neuron and rat hippocampal slice cultures. We also found that the two *Dyrk1a* nonsense truncations were unable to rescue defects caused by *Dyrk1a* knockdown. Therefore, we conclude that nonsense mutations caused a *loss of function* of the DYRK1A protein, which had effects on dendritic outgrowth in neurons, dendritic spine development and embryonic cortical migration. Moreover, upregulation and downregulation of *Dyrk1a* also reduced the dendritic spine density in rat hippocampal slice cultures. Importantly, cortical migration during the mouse embryonic stage was also altered by the overexpression of WT *Dyrk1a* from E14.5 to P0. Based on these results, we conclude that *Dyrk1a* has a key role in embryonic development.

Furthermore, overexpression of the two nonsense truncations resembled defects caused by *Dyrk1a* knockdown, suggesting that patients carrying *Dyrk1a* nonsense mutations show haploinsufficiency of *Dyrk1a*. Reduced DYRK1A proteins levels of in patients likely lead to defects in neural development and further ASD symptoms, providing new evidence that the dosage of *Dyrk1a* is critical for proper neurite and axonal outgrowth.

To date, the impact of *Dyrk1a loss of function* on neural development remains limited. For example, mice expressing only one copy of *Dyrk1a* exhibit a microcephalic phenotype.<sup>45,46</sup> However, the functional consequences of *Dyrk1a loss of function* have not been fully addressed. *Dyrk1a* knockout was reported to be lethal during the embryonic period, whereas *Dyrk1a*<sup>+/-</sup> mice survive but display severe defects in cognition, DDs and abnormalities in brain size and structure.<sup>47,48</sup> Thus it is difficult to undertake further analyses of this gene in a *loss-of-function* mouse model. The two *Dyrk1a* truncations we found in ASD patients provide important insights into the role of *loss-of-function* forms of *Dyrk1a* in various aspects of neural development.

DYRK1A phosphorylates multiple substrates. For instance, P53 Ser15 is directly phosphorylated by DYRK1A, and P53 phosphorylation at this site inhibits the proliferation of neural progenitors.<sup>10</sup> DYRK1A has been shown to selectively phosphorylate RCAN1 at Ser112, prompting the phosphorylation of another residue, Ser108, by GSK3 $\beta$  and resulting in the formation of insoluble aggregates that contribute to the development of Alzheimer disease.<sup>11</sup> N-WASP Thr192 may also be a substrate for DYRK1A phosphorylation, and spine formation can be inhibited by phosphorylation at this site.<sup>12</sup> DYRK1A phosphorylates Tau at multiple sites, leading to decreased microtubule assembly and the formation of interneuron aggregates.<sup>13,23</sup> These observations reveal the potential role of *Dyrk1a* in dendritic outgrowth. In addition, DYRK1A negatively regulates nuclear factor of activated T cells by phosphorylating the serine-proline repeats 3 domain.<sup>49</sup>

Further studies are required to illustrate the exact substrates of DYRK1A that are responsible for various aspects of neural development.

In the present study, we showed that both excessive and reduced *Dyrk1a* activity yield similar phenotypes of delayed neural development that were distinct from the phenotypes associated with other candidate genes in neurodevelopment.<sup>50</sup> Although ASD and DS are both neuronal development disorders and although previous studies have mostly focused on the relationship between *DYRK1A* and DS,<sup>9,15,21,23</sup> there are essential differences between ASD and DS. For example, the incidence stage and the specific phenotypes differ; severe mental retardation and delayed development is observed in DS patients, whereas verbal dysfunction and social problems are often observed in ASD patients.<sup>2</sup> Genes in the DSCR region of chromosome 21 have clear connections with DS, whereas genetic factors other than chromosome 21 are associated with ASD. Thus it is important and necessary to identify the mechanisms by which *DYRK1A* contributes to ASD.

In summary, we found that *Dyrk1a* strongly affected various aspects of neuronal development, such as dendritic outgrowth, dendritic spine density and cortical migration, during early postnatal development. Importantly, R205X and E239X, two *DYRK1A* truncations that were identified in ASD/ID/DD patients, functioned as *loss-of-function* mutants.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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#### AUTHOR CONTRIBUTIONS

BL Wu and ZL Qiu conceived, designed and supervised the study. T Dang drafted the manuscript. BL Wu and ZL Qiu revised the manuscript. T Dang performed the neuroscience experiments and analyzed the data. B Yu participated in the IUE assay. DL Tong participated in the slice culture assay. C Cheng participated in the data analysis. YF Zhang prepared the experimental mice. WY Duan, W Wu, K Ye, WX Zhang and M Wu performed the exome sequencing. WY Duan performed the primary analysis of the data and mutation validations. Y An and BB Wu assisted in supervising the study. All authors read and approved the final manuscript.

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