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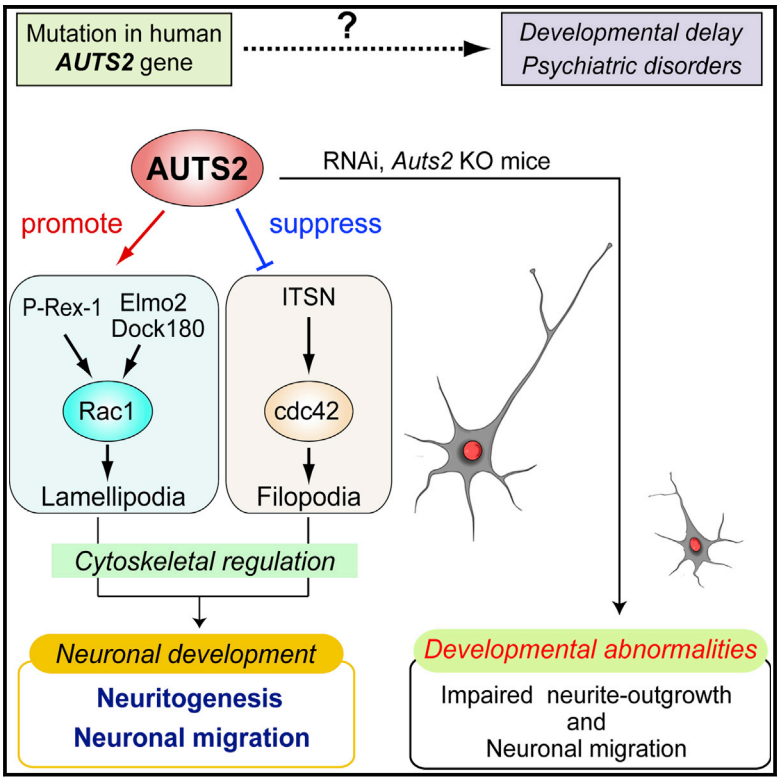
Cytoskeletal Regulation by AUTS2 in Neuronal Migration and Neuritogenesis

Graphical Abstract



Highlights

- AUTS2 localizes in both the nuclei and cytoplasm of neurons
- Cytoplasmic AUTS2 regulates Rho family GTPases to control actin dynamics
- AUTS2 is involved in neurite outgrowth and branch formation in neurons
- AUTS2 controls neuronal migration via Rac1 signaling pathway

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In Brief

Hori et al. demonstrate a role for cytoplasmic AUTS2 in the regulation of Rho family GTPases contributing to neural development. AUTS2 activates Rac1 to induce lamellipodia but downregulates Cdc42 to suppress filopodia. Loss-of-function experiments show that this AUTS2-Rac1 pathway is required for neuronal migration and neuritogenesis in the developing brain.

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Cytoskeletal Regulation by AUTS2 in Neuronal Migration and Neuritogenesis

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SUMMARY

Mutations in the *Autism susceptibility candidate 2 gene* (*AUTS2*), whose protein is believed to act in neuronal cell nuclei, have been associated with multiple psychiatric illnesses, including autism spectrum disorders, intellectual disability, and schizophrenia. Here we show that cytoplasmic AUTS2 is involved in the regulation of the cytoskeleton and neural development. Immunohistochemistry and fractionation studies show that AUTS2 localizes not only in nuclei, but also in the cytoplasm, including in the growth cones in the developing brain. AUTS2 activates Rac1 to induce lamellipodia but downregulates Cdc42 to suppress filopodia. Our loss-of-function and rescue experiments show that a cytoplasmic AUTS2-Rac1 pathway is involved in cortical neuronal migration and neuritogenesis in the developing brain. These findings suggest that cytoplasmic AUTS2 acts as a regulator of Rho family GTPases to contribute to brain development and give insight into the pathology of human psychiatric disorders with *AUTS2* mutations.

INTRODUCTION

The *Autism susceptibility candidate 2* (*AUTS2*) gene was originally associated with autism spectrum disorders (ASDs) in a study that described a de novo balanced translocation disrupting the *AUTS2* locus in a pair of monozygotic twins (Sultana et al., 2002). Thereafter, more than 30 unrelated patients with multiple neuropsychiatric disorders such as ASD, intellectual disability (ID), attention deficit hyperactivity disorder, dyslexia, and epilepsy as well as developmental delay (DD), visual impairment, and microcephaly have been shown to carry distinct heterozygous disruptions of the *AUTS2* locus (Bakkaloglu et al., 2008; Ben-David et al., 2011; Elia et al., 2010; Glessner et al., 2009; Huang et al., 2010; Kalscheuer et al., 2007; Pinto et al., 2010; Talkowski et al., 2012). In addition, 24 exonic microdeletions were identified in the *AUTS2* gene in individuals with various psychiatric disorders (Beunders et al., 2013). Moreover, very recent studies suggested that this gene is also associated with schizophrenia (Zhang et al., 2014; McCarthy et al., 2014). The wide spectrum of phenotypes observed in patients with *AUTS2* mutations suggests that this gene is not associated with a specific type of ASD but with a broader range of psychiatric disorders, thus highlighting *AUTS2* as a current gene of interest (Oksenberg and Ahituv, 2013).

AUTS2 encodes a relatively large protein (AUTS2 protein, 1,259 amino acids in human). In addition to two proline-rich regions (PR1 and PR2), AUTS2 possesses motifs that suggest nuclear localization, including a few putative nuclear localizing sequences, eight histidine repeats, and one predicted PY motif (Oksenberg and Ahituv, 2013; Salichs et al., 2009; Zhi and Chen, 2012) (Figure 2C). Moreover, immunostaining as well as forced expression of AUTS2 protein in cultured cells revealed nuclear localization of this protein (Bedogni et al., 2010b), which led many scientists to believe that AUTS2 functions in cell nuclei (Oksenberg and Ahituv, 2013).

In the developing mouse brain, *Auts2* is highly expressed in many regions such as the frontal cortex, hippocampus, and cerebellum (Bedogni et al., 2010a, 2010b; Lancaster et al., 2013), key areas for high order brain functions. However, little is known with regard to the physiological role of AUTS2, due to lack of functional analysis for this gene, except for a few studies in zebrafish. In zebrafish, *Auts2* is highly expressed in the brain during development (Beunders et al., 2013; Oksenberg et al., 2013), and knockdown of *Auts2* by morpholino studies resulted in microcephaly and reduction of neurons in embryos (Beunders et al., 2013; Oksenberg et al., 2013). While these results suggest that AUTS2 functions in cell proliferation, survival, and maturation of neurons in zebrafish, it did not explain the pathology of psychiatric patients with *AUTS2* mutations. Therefore, we suspected AUTS2 might have other functions in the mammalian brain, which might be more directly linked with the phenotypes of

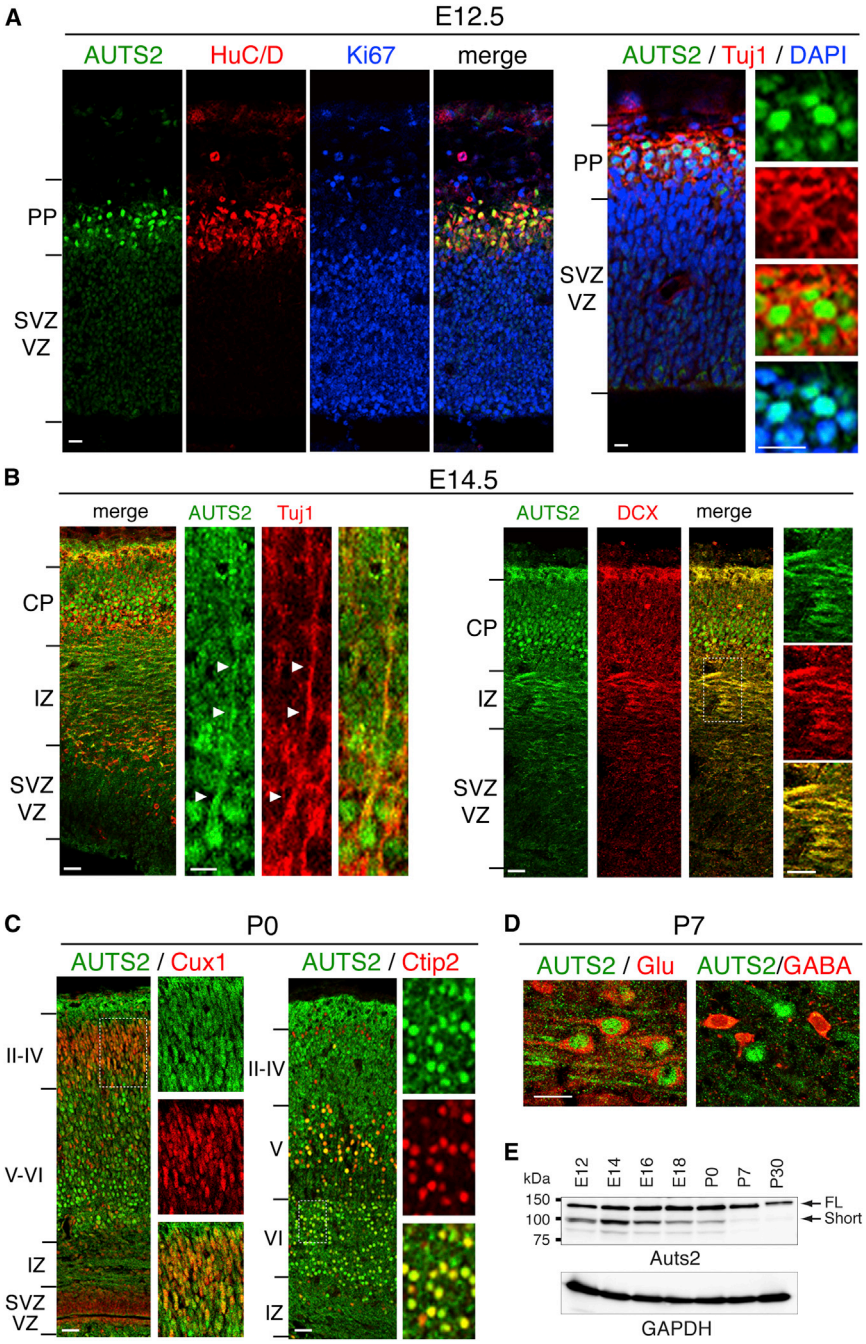

Figure 1. AUTS2 Expression in the Developing Cerebral Cortex

(A) Triple-immunofluorescent staining of AUTS2 with postmitotic neuronal markers HuC/D and Tuj1 or proliferating cell marker Ki67 in E12.5 mouse cerebral cortex.

(B) Immunostaining of E14.5 mouse cerebral cortex shows the colocalization of AUTS2 with Tuj1 in CP (arrowheads) and DCX in the IZ.

(C) Immunostaining for AUTS2 and the layer-specific marker Cux1 (upper layer; left) and Ctip2 (deep layer; right) at P0 mouse neocortex.

(D) In P7 mouse neocortex, AUTS2 is specifically expressed in glutaminase (Glu)-positive excitatory but not GABA-expressing inhibitory neurons.

(E) Expression of AUTS2 protein in brain development; 30 μg of neocortical lysates were blotted with anti-AUTS2 Ab.

PP, preplate; SVZ: sub-VZ. Scale represents 50 μm. See also Figure S1.

psychiatric disorders. To uncover the physiological roles of AUTS2 in brain development, we investigated its molecular properties in neurons in the developing cerebral cortex.

RESULTS

Localization of AUTS2 in the Brain

Western blotting analysis with an antibody against AUTS2 (1,016–1,116 amino acids) revealed that the expected full-length (FL) mouse AUTS2 protein (approximately 140 kDa) initially appears in the cerebral cortex at early embryonic stages (E12.5) and continues throughout development (Figure 1E). This antibody also detected an additional shorter band (~100 kDa), which was transiently expressed in early development, plateaued at E14.5, and was undetectable postnatally. The molecular weight for the short band matches that of a short isoform of AUTS2 predicted by UCSC (the University of California Santa Cruz) Genome bioinformatics. Immunohistochemistry of the developing mouse cerebral cortex at E12.5 showed that AUTS2 colocalized with HuC/D and Tuj1, markers for postmitotic neurons, but not with the proliferating cell marker Ki67 in the ventricular zone (VZ) (Figure 1A).

In early embryonic stages, AUTS2 is exclusively found in nuclei of preplate neurons (Figure 1A). At later stages, however, AUTS2 is also detected in neurites labeled by Tuj1 at the cortical plate (CP) as well as in doublecortin (DCX)-positive axonal tracts of projection neurons in the intermediate zone (IZ) (Figure 1B). Postnatally, AUTS2 is broadly expressed in the CP from deep- to upper-layer neurons (Figure 1C). We also found that AUTS2 is specifically expressed in glutamatergic neurons but not GABAergic inhibitory neurons in the cerebral cortex (Figure 1D). In the hippocampus, AUTS2 is expressed in glutamatergic neurons such as pyramidal neurons and granule cells, but not in GABAergic inhibitory neurons (data not shown).

Although previous studies implied that AUTS2 is a nuclear protein (Bedogni et al., 2010b; Sultana et al., 2002), our immunohistochemical observations suggested that AUTS2 localizes in both nuclear and extranuclear regions. To further characterize the

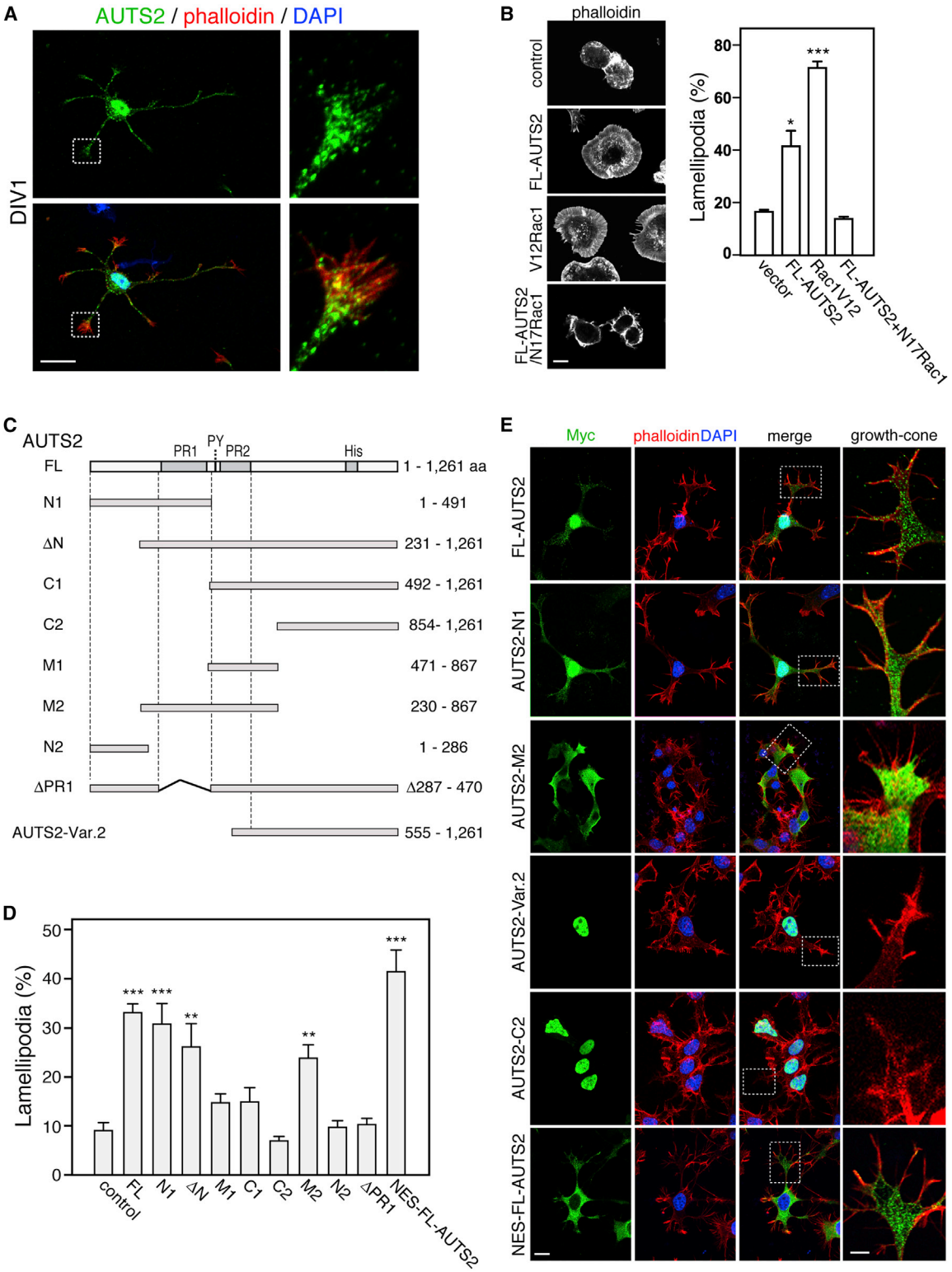

Figure 2. Rac1-Dependent Lamellipodia Formation by FL-AUTS2

(A) Immunocytochemistry of primary hippocampal neurons at DIV1 shows the localization of AUTS2 at growth cones.

(B) FL-AUTS2 induces lamellipodia in N1E-115 cells that is suppressed by coexpression of N17Rac1. Graph shows the percentage of cells with lamellipodia in total cells transfected.

(legend continued on next page)

subcellular localization of AUTS2, fractionated brain lysates were prepared from E18.5 mouse cerebral cortices. Immunoblot data revealed that FL-AUTS2 bands were present not only in nuclei but also in the cytoplasmic fraction as well as the cytoskeleton-containing insoluble fractions (Figure S1C). In contrast, the shorter band was exclusively found in the nucleic fraction (Figure S1C). This suggests that the FL-AUTS2 localizes in both nuclear and extranuclear subcellular regions in contrast with the nuclear-specific short isoform.

In primary cultured hippocampal neurons, AUTS2 was observed not only in the nuclei but also in neurites and cytoplasm (Figure 2A, left). In particular, AUTS2 appeared to accumulate at actin-rich growth cones (Figure 2A, right). Consistent results were observed in primary cortical neurons (data not shown). We also confirmed that another anti-AUTS2 antibody showed similar immunofluorescence patterns (data not shown, see Experimental Procedures). In the neuroblastoma N1E-115 cell line, punctate AUTS2 immunostaining was also detected in growth cones partially colocalized with Rac1, a member of the Rho family small GTPases (Figure S1A). Furthermore, the labeled AUTS2 immunoclusters were maintained after in situ extraction with Triton X-100 in N1E-115 cells, suggesting that AUTS2 is associated with the cytoskeleton (Figure S1B). This is consistent with the detection of FL-AUTS2 in the cytoskeleton-containing insoluble fractions (Figure S1C). These findings suggested that AUTS2, in particular the full-length isoform, may have a cytoplasmic role in cytoskeletal regulation, in addition to its nuclear function.

AUTS2 Regulates Rho-family GTPases

In the presence of serum, N1E-115 cells usually appear spherical in shape (Figure 2B). Overexpression of FL-AUTS2, however, dramatically induced lamellipodia, similar to cells expressing an active Rac1 (V12Rac1) (Figure 2B). In contrast, coexpression of FL-AUTS2 with a dominant-negative Rac1 (N17Rac1) suppressed AUTS2-dependent lamellipodial formation (Figure 2B). Furthermore, we observed the activation of endogenous Rac1 in FL-AUTS2-overexpressed N1E-115 cells using GTPase pull-down assays (Figure S2A; ΔN-STEF was used as positive control for activation for Rac1) (Matsuo et al., 2002). These results suggest that FL-AUTS2 acts as an upstream positive regulator of Rac1 in the regulation of actin cytoskeleton.

We next expressed a series of AUTS2 deletion mutant proteins in N1E-115 cells. Only mutant AUTS2 proteins containing the N-terminal PR1 region (288–471 amino acids) such as AUTS2-N1, AUTS2-ΔN, and AUTS2-M2 exhibited lamellipodia formation to a similar extent as FL-AUTS2 (Figures 2C and 2D), suggesting that the PR1 region is important for the regulation of actin remodeling. AUTS2 deletion mutants that lack a PR1 domain, including AUTS2-Var.2 (corresponding to a short isoform of AUTS2, as described below; see Figure S5), did not exhibit the lamellipodia-inducing ability.

We examined the subcellular localization of the recombinant AUTS2 and its deletion mutant proteins in differentiated N1E-115 cells. FL-AUTS2 is localized in the cytoplasm, including neurites and growth cones, as well as nuclei (Figure 2E), exhibiting immunostaining patterns similar to endogenous AUTS2 (Figures 2A, S1A, and S1B). The AUTS2-N1 mutant exhibited the same distribution patterns as FL-AUTS2, whereas the AUTS2 short-form variant (lacking the N terminus as described below; AUTS2 variant 2; see Figure S5) and AUTS2-C2 were exclusively nuclear (Figure 2E). Interestingly, the AUTS2-M2 mutant, which is a minimal fragment for lamellipodia induction, was mainly cytoplasmic, with some slight nuclear localization observed (Figure 2E). To further investigate the cytoplasmic function of AUTS2, we fused a nuclear export sequence (NES) derived from MAPKK at the N terminus of FL-AUTS2 (NES-FL-AUTS2). We found that NES-FL-AUTS2 is exclusively localized in the cytoplasm of N1E-115 cells (Figure 2E). NES-FL-AUTS2 exhibited greater lamellipodia-inducing activity compared with FL-AUTS2 in N1E-115 cells (Figure 2D, $p = 0.04$ versus FL-AUTS2). These findings revealed a correlation of "lamellipodia-inducing ability" and "cytoplasm localizing ability" among the mutant proteins and therefore suggest that cytoplasmic AUTS2 may induce lamellipodia via activation of Rac1. Furthermore, these results imply that PR1 is a critical region for Rac1 activation and cytoplasmic localization.

It has been reported that Rac1 is activated by a variety of guanine nucleotide-exchange factors (GEFs), including STEF, Tiam1, P-Rex1, and Dock180/Elmo2 complex (Buchsbaum, 2007; Matsuo et al., 2003; Yoshizawa et al., 2005). While DN forms for P-Rex1 (ΔDH-P-Rex1), Elmo2 (Elmo2-ΔN), and Dock180 (Dock180-ISP) suppressed FL-AUTS2-induced lamellipodia in N1E-115 cells, DN forms for STEF and Tiam1 (STEF-PHnTss) did not (Figure S2B) (Katoh and Negishi, 2003; Matsuo et al., 2002; Yoshizawa et al., 2005). Furthermore, we confirmed by immunoprecipitation experiments that FL-AUTS2 interacts with P-Rex1 and the Elmo2/Dock180 complex (Figures 3D and 3E), but not STEF or Tiam1 (data not shown). These results suggest that FL-AUTS2 can activate Rac1 via interaction with P-Rex1 and the Elmo2/Dock180 complex to regulate actin dynamics in N1E-115 cells.

As was observed in N1E-115 cells, we found that introduction of FL-AUTS2 caused an increase in the Rac1 activity in primary hippocampal neurons (Figure 3A). We previously demonstrated that the activation of the Rac1 signaling pathway in primary hippocampal neurons promotes neurite outgrowth (Nishimura et al., 2005). Consistently, at 4 days in vitro (DIV4), primary hippocampal neurons transfected with FL-AUTS2 had longer neurites compared with the control neurons; this could be suppressed

(C and D) Determination of the functional domain of AUTS2 for lamellipodia formation. (C) Schematic diagram of mouse FL-AUTS2 and the deletion mutant constructs. PR, proline-rich region; PY, PY motif; His, eight histidine repeats. (D) N1E-115 cells were transfected with indicated plasmids. Cells with lamellipodia scored as a percentage of the total number of transfected cells ($n > 500$ cells from three to six independent experiments for each construct).

(E) Subcellular localization of Myc-tagged AUTS2 variants and its truncated mutants in the differentiated N1E-115 cells. AUTS2-Var.2, AUTS2 short-form variant 2 (see Figure S3F).

Data are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, t test. Scale bar represents 20 μm (A, B, and E) and 5 μm (E, high magnifications). See also Figures S1 and S2.

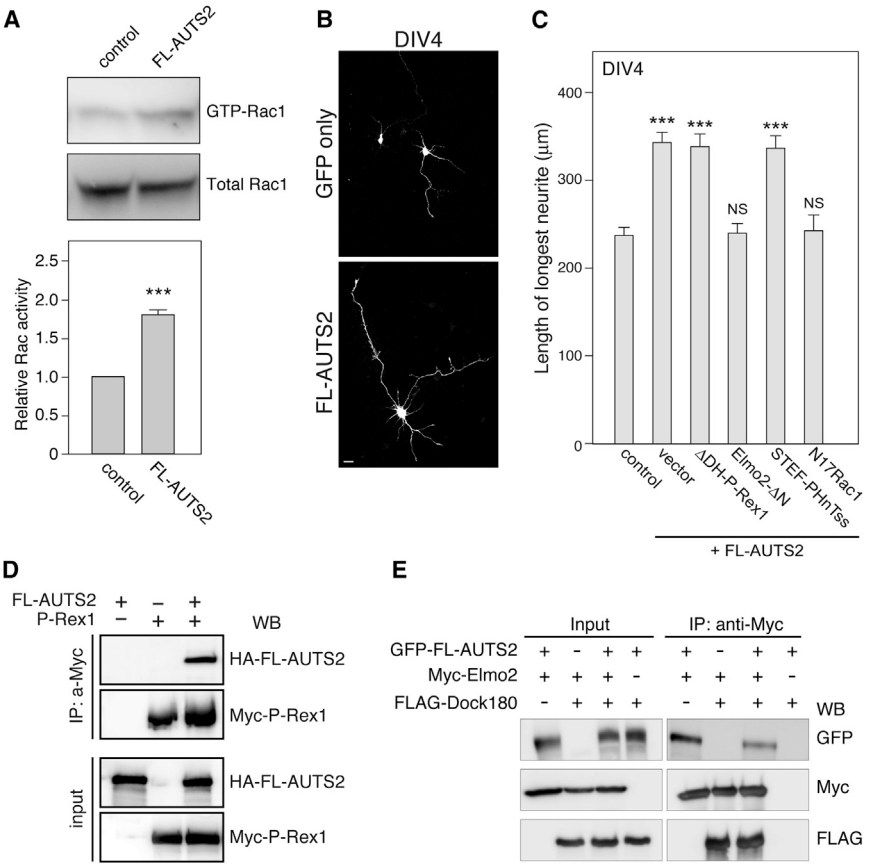

Figure 3. FL-AUTS2 Induces Neurite Elongation via Activating Rac1

(A) Rac1 activity in primary cultured cortical neurons at DIV7, transduced with control or FL-AUTS2 expression plasmid, was analyzed by GST-CRIB pull-down assay.

(B) Representative images of primary hippocampal neurons at DIV4 coelectroporated with control or FL-AUTS2 plus GFP expression vector.

(C) Effects of the dominant-negative forms for Rac1 and its upstream regulators on AUTS2-induced neurite outgrowth. The length of the longest neurite was measured in primary cultured hippocampal neurons transfected with the indicated vectors at DIV4 (n = 80∼100 neurons).

(D and E) Interaction of AUTS2 and Rac-GEFs. HEK293T cell lysates transfected with the indicated plasmids were immunoprecipitated with an antibody against Myc. The precipitants were analyzed with anti-GFP, Myc, HA, and FLAG antibodies, respectively.

All graphs are mean ± SEM of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, t test. Scale bar represents 20 μm. See also Figure S2.

by cointroduction of DN-Rac1 (Figures 3B and 3C). Furthermore, this neurite outgrowth-inducing ability of FL-AUTS2 was blocked by cotransfection of the DN form for Elmo2 but not by that for P-Rex1 or STEF/Tiam1 (Figure 3C). These findings suggest that FL-AUTS2 can induce neurite outgrowth of primary hippocampal neurons through the activation of the Elmo2/Dock180-Rac1 pathway.

Physiological Roles of AUTS2 in Neuritogenesis and Neuronal Migration

We prepared shRNA constructs specifically targeting *FL-Auts2* mRNA (Figure S5F) that effectively downregulated expression of FL-AUTS2 but not the short isoform (Figures S3A and S3B). The shRNA and GFP expression vectors were cointroduced into mouse embryonic brains by in utero electroporation (Kawauchi et al., 2003) at E15.5, and dissociated cortical neurons were prepared at E16.5 and cultured for DIV2 to DIV6. In the *Auts2* shRNA-expressing neurons, the length and branch number of axons and dendrites were dramatically reduced (Figures 4A and 4B). The impairment of neurite formation was, however, restored by the expression of the shRNA-resistant FL-AUTS2 ($FL\text{-}AUTS2^{R}$), indicating that such a defect is the result of specific knockdown of *Auts2*. The impairment of neurite formation by *Auts2* knockdown was also reversed by WT Rac1 (Figure 4B). Together with the fact that AUTS2 has the ability to promote neurite extension by activating Rac1 (Figure 3C), these results suggest that AUTS2 regulates neuritogenesis via activation of Rac1 signaling.

Moreover, all GFP-positive cells showed immunoreactivity with Tuj1, a neuronal marker, indicating that misspecification to other cell types such as glial cells by *Auts2*-silencing does not occur (data not shown). Similarly, introduction of short interfering RNAs (siRNAs) resulted in inhibition of neurite outgrowth in N1E-115 cells that normally elongate neurites in serum-free media (Figure 4C). These findings suggest that AUTS2 is required for neurite extension and branch formation.

Interestingly, in both primary cortical neurons and N1E-115 cells where *Auts2* was knocked down, filopodia-like protrusions were extensively formed at the cell periphery and neurites (Figures 4D and 4E). Filopodia formation in those cells was effectively suppressed by introduction of siRNA for another Rho-family GTPase, Cdc42 (Figures 4F and S3B), a well-known key molecule for filopodial formation (Etienne-Manneville and Hall, 2002). Furthermore, introduction of siRNA for either (or both) Intersectin 1 (ITSN 1) and ITSN 2, which are GEFs specific for Cdc42 (Hunter et al., 2013), also suppressed the filopodia formation induced by *Auts2* knockdown (Figures S3C and S3D). In addition, GTPase pull-down assays revealed upregulation of Cdc42 activity in the *Auts2*-knockdown N1E-115 cells (Figure 4G). These results suggest that AUTS2 acts as an inhibitory factor against Cdc42 signaling for filopodia formation, presumably via suppression of ITSN 1 and ITSN 2. Thus, this molecule seems to have the very unique property of inversely regulating the activities of Rac1 and Cdc42. However, because the physiological relevance of AUTS2 in suppressing Cdc42 is unclear as described below, we do not focus on the AUTS2-Cdc42 pathway in this manuscript.

We then introduced *Auts2* shRNA with a GFP indicator into mouse embryonic cerebral cortices at E14.5 by in utero electroporation. At P0, in control brains, more than 80% of neurons have migrated into layers II–IV of the CP (Figure 5A). However, the majority of the shRNA-expressing cells were observed to exhibit a drastic migratory defect (Figure 5A). This abnormality could be rescued by not only FL-AUTS2 but also by NES-FL-AUTS2 (Figure 5A). These facts indicate that cytoplasmic AUTS2 is involved in cortical neuronal migration.

During the early migration process from lower IZ (loIZ) to upper IZ (uIZ) at E17.5, cortical neurons usually transform from multipolar to spindle-like bipolar shapes with a long leading process extending along radial glial fibers (Noctor et al., 2004; Tabata and Nakajima, 2003) (arrows in Figure 5B). However, in the shRNA-expressing neurons in uIZ and CP, the leading process appeared twisted and irregular with branching (arrowheads in Figure 5B). It has been reported that Rac1 not only regulates actin cytoskeleton but also activates JNK in migrating neurons in the IZ (Kawauchi et al., 2003). While activated JNK (phosphorylated-JNK) was strongly observed in migrating neurons in the IZ of control cortices at E17.5, it was almost completely absent in shRNA-introduced neurons (Figure 5C), suggesting that Rac1 signaling is downregulated in those migrating neurons, although we cannot exclude the possibility that AUTS2 may regulate the activation of JNK via the Rac1-independent signaling pathway.

As P-Rex1 was previously found to participate in cortical neuronal migration by activating Rac1 (Yoshizawa et al., 2005), and because AUTS2 can activate Rac1 via P-Rex1 (Figure S2B), these findings may imply that in migrating neurons AUTS2 activates the P-Rex1-Rac1 pathway to elicit normal cortical neuronal migration.

We subsequently performed coimmunostaining of GFP with cortical layer-specific markers in the shRNA-introduced brain sections. Almost all the shRNA-expressing cells in the deep-layer expressed Cux1, a marker for upper cortical layer neurons (Figures 5D and S3F). In contrast, we did not find any GFP-positive cells expressing Ctip2, a marker for deep-cortical layer neurons in the brain sections electroporated with the scramble or *Auts2* shRNA (Figure S3E; n = 400 cells were counted from four embryos). Moreover, there was no colocalization of shRNA-expressing cells with cleaved caspase 3, an apoptotic cell death marker (data not shown). These findings suggest that knockdown of *Auts2* affected neither neuronal layer identity nor cell survival.

Generation of Mouse *Auts2* Mutants

To genetically investigate the role of AUTS2 in vivo, we engineered knockout (KO) mice in which exon 8 of the *Auts2* gene can be removed by Cre-recombinase activity, creating a frameshift mutation that would disrupt coding for the downstream portion of AUTS2 containing the PR1 region (Figures S4A and S5A). Information from various databases and previous papers indicated that the shorter AUTS2 band likely corresponded to a splicing variant (variant 1; Figure S5F) originating from a translational start site (ATG) in the middle of exon 8. We predicted that this targeting strategy would knock out both FL-*Auts2* and the short isoforms. Surprisingly, we found that both FL and shorter AUTS2 proteins were almost completely eliminated in homozygotes of $Auts2^{neo}$ ($Auts2^{neo/neo}$; Figure S5C), which still possessed exon 8 plus the *pgk-neo* selection cassette in the genome (Figure S4A). Consistent with this, we could barely detect AUTS2 protein by immunohistochemistry in the $Auts2^{neo/neo}$ brains (Figure S5D). AUTS2 was decreased by ~50% in heterozygotes compared with WT mice (Figure S5C). The reduction of AUTS2 in this allele is likely caused by insertion of the *pgk-neo* cassette into intron 9 because excision of the cassette by FLP recombinase ($Auts2^{flox}$ allele) rescued the expression of AUTS2 (Figures S4A and S5C). Therefore, we used $Auts2^{neo}$ as a strong loss of function allele for *Auts2* in this study.

Next we generated an $Auts2^{del8}$ allele, which lacks exon 8, by crossing $Auts2^{flox}$ mice with Cre recombinase mice (CAG-Cre; Figures S4A and S5B). As expected, the FL-AUTS2 was completely eliminated in the homozygous brain ($Auts2^{del8/del8}$; Figure S5C). However, the expression level of an AUTS2 short isoform was alternatively increased, which was also observed in heterozygotes ($Auts2^{del8/+}$; Figure S5C). Beunders et al. (2013) recently identified a short 3′ human *AUTS2* mRNA transcript that originates from the middle of exon 9. We confirmed by 5′ rapid amplification of cDNA ends analysis (5′RACE) the existence of a similar transcriptional start site in exon 9 (variant 2) in the mouse brain, in addition to the previously predicted start site in exon 7 (variant 1; Figures S5E–S5G). According to our experimental design (Figure S4A), the $Auts2^{del8}$ allele should express variant 2 but not variant 1, suggesting that the strongly detected shorter band probably corresponds to variant 2. We suspect that loss of the FL *Auts2* may lead to compensatory enhanced expression of the short variant 2, although we lack direct evidence.

Developmental Abnormalities in *Auts2* Mutants

Because homozygotes of both lines ($Auts2^{neo/neo}$ and $Auts2^{del8/del8}$) were neonatally lethal, we examined their embryonic brains. There were no gross morphological or histological differences between brains from $Auts2^{neo}$ or $Auts2^{del8}$ mutants and WT embryos (Figure S4B, data not shown). A previous study demonstrated the involvement of *Auts2* in the regulation of cell proliferation and neuronal differentiation in zebrafish (Beunders et al., 2013). To determine whether *Auts2* deficiency in mice also affects these developmental events, we examined the cell cycle exit rate in the developing cerebral cortices of *Auts2* mutant mice. E15.5 brain sections were prepared 24 hr after pulse labeling with 5-ethynyl-2′-deoxyuridine (EdU) and costained with Ki67 (Figure S4C). The number of cells that remain in progenitor state in the VZ was quantified by counting the $EdU^{+}Ki67^{+}$ cells. As shown in Figures S4D and S4E, there was no significant difference in the rate of cell cycle exit or the thickness of VZ between WT and *Auts2* mutant mice ($Auts2^{neo/neo}$ or $Auts2^{del8/del8}$). Together with the observation that AUTS2 is not expressed in proliferating cells in the VZ (Figures 1A and 1B), these findings suggest that AUTS2 is not involved in the proliferation of progenitor cells in the mouse cerebral cortex.

To estimate neuronal migration, pregnant mice with E14.5 embryos were injected with bromodeoxyuridine (BrdU), and embryos were harvested at E18.5. We found that a significant proportion of the BrdU-positive cells abnormally remained in

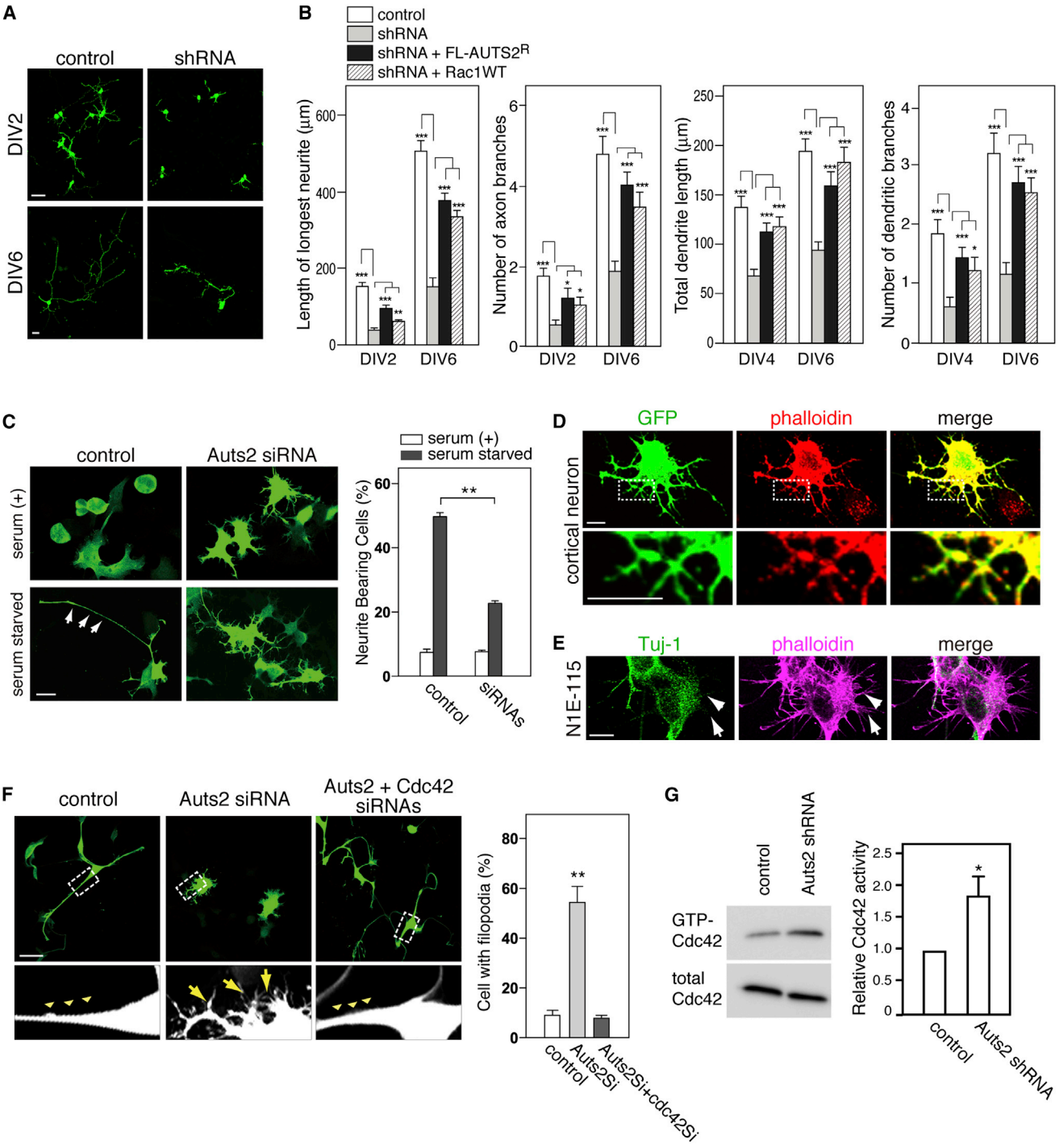

Figure 4. Knockdown of *Auts2* Affects Neural Morphogenesis

(A) Primary cortical neurons cotransfected with *Auts2*-shRNA or control-shRNA and GFP vector.

(B) The measurement of neurite length and number of branching points of individual neurons (n = 30 neurons). Expression of the shRNA-resistant FL-AUTS2 (FL-AUTS2^{R}) or Rac1-WT restores the impairment of neurite formation in *Auts2*-deficient neurons.

(C) Knockdown of *Auts2* with siRNA suppresses the neurite elongation of N1E-115 cells induced by serum starvation. Cells with neurites were scored as a percentage of the total number of transfected cells (n > 500 cells from three independent experiments).

(D and E) *Auts2* knockdown induces F actin (phalloidin) but not tubulin (Tuj-1)-based filopodia-like protrusions (white arrows) in primary cortical neurons (D) at DIV2 and N1E-115 cells (E).

(legend continued on next page)

the deep layer of *Auts2*^{neo} heterozygous and homozygous mutant brains at E18.5 in a gene dose-dependent manner (Figure S6), suggesting that *Auts2* is involved in cortical neuronal migration. Cerebral cortices of *Auts2*^{del8} mutants electroporated in utero with a GFP vector at E14.5 were subsequently examined at E18.5 for the distribution of GFP-positive neurons. In WT cortices, a large portion of GFP-positive neurons reached layers II–IV within 4 days of labeling, whereas a considerable number of neurons exhibited migration defects in *Auts2*$^{del8/+}$and *Auts2*$^{del8/del8}$ mutant brains in a gene dosage-dependent manner (Figure 6A). Because the short isoform of AUTS2 (variant 2) is maintained in the homozygous *Auts2*$^{del8/del8}$ brains (Figure S5C), this result indicates that FL-AUTS2, but not variant 2, is involved in cortical neuronal migration. Accordingly, this phenotype was clearly rescued by electroporation of a FL-AUTS2 vector (Figure 6A).

To further investigate the physiological significance of AUTS2-Rac1 signaling in neuronal migration, we performed the coelectroporation of a Cre recombinase vector into *Auts2*$^{flox/flox}$ brains with a series of AUTS2 deletion constructs and small GTPase expression vectors. Consistent with the results observed in the *Auts2*$^{del8/del8}$ brains, the acute deletion of Auts2 by Cre recombinase in *Auts2*$^{flox/flox}$ brains recapitulated neuronal migration defects, as expected. Interestingly, this abnormality was rescued not only by FL-AUTS2 but also WT-Rac1 (Figure 6B), indicating that the AUTS2-Rac1 pathway is involved in cortical neuronal migration. Consistently, a short AUTS2-M2 mutant protein that can induce lamellipodia is also able to rescue the migration defects, while AUTS2-ΔPR1, lacking the lamellipodia-inducing ability, failed to rescue the phenotype (Figure 6B). The migration phenotype was not rescued by expression of the DN form for Cdc42 (N17Cdc42) (Figure 6B). This implies that the inhibitory effect of AUTS2 against Cdc42 does not play an important role in the regulation of neuronal migration.

Next, we examined the contralateral projecting axons of layer II/III cortical neurons of embryos electroporated at E14.5. In E18.5 WT brains, GFP-labeled axons were elongated and found to cross the midline to reach the contralateral cortex. In contrast, *Auts2*$^{del8/+}$ and *Auts2*$^{del8/del8}$ mutant brains exhibited severe impairment of axonal elongation in a gene-dosage dependent manner (Figure 7A), suggesting that the FL-AUTS2 is required for elongation of the cortical commissural axons. The axonal elongation defects were also observed in *Auts2*$^{flox/flox}$ brains electroporated with the Cre-expression vector (Figure S7). Consistent with the neuronal migration results in Figure 6B, this abnormality was rescued by FL-AUTS2, AUTS2-M2, and WT-Rac1, but not by AUTS2-ΔPR1 or DN-Cdc42 (Figure S7). These findings strongly suggest that AUTS2 activation of Rac1 is involved in cortical axonal elongation in vivo.

Next, cortical neurons of embryonic brains were labeled with GFP at E15.5 by in utero electroporation and harvested at E16.5. When cultured in dissociated cell culture in vitro for 6 days, the length and branch number of putative axons and dendrites in GFP-positive neurons were significantly reduced in *Auts2*$^{del8/+}$ and *Auts2*$^{del8/del8}$ in a gene dose-dependent manner (Figure 7B), confirming that AUTS2 is required for neuritogenesis in a cell autonomous manner.

DISCUSSION

Although the AUTS2 protein is generally believed to reside and function in cell nuclei, we found that AUTS2 also localizes extranuclearly, in regions such as the cytoplasm of processes and cell bodies of neurons, as a regulator of Rho family small G protein signaling. In our protein fractionation study, we detected a considerable amount of FL-AUTS2 in the cytoplasmic as well as the cytoskeleton-containing insoluble fractions, while the shorter isoform was found only in the nucleic fraction. Immunohistochemical staining of the developing brain revealed that AUTS2 was present not only in nuclei but also in the cytoplasm and neurites, especially at the growth cone, partially colocalized with Rac1. Furthermore, in situ extraction experiments revealed that AUTS2 is associated with the cytoskeleton. These results suggest that the AUTS2 protein, at least FL-AUTS2, is not always predominantly nuclear but resides in extranuclear regions of neurons.

We showed that FL-AUTS2 could activate Rac1 to induce lamellipodia and neurite extension in N1E-115 cells and primary hippocampal neurons, respectively. The former ability is believed to be transmitted via P-Rex1 and Elmo/Dock180, while the latter is via Elmo/Dock180. In addition, AUTS2 appears to downregulate Cdc42 activity via suppression of INST1 and INST2.

Cdc42 and Rac1 activities have been shown to be similarly regulated by sequential activation pathways from Cdc42 to Rac1 (Gonzalez-Billault et al., 2012). In contrast, AUTS2 uniquely regulates Rac1 and Cdc42 activities in opposing directions in neuronal cells. We suspect that this protein might be involved in the fine tuning of morphological changes of specialized parts in neurons, such as processes, where precise, fine, and distinct control of Rac1 and Cdc42 activities as well as actin dynamics may be required. However, although we have uncovered some of the roles of AUTS2-Rac1 in neuronal migration and neuritogenesis, the physiological relevance of the AUTS2-Cdc42 pathway remains to be determined.

Using protein deletion studies, we analyzed the relationship between the protein structure, Rac1-activating/lamellipodia-inducing ability, and the subcellular localization. Interestingly, we found that the lamellipodia-inducing ability was restricted to deletion proteins with cytoplasmic-localizing ability. Moreover, NES-FL-AUTS2, an exclusively cytoplasmic-localizing FL-AUTS2, possesses lamellipodia-inducing ability. These findings strongly indicate that AUTS2 can function extranuclearly to induce cytoskeletal reorganization via Rac1 activation. Our

(F) Simultaneous knockdown of *Auts2* and Cdc42 suppresses the filopodia formation in N1E-115 cells. Graph shows the percentage of cells with filopodia, with total number of transfected cells indicated by GFP (n = 3, more than 500 cells per experiment).

(G) Lysates from N1E-115 cells transfected with indicated constructs were incubated with GST-CRIB, and bound Cdc42 was detected with an antibody against Cdc42 (n = 3).

All graphs are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, t test. Scale bars represent (A, C, and F) 20 μm and (D) 10 μm. See also Figure S3.

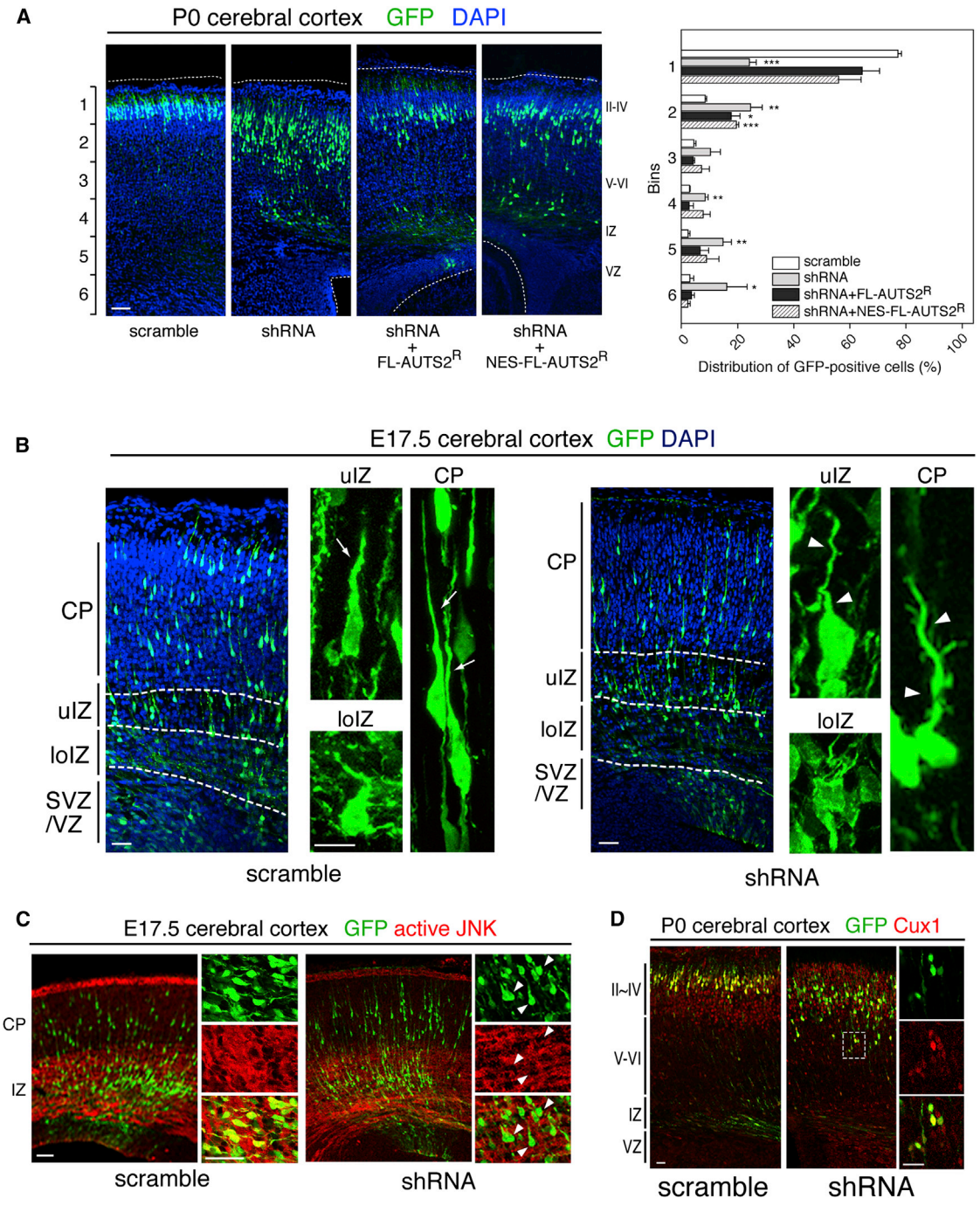

Figure 5. AUTS2 Is Necessary for Neocortical Neuronal Migration

(A) Cortical neuronal migration is impaired by knockdown of *Auts2* with shRNA construct in P0 brains. This defect is rescued by expressing FL-AUTS2^R and NES-FL-AUTS2^R. Distribution of GFP-positive cells in each region is presented by the percentage of total number of GFP-positive cells (n = 4 embryos).

(B) Effects of *Auts2* knockdown on morphology of migrating neurons in E17.5 brain sections.

(C) Knockdown of *Auts2* in cortical neurons suppressed the activation of JNK in IZ of E17.5 brains.

(D) The effect of *Auts2* shRNA in neocortex at P0 as shown by immunostaining with anti-Cux1 antibody. All electroporation with indicated plasmids was performed at E14.5.

Data are presented as the means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, t test. Scale bar represents 50 μm. See also Figure S3.

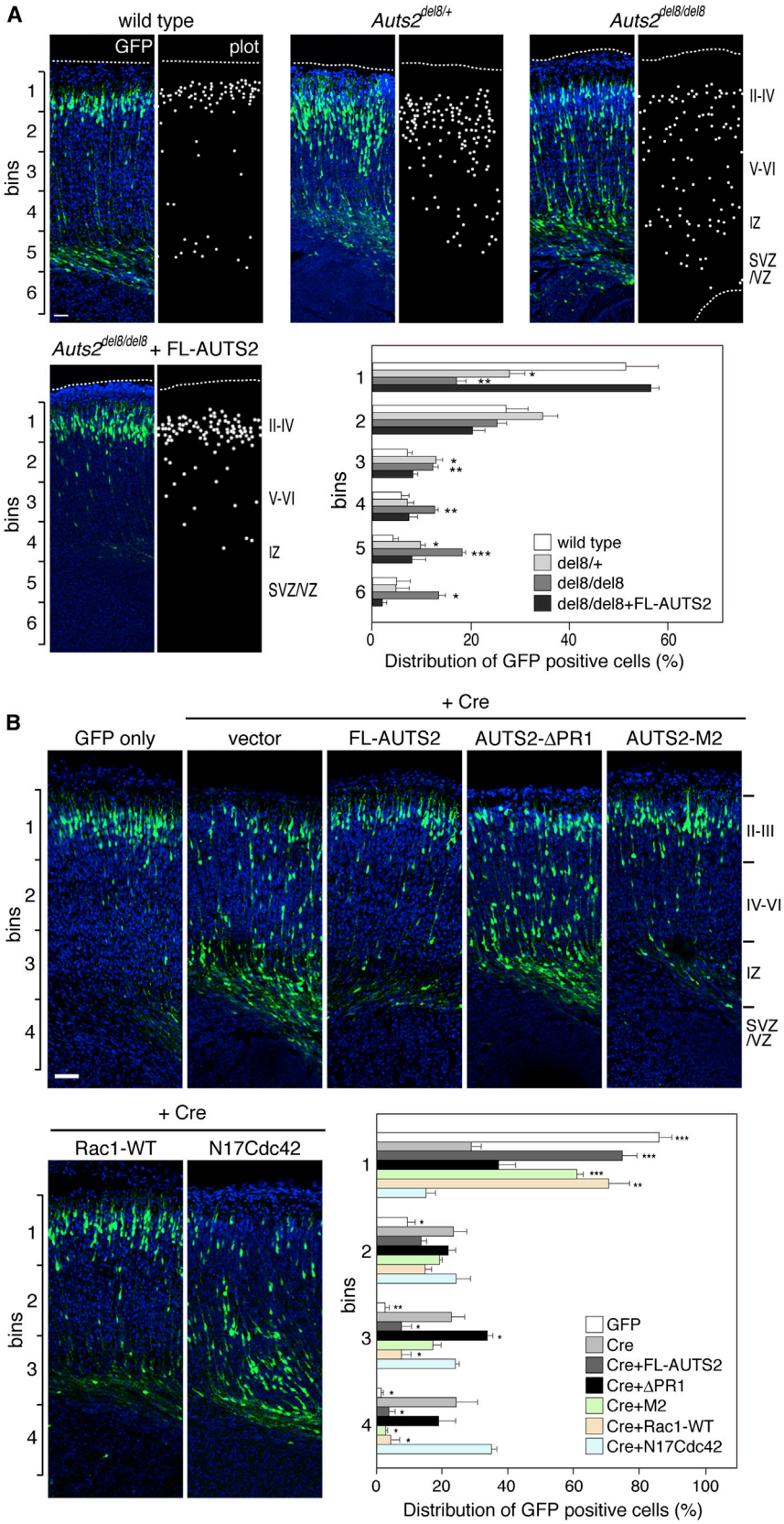

Figure 6. Impairment of Cortical Neuronal Migration in *Auts2*-Deficient Mice

(A) Cortical brain sections at E18.5 prepared from WT and *Auts2*^{del8} mutant mice electroporated with GFP vector (upper) or coelectroporated with GFP and FL-AUTS2 expression vectors at E14.5 (lower). Nuclei of GFP-positive cells plotted with white dots are shown in each right panel. The graph shows the percentage of GFP$^+$ cells in each of six bins versus the total number of GFP$^+$ cells.

(B) Cortical brain sections of E18.5 *Auts2*$^{flox/flox}$ mice electroporated with GFP and/or Cre recombinase at E14.5 show that acute KO of *Auts2* results in a defect in neuronal migration. Coexpression of FL-AUTS2, AUTS2-M2, or Rac1-WT, but not AUTS2-ΔPR1 or N17Cdc42 can rescue this defect. Graph shows the percentage of GFP$^+$ cells in each of four bins versus the total number of GFP$^+$ cells.

Data are presented as the mean ± SEM (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001 versus control (A) and Cre (B), t test. Scale bar represents 50 μm. See also Figures S5 and S6.

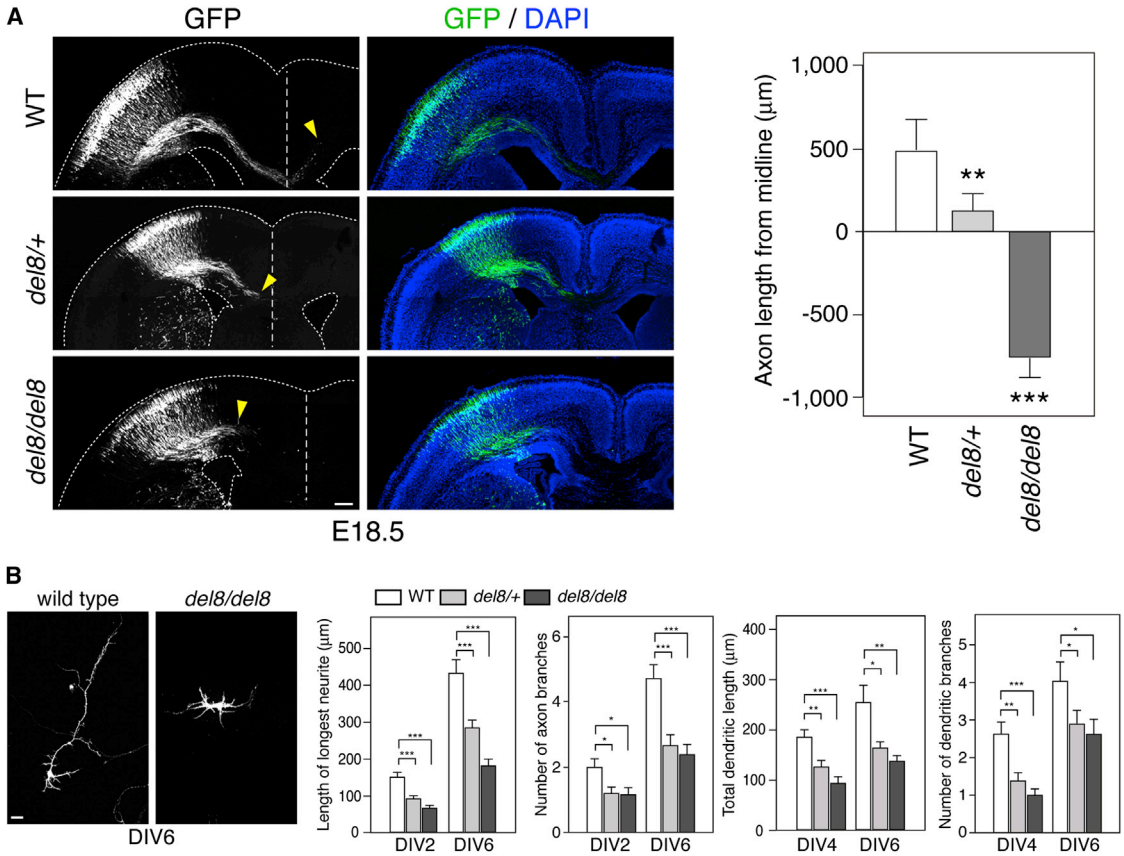

Figure 7. Loss of *Auts2* Impairs Axon Elongation In Vivo

(A) Contralateral axons projecting from layer II/III neurons in WT and *Auts2*^{del8} mutant cerebral cortex at E18.5. Visualization of GFP fluorescence is from vectors electroporated at E14.5. Yellow arrowheads indicate tips of axonal fibers. The photos were taken with a longer exposure period than the ones in Figure 6 in order to obtain stronger immunofluorescent signals at extending axons. Graph shows the distance from the tip of axons to midline along the corpus callosum (n = 4).
(B) Primary cortical neurons from WT and *Auts2*^{del8} mutant brains electroporated with GFP vector at E15.5. The graph shows the neurite length and the number of branching points of WT and *Auts2*^{del8} mutant individual cortical neuron (each of n > 30 neurons from multiple embryos).
Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, t test. Scale bars represent (A) 50 μm and (B) 20 μm. See also Figure S7.

protein deletion analyses revealed the PR1 domain to be the essential region for cytoplasmic localization and Rac1 activation.

In this study, we demonstrated that AUTS2 physiologically functions in cytoplasm for neuronal migration and neuritogenesis via activation of Rac1. In the loss-of-function experiments using shRNA in animals and *Auts2* mutants (*Auts2*^{neo}, *Auts2*^{del8}, *Auts2*^{flox}+Cre), cortical neuronal migration was significantly disrupted. As this abnormal migration was rescued by NES-FL-AUTS2, it suggests that cytoplasmic AUTS2 is involved in cortical neuronal migration. Furthermore, as this abnormality could also be rescued by Rac1, it suggests that the AUTS2-Rac1 pathway participates in this developmental process. Similarly, in the loss-of-function studies in vivo and in vitro, neurite extension and branch formation were impaired, which were also rescued by Rac1. This suggests that the AUTS2-Rac1 pathway is also physiologically involved in neuritogenesis. As previous studies have demonstrated, a variety of Rac-GEFs, including P-Rex1, STEF, Tiam1, and Elmo2/Dock180 complex, are involved in Rac1 activation during neural development (Buchsbaum, 2007; Matsuo et al., 2003; Yoshizawa et al., 2005). We found in this study that AUTS2 is involved in Rac1 activation via P-Rex1 and the Elmo2/Dock180 complex, but not STEF or Tiam1, for the lamellipodia formation in N1E-115 cells. However, the enhancement of neurite elongation in primary neurons by AUTS2 expression is specifically mediated by the Elmo2/Dock180 complex. These results suggested that several Rac-GEFs differentially or cooperatively participate in Rac1 activation to promote neuronal migration and neurite outgrowth. Interestingly, Yang et al. (2012) previously reported that overexpression of WT-Rac1 dramatically promoted cortical neuronal migration. Taken together, these results suggest that overexpression of WT-Rac1 increases the basal level of the activated-form of Rac1 that is likely mediated through AUTS2-independent Rac-signaling pathways such as STEF and Tiam1 and that it may compensate for the downregulation of Rac1 activation by disruption of AUTS2-Rac1 signaling pathways in *Auts2*-deficient neurons for neuronal migration and neurite outgrowth. On the other hand, neither impaired migration nor axonogenesis could be rescued by DN-Cdc42, implying that the Cdc42-suppressing ability of AUTS2 does not physiologically play an important role in neuronal migration or neuritogenesis.

In the *Auts2* knockdown brains, we realized that the twisted and irregular morphology of the leading process of migrating neurons (Figure 5B) resembled that of migrating neurons in which JNK function is suppressed (Kawauchi et al., 2003). As JNK controls leading process morphology by regulating microtubule dynamics via phosphorylation of MAP1B (Kawauchi et al., 2003), a similar regulatory machinery for leading process morphology might exist downstream of AUTS2-Rac1, in addition to the regulatory machinery for actin cytoskeleton (Kawauchi and Hoshino, 2008).

We did not observe abnormal apoptosis or proliferation of cells in any of the *Auts2* mutant brains (*Auts2*$^{neo/neo}$ and *Auts2*$^{del8/del8}$), in contrast to what was observed in *Auts2* knockdown zebrafish embryos (Beunders et al., 2013; Oksenberg et al., 2013). Another discrepancy between mouse and zebrafish AUTS2 is the location of the functional domain. While RP1 is the central functional domain of mouse AUTS2 for regulating actin cytoskeleton and Rac1 activity, the zebrafish functional domain seems to be the C-terminal region that does not contain the RP1. These findings imply that the molecular function of AUTS2 is different between mammals and fish. It is also possible that the C-terminal region of mouse AUTS2 may have a function other than regulation of actin dynamics and Rac1.

In conjunction with the predicted AUTS2 structure, our immunoblot analyses and 5′ RACE experiments suggested the existence of short isoforms (variants 1 and 2), which lack the functional PR1-containing N-terminal region. We observed impairment of neuronal migration and neuritogenesis not only in *Auts2*$^{neo/neo}$ but also in *Auts2*$^{del8/del8}$ animals where a short isoform of AUTS2 is maintained. This also confirms that the critical region for neuronal migration and neuritogenesis resides in the RP1-containing N-terminal region. We cannot exclude the possibility that the C-terminal region or the short isoform may have other functions, probably in nuclei, such as cell proliferation or differentiation because AUTS2 short isoform exclusively localizes in the nuclei. It will be interesting in future work to elucidate the molecular function of nucleic AUTS2 in brain development.

Individuals with heterozygotic disruptions of the human *AUTS2* gene occasionally present with brain malformations, including corpus callosum hypoplasia, in addition to psychiatric phenotypes (Kalscheuer et al., 2007; Oksenberg and Ahituv, 2013). Consistent with human cases, *Auts2* heterozygotic mutant mice also exhibit developmental defects, such as abnormal neuronal migration and neuritogenesis. Therefore, our mutant *Auts2* mouse lines represent good animal models for human psychiatric disorders with *AUTS2* mutations. Further analyses of the mouse mutants should help us to understand better the physiological function of *Auts2* in brain development and the pathology of the *AUTS2*-related psychiatric diseases.

EXPERIMENTAL PROCEDURES

Experimental Animals

All animal experiments in this study have been approved by the Animal Care and Use Committee of the National Institute of Neuroscience, Japan, and the guidelines established by the Institutional Animal Care and Use Committee of Nagoya University, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the NIH Guide for the Care and Use of Laboratory Animals.

In Utero Electroporation

ICR pregnant mice were purchased from SLC Japan. In utero electroporation experiments were performed as described previously (Kawauchi et al., 2003). pmU6-*Auts2* or scramble shRNA vector (0.5∼2 μg/μl) was cotransfected with pEGFP (0.5 μg/μl) (Kawauchi et al., 2003). To perform the rescue experiments in Figure 6B, pCAG-Cre (1.25 μg/μl) and the indicated expression plasmids (0.1∼1.25 μg/μl) were cotransfected with pEGFP.

Primary Culture of Cortical and Hippocampal Neurons

At E16.5, cortices and hippocampi were dissected from ICR mice or *Auts2*^{del8} mutant and WT mouse embryos that had been electroporated at E15.5 and treated with 0.25% trypsin-EDTA in Hank's balanced salt solution (Sigma) for 15 min at 37°C. The dissociated neurons were plated at a density of 7,500–10,000 cells/cm^2 on cover glasses coated with 1 mg/ml poly-L-lysine (Sigma) and maintained in modified Eagle's medium containing 2% B27 supplement (Invitrogen). Neurons were electroporated with the expression plasmids using the NEPA21 electroporator (Nepa Gene) according to the manufacturer's instructions.

GTPase Pull-Down Assay

Rac1/Cdc42 pull-down assays were performed as described previously (Matsuo et al., 2003). Briefly, the active, GTP-bound form of Rac1 or Cdc42 was pulled down from the cell lysates with 20 μg of PAK-GST proteins beads (Cytoskeleton). Bound GTP-Rac1 and -Cdc42 proteins were detected by western blotting using anti-Rac1 (ARC03; Cytoskeleton) and anti-Cdc42 (610928; BD Transduction Labs), respectively. Densitometry analysis was performed with ImageJ free image analysis software (NIH), and relative small GTPase activity was determined by the amount of GTP-bound Rac1 or Cdc42 bound to PAK-GST protein beads normalized to the amount of total Rac1 or Cdc42 in cell lysates, respectively. Statistical significance was established by Student's t test using GraphPad Prism 5.0 Software.

Immunofluorescence

Embryos at E12.5 and E14.5 were dissected in ice-cold PBS and fixed in 4% paraformaldehyde (PFA) for 2 hr at 4°C. For E18.5 and postnatal brains, tissues were fixed with 4% PFA at 4°C overnight. Fixed embryos and dissected tissues were cryoprotected by overnight immersion in 30% sucrose in PBS, embedded in optimum cutting temperature (Tissue-Tek O.C.T. compound) and cryosectioned at 14∼20 μm. For Immunocytochemistry, cells were fixed with 4% PFA for 20 min at room temperature. Immunofluorescence was performed using the following antibodies: rabbit anti-AUTS2 (1:300; HPA000390; Sigma-Aldrich), goat anti-AUTS2 (1:300; EB09003; Everest Biotech), mouse anti-β-III tubulin (Tu-20) (1:500; Millipore/Chemicon), goat anti-doublecortin (C-18) (1:200; sc-8068; Santa Cruz), anti-Cux1 (M-222) (1:3,000; sc-13024; Santa Cruz), anti-Ctip2 (1:5,000; ab18465; Abcam), rabbit anti-glutaminase (Hoshino et al., 2005) (1:500), mouse anti-HuC/D (16A11) (1:500; Invitrogen), rat anti-Ki67 (1:50; 14-5698; eBioscience), rabbit anti-GABA (1:500; A2052; Sigma-Aldrich), rabbit anti-active-JNK (1:200: V793A; Promega), rat anti-GFP (RQ1) (1:100; gift from A. Imura, BRI, Kobe), rabbit anti-cleaved Caspase3 (1:500; 9661S; Cell Signaling), rabbit anti-Intersectin 1 (LS-C186666; LifeSpan BioSciences), and rabbit anti-Intersectin 2 (bs-13646R; Bioss). For immunohistochemical analysis including in utero electroporation experiments, we used the somatosensory cortex area in the coronal sections between the level of caudal half of caudate putamen in which the corpus callosum fully appears, and the sections just before the hippocampus starts to appear. Fluorescence imaging, cell counts, and measurement of neurite length were carried out using a Zeiss LSM 780 confocal microscope system (Carl Zeiss) and ZEN 2009 software (Carl Zeiss) or Keyence All-in-One fluorescence microscope (BZ-X700).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.11.045.

AUTHOR CONTRIBUTIONS

K.H. and M.H. designed this study, wrote the manuscript, and supervised and coordinated the project. K.H. and A.S. performed all of biochemical and imaging experiments and statistical analysis. K.H., M.A., and M.Y. generated and K.S., M.H., T.N., and K.Y. supervised the designs, and W.S. and K.N. helped with breeding of *Auts2* mutant mice. A.S., R.H., and T.H. performed histological and immunohistochemical experiments. S.T., T.N., and K.K. helped with and supervised the biochemical experiments.

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