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Autism-associated protein kinase D2 regulates embryonic cortical neuron development

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ABSTRACT

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder, characterized by impaired social interaction, repetitive behavior and restricted interests. Although the molecular etiology of ASD remains largely unknown, recent studies have suggested that *de novo* mutations are significantly involved in the risk of ASD. We and others recently identified spontaneous *de novo* mutations in *PKD2*, a protein kinase D family member, in sporadic ASD cases. However, the biological significance of the *de novo PKD2* mutations and the role of PKD2 in brain development remain unclear. Here, we performed functional analysis of PKD2 in cortical neuron development using *in utero* electroporation. PKD2 is highly expressed in cortical neural stem cells in the developing cortex and regulates cortical neuron development, including the neuronal differentiation of neural stem cells and migration of newborn neurons. Importantly, we determined that the ASD-associated *de novo* mutations impair the kinase activity of PKD2, suggesting that the *de novo PKD2* mutations can be a risk factor for the disease by loss of function of PKD2. Our current findings provide novel insight into the molecular and cellular pathogenesis of ASD.

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1. Introduction

Autism spectrum disorder (ASD) is a group of intractable neurodevelopmental disorders with impairments in social interactions and verbal communication abilities, restricted interests and stereotyped repetitive behaviors [1]; the prevalence rate of ASD is 1 in 40 children [2]. Previous studies have suggested that aberrant brain development is critically involved in ASD [3–6]; however, the molecular etiology underlying the pathological phenotypes of ASD remains largely unclear. Currently, there are no established treatment strategies for the core symptoms of ASD. Thus, the development of new treatment approaches based on the molecular etiology and pathophysiology of ASD is urgently needed [7,8].

Molecular genetic studies have suggested that ASD is highly heterogeneous, with many types of genetic abnormalities [9]. The concordance rate for ASD in monozygotic twins was more than 90%, which is significantly higher than the rate in dizygotic twins, suggesting that ASD has a strong genetic component [10,11]. However, numerous cases of ASD are sporadic, and the genetic cause of approximately 90% of sporadic ASD cases remains unidentified [12,13]. Recently, in addition to heritable mutations, *de novo* mutations, new genomic mutations found in a child but found in neither of the parent, have been investigated as a risk factor for ASD [14–17].

Protein kinase D (PKD) is a serine/threonine protein kinase with a catalytic domain and two cysteine-rich phorbol ester binding domains similar to those of protein kinase C [18,19]. The PKD family members consist of PKD1, PKD2 and PKD3, which are ubiquitously but differentially expressed depending on the cell type and external stimulation [20,21]. In peripheral proliferative tissues, PKD2 plays important roles in cell growth and differentiation [22,23]. In addition to peripheral tissues, PKD2 is highly expressed in the brain, where it regulates the establishment and maintenance of neuronal polarity [24,25]. Recently, we and others have identified *de novo* mutations in *PKD2* from patients with sporadic ASD [26,27], suggesting that the *de novo* mutations in *PKD2* can be associated with the risk of ASD. Considering that ASD is suggested to be caused by aberrant cortical neuron development [3–6], PKD2 may regulate cortical neuron development.

In this study, we conducted a functional analysis of PKD2 in cortical neuron development *in vivo*. We found that PKD2 was highly expressed in neural stem cells (NSCs) in the embryonic cerebral cortex and that *Pkd2* knockdown disrupted neuronal development in the cerebral cortex in mouse embryos. Furthermore, we showed that the ASD-associated *de novo PKD2* mutations decreased the autophosphorylation levels of PKD2 and its downstream kinases, ERK1/2, suggesting that the *de novo* mutations impaired the kinase activity of PKD2. Taken together, our current results strongly suggest that PKD2 regulates cortical neuron development and that ASD-associated *de novo PKD2* mutations can be a risk factor for the disease by loss of function of PKD2.

2. Materials and methods

2.1. Reverse transcription and real-time PCR

Reverse transcription of total RNA isolated from cells and tissues was performed using Superscript III (Life Technologies, CA, USA). The RNA transcription levels were measured by real-time PCR with SYBR Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan) using a CFX96 real-time PCR detection system (Bio-Rad Laboratories, CA, USA) as described previously [26]. The expression levels of *Pkd2* (forward primer sequence: 5′-CTGTTCTATCGTGGACCAGAAGT-3'; reverse primer sequence: 5′-GGCTGATGTTGGGTCATGTT-3′) were normalized to the expression levels of *Gapdh* (forward primer sequence: 5′-GTGTTCCTACCCCCAATGTG-3'; reverse primer sequence: 5′-TACCAGGAAATGAGCTTGAC-3′) and were determined in accordance with the $2^{-\Delta\Delta Ct}$ method.

2.2. Plasmid preparation

A plasmid vector expressing Myc-tagged wild-type (WT) PKD2 was generated by subcloning a PCR-amplified PKD2 cDNA (Kazusa DNA Res. Inst., Chiba, Japan, clone ID: cp93316) into the pcDNA3 expression vector. *De novo* mutations and kinase active mutations of PKD2 ($PKD2^{S706/710E}$) [28,29] were generated using a KOD mutagenesis kit according to the manufacturer's instructions (Toyobo, Osaka, Japan). The MISSION shRNA against PKD2 construct (shPKD2; TRCN0000024328, hairpin sequence of 5′ flanking-sense strand-loop-antisense strand-3′ flanking: 5′-CCGG-GTACGACAA-GATCCTGCTCTT-CTCGAG-AAGAGCAGGATCTTGTCGTAC-TTTTT-3′) was purchased from Sigma-Aldrich (MO, USA) [26]. The hairpin sequence of miR30-based shRNA against PKD2 ($shPKD2^{miR30}$) (hairpin sequence of 5′ flanking-sense strand-loop-antisense strand-3′ flanking: 5′-TGCTGTTGACAGTGAGCG-CGCAGTAAAGGT-CATTGACAAA-TAGTGAAGCCACAGATGTA-TTTGTCAATGACCTT-TACTGCA-TGCCTACTGCCTCGGA-3′) was designed using a design tool (http://cancan.cshl.edu/RNAi_central/RNAi.cgi?type=shRNA). The template oligonucleotide carrying the $shPKD2^{miR30}$ sequence purchased from Eurofins Genomics (Tokyo, Japan) was amplified via PCR and subcloned into a pCAG-miR30 vector.

2.3. Measuring the knockdown efficiency of shRNA

The pCAG-GFP vector, MISSION shRNA or $shRNA^{miR30}$ constructs and the pcDNA3 expression construct encoding WT mouse PKD2 were transfected into HEK293T cells. Three days later, total RNA was isolated from the transfected cells, and the mouse *Pkd2* mRNA levels were measured by real-time PCR. The knockdown efficacy was normalized by transfection efficiency.

2.4. In utero electroporation

In utero electroporation was conducted on embryonic day 14.5 (E14.5) ICR embryos (SLC, Shizuoka, Japan) as described previously [30]. MISSION shRNA or $shRNA^{miR30}$ constructs (1 μg/μL), the pcDNA3 expression construct encoding kinase active mutant of PKD2 ($PKD2^{S706/710E}$) (1 μg/μL) and the pCAG-GFP vector (0.5 μg/μL) were injected into the lateral ventricles. The embryos were harvested at E16.5 or E18.5, and three non-adjacent coronal sections per brain were imaged for quantification. The images were acquired with an FV1000 confocal microscope (Olympus, Tokyo, Japan) and analyzed with ImageJ software (NIH, MD, USA) and Adobe Photoshop CS (Adobe Systems, CA, USA).

2.5. Immunohistochemistry

Mouse embryonic brains were fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4 °C. The brains were sectioned at a 20 μm thickness using a cryostat (CM1520, Leica, Welzlar, Germany). The brain slices were permeabilized with blocking solution containing 0.25% Triton X-100 (Wako, Osaka, Japan), 1% normal goat serum (Thermo Fisher Scientific, MA, USA) and 1% bovine serum albumin (Sigma-Aldrich) in PBS for 1 h at room temperature and then incubated in the blocking solution containing primary antibody at 4 °C overnight. The following day, the slices were incubated with the blocking solution containing biotinylated secondary antibody and Hoechst 33258 dye (Calbiochem, CA, USA) for 1 h at room temperature. The biotinylated secondary antibody was detected using Texas Red-conjugated streptavidin (#SA-5006, Vector Labs, CA, USA). The primary antibodies were chicken anti-GFP

(#ab13970, 1:500, Abcam, Cambridge, UK), rabbit anti-GFP (#598, 1:200, MBL, Aichi, Japan), rabbit anti-PAX6 (#901301, 1:50, BioLegend, CA, USA), rabbit anti-TBR2 (#ab23345, 1:50, Abcam) and mouse anti-SATB2 (#ab51502, 1:50, Abcam). The secondary antibodies were Alexa Fluor 488-conjugated goat anti-chicken IgY (#A-11039, 1:500, Life Technologies), Alexa Fluor 488-conjugated goat anti-rabbit IgG (#A-11008, 1:200, Life Technologies), biotinylated goat anti-rabbit IgG (#BA-1000, 1:200, Vector Labs) and biotinylated goat anti-mouse IgG (#BA-9200, 1:200, Vector Labs).

2.6. Cell culture and transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with high glucose, GlutaMAX (Life Technologies) and 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. On day 2, transient transfections were performed using TransIT-LT1 Transfection Reagent (Mirus Bio LLC., WI, USA) as described previously [31]. On day 5, the transfected cells were harvested and lysed with radioimmunoprecipitation assay buffer.

2.7. Immunoblotting

Cell lysates were analyzed by SDS-PAGE followed by transfer to polyvinylidene difluoride membranes and blotting with the indicated antibodies as described previously [32]. The primary antibodies were mouse anti-Myc (9E10) (#sc-40, 1:400, Santa Cruz Biotechnology, CA, USA), rabbit anti-PKD2 (phospho-S876)

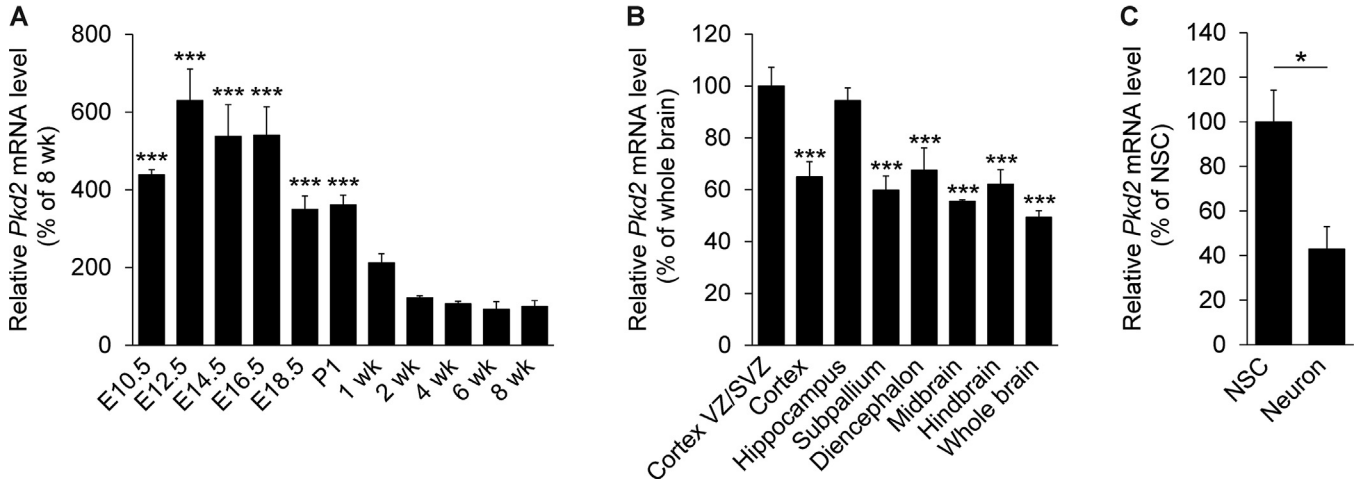

Fig. 1. *Pkd2* is highly expressed in the NSCs of the mouse embryonic cerebral cortex. (A) qRT-PCR analysis of the temporal expression patterns of *Pkd2* mRNA in the mouse brain (each n = 3). E, embryonic day; wk, week-old. (B) qRT-PCR analysis of the regional expression patterns of *Pkd2* mRNA in the mouse brain at E16.5 (each n = 3). VZ, ventricular zone; SVZ, subventricular zone. (C) qRT-PCR analysis of the expression levels of *Pkd2* mRNA in NSCs and neurons (each $n = 3$). Data are presented as the mean ± SEM. Statistical significance was analyzed using one-way ANOVA with Bonferroni-Dunn *post hoc* tests (A and B) and Student's *t*-test (C). $^{*}P < 0.05$, $^{***}P < 0.001$ (A, vs 8 wk; B, vs cortex VZ/SVZ).

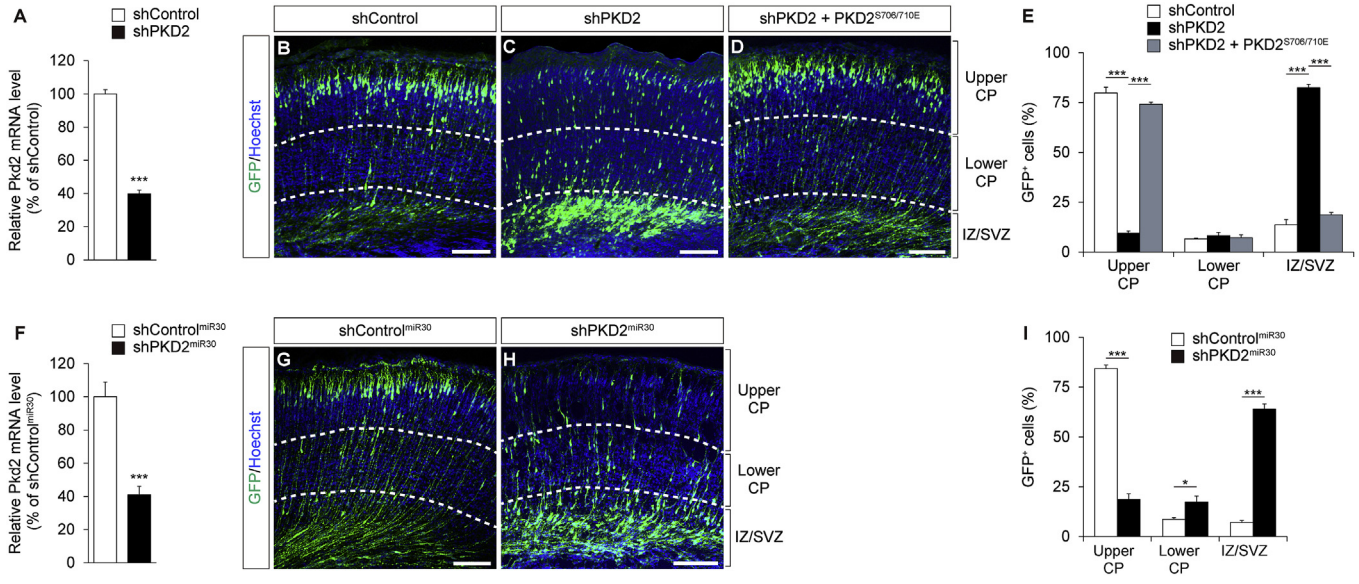

Fig. 2. Neuronal migration is impaired by *Pkd2* knockdown in the mouse developing cerebral cortex. (A and F) Knockdown efficacy of shRNA against PKD2 (shPKD2) measured in HEK293T cells transfected with MISSION shRNA vectors (A) or $shRNA^{miR30}$ vectors (F) together with pcDNA3 encoding WT mouse PKD2 (each $n = 4$). (B, C, D, G and H) Representative images of embryonic cortical sections at E18.5, 4 days after *in utero* electroporation with shControl (B), shPKD2 (C), shPKD2 and $PKD2^{S706/710E}$ (D), $shControl^{miR30}$ (G) or $shPKD2^{miR30}$ (H). Immunohistochemistry for GFP with Hoechst 33258 counterstaining is shown. Scale bars, 100 μm. (E and I) Quantification of GFP^{+} cell position in the indicated zone of the developing cortex as a percentage of total GFP + cells (each $n = 4$). CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone. Data are presented as the mean ± SEM. Statistical significance was analyzed using Student's *t*-test (A and F) and two-way ANOVA with Bonferroni-Dunn *post hoc* tests (E and I). $^{*}P < 0.05$, $^{***}P < 0.001$.

(#ab51251, 1:1000, Abcam), mouse anti-ERK2 (D-2) (#sc-1647, 1:500, Santa Cruz Biotechnology), and rabbit anti-phospho-p44/42 MAPK (ERK1/2) (T202/Y204, T185/Y187) (#9101, 1:500, Cell Signaling Technology, MD, USA). The secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (#sc-2004, 1:1000, Santa Cruz Biotechnology) and alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (#sc-2008, 1:1000, Santa Cruz Biotechnology).

2.8. Data analysis and statistics

Statistical analysis was performed using Stat-View (SAS Institute, NC, USA). The quantified data were analyzed using Student's *t*-test, one-way ANOVA followed by Bonferroni-Dunn *post hoc* tests and two-way repeated-measures ANOVA followed by Bonferroni-Dunn *post hoc* tests.

3. Results

3.1. Temporal, regional and cell type-specific expression patterns of *Pkd2* in the brain

To reveal the function of PKD2 in the brain, we first analyzed the temporal and regional expression patterns of *Pkd2* mRNA in the mouse brain. *Pkd2* expression levels were elevated during embryonic neurogenesis between E10.5 and E16.5 and gradually decreased after birth (Fig. 1A). At E16.5, *Pkd2* was relatively highly expressed in the ventricular and subventricular zones (VZ/SVZ) of the cerebral cortex, where radial glial cells, embryonic neural stem cells (NSCs), differentiate into young neurons during embryonic cortical development (Fig. 1B). We then analyzed the *Pkd2* mRNA levels in NSCs and neurons and found that *Pkd2* expression was higher in NSCs than neurons (Fig. 1C). These expression patterns of *Pkd2* suggest that PKD2 is involved in cortical neuron development.

3.2. The effects of Pkd2 knockdown on cortical neuron development

Next, to investigate the role of PKD2 in embryonic neurogenesis, we knocked down the expression of *Pkd2* using shRNA (shPKD2; MISSION TRC shRNA library SP1, Sigma-Aldrich) and miR30-based shRNA (shRNA^{miR30}), both of which target *Pkd2*. The knockdown efficacies of shPKD2 and shPKD2^{miR30}, whose target sites are different from each other, were validated, and we found that these constructs effectively decreased the *Pkd2* mRNA level (Fig. 2A and F). Each shRNA vector together with a GFP expression construct was introduced into VZ cells by *in utero* electroporation at E14.5. Four

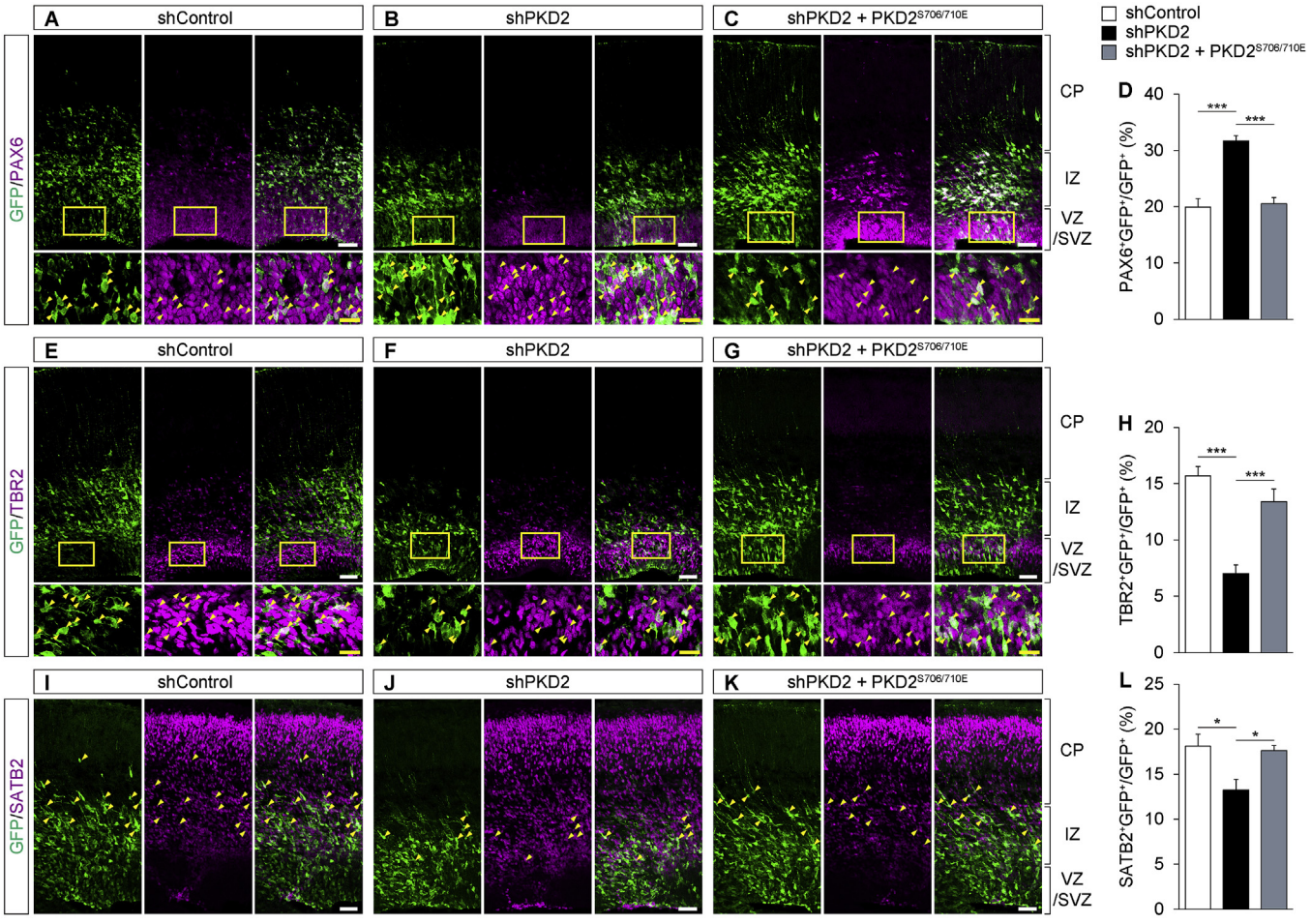

Fig. 3. The neuronal differentiation of NSCs is impaired by *Pkd2* knockdown in the mouse developing cerebral cortex. (A-C, E-G and I–K) Representative images of embryonic cortical sections at E16.5, 2 days after *in utero* electroporation with shControl (A, E and I), shPKD2 (B, F and J) or shPKD2 and PKD2$^{S706/710E}$ (C, G and K). Immunohistochemistry for GFP and PAX6 (A–C), TBR2 (E–G) or SATB2 (I–K) is shown. Yellow boxes indicate areas enlarged in the lower panels. Arrowheads indicate colabeled cells. White scale bars, 50 μm; yellow scale bars, 20 μm. CP, cortical plate; IZ, intermediate zone, VZ, ventricular zone; SVZ, subventricular zone. (D, H and L) Quantification of cell marker$^+$ GFP$^+$ cells as a percentage of total GFP$^+$ cells (each $n = 4$). Data are presented as the mean ± SEM. Statistical significance was analyzed using one-way ANOVA with Bonferroni-Dunn *post hoc* tests. $^*P < 0.05$, $^{***}P < 0.001$.

days later, we harvested the embryos, sectioned the brains and analyzed the migration of GFP$^+$ neurons. At E18.5, the migration of GFP$^+$ cells was impaired by MISSION shPKD2-mediated *Pkd2* knockdown (Fig. 2B, C and E). Overexpression of a kinase active mutant of PKD2 (PKD2$^{S706/710E}$) [28,29], but not WT-PKD2, rescued the impaired migration (Fig. 2D and E and data not shown). Likewise, knockdown of *Pkd2* by shPKD2^{miR30} also impaired the migration of GFP$^+$ cells (Fig. 2G–I). These results suggest that PKD2 regulates embryonic neuronal development in the cerebral cortex.

To further examine the function of PKD2 in neuronal differentiation, at E16.5, 2 days after *in utero* electroporation, we stained the brains of embryos with antibodies against PAX6 (NSC marker), TBR2 (intermediate progenitor cell marker) and SATB2 (neuron marker). With *Pkd2* knockdown, the proportion of GFP$^+$ PAX6$^+$ NSCs was increased (Fig. 3A, B and D). The proportions of GFP$^+$ TBR2$^+$ intermediate progenitor cells and GFP$^+$ SATB2$^+$ neurons were decreased and correlated with the increased NSCs (Fig. 3E, F, H, I, J and L). The altered proportion of each type of cell was rescued by overexpression of PKD2$^{S706/710E}$ (Fig. 3C, D, G, H, K and L). These results suggest that PKD2 regulates the differentiation of NSCs into neurons during embryonic neuron development.

3.3. The effects of ASD-related de novo mutations on the kinase activity of PKD2

Previously, we identified a *de novo* mutation, S183 N, within the protein kinase C conserved phorbol esters/diacylglycerol binding (C1a) domain of PKD2 from an ASD patient [26] (Fig. 4A). Another group also identified an ASD-related *de novo* mutation, R232C, between the C1a and C1b motifs [27] (Fig. 4A). To investigate the effect of the *de novo* mutations on the kinase activity of PKD2, we assessed the autophosphorylation levels at the S876 residue, a phosphorylation site related to the kinase activity of PKD2 [33]. We found that the S876 phosphorylation levels of S183N-mutated- and R232C-mutated-PKD2 were decreased compared to WT-PKD2 in HEK293T cells (Fig. 4B and C). Since previous reports have found that PKD2 is an upstream regulator of ERK [34,35], we next analyzed the phosphorylation levels of ERK in HEK293T cells expressing Myc-tagged WT-, S183N-mutated- and R232C-mutated-PKD2. We found that the phosphorylation levels of ERK were decreased in the lysates from HEK293T cells expressing the *de novo* mutated variants compared to WT-PKD2 (Fig. 4D and E), confirming that ASD-related *de novo* mutations impaired the kinase activity of PKD2.

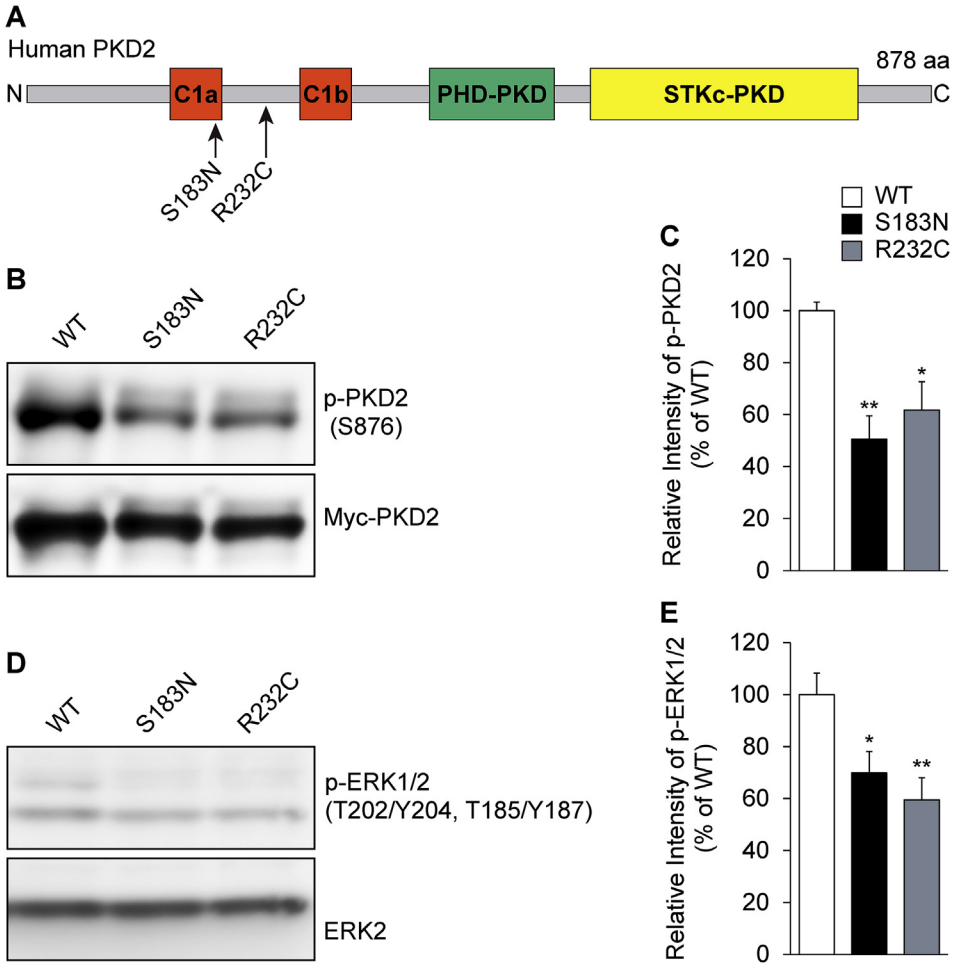

Fig. 4. The kinase activities of ASD-associated *de novo* mutated PKD2 variants are impaired. (A) Schematic image of human PKD2 protein (NP_057541.2) and ASD-associated *de novo* mutations. C1a and C1b, protein kinase C conserved phorbol esters/diacylglycerol binding domain; PH-PKD, pleckstrin homology domain; STKc-PKD, catalytic domain of the serine/threonine kinase. (B) Representative western blots of phosphorylated PKD2 (p-PKD2) (S876) and myc-tagged PKD2 (myc-PKD2). (C) Quantification of the relative band intensity of p-PKD2 (S876) normalized by each myc-tagged PKD2 level (each $n = 4$). (D) Representative western blots of p-ERK1/2 (T202/Y204 and T185/Y187) and ERK2. (E) Quantification of the relative band intensity of p-ERK1/2 normalized by each ERK2 level (each $n = 4$). Data are presented as the mean ± SEM. Statistical significance was analyzed using one-way ANOVA with Bonferroni-Dunn *post hoc* tests. $^*P < 0.05$, $^{**}P < 0.01$ (vs WT).

4. Discussion

Although previous studies have shown that PKD2 positively regulates retinoic acid-induced neuronal development in mouse neuroblastoma Neuro2a cells [26] and the establishment and maintenance of neuronal polarity in neurons [24,25], the role of PKD2 in neurogenesis is largely unknown. In this study, we found that PKD2 was highly expressed in the VZ/SVZ of the cerebral cortex during embryonic neurogenesis (Fig. 1). Our current results showed for the first time that PKD2 regulated the neuronal differentiation of NSCs and the migration of newborn neurons in the developing cerebral cortex (Figs. 2 and 3). Importantly, the ASD-associated *de novo* mutations impaired the kinase activity of PKD2 (Fig. 4). Since previous studies have identified an association between the disrupted development of the cerebral cortex and ASD [3–6], the impaired neuronal development caused by *de novo* mutations in PKD2 may be an underlying mechanism of ASD development.

ERK phosphorylation is a key event for neuronal development, such as neuronal differentiation [36,37] and migration [38,39]. ERK signaling regulates the balance between the self-renewal and neuronal differentiation of NSCs; while phosphorylated ERK activates the STAT3 pathway to induce neuronal differentiation [37,40]. Inhibition of ERK phosphorylation drives the Akt pathway to shift the balance toward the self-renewal of NSCs [41]. PKD2 may regulate embryonic cortical neuron development by changing the balance between the self-renewal and neuronal differentiation of NSCs through modulating ERK activity. Since a previous report found that the hippocampal structure of adult *Pkd2* knockout mice was virtually normal compared to that of WT mice [24], the delayed neuron development induced by *Pkd2* knockdown may catch up during development.

While overexpression of the kinase active variant of PKD2 ($PKD2^{S706/710E}$) rescued the impaired cortical neuron development (Figs. 2 and 3), overexpression of WT-PKD2 could not (data not shown). Although the precise underlying mechanism remains unknown, overexpressed mRNA might not be able to act as an endogenous mRNA encoding PKD2 in terms of mRNA localization in the cells, mRNA splicing events, etc. Alternatively, the amount of overexpressed PKD2 may not be enough to rescue the knockdown phenotype.

In summary, our current findings suggest that *de novo* mutations in *PKD2* may impair cortical neuron development, resulting in ASD development. In addition to *PKD2*, *de novo* mutations were identified in other PKD family members, such as *PKD1* and *PKD3*, as well as distantly related PKC family members, such as *PKCA*, *PKCB*, *PKCE, PKCQ* and *PKC2* [16,17,27,42–46] (Supplementary Table 1). In addition to genetic studies, recent molecular studies suggest that aberrant PKC signaling is involved in ASD [47–49]. Further analysis focusing on *de novo* mutations in PKD family members and distantly related PKC family members will provide novel insights into the molecular etiology and pathophysiology of ASD.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.09.048.

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