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WAVE2–Abi2 Complex Controls Growth Cone Activity and Regulates the Multipolar–Bipolar Transition as well as the Initiation of Glia-Guided Migration

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Glia-guided migration (glia-guided locomotion) during radial migration is a characteristic yet unique mode of migration. In this process, the directionality of migration is predetermined by glial processes and not by growth cones. Prior to the initiation of glia-guided migration, migrating neurons transform from multipolar to bipolar, but the molecular mechanisms underlying this multipolar–bipolar transition and the commencement of glia-guided migration are not fully understood. Here, we demonstrate that the multipolar–bipolar transition is not solely a cell autonomous event; instead, the interaction of growth cones with glial processes plays an essential role. Time-lapse imaging with lattice assays reveals the importance of vigorously active growth cones in searching for appropriate glial scaffolds, completing the transition, and initiating glia-guided migration. These growth cone activities are regulated by Abl kinase and Cdk5 via WAVE2–Abi2 through the phosphorylation of tyrosine 150 and serine 137 of WAVE2. Neurons that do not display such growth cone activities are mispositioned in a more superficial location in the neocortex, suggesting the significance of growth cones for the final location of the neurons. This process occurs in spite of the "inside-out" principle in which later-born neurons are situated more superficially.

Keywords: actin, Cdk5, cortex, development, radial migration

Introduction

Precise control of neuronal migration is essential for the development of the neocortex. In higher animals, most excitatory neurons and some inhibitory neurons are supplied by radial migration (Rakic 1990; Bayer and Altman 1991; Miyata et al. 2001; Letinic et al. 2002; Noctor et al. 2004; Marin et al. 2010). Recent studies using time-lapse imaging have revealed that radially migrating neurons, especially those destined for superficial layers, follow a trajectory in which they transform from a multipolar shape to a bipolar shape after the final division and then slide up along the radial glial process to the cortical plate, which is called locomotive movement, glia-guided migration, or glia-guided locomotion (Noctor et al. 2001; Nadarajah and Parnavelas 2002; Tabata and Nakajima 2003). The involvement of several molecules, such as Cdk5 (Gilmore et al. 1998; Ohshima et al. 2007), CRIMP2 (Sun et al. 2010), filamin A (Nagano et al. 2002, 2004; LoTurco and Bai 2006), and SCG10/Stathmin-2 (Westerlund et al. 2011), in this multipolar–bipolar transition has been reported. However, how neurons undergo this transition and resume their migration as well as the underlying molecular mechanisms have not been fully elucidated.

Radial migration, especially glia-guided locomotion (glia-guided migration), is a unique mode of cell migration because the direction of a migrating neuron is restricted by contact with glial processes, whereas most other directed migrating cells sense the surroundings with special protrusions (growth cones or lamellipodia and filopodia) that are formed at the leading edge and determine the direction of the cells. Because focal adhesion kinase accumulates in a region proximal to the growth cone in a neuron undergoing radial migration (Xie et al. 2003; Marin et al. 2010), it is likely that a neuron contacts radial glia through this region, and then the actual role of the growth cone for radial migration is unclear.

Abi2 is a protein that is enriched in growth cones and belongs to the Abi protein family, which was initially identified as a binding partner for Abelson tyrosine kinase (Abl kinase) (Dai and Pendergast 1995). It has been demonstrated that the deletion of Abi2 leads to cobblestone-like cortical malformation in mice; invagination of layer 1 and neurons with aberrant cellular orientation were observed (Grove et al. 2004), suggesting a significant contribution of Abi2 to radial migration.

Previous studies have shown that the Wiskott–Aldrich syndrome protein (WASP) family is critical for dynamic cytoskeleton regulation in a growth cone (Nozumi et al. 2003; Takenawa and Suetsugu 2007). In particular, WASP family verprolin homologous proteins (WAVEs) are important for the formation of lamellipodia because it is essential to generate branched F-actin by binding and activating the Arp2/3 complex through its C-terminal verprolin central acidic domain (Takenawa and Suetsugu 2007). It has been demonstrated that WAVEs (WAVE1, WAVE2, or WAVE3) are associated with Abi together with other members of the WAVE regulatory complex (WRC) such as Sra1/Cyfip1, Nap1/Hem-2, and HSPC300 (Eden et al. 2002; Sossey-Alaoui et al. 2005; Stovold et al. 2005; Chen et al. 2010) in cells. Therefore, Abi is apparently important for regulating the intracellular network of actin filaments (F-actin) in the growth cone (Courtney et al. 2000). Indeed, it has been demonstrated that Abi1, one of the Abi family molecules, is localized to sites of actin polymerization in protrusive membrane structures (spines) and regulates F-actin dynamics in vitro (Proepper et al. 2007).

In this study, we observed that multipolar neurons searched for glial processes with growth cones in the course of the multipolar–bipolar transition and that this activity was important for the determination of the starting order of glia-guided locomotion. We found that WAVE2–Abi2 plays a

critical role in the regulation of growth cone activity and that Abl kinase and Cdk5 regulate its activity by phosphorylating WAVE2.

Materials and Methods

Animals

C57BL/6 mice were purchased from a local vendor (SLC), housed at a constant temperature and humidity, and provided with food and water. Embryonic day 0.5 (E0.5) was defined as the day of confirmation of the vaginal plug. All experiments were conducted in compliance with the Guidelines for Use of Laboratory Animals of University of Fukui, and all efforts were made to minimize both the number of animals used and their suffering.

Expression Constructs

The full-length mouse Abi2 (GenBank accession number NM_198127) and WAVE2 cDNA (GenBank accession number AY135643) were cloned from E17 mouse embryonic cerebral cortex by reverse transcriptase–polymerase chain reaction using primeSTAR polymerase (Takara Biotechnology). Full-length cDNA was subcloned into expression plasmids. For details, see Supplementary Material.

RNA Interference

Constructs of various short hairpin RNAs (shRNAs) were prepared in the mouse U6 shRNA promoter (mU6pro) vector, which has a mouse U6 promoter (Nagano et al. 2004) or in the pSUPER RNAi system with the H1 RNA polymerase III promoter (Oligoengine). For details, see Supplementary Material.

In Utero Electroporation Gene Transfer

Plasmids were transfected by in utero electroporation using previously described methods (Nagano et al. 2004). Briefly, E14.5 or E15.5 pregnant female C57BL/6 mice were anesthetized by intraperitoneal injection of 2,2,2-tribromoethanol (200–300 mg/kg body weight) before the experiments. A total of 1 μg of plasmid was injected by transuterine pressure microinjection into the lateral ventricle of the embryos by delivering 5 electrical pulses (40 V; 50 ms duration) at intervals of 950 ms with a square-pulse electroporator (CUY21EDIT; Nepa Gene) and a tweezer-type electrode with disc electrodes (5 mm in diameter) at the tip (CUY650-5; Nepa Gene). For the analysis of migration and cell shape, brains were fixed with 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.4, cut coronally into 100 μm slices with a Vibratome (VT1000S; Leica Microsystems), and imaged on a laser-scanning confocal microscope (LSM 5 PASCAL; Carl-Zeiss). For quantification, the neocortical wall was divided into 5 vertical bins of equal size; the number of fluorescence-labeled cells in each bin was counted and presented as a percentage of the total number of electroporated cells in all 5 bins±standard error of the mean (SEM). A statistical analysis was performed using 1-way analysis of variance (ANOVA), followed by a Fisher's protected least significant difference (PLSD) post hoc test.

Preparation of Acute Brain Slices and Time-Lapse Imaging

An shRNA vector, together with the Tα-LPL-Lyn-GFP, Tα-Cre, and tdTomato expression vectors, was transfected by in utero electroporation on E14.5, and slices were obtained at E15.5. The slices were mounted onto 8-well chamber cover glass (Nalgene Nunc) and incubated in a medium consisting of 50% Dulbecco's modified Eagle's medium (Wako), 25% horse serum (Nichirei), 25% Hanks' balanced salt solution (Invitrogen) and 100 μg/mL penicillin/100 mg/mL streptomycin (PS). Followed by incubation for 2 h in a humidified chamber (5% CO_2/95% air, 37°C), media were changed to culture media consisting of neuronal basal medium (Invitrogen), supplemented with 1:50 B27 (Invitrogen), 0.2 μM L-glutamate, and PS. Time-lapse observation was performed with a FluoView FV10i incubator (5% CO_2/95% air at 37°C) (Olympus). Fluorescence-labeled cells were observed every 30 min for 9.5 h.

Primary Neuron Culture

Various vectors were transfected by in utero electroporation at E14.5, and dissociated cortical neuron cultures were prepared on E15.5–17.5, as described previously (Nagano et al. 2004). Neurons were plated on a polyethyleneimine-coated 8-well chamber cover glass and maintained in culture media. For quantification of active growth cones, fluorescence-labeled cells were observed every 15 min for 12–16 h with automated multiarea time lapse. The numbers of active growth cones and neurites of each neuron were counted and presented as the mean frequency of neurites with active growth cones ±SEM. Statistical significance (*P*-value) was calculated with Student's *t*-test.

Immunostaining and Immunohistochemistry

Primary neuronal cultures were fixed with 4% paraformaldehyde, and staining was performed as described previously (Takabayashi et al. 2010). For immunohistochemistry, 14 μm thick frozen sections were made on a cryostat and subjected to antibody reaction. Staining was performed as described previously (Nagano et al. 2004). Images were obtained on a laser-scanning confocal microscope (LSM 5 PASCAL). For details, see Supplementary Material.

Immunoprecipitation and Western Blot Analysis

Forebrains were dissected out from E17.5 mouse brains, followed by homogenization with RIPA buffer [0.1% sodium deoxycholate, 0.5% Nonidet P-40, 150 mM NaCl, 50 mM Tris–HCl with 1% Protease Inhibitor Mixture Set I (Sigma)], and incubated with anti-Abi2 (sc-20327, Santa Cruz Biotechnology) or anti-WAVE2 antibodies with Dynabeads (Dynal). Immunoprecipitation and immunoblot analyses with anti-Nap1 (07-515, EMD Millipore), anti-Sra1 (309011, Synaptic Systems), and anti-WAVE2 antibodies were performed as described. For details, see Supplementary Material.

Lattice Culture and Time-Lapse Imaging

Various vectors were transfected by in utero electroporation at E14.5, and cortical cells were dissociated for primary culture on E15.5 or 16.5. Cells were then plated at a density of 1×10^6 cells/well for primary neuronal cultures. At 1 day in vitro (DIV), 5 kDa dextran sulfate (final concentration of 10 μg/mL, Wako) was added to the medium to form lattice fascicles (Nichols et al. 2008), and images were then taken every 10–20 min for 12–18 h with automatic multiarea time-lapse imaging. The lattice network was stabilized prior to immunostaining (Nichols et al. 2008). An aliquot of 20 mL of a 2% solution of calfskin gelatin (Sigma) was gently added to each well and allowed to solidify before fixation in 4% paraformaldehyde. Immunostaining was performed following a standard protocol. We began to count the number of neurons apposed to lattice fascicles approximately 5 h after the addition of dextran sulfate, when the lattice was almost formed, and we counted the neurons for about 7–11 h. Results are indicated as mean ± SEM. Statistical significance was calculated with Student's *t*-test. For latrunculin B (Merck) treatment, neurons were incubated for 1 h with various concentrations of latrunculin B in a buffer consisting of 140 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$, and 2 mM $MgCl_2$ in 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, before the addition of dextran sulfate at 1 DIV. Neurons were then cultured without latrunculin B. For quantification of the migration distance of green fluorescent protein (GFP)-labeled neurons along lattice fascicles, time-lapse images of neurons apposed to lattice fascicles were obtained every 3 min for 60 min at 2 DIV. We measured the distance between the starting position (at 0 min) and the final position (at 60 min) of each neuron.

Cell Culture

COS-7 cells and NIH3T3 cells were used. Vectors were transfected using FuGENE 6 transfection reagent (Roche Diagnostics Corporation) and PolyFect transfection reagent (Qiagen).

Results

Neurons Search for Glial Scaffolds with Growth Cones Prior to Completing the Multipolar–Bipolar Transition and Initiating Glia-Guided Locomotion

Radially migrating neurons momentarily stall in the intermediate zone, undergo the multipolar–bipolar transition, and then initiate glia-guided locomotion (Tabata and Nakajima 2003). To elucidate how neurons proceed to glia-guided locomotion in the course of the multipolar–bipolar transition and to determine its underlying mechanisms, we examined the behavior of neurons using time-lapse imaging. For unambiguous visualization of cell morphology, GFP tagged with the membrane localization sequence of Lyn (Lyn-GFP) was generated and expressed in the cells of interest. We transfected neuron-specific tubulin α-driven LPL-Lyn-GFP vector (Tα-LPL-Lyn-GFP), which has 2 loxP sequences upstream of its multiple cloning site and allowed Lyn-GFP to be transcribed only in the presence of Cre activity. We diluted the Tα-Cre-expressing vector so that Lyn-GFP was expressed in a few neurons and coexpressed it with a CAG-driven tdTomato expression vector for the identification of cells transfected by in utero electroporation gene transfer to E14.5 mice. Brain slices were made at E15.5, and time-lapse imaging was performed. In the intermediate zone, multipolar neurons extended and retracted neurites vigorously. A neurite with a distinct growth cone was then elongated, and the neuron transformed into a bipolar shape and then resumed migration (Fig. 1*A* and Supplementary Movie S1).

Because dynamic growth cones were observed in the late phase of multipolar–bipolar transition in vivo, we further examined how neurons interact with glial processes and initiate glia-guided locomotion. To address this question, we utilized the lattice assay (Nichols et al. 2008). It has been demonstrated that dissociated cortical neurons form many islands of cell clusters interconnected by processes (lattice fascicles) upon the addition of dextran sulfate in the medium (Nichols et al. 2008). First, we explored whether we could reproduce the multipolar–bipolar transition in this assay. Cortices that had been transfected with a GFP-expressing vector by in utero electroporation at E14.5 were dissociated and cultured for a few days. Dextran sulfate was added and lattice fascicles formed. Many free multipolar-shape neurons were found at the beginning of the assay. In the course of lattice fascicle formation, bipolar neurons that were apposed to the lattice fascicles appeared (Supplementary Fig. S1*A* and Supplementary Movie S2). Some bipolar neurons moved along the lattice fascicles (Supplementary Fig. S1*A*, lower panels). Most lattice fascicles were nestin-positive, suggesting that the radial glia extended their processes to other islands of cell clusters as lattice fascicles (Supplementary Fig. S1*B*). Bipolar neurons were apposed along nestin-positive glial processes (Supplementary Fig. S1*C*). Therefore, this lattice assay enabled us to study the behavior of neurons and how they interact with glial processes in the late phase of the multipolar–bipolar transition in vitro. Distinct and dynamic growth cone activities were observed when growth cones were probing and latching onto lattice fascicles during the multipolar–bipolar transition (Fig. 1*B*; Supplementary Fig. S2*A* and Supplementary Movie S3). It was particularly interesting that a neurite with an apparent growth cone ("apparent" growth cone: a structure whose lamellipodium is at least 2 times larger in diameter than its following neurite) first touched and then extended along the lattice fascicles; 5–8 h later, the cell body started to move forward (Fig. 1*B*,*C*). In some cases, a multipolar neuron extended a long neurite with an active growth cone ["active" growth cone (Zheng and Poo 2007): apparent and morphologically dynamic growth cone whose lamellipodium changes its morphology completely for an hour], but soon retracted it and extended another new neurite for the future leading process and then started to move along the lattice fascicles (Fig. 1*D*–*F*; Supplementary Fig. S2*B* and Supplementary Movie S4).

Abi2 and WAVE2 are Important for Radial Migration

We next studied the importance of growth cones for completing the multipolar–bipolar transition and/or initiating glia-guided locomotion. First, we searched for molecules that control the growth cone activities of radially migrating neurons. F-actin branching is a fundamental process for growth cone activity and is regulated by the WAVE family proteins together with the WRC (Nozumi et al. 2003). Deletion of Abi2, which is a member of the WRC, results in brain malformation (Grove et al. 2004). Therefore, we assumed that Abi2 is involved in growth cone regulation and explored this possibility by acutely knocking down Abi2. After confirming the Abi2 mRNA expression in the developing neocortex (Supplementary Fig. S3*A*), we generated 3 different knockdown vectors against Abi2 (sh-Abi2): sh-Abi2-1, sh-Abi2-2, and sh-Abi2-3. The 3 constructs had similar Abi2 knockdown efficiencies (Fig. 2*A*). sh-Abi2-1, sh-Abi2-2, or sh-Abi2-3 was transfected together with an enhanced GFP (EGFP) expression vector into cells lining the ventricle of the mouse neocortex by in utero electroporation at E14.5, so that transfected cells could be visualized by EGFP. A large fraction of sh-Abi2-treated cells still stayed in the intermediate zone or in the ventricular zone at E17.5 (Fig. 2*B*). Hereafter, we used sh-Abi2-1 to knock down Abi2 and called sh-Abi2-1 as sh-Abi2. The degree of Abi2 knockdown and the severity of radial migration impairment were proportional to the amount of transfected sh-Abi2 (Fig. 2*C*). In contrast, the knockdown of Abi1, which is another Abi protein, did not alter radial migration (Fig. 2*B*). Four days after transfection, most Abi2-deficient neurons migrated upward to the cortical plate, which suggests that the knockdown of Abi2 results in a delay of migration (Fig. 2*D*,*E*). This migration delay due to Abi2 deficiency was rescued by coexpression of RNAi-resistant Abi2 whose nucleotide sequence, but not amino acid sequence, was changed (Abi2 R) (Fig. 2*D*,*E*).

Because WAVE is a component of the WRC together with Abi (Chen et al. 2010), we next investigated whether WAVE proteins are involved in radial migration. A knockdown vector against WAVE1, WAVE2, or WAVE3 was transfected together with an EGFP expression vector to cells lining the ventricle by in utero electroporation at E14.5. The cortices were observed on E17.5. Acute deficiency of WAVE2 induced by transfection of a knockdown vector against WAVE2

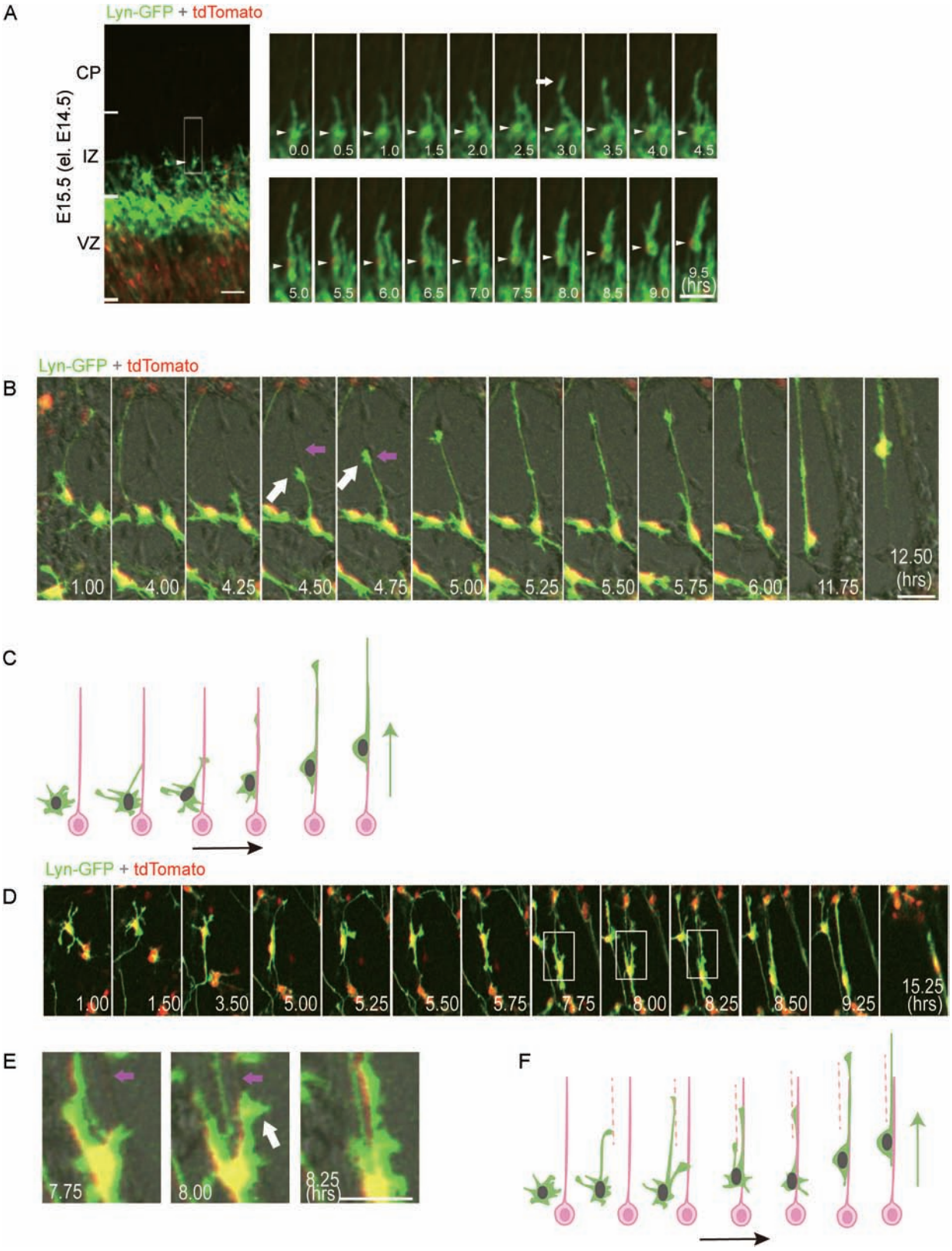

Figure 1. Multipolar neurons probe scaffolds for future guided migration with active growth cones by extending a neurite; they then resume migration after temporarily maintaining a bipolar shape. (*A*) Tα-LPL-Lyn-GFP, Tα-Cre, and tdTomato expression vectors were transfected by in utero electroporation at E14.5, and slices were obtained on E15.5. The morphology of tdTomato-expressing neurons (red) was visualized with coexpressed GFP with a membrane-targeting motif (Lyn-GFP) (green). White arrowheads indicate a neuron of interest. Small panels are higher magnifications of the temporal profiles of the indicated regions in the left panel. A white arrow indicates the active growth cone. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone. (*B*) Lattice assay. Tα-LPL-Lyn-GFP, Tα-Cre, and tdTomato expression vectors were transfected by in utero electroporation at E14.5, and primary cultures were generated on E15.5. After 1 DIV (the plating day is defined as 0), images were obtained after the addition of dextran sulfate (time = 0). The activity of growth cones (white arrow) was dynamic during the multipolar–bipolar transition to allow probing and latching onto the lattice fascicles (purple

(sh-WAVE2), but not WAVE1 or WAVE3, resulted in radial migration arrest on E17.5, suggesting that WAVE2 plays a specific role in radial migration (Fig. 2*F–H* and Supplementary Fig. S4). Indeed, WAVE2 mRNA was expressed in the developing neocortex (Supplementary Fig. S3*B*). Moreover, Abi2 and WAVE2 were well colocalized there (Supplementary Fig. S3*C*). We confirmed that the knockdown of WAVE2 and Abi2 did not influence cell proliferation, apoptosis, neuronal differentiation in the ventricular zone (Fig. 2*I*,*J* and Supplementary Fig. S5*A*,*B*), or neuronal subtype specification (Supplementary Fig. S6*A–C*), although it has been demonstrated that WAVE1 is important for apoptosis in some cells (Danial et al. 2003).

Abi2 and WAVE2 are Important for Growth Cone Activities of Radially Migrating Neurons and Essential for Completing the Transition from Multipolar to Bipolar on Time

We next evaluated whether Abi2 and/or WAVE2 plays an essential role in the growth cone activity of radially migrating neurons in the multipolar–bipolar transition. To study the importance of Abi2 and WAVE2 in growth cone activities, we knocked down Abi2 or WAVE2 by transfecting sh-Abi2 or sh-WAVE2 together with Tα-LPL-Lyn-GFP, Tα-Cre, and CAG-tdTomato expression vectors and observed the behavior of transfected neurons. Abi2- or WAVE2-deficient neurons transiently transformed into a bipolar shape from a multipolar shape, but quickly retracted their long neurites and became multipolar again. Whereas the motility of the neurites was dynamic, active growth cones were rarely seen at their tips (Fig. 3*A*,*B* and Supplementary Movies S5 and S6), implying that Abi2 and WAVE2 are crucial for forming growth cones and that growth cones are important for neurons to complete the multipolar–bipolar transition.

The WAVE2–Abi2 Complex is Crucial for Growth Cone Activity

Abi2 formed a complex with Sra1, Nap1, and WAVE2 in the developing neocortex (Fig. 4*A*). Moreover, Abi2 knockdown resulted in a decrease in WAVE2 but not Nap1 (Fig. 4*B*), suggesting that Abi2 and WAVE2 are regulated en bloc. We further examined whether Abi2 binds to WAVE2 in a manner similar to that of Abi1 (Leng et al. 2005). Abi2 or its truncated forms were tagged with FLAG and coexpressed with GFP-tagged WAVE2 in COS-7 cells. Immunoprecipitation was performed with an anti-FLAG antibody, and precipitates were probed with an anti-GFP antibody. Abi2 without its C-terminus was coimmunoprecipitated with WAVE2, but not Abi2 without its N-terminus, suggesting that Abi2 binds to WAVE2 through its N-terminus in a manner similar to that used by Abi1 to bind to WAVE1 or WAVE2 (Echarri et al. 2004; Leng et al. 2005) (Supplementary Fig. S7*A*,*B*). Indeed, Abi2 without its C-terminus, but not Abi2 without its N-terminus, colocalized well with WAVE2 in COS-7 cells (Supplementary Fig. S7*C*).

We also asked whether or not an acute deficiency of Abi2 or WAVE2 reduced growth cone activity. After being transfected with sh-Abi2, sh-WAVE2, or empty shRNA vectors (sh-con), together with Tα-LPL-Lyn-GFP, Cre, and tdTomato expression vectors using in utero electroporation, neurons were dissociated and cultured. Large lamellipodia were observed at the tips of neurites (Fig. 4*C*). Colocalization of endogenous Abi2 and phalloidin (F-actin) and colocalization of endogenous Abi2 and endogenous WAVE2 were confirmed to be present in growth cones (Fig. 4*C*,*D*). Our time-lapse imaging revealed that the growth cone morphology was dynamically changed (active growth cones) (Zheng and Poo 2007), whereas that of neurons with knocked-down Abi2 or WAVE2 was not (Fig. 4*E–G*). We counted the number of neurites with active growth cones among tdTomato-expressing cells. The mean frequency of neurites with active growth cones was reduced to a similar degree when either Abi2 or WAVE2 was knocked down (Fig. 4*H*). Moreover, the knockdown of Abi2 or WAVE2 impaired the elongation of neurites of cortical neurons to a similar degree, suggesting that they work together in cortical neurons (Supplementary Fig. S7*D*,*E*).

We next asked whether Abi2 and/or WAVE2 is involved in the regulation of growth cone activities in migrating neurons in vivo. To address these issues, we counted the number of neurites with active growth cones of migrating neurons in explants. The mean frequency of neurites with active growth cones of GFP-labeled neurons in the intermediate zone was reduced to a similar degree when either Abi2 or WAVE2 was knocked down (Fig. 5*A*), which is consistent with the results in vitro (Fig. 4*H*), whereas the length of the leading processes was not significantly different due to Abi2 or WAVE2 acute deficiency (Fig. 5*B*). Therefore, it is likely that Abi2 and WAVE2 are important for growth cone activities of migrating neurons in vivo.

WAVE2–Abi2 Regulates Growth Cone Activities and is Important for Neuronal Latching onto Lattice Fascicles

We further studied whether the WAVE2–Abi2 complex was involved in the completion of the multipolar–bipolar transition and in the initiation of glia-guided locomotion with the lattice assay. Whereas multipolar neurons extended their neurites with dynamic growth cones and then latched onto lattice fascicles (Figs 1*B*,*D* and 6*A* and Supplementary Movies S3 and S4), knockdown of Abi2 or WAVE2 made growth cones inactive, and most knockdown neurons remained multipolar for a longer period (Fig. 6*B*,*C* and Supplementary Movies S7 and S8). The number of sh-Abi2- or sh-WAVE2-treated neurons that apposed to lattice fascicles decreased, compared with sh-con (Fig. 6*D–G*). Because WAVE2 regulates F-actin dynamics (Takenawa and Suetsugu 2007), we evaluated whether the disruption of F-actin led to a decrease in the number of neurons that apposed to lattice fascicles. The number of neurons apposed to lattice fascicles decreased in the presence of the actin filament modulator latrunculin B (Lat B) (Supplementary Fig. S8*A*,*B*), which forms complexes with actin monomers and thereby inhibits actin polymeriz-

arrow). A neuron first extended a leading process and then initiated migratory movement approximately 8 h after plating. (*C*) A schematic drawing of neuronal behavior in (*D*). (*D*) Another example. A neuron extended a probing process, then retracted it, and extended another as a future leading process. (*E*) High-magnification images of the rectangles of (*D*) showing how an active growth cone latched onto lattice fascicles. A white arrow indicates the growth cone, whereas purple arrows indicate lattice fascicles. (*F*) A schematic drawing of the neuronal behavior in (*D*). Scale bars = 100 μm (left), 20 μm (right small panels) (*A*), and 30 μm (*B*, *D*, and *E*). See (*A*), (*B*), and (*D*) and also Supplementary Movies S1, S3, and S4, respectively.

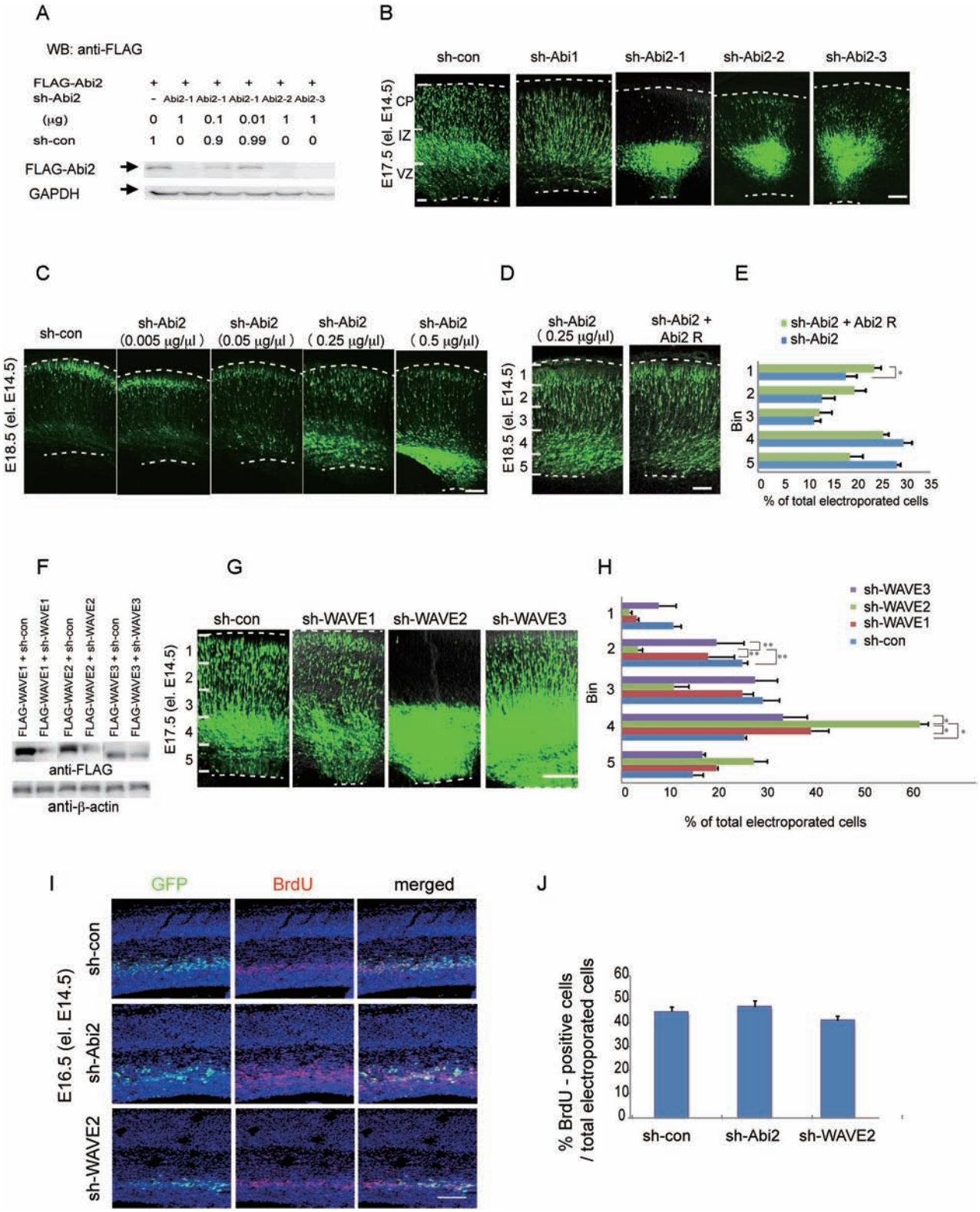

Figure 2. WAVE2 and Abi2 are pivotal for radial migration but do not interfere with cell proliferation. (*A*) Three different sh-Abi2 vectors at various concentrations were transfected in COS-7 cells with the FLAG-Abi2 expression vector. An empty shRNA vector was used for the control (sh-con). The total amount of the shRNA vector was adjusted to 1 μg in each transfection with the sh-con vector. The knockdown efficiency was evaluated by western blotting for FLAG. GAPDH was used as a loading control. (*B*) The various shRNA vectors were transfected together with the GFP expression vector into cells lining the ventricle of the cortex by in utero electroporation at E14.5 (el. E14.5). Cortices were observed on E17.5. The sh-Abi1-treated cells migrated to the cortical plate (CP) similar to the sh-con, whereas a larger fraction of the sh-Abi2-treated cells was still localized in the intermediate zone (IZ) or in the ventricular zone (VZ) at E17.5. The edges of the cortex are shown with dotted lines. (*C*) Cortices were observed on E18.5. Neurons migrated more than 4 days after transfection. The degree of impairment of radial migration was dependent on the amount of shRNA vectors against Abi2 that were transfected. (*D*) To confirm the specificity of shRNA against Abi2, sh-Abi2 (0.25 μg/μL) was transfected together with an empty CAG vector ($n = 3$) or an RNAi-resistant Abi2-expressing CAG vector (Abi2 R) ($n = 4$), in which the cDNA sequence but not the amino acid sequence for Abi2 was changed. Representative examples are shown. The

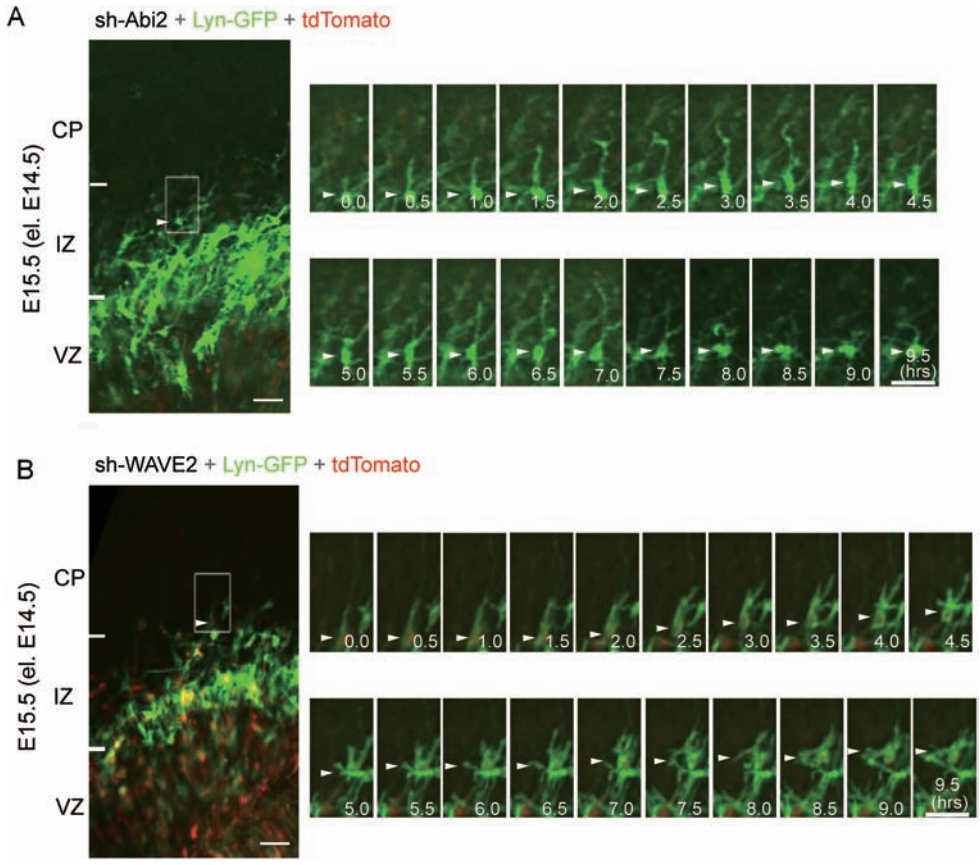

Figure 3. Abi2 and WAVE2 are indispensable to accomplish the transition from a multipolar shape to a bipolar shape. sh-Abi2 vector (*A*) or sh-WAVE2 vector (*B*), together with Tα-LPL-Lyn-GFP (green), Tα-Cre, and tdTomato (red) expression vectors, was transfected by in utero electroporation at E14.5, and slices were obtained on E15.5. White arrowheads indicate the neurons of interest. Small panels are higher magnifications of the temporal profiles of the regions indicated in the left panels. Scale bars = 100 μm (left) or 20 μm (right small panels). See (*A*) and (*B*) and also Supplementary Movies S5 and S6.

ation in proportion with its concentration (Wakatsuki et al. 2001). Moreover, we found that no additional effects were observed in Abi2 knockdown or WAVE2 knockdown neurons by the addition of Lat B (Supplementary Fig. S8*C*). These results suggest that F-actin possibly mediates the WAVE2–Abi2-dependent control of growth cone activity.

Knockdown of Abi2 or WAVE2 Results in Retarded Migration

Additionally, we asked whether WAVE2–Abi2 plays a role in migration. We measured how far neurons migrated on the lattice fascicles in 60 min. Although the distance migrated in 60 min varied significantly, the average speed of neurons with knocked-down Abi2 or WAVE2 was slower than that of controls, suggesting that Abi2 and WAVE2 also play a role in locomotion. Notably, a small fraction of control neurons migrated quickly (>60 μm in an hour) (Supplementary Fig. S9*A*–*D*).

Neurons Without Active Growth Cones were Located More Superficially in the Cortex

We next evaluated whether growth cone activities affected neocortical development. Prior to our study, we examined whether the deficiency of Abi2, or WAVE2, influenced the adherens junction of the ventricular zone of the brain because the deletion of Abi2 results in impaired adherens junction formation in the developing lens (Grove et al. 2004). No such changes were observed (Fig. 7*A*). We then studied the morphology and localization of Abi2- or WAVE2-deficient neurons in the postnatal cortex. At postnatal day 21, Abi2- or WAVE2-

degree of impairment of migration due to sh-Abi2 was decreased in the presence of the resistant vector. (*E*) Cortices were divided into 5 equal-size vertical bins. The proportion of cell numbers of the sh-Abi2-treated cells in the presence of the resistant vector increased in bin 1. Data are presented as mean ± SEM. $*P < 0.05$ by 1-way ANOVA test. (*F*) sh-WAVE1, WAVE2, or WAVE3 vectors were cotransfected with the FLAG-WAVE1, FLAG-WAVE2, or FLAG-WAVE3 expression vectors in COS-7 cells. Knockdown was confirmed by western blotting for FLAG. β-actin was used as an internal control. (*G*) In utero electroporation gene transfer was performed at E14.5, and the cortices were observed on E17.5. sh-con-, sh-WAVE1-, or sh-WAVE3-treated cells migrated up to the CP, whereas a larger fraction of the sh-WAVE2-treated cells were still localized in the IZ or VZ. (*H*) Cortices were divided into 5 vertical bins of equal size, as shown in (*G*). The proportion of cell numbers of the sh-WAVE2-treated cells increased significantly in bin 4 compared with other shRNA-treated cells, whereas it decreased significantly in bin 2. Data are presented as mean ± SEM ($n = 3$). $**P < 0.005$ and $*P < 0.05$ versus other shRNA after a significant ANOVA followed by a Fisher's PLSD post hoc test. (*I*) Cells lining the lateral ventricle were transfected with various shRNA vectors together with the GFP expression vector at E14.5 using in utero electroporation. BrdU (50 mg/kg) was injected intraperitoneally on E14.5, and immunostaining of BrdU (red) was performed at E16.5. Nuclei were visualized with Hoechst stain (blue). (*J*) Quantification of the number of BrdU-positive cells in the total GFP-positive electroporated cells. No difference was found between the sh-con and sh-Abi2 or sh-WAVE2-treated neurons. Data ($n = 3$) are presented as mean ± SEM. Scale bars = 30 μm (*B–D*, *G*, *I*).

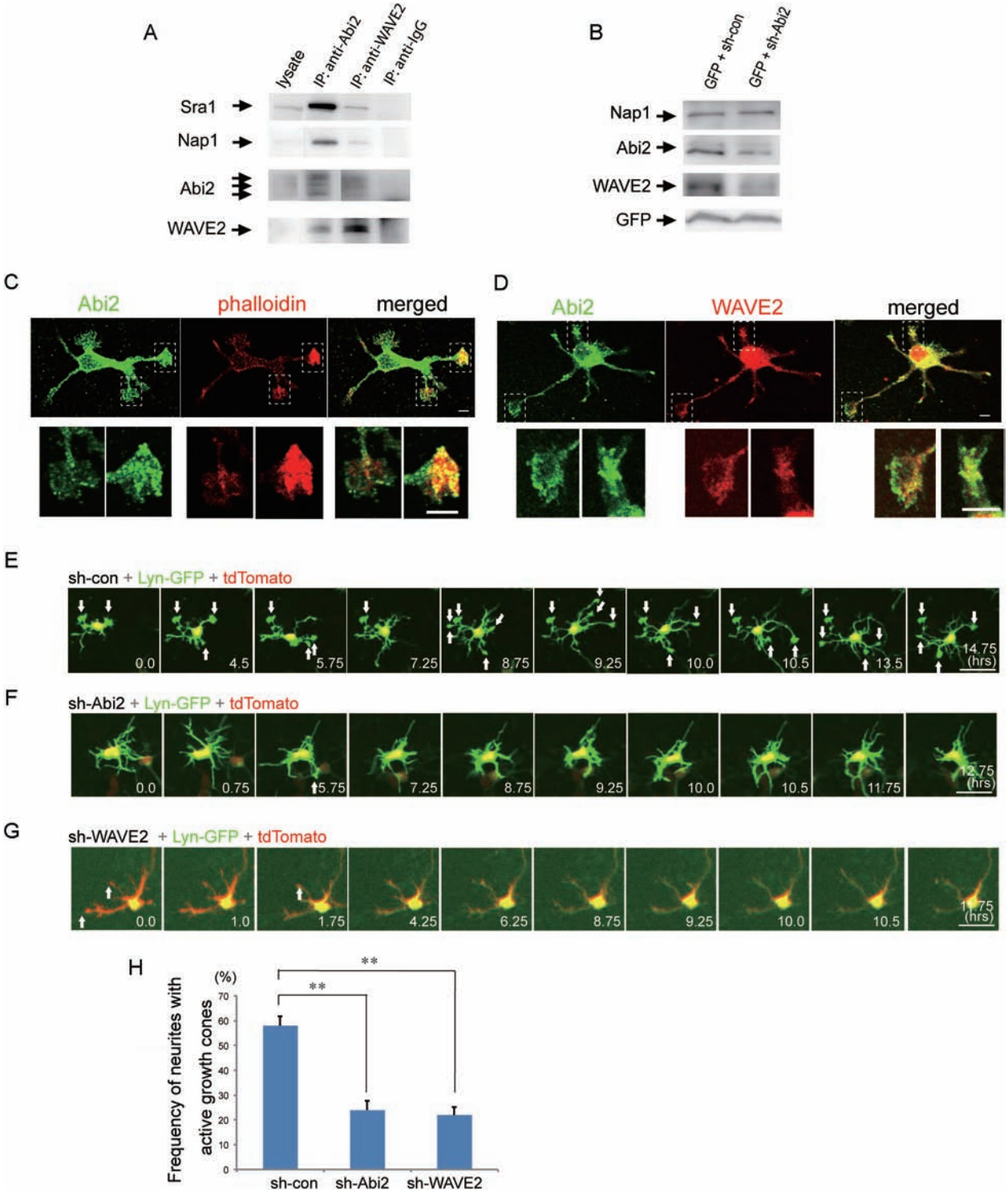

Figure 4. The WAVE2–Abi2 complex is essential for dynamic growth cone activity. (*A*) The WAVE2 complex (Sra1, Nap1, Abi2, and WAVE2) was observed in the mouse embryonic (E17.5) cortex. Lysates were immunoprecipitated with anti-Abi2 antibody or anti-WAVE2 antibody and probed with antibodies against Sra1, Nap1, Abi2, or WAVE2. Anti-IgG antibody was used as a negative control. (*B*) sh-Abi2 or sh-con and GFP expression vectors were cotransfected in NIH3T3 cells. shRNA-treated cells were collected using GFP detection by fluorescence-activated cell sorting. Compared with the sh-con, endogenous Abi2 and WAVE2, but not Nap1, decreased in the Abi2 shRNA-treated cells. (*C* and *D*) Abi2 colocalized well with phalloidin (F-actin) and WAVE2 in growth cones. (*E–G*) Empty shRNA vector (sh-con) (*E*), sh-Abi2 (*F*), or sh-WAVE2 (*G*), together with Tα-LPL-Lyn-GFP (green), Tα-Cre, and tdTomato (red) expression vectors, was transfected by in utero electroporation at E14.5. Neurons were dissociated on E15.5 and cultured. After 3 DIV, time-lapse images were collected. White arrows indicate active growth cones. (*H*) The number of neurites with an active growth cone per transfected neuron was counted in the sh-Abi2-treated ($n = 14$) and sh-WAVE2-treated cells ($n = 10$) as well as in sh-con-treated cells ($n = 42$). Data represent mean ± SEM. $^{**}P < 0.01$ by Student's *t*-test. Active growth cones were much less frequent in Abi2 or WAVE2 knockdown neurons. Scale bars = 5 μm (*C* and *D*) or 30 μm (*E–G*).

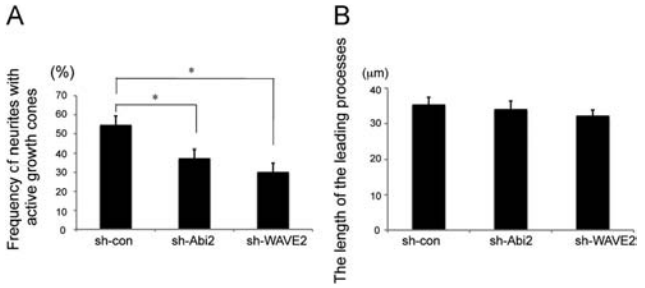

Figure 5. The WAVE2–Abi2 complex is essential for dynamic growth cone activity, but does not interfere with extension of leading processes. sh-Abi2 vector or sh-WAVE2 vector, together with Tα-LPL-Lyn-GFP (green), Tα-Cre, and tdTomato (red) expression vectors, was transfected by in utero electroporation at E14.5, and slices were obtained on E15.5. The examples are shown in (*A*) of Figures 1 and 3. (*A*) The number of neurites with an active growth cone per transfected neuron was counted in the sh-Abi2-treated and sh-WAVE2-treated cells as well as in sh-con-treated cells. Active growth cones were much less frequent in Abi2 or WAVE2 knockdown neurons. Quantification data are presented as mean ± SEM. $*P < 0.05$ by Student's *t*-test. sh-con ($n = 5$), sh-Abi2 ($n = 5$), and sh-WAVE2 ($n = 5$). (*B*) The length of the leading neurites was measured. No difference was detected between control and knockdown of Abi2 or WAVE2. Quantification data are presented as mean ± SEM. sh-con ($n = 5$), sh-Abi2 ($n = 5$), and sh-WAVE2 ($n = 5$).

knockdown neurons were located more superficially than control neurons in the neocortex (Fig. 7*B–D* and Supplementary Fig. S6*D*).

WAVE2–Abi2 Activity is Regulated by Abl Kinase and Cdk5 Through the Phosphorylation of WAVE2

Because the growth cone activity, in which WAVE2–Abi2 is crucial, plays an important role in completing the multipolar–bipolar transition, and its impairment eventually results in the misplacement of neurons in the cortex, we examined how the activities of growth cones are regulated. It has been reported that the phosphorylation of Tyr151 of WAVE3 (Sossey-Alaoui et al. 2007) [corresponding to Tyr150 of mouse WAVE2 (Leng et al. 2005)] by Abl kinase is important for WAVE-regulatory-complex-mediated actin assembly and lamellipodium formation in adenocarcinoma cells. Additionally, the phosphorylation of WAVE1 Thr138 by Cdk5 (Chen et al. 2010) [corresponding to Ser137 of mouse WAVE2 (Miyamoto et al. 2008)] alters cellular actin dynamics, and the phosphorylation of WAVE2 is important for oligodendrocyte migration (Miyamoto et al. 2008). Although phosphorylation of WAVE1 Ser310, Ser391, and Ser441 by Cdk5 is important for actin polymerization as well as for dendrite formation, these are not conserved in WAVE2 or WAVE3 (Kim et al. 2006). We first evaluated whether or not the phosphorylation of Tyr150 of WAVE2 is essential for radial migration. Mutated WAVE2 in which Tyr150 was changed to phenylalanine (WAVE2 Y150F) was overexpressed in the ventricular zone, together with a GFP expression vector on E14.5. Overexpression of WAVE2 impaired radial migration to some extent, whereas overexpression of WAVE2 Y150F resulted in an accumulation of neurons in the intermediate zone, which suggests that the phosphorylation of Tyr150 is important for radial migration and especially for the multipolar–bipolar transition (Supplementary Fig. S10*A*,*B*). Because Abi1-mediated coupling of Abl kinase to WAVE2 enhances Abl-evoked WAVE2 tyrosine phosphorylation (Leng et al. 2005), we asked whether Abi2 also promotes WAVE2 phosphorylation by Abl kinase. Phosphorylation of Tyr150 by Abl kinase increased in the presence of Abi2 (Fig. 8*A*). We next assessed whether the phosphorylation of Tyr150 is required for neurons to appose to the glial processes using the lattice assay. The proportion of neurons on the lattice fascicles decreased with exogenous WAVE2 Y150F expression, suggesting that the phosphorylation of Tyr150 is important (Fig. 8*B*). In addition, we carried out the lattice assay with WAVE2 S137A, in which Ser137 is changed to alanine, and with WAVE2 S137E, in which Ser137 is changed to glutamic acid. Our lattice assay revealed the importance of the phosphorylation of Ser137 for neurons to latch onto cellular processes, as overexpression of WAVE2 S137A resulted in a decreased proportion of neurons apposed to lattice fascicles (Fig. 8*C*). Furthermore, such activity was more severely impaired when Cdk5 was knocked down, or kinase-dead Cdk5 (Cdk5 D144N, in which aspartic acid is changed to asparagine) (Ohshima et al. 2007) was overexpressed (Fig. 8*D* and Supplementary Fig. S10*C–E*). A summary of our findings is depicted in Figure 8*E*,*F*.

Discussion

We demonstrated here that WAVE2–Abi2 complex, which controls growth cone activity, is crucial for completing transition from a multipolar to bipolar shape. Our data suggest that the transformation from multipolar to bipolar is not solely a cell-autonomous event; rather, external molecules and/or mechanical stimuli transduced through growth cones are important for this transformation, although the significance of the growth cone for radial migration has not been appreciated so far. In addition, WAVE2–Abi2 complex is important for the determination of the final location of migrating neurons in the neocortex. If the activity of the growth cone that is regulated by the WAVE2–Abi2 complex is not sufficiently dynamic to search for a glial process and/or contact and translocate onto a glial process, the neurite takes a long time to appose to glial process and the initiation of glia-guided locomotion is delayed, possibly being passed by later generated neurons. It has been considered that radially migrating neurons settle in layers depending on their birthdates, that is, early-born neurons settle in deeper positions whereas late-born neurons settle in more superficial positions in a so-called "inside-out" fashion (Marin et al. 2010). In contrast, our study revealed that the order of starting locomotive movement was also crucial for the determination of the final position in the cortex.

Notably, because sh-Abi2 was transfected into some neurons by in utero electroporation, neurons with Abi2 knockdown migrated among other neurons with no Abi2 knockdown. Thus, the influence of knockdown on cell motility is easily visible. In contrast, Abi2 deletion affects all neurons in the case of Abi2 knockout mice (Grove et al. 2004). Therefore, the final phenotypes seen in our knockdown experiments and the reported knockout mice are not the same.

It has been reported that WAVE2 is important for oligodendrocyte migration (Miyamoto et al. 2008), but the significance of WAVE2 for radial migration has remained elusive. Unlike Abi2, knockout of WAVE2 results in early embryonic lethality (Yamazaki et al. 2003; Yan et al. 2003), indicating that WAVE2 plays a more critical role in early development than Abi2. However, the significance of WAVE2 in later stages is still an

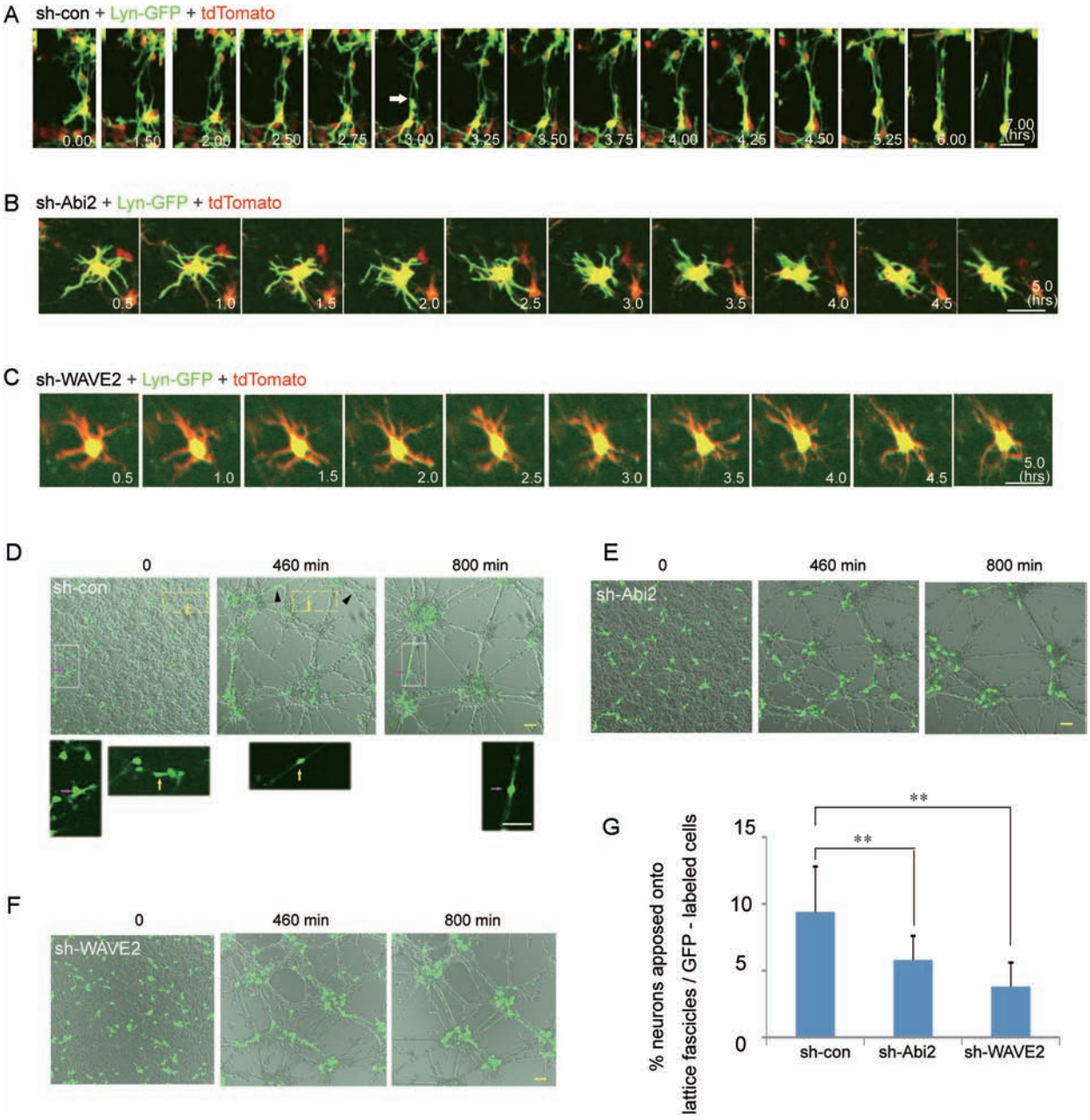

Figure 6. Abi2 and WAVE2 are essential for probing and apposing lattice fascicles. Various shRNA vectors were transfected with other expression vectors. Lattice cultures were generated under the same conditions as in (*B*) of Figure 1. (*A*) A multipolar neuron probed lattice fascicles with an active growth cone (white arrow) and then transformed into a bipolar shape. Similar examples are shown in (*B*) of Figure 1. (*B* and *C*) Abi2 knockdown or a WAVE2 knockdown did not transform neurons into a bipolar shape or result in apposition onto lattice fascicles, although it resulted in active extension and retraction of neurites. (*D–F*) Low-magnification images of lattice assays. Cortical neurons transfected with various vectors by in utero electroporation were labeled (green). Two typical neurons were tracked in the course of lattice formation (a purple or yellow arrow). They transformed from multipolar to bipolar and apposed onto lattice fascicles, and Abi2 or WAVE2 knockdown reduced the number of these neurons. Arrowheads indicate bipolar neurons moving along the lattice fascicles. (*G*) Five hours after the addition of dextran sulfate, the number of neurons apposed onto lattice fascicles was counted for 9 h. Data ($n = 18$) represent the mean ± SEM. $^{**}P < 0.01$ by Student's *t*-test. Scale bars are 30 μm (*A* and *B*) or 100 μm (*D–F*). See (*B*) and (*C*) and also Supplementary Movies S7 and S8, respectively.

open question. In this study, we demonstrated that an acute deficiency of Abi2 or WAVE2 leads to the impairment of radial migration to similar degrees, suggesting that these molecules regulate radial migration en bloc. It is of interest that when Nap1, a component of the WRC (Chen et al. 2010), is deleted, there is no obvious impairment of migration (Yokota et al. 2007). A recent structural study revealed that the WRC is divided into 2 subcomplexes: a WAVE:Abi:HSPC300 trimer and an Sra1:Nap1 dimer (Chen et al. 2010). Thus, it is possible that there are other molecules that are capable of regulating WAVE activity together with the WAVE:Abi:HSPC300 trimer instead of an Sra1:Nap1 dimer.

Because Abl kinase is involved in the remodeling of the actin cytoskeleton in response to extracellular stimuli, external signals are likely to influence the cortical formation through the regulation of growth cone activity in the course of the

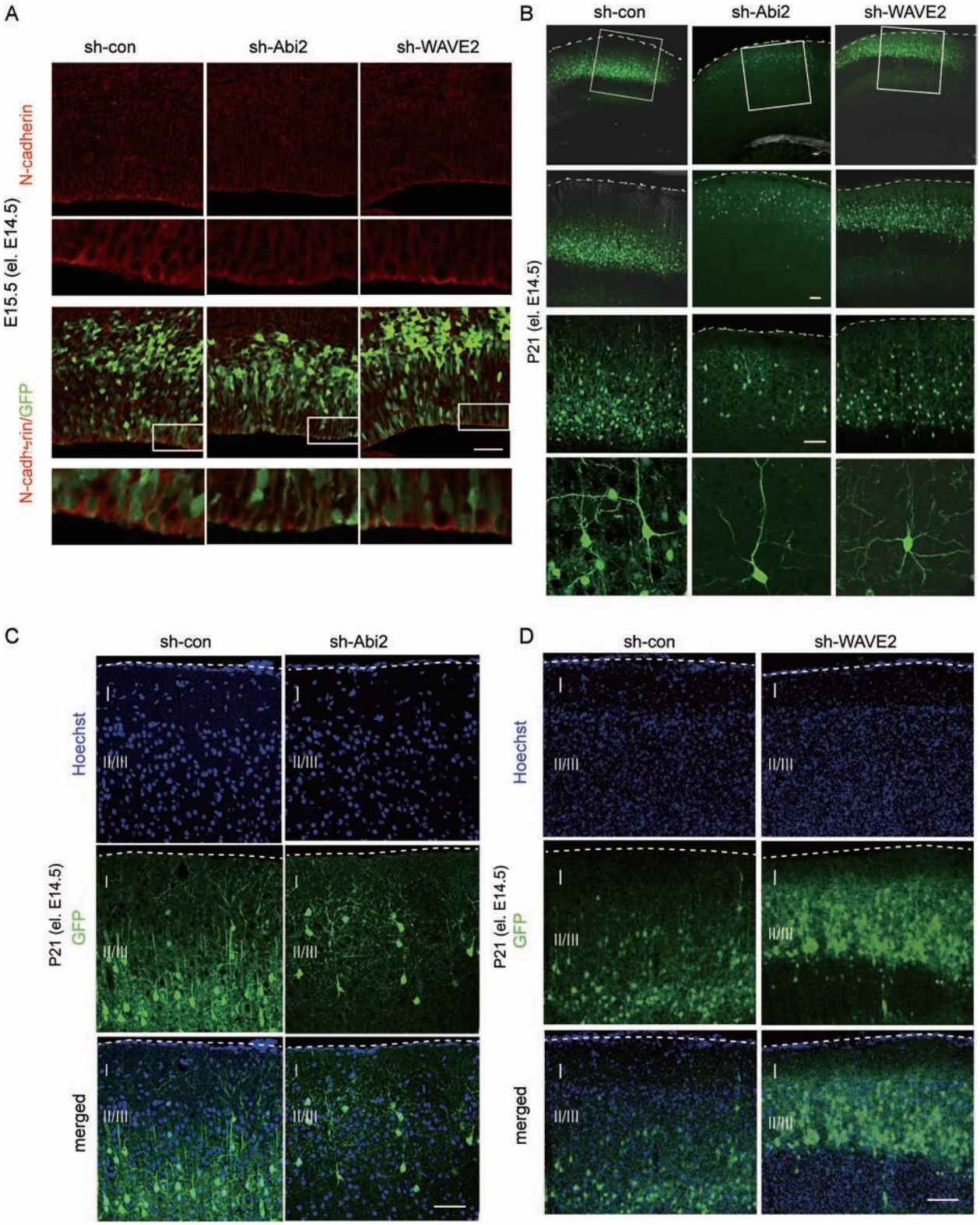

Figure 7. Abi2 and WAVE2 knockdown result in the misplacement of neurons but do not interfere with adherens junctions. (*A*) Immunostaining for the adherens junction component N-cadherin (red). In utero electroporation was performed at E14.5, and cortices were observed on E15.5. No difference was found between the sh-con and sh-Abi2 or sh-WAVE2-treated neurons in terms of N-cadherin staining in the ventricular zone. Small panels are high-magnification images of the rectangles. (*B–D*) In utero electroporation gene transfer was performed at E14.5, and the cortices were observed on postnatal day 21 (P21). sh-Abi2- or sh-WAVE2-treated neurons were finally situated more superficially in the cortical plate compared with sh-con, in which most labeled neurons were located in Layer 3. Areas shown in the rectangles were magnified in the lower panels. sh-Abi2-treated neurons showed poor dendritic development. Nuclei of cells in (*C* and *D*) were stained with Hoechst (blue). Scale bars = 10 μm (*A*) and 30 μm (*B–D*).

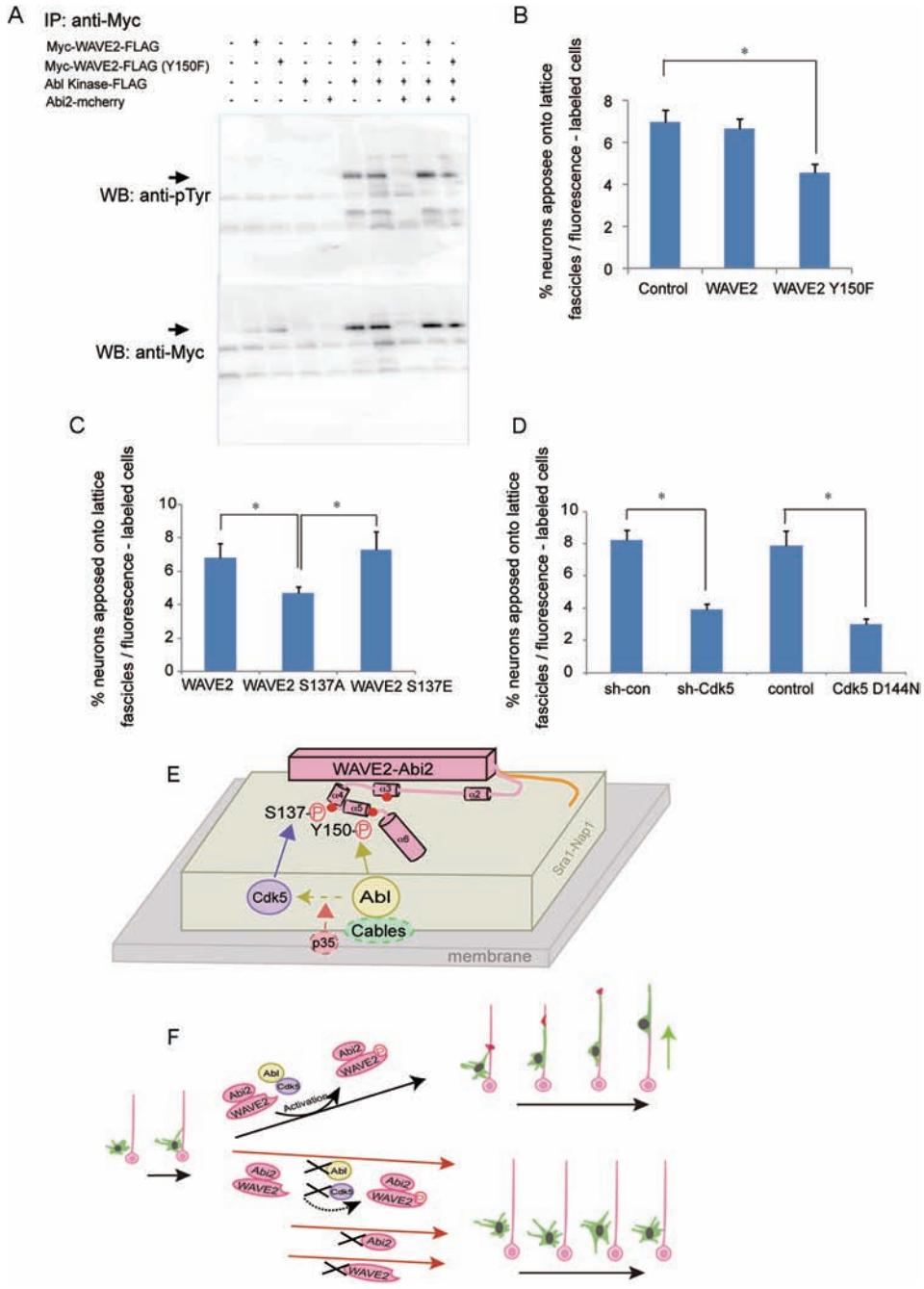

Figure 8. Abl kinase and Cdk5 regulate WAVE2 activity through phosphorylation at tyrosine 150 and serine 137, respectively. (*A*) An expression vector for Myc-tagged WAVE2 or mutated WAVE2 in which tyrosine 150 is changed to phenylalanine (WAVE2 Y150F) was transfected into COS-7 cells with/without Abl kinase and/or Abi2 expression vectors. Immunoprecipitation was performed with an anti-Myc antibody, and the precipitates were immunoblotted sequentially with an anti-pTyr or anti-Myc antibody. The pTyr signal of WAVE2, but not WAVE2 Y150F, was stronger in the presence of Abi2, suggesting that the phosphorylation of tyrosine 150 of WAVE2 was enhanced due to Abi2. (*B*) WAVE2 Y150F overexpression resulted in a decrease in the number of neurons that apposed to lattice fascicles. Five hours after the addition of dextran sulfate, the number of neurons apposed to lattice fascicles was measured for 9 h. Data ($n = 18$) represent mean ± SEM. (*C*) The ability of neurons to latch onto lattice fascicles was impaired in WAVE2 S137A-overexpressing neurons. Expression vectors for WAVE2, mutant WAVE2 S137A or WAVE2 S137E, in which serine 137 is substituted with alanine or glutamate, were transfected on E14.5. Five hours after the addition of dextran sulfate, the number of neurons apposed to lattice fascicles was measured for 9 h. The data for WAVE2 ($n = 17$), WAVE2 S137A ($n = 15$), and WAVE2 S137E ($n = 9$) are presented as mean ± SEM. (*D*) The ability to latch onto lattice fascicles was impaired in neurons overexpressing sh-Cdk5 or kinase-dead Cdk5 (Cdk5 D144N). Five hours after the addition of dextran sulfate, the number of neurons apposed to lattice fascicles was measured for 9 h. The data of sh-con ($n = 14$), sh-Cdk5 ($n = 16$), control ($n = 10$), or Cdk5 D144N ($n = 20$) are presented as mean ± SEM. *$P < 0.05$ by Student's *t*-test (*B*–*D*). (*E*) Structure of the WAVE2 and WRC based on the structural study on WAVE1 (Chen et al. 2010). A schematic diagram illustrating the WAVE2–Abi2 at the membrane, its possible phosphorylation sites (red dots) and regions of the a-helix are shown (pink box). Abi2 (except for its a-helix) is shown in orange, whereas WAVE2 (except for its a-helix) is shown in pink. In this study, we demonstrated the involvement of Cdk5 and Abl kinase in radial migration through the phosphorylation of WAVE2, whereas an involvement of Cables and p35 for Abl kinase action on Cdk5 was previously reported (Zukerberg et al. 2000). (*F*) Summary of the present study. Dendritic growth cone activity is indispensable for the commencement of glia-guided locomotion. WAVE2 is activated by phosphorylation and then works together with Abi2 for F-actin branch formation. However, activation of WAVE2 by Abl kinase and Cdk5 is not sufficient; if the growth cones do not become active, multipolar neurons that are ready to transform into bipolar are not able to complete the transformation.

multipolar–bipolar transition (Zukerberg et al. 2000). Our data show that the phosphorylation of Tyr150 of WAVE2, which has been demonstrated to be performed by Abl kinase, is essential for radial migration. In addition to this direct effect of Abl kinase, it has been demonstrated that Abl kinase controls Cdk5 activity together with other regulators such as Cables and p35 (Zukerberg et al. 2000). Therefore, it is likely that Abl kinase is involved in WAVE2 phosphorylation in at least 2 different pathways: direct phosphorylation of WAVE2 and indirect phosphorylation by Cdk5. Several lines of evidence suggest that Cdk5 is involved in the multipolar–bipolar transition, and its deletion results in inappropriate lamination (Ohshima et al. 2007; Nishimura et al. 2010). In this study, we reveal an important downstream consequence of Cdk5 activity. Additionally, our lattice assay suggests that WAVE2 is not the sole substrate for Cdk5 because knockdown of Cdk5 or overexpression of the dominant negative form of Cdk5 leads to a more severe impairment of neuronal latching activity compared with the overexpression of WAVE2 S137A. Therefore, it is likely that Cdk5 regulates radial migration through several pathways.

The lattice assay enables us to study how a single neuron interacts with a single nearby glial process, which is difficult to study with standard explant cultures. Some neurons probed a couple of fascicles with neurite-extending or -retracting activities and then chose one fascicle, suggesting that there is some preference for particular fascicles. Neurons are generated from radial glia (Bayer and Altman 1991). Radial glial processes and their descendant neurons are found close to each other in vivo, whereas the cells are mixed during the dissociation procedure in our lattice assay. Notably, most GFP-expressing cells are progenitors, as they are located in the ventricle where we transfect the plasmids. Thus, their descendants are generated in the culture. Therefore, in some cases, siblings are located close to each other even in our lattice assay. How this preference is established is interesting but remains an open question.

Author Contribution

M.-J.X. performed almost all experiments including in utero electroporation experiments. H.Y. carried out preliminary explant cultures and set up for time-lapse imaging. K.K. set up lattice assays together with H.Y. C.-C.W. performed some in utero electroporation experiments. M.K. performed experiments with BrdU. H.Z. advised experimental conditions on callosal axonal extension. A.S. and T.M. generated LPL-Lyn-GFP vector. K.N. provided some Abi1 materials. Y.O. constructed WAVE2 and Abi2 probes for in situ hybridization. T.I. performed experiments with in situ hybridization. M.S. conceived the project, organized the experiments, and wrote the article together with M.-J.X. All listed members provided invaluable comments on the article.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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