DAB2IP REGULATES NEURONAL MIGRATION AND NEURITE DEVELOPMENT

IN THE DEVELOPING NEOCORTEX

by

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ABSTRACT OF THE DISSERTATION

DAB2IP REGULATES NEURONAL MIGRATION AND NEURITE DEVELOPMENT

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Dab2ip (DOC-2/DAB2 interacting protein) is a member of the Ras GTPase-activating protein (GAP) family that has been previously shown to function as a tumor suppressor in several systems. Dab2ip is also highly expressed in the brain where it interacts with Dab1, a key mediator of the Reelin pathway that controls several aspects of brain development and function. I found that Dab2ip is highly expressed in the developing cerebral cortex, but that mutations in the Reelin signaling pathway do not affect its expression. To determine whether Dab2ip plays a role in brain development, I knocked down or over expressed it in neuronal progenitor cells of the embryonic mouse neocortex using the *in utero* electroporation technique. Dab2ip down-regulation severely disrupts neuronal migration, affecting preferentially late-born principal cortical neurons. Dab2ip overexpression also leads to migration defects. Structure-function experiments *in*

vivo further show that both PH and GRD domains of Dab2ip are important for neuronal migration. A detailed analysis of transfected neurons reveals that Dab2ip down- or up-regulation disrupts the transition from a multipolar to a bipolar neuronal morphology in the intermediate zone. Knock down of Dab2ip in neurons *ex-vivo* indicates that this protein is necessary for proper neurite development and for the expression of several major neuronal microtubule associated proteins (MAPs), which are important for neurite growth and stabilization. To further investigate the role of Dab2ip in the developing brain, I also conducted Western blot analysis of brain lysates obtained from *Dab2ip* knockout mice. I found that Dab2ip deficiency results in the abnormal activity of signal transduction pathways involving the Erk1/2 and Akt kinases during early embryonic brain development, and confirmed that Dab2ip is necessary for the expression of MAPs. Furthermore, I discovered that Dab2ip is required for maintaining the normal levels of Dab1 specifically during embryonic, but not postnatal, brain development. Thus, this study identifies, for the first time, a critical role for Dab2ip in mammalian cortical development and begins to reveal molecular mechanisms that underlie this function.

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Chapter 1. Introduction

1. Development of neocortex

The neocortex is the most recently evolved part of the cerebral cortex, which is derived from the superficial neural tissue of the mammalian forebrain. In humans, it functions in motor commands, sensory perception, and higher cognitive tasks. The neocortex develops into a laminated structure containing six cortical layers (I-VI) through an elaborate interplay between the processes of neurogenesis and neuronal migration, and through further neuronal differentiation and connectivity to form the synapses that underlie the formation of neural circuits. Each cortical layer contains excitatory glutamatergic pyramidal neurons and inhibitory γ-aminobutyric acid-expressing (GABAergic) interneurons (DeFelipe and Farinas, 1992; Jones, 1986). The pyramidal neurons make up over 80 % of all cortical neurons, they display a pyramidal-shaped soma, an axon traveling intracortically or subcortically, apical dendrites directed toward the pial surface, and basal dendrites directed toward the ventricular surface. The dendrites develop spines, which are membraneous protrusions that receive and integrate synaptic inputs from axons. The interneurons represent approximately 20% of the cortical neurons and project axons that terminate in local cortical circuits. The neurons in each cortical layer acquire distinct neuronal identities defined by the expression of layer-specific molecular markers and axon projection patterns (DeFelipe and Farinas, 1992; Hevner et al., 2003; Molyneaux et al., 2007). Layer I is the outermost superficial layer of the neocortex, also called as the marginal zone in the developing cortex. The marginal zone contains Cajal-Retzius cells that produce Reelin, a critical extracellular matrix protein

essential for radial migration. Cortical layers (II-VI) form the cortical plate, and are vertically assembled according to the time of birth, with layer VI been form first. Thus, cortical layers are generated in an inside-out manner (Marin-Padilla, 1978; Rakic, 1974). Supragranular layers (II-III) pyramidal neurons project intracortically (either intra-hemispherically or contralaterally) through the corpus callosum (Howell et al., 1999). Layer IV, a granular layer, is composed of pyramidal neurons and spiny stellate neurons. This layer is the main site that is targeted by thalamocortical axon to develop the barrel cortex for the interpretation of somatosensory information (Wu et al., 2011). Layer V, one of the deep-layers of the neocortex with layer VI, contains pyramidal neurons that develop the major corticofugal axons targeting subcerebral structures including corticobulbar, corticospinal, corticotectal and corticopontine tracts (Howell et al., 1999; Molnar and Cheung, 2006). Also, Layer V neurons form the collateral axons innervating the striatum and thalamus (Molnar and Cheung, 2006).

The correct positioning of neurons in the appropriate cellular layers is not absolutely required to establish proper target connectivity, but it is generally believed to be important for the refinement of cortical circuitries. Therefore, the impairment in cortical lamination and synaptic connectivity is thought to result in a wide range of neurodevelopmental disorders, such as lissencephaly, austism, schizophrenia, and some forms of childhood epilepsy (Barkovich et al., 2012; Kazdoba et al., 2012; Manzini and Walsh, 2011). The molecular mechanisms that regulate neuronal development have been only partially uncovered, and still much remains to be elucidated in order to improve the prevention and treatment of neurodevelopmental disorders.
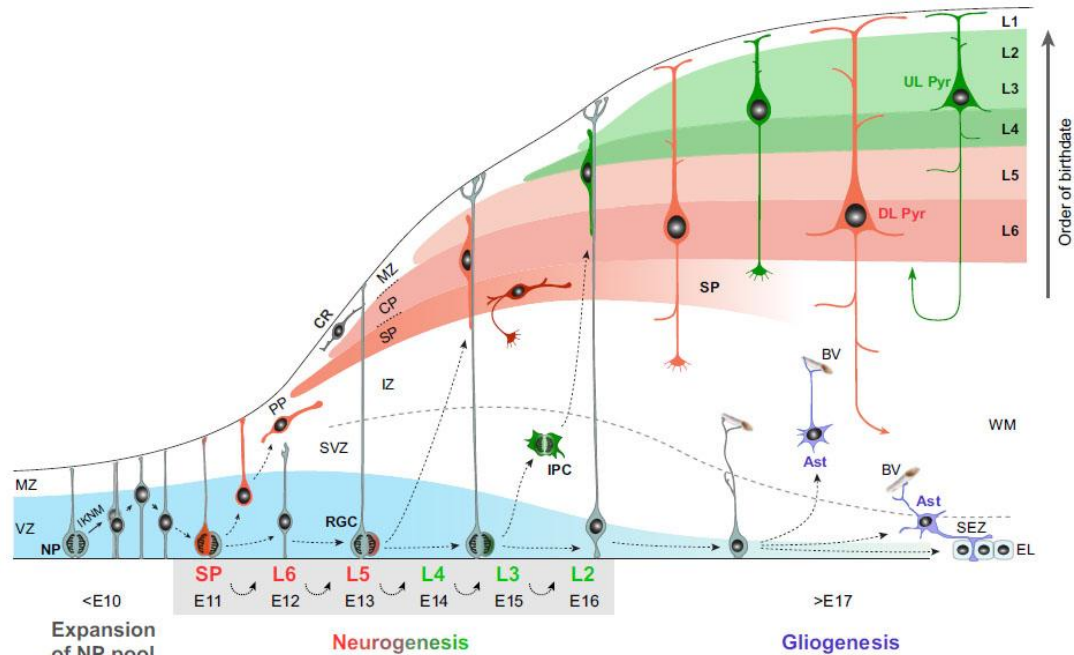
Figure 1. Generation and migration of cortical pyramidal neurons in the mouse developing neocortex.

Prior to neurogenesis, neural progenitor (NP), divides symmetrically to make a pool of progenitors. Starting from around E11.5, radial glial cell (RGC), originated from NP in the ventricular zone (VZ), divides asymmetrically to generate self-renewed RGC and neuron that is turned to be the preplate (PP) neurons migrating to the pia. The subsequently generated neurons migrate toward the pia and split the PP into the subplate (SP) and the marginal zone (MZ, Layer I later) and become layer VI neurons as cortical plate (CP) neurons. At this time MZ contains Cajal-Retzius cells (CR) producing Reelin for further layers formation. Subsequently born neurons keep migrating to the pia passing previously born neurons to reside in the outer layer of the CP, which is inside-out manner. Thus pyramidal neurons (Pyr) of the upper layers (II-IV) are born and positioned at a late stage and the one of the deep layers (V-VI) resides early. During the corticogenesis, the

RGC is also divided asymmetrically to generate a neuron and intermediate progenitor cell (IPC) that migrates to the subventricular zone and generates more neurons. When corticogenesis fades around the time when an animal is born, RGCs are differentiated to astrocytes (Ast) for the CP and subependymal zone (SEZ) and they form a layer of ependymal cells (EL). BV, blood vessel, WM, white matter.

1-1.   Generation of excitatory pyramidal neurons

Cortical pyramidal (principal) neurons are generated from the radial glia in the ventricular zone (VZ) and intermediate progenitors in the subventricular zone (SVZ) (Angevine and Sidman, 1961). The radial glia stem cell is a precursor cell during neurogenesis (Hartfuss et al., 2001; Malatesta et al., 2000; Noctor et al., 2001; Noctor et al., 2004). This cell type is derived from neuroepithelial cells which reside in the wall of the ventricle during the development of cerebral cortex, and is thought to undergo multiple rounds of self-renewal to expand the population of progenitor cells by symmetric division prior to neurogenesis (Haubensak et al., 2004; Rakic, 1982). As neurogenesis begins, progenitor cells produce a self-renewing radial glia and a postmitotic daughter neuroblast or, alternatively, it produces an intermediate progenitor cell by asymmetric division (Anthony et al., 2004; Malatesta et al., 2000; Noctor et al., 2004). Concurrently, the radial glia serves as a radial scaffold that guides migrating neurons along with its radial fiber, which spans from the wall of neural tube to the pial surface of the cortex, while its cell body resides in the VZ (Ayala et al., 2007; Rakic, 1971). At postnatal ages, as neurogenesis fades, the radial glia differentiates into an ependymal cell or an astrocyte. These glial cells play active roles in brain maturation, ranging from structural support to the modulation of synaptic activity in neuronal networks (Noctor et al., 2008; Schmechel and Rakic, 1979). The intermediate progenitors, born from the radial glia in the ventricular zone, migrate to the subventricular zone and generate more pyramidal neurons. These neurons contribute to the formation of all layers of the neocortex (Caviness and Takahashi, 1995; Haubensak et al., 2004; Kowalczyk et al., 2009; Noctor et al., 2008).

The development of the mammalian neocortex takes place in multiple steps. The first postmitotic neurons in the mouse neocortex are born at embryonic day 10.5 (E10.5) and form the preplate, which then produces the subplate and the marginal zone (De Carlos and O'Leary, 1992; Marin-Padilla, 1978). The subplate is developmentally transient and plays a key role in the initial establishment of thalamo-cortical afferent and efferent projections (Herrmann et al., 1994; Kostovic and Rakic, 1990). The marginal zone, which is destined to become layer I at postnatal days, contains specialized neurons, called Cajal-Retzius cells, that produce Reelin, a key molecule that promote cortical lamination (D'Arcangelo et al., 1995). The next generation of neurons, born around E11.5 as the first cortical pyramidal cells, migrates toward the pia and splits the preplate into the subplate and the marginal zone to form the cortical plate (Marin-Padilla, 1978). This subset of neurons eventually forms layer VI, the deepest layer in the cortical plate (Marin-Padilla, 1978). The next newly generated neurons, upon entering the cortical plate, travel past older neurons to become positioned in the most superficial layer of the developing cortex, where they settle and differentiate into layer-specific neurons (Angevine and Sidman, 1961; D'Arcangelo and Curran, 1998; Frantz and McConnell, 1996; Rakic, 1974). Thus, the sequential generation and migration of neurons in the radial dimension of the neocortex results in the formation of inside-out cortical layers, meaning that the inside layers are formed first and the outside layers are formed last. The birth date of cortical pyramidal neurons is closely associated with their laminar identity, which consists of the expression of specific markers, and defined synaptic connectivity, axon projections, and physiological functions (Howell et al., 1999; Molyneaux et al., 2007).

1-2.   Radial migration of excitatory pyramidal neurons

Based on the origin and destination of the neurons, two kinds of neuronal migration modes have been described: radial migration, by which pyramidal excitatory neurons migrate from the VZ/SVZ of the dorsal telencephalon toward the surface of the brain; and tangential migration, by which GABAergic inhibitory neurons migrate from subpallium structures toward the neocortex (Ayala et al., 2007). All principal cortical neurons are born in ventricular or subventricular zone of the neocortex and migrate radially toward the pial surface to become positioned in cellular layers (Rakic, 1972). At the early stage of corticogenesis, principal neurons migrate mostly by somal translocation, using their long leading processes contacting the pia to move efficiently into the upper cortical plate and later settle in deep cortical layers (Nadarajah et al., 2001). As the cortex develops further, the migration of later-born principal neurons has multiple phases, including a temporary migration arrest and morphological changes that take place before neurons finally travel into the cortical plate (Noctor et al., 2004; Tabata and Nakajima, 2003). Many postmitotic neurons move up radially from the VZ/SVZ and acquire a multipolar morphology in the SVZ/intermediate zone (IZ) , where they pause for a day or more (in case of mouse) displaying dynamic behaviors such as changing orientation and retracting or extending processes (Noctor et al., 2004). More than a half of migrating neurons go through a retrograde migration phase, in which the cell body moves backword, extending the leading process toward the ventricle (Noctor et al., 2004). After that, neurons change their polarity to set off glia-guided locomotion into the developing cortical plate , which requires a leading process that wraps around a radial glia fiber

(Noctor et al., 2004; Rakic, 1971; Tabata and Nakajima, 2003). This morphological transition has been proposed to facilitate the initial extension of the axon (Noctor et al., 2004). When the leading process of migrating neurons approaches the marginal zone, the late-born neurons detach from the glial fiber and use somal translocation to complete their migration and become properly positioned in the superficial region of the developing cortex (Elias et al., 2007; Gongidi et al., 2004; Nadarajah et al., 2001).
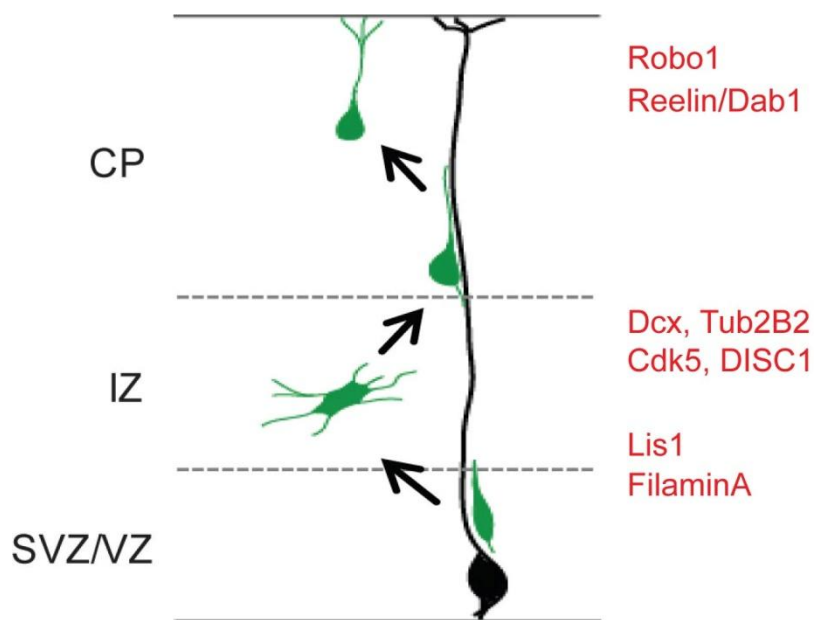
Figure 2. Radial migration of late-born neuron in the developing neocortex.

Principal cortical neurons born in the VZ/SVZ migrate to the SVZ/IZ changing their morphology to multipolar cells, and then pause the migration for a day (mouse) before they get to the IZ. In the upper IZ, neurons change morphology again from multipolar to bipolar cells, and then migrate by glia-guided locomotion into the developing CP. When the leading process of the migrating neurons reaches the marginal zone, neurons switch migrating mode to somal translocation, moving their cell bodies through previously resided neurons and then becoming positioned in the outermost layers of the CP. The proteins colored in red are described in detail in the molecular mechanism section, Chapter 1. 1-2 and depicted here at the migration phases that they mostly regulate.

Molecular mechanisms

Reelin and the Control of Somal translocation

The molecular mechanisms that control the distinct steps in neuronal radial migration have been partially elucidated by several investigators. Genetic studies in mouse models demonstrated that the extracellular protein Reelin (Reln) is required for the formation of cellular layers of the neocortex by regulating mostly somal translocation and neuronal orientation during radial migration (D'Arcangelo et al., 1995; Franco et al., 2011; Jossin and Cooper, 2011; Sekine et al., 2011). Since the inside-out pattern of lamination is mainly determined by somal translocation, which forces migrating neurons past older neurons during early corticogenesis and the final step of late-born neurons' migration, Reelin signaling deficiency causes the deposition of migrating neurons below the older neurons, disrupting normal cortical lamination (Caviness, 1982; Franco et al., 2011). In addition, a recent study has shown that Reln also plays a role in the polarization of multipolar neurons during the radial migration of late-born neurons in the IZ through regulating the activity of Rap1 (Jossin and Cooper, 2011). The signal transduction machinery that mediates the activities of Reln includes the ApoER2 and VLDLR receptors, and the adapter protein Dab1 (D'Arcangelo et al., 1999; Hiesberger et al., 1999). Thus, genetic disruptions of the signaling pathway, such as those present in *ApoER2*/*VLDLR* double knockout and *Dab1* knockout mice, lead to a migration defect that is similar to that caused by *Reln* deficiency in the *reeler* homozygous mutant mouse (Boycott et al., 2005; Hiesberger et al., 1999; Rice et al., 1998). Similarly, in humans mutations in the *RELN* or *VLDLR* genes cause a severe migration disorder known as

lissencephaly and cerebellar hypoplasia, which leads to intellectual disability, motor dysfunction and epilepsy (Boycott et al., 2005; Hong et al., 2000; Ozmen et al., 2000).

Robo1-Terminal positioning

Roundabout (Robo) and its ligand Slit are also proposed to regulate neuronal migration. Robo signaling has been identified as a critical regulator of axonal pathfinding and is also involved in the control of proliferation and migration of neocortical interneurons (Andrews et al., 2008; Andrews et al., 2006; Lopez-Bendito et al., 2007). Recently, Robo1 has been demonstrated to regulate final positioning of layer II/III pyramidal neurons during radial migration (Gonda et al., 2012). In this study, neurons in which Robo1 was suppressed by RNAi exhibited delayed migration into the cortical plate and abnormal positioning of layer II and III underneath the marginal zone, regardless of their birth date (Gonda et al., 2012).

Doublecortin-Morphological transition

Several cytoskeletal regulators, such as microtubule-associated proteins (MAPs), actin-binding protein and tubulin subunits have been identified as mediators of the morphological transition from multipolar to bipolar stage during the radial migration of late-born neurons,. MAPs are important for microtubule assembly, stabilization, and interaction with other cytoskeleton proteins (Baas and Heidemann, 1986; Hirokawa, 1994). Doublecortin (Dcx) is the MAP that has been best characterized for its function in neuronal migration and differentiation. Genetic mutations in human *DCX* cause X-linked lissencephaly (a migration disorder characterized by a paucity of cortical gyri or smooth brain) in males and double cortex syndrome (also called subcortical band heterotopia) in females (des Portes et al., 1998). Surprisingly, genetic deletion of *Dcx* in a mouse model

did not show any defect in neocortical lamination. This is likely due to genetic compensation by Doublecortin-like kinase (Dclk) since double knockout of *Dcx* and *Dclk* causes a neuronal migration deficit in the mouse neocortex (Corbo et al., 2002; Deuel et al., 2006; Koizumi et al., 2006). The knockdown of Dcx or Dclk mRNA in migrating neurons by *in utero* electroporation revealed that the acute loss of these genes causes a migration arrest in the IZ as multipolar cells, which indicates that Dcx and Dclk are required at the morphological transition stage during radial migration (Bai et al., 2003; Koizumi et al., 2006). In accordance with this view, Dcx overexpression makes the neurons retain their bipolar morphology in the IZ, a result which is opposite to the multipolar morphology in Dcx knockdown neurons (LoTurco and Bai, 2006).

Lis1-Morphological transition

Heterozygous mutations in the *PAFAH1B1* gene is the main cause of type I lissencephaly in humans. The gene product, Lis1, interacts with dynein, dynactin, NudE1, and NudEL1 to regulate dynein motor function and microtubule dynamics, and thus plays a key role in nuclear movement and neuronal migration (Cardoso et al., 2002; LoTurco and Bai, 2006; Smith et al., 2000; Sweeney et al., 2001; Tsai and Gleeson, 2005). Lis1 is a regulatory subunit of the platelet-activating factor acetylhydrolase Ib (PAFAH1B) complex that hydrolyzes the platelet-activating factor (PAF), which in turn functions as a second messenger involved in long-term potentiation (LTP) (Escamez et al., 2012; Kato et al., 1994). Knockdown assays using Lis1 RNAi (RNA interference) demonstrated that a reduced level of Lis1 causes the accumulation of multipolar neurons in the SVZ in the developing rat brain, which suggests that Lis1 is required for the progression from

multipolar to bipolar stage during radial neuronal migration (Tsai et al., 2005). It is interesting that Lis1 also interacts with phosphorylated Dab1 resulting from the activation of Reelin-VLDLR receptor signaling, which controls somal translocation and the orientation of multipolar neurons in the IZ as discussed above (Assadi et al., 2008; D'Arcangelo, 2006; Zhang et al., 2007a; Zhang et al., 2009). Although genetic studies have revealed that the loss of catalytic subunits of the PAFAH1B1 complex, PAFAH1B3 (ALPHA1) and PAFAH1B2 (ALPHA2), does not affect brain development, genetic loss of other components of the dynein/dynactin pathway, such as *Ndel1* and *14-3-3ε,* causes neuronal migration defects in the mouse (Koizumi et al., 2003; Sasaki et al., 2005; Toyo-oka et al., 2003; Yan et al., 2003). Interestingly, Dcx was also found to participate in the Dynein complex with Lis1, and their genetic interaction has been described (Pramparo et al., 2010; Tanaka et al., 2004a).

Filamin A-Morphological transition

Unlike Dcx and Lis1, which function in the control of microtubule dynamics, Filamin A is an actin-binding protein that crosslinks actin and interacts with integrins for the control of cell adhesion and migration (Liu, 2011). In humans, mutations in *FLNA* (X-linked) cause periventricular nodular heterotopia, which is associated with epilepsy and cognitive defects (Chang et al., 2005; Liu, 2011; Sheen et al., 2001). RNA interference studies revealed that Filamin A is required for the migration of multipolar cells and for the transition to bipolar morphology. Migrating neurons that exhibit loss of Filamin A accumulated in the SVZ (Nagano et al., 2004; Nagano et al., 2002). This defect was rescued by introducing RNAi targeting Filip1, a protein that binds and

degrades Filamin A, indicating that adequate amounts of Filamin A are necessary for radial migration (Nagano et al., 2004).

Tubulin-Morphological transition

The microtubule (MT) is a key structural component of the cell that enables important biological processes such as cell cycle, cell morphology acquisition, motility, and intracellular trafficking (Jaglin and Chelly, 2009). MTs are produced by the polymerization of dimerized tubulins whose isoforms belong to a large family of proteins (α-, β-, γ-, δ-, ζ-, η-tubulin). α- and β-tubulins have different isotypes whose expression is regulated spatially and temporally (Lewis et al., 1985; Miller et al., 1987). As migrating neurons undergo multiple morphological changes during radial migration which require modulation of cytoskeletal dynamics, mutations in tubulin subunits can be expected to cause neurodevelopmental disorders. Indeed, mutations in the *TUB1A1* gene cause a lamination defect in the developing mouse cortex and are associated with type I lissencephaly in humans (Keays et al., 2007). In addition, knockdown of Tubb2b by *in utero* electroporation revealed that the loss of this gene results in the accumulation of migrating neurons in the IZ of the developing rat neocortex (Jaglin and Chelly, 2009; Jaglin et al., 2009).

Cdk5-Morphological transition

Cyclin-dependent kinase 5 (Ckd5) is a serine/threonine kinase protein that was discovered based on its sequence homology to other cyclin-dependent kinases such as cdc2, cdk2, and cdk3 (Lew et al., 1992; Su and Tsai, 2011). However, unlike other cyclin-dependent kinases, its function is not directly involved in the regulation of cell cycle, but is highly associated with neurodevelopment, synaptic plasticity, and synaptic

homeostasis (Su and Tsai, 2011). Cdk5 is activated by binding of p35 and p39, whose double null-deletion mutants show a phenotype similar to Cdk5 knockout mice, including perinatal lethality and severe lamination deficits (Ko et al., 2001; Ohshima et al., 1996). An IUE study with conditional Cdk5 mutant mice has shown that Cdk5 is required for neuronal migration, especially for the morphological transition stage from multipolar to bipolar neurons in the IZ during radial migration (Ohshima et al., 2007a). This kinase has been also extensively studied because its substrates regulate multiple aspects of cellular functions (Su and Tsai, 2011). Related to neuronal migration, Cdk5 phosphorylates multiple proteins including neurofilament, Dab1, MAPs (Map1b, Tau, Dcx, Ndel1), DISC1, and actin regulatory proteins (PAK1) (Ayala et al., 2007; Su and Tsai, 2011). In addition, Cdk5 phosphorylates Dcx and lowers the affinity of Dcx for the microtubule, thereby affecting Dcx-dependent neuronal migration (Tanaka et al., 2004b).

DISC1-Morphological transition

Disrupted in Schizophrenia-1 (DISC1) is one of the best known risk factors for schizophrenia, which was originally identified at the breakpoint of a balanced chromosomal translocation that segregates with psychiatric disorders in a large Scottish family (Blackwood et al., 2001; Chubb et al., 2008). DISC1 regulates differentiation and proliferation of neural progenitors and is also involved in the neuronal migration and orientation of migrating neurons in the developing neocortex via the interaction with several proteins including Ndel1, Lis1, and Gsk3β (Chubb et al., 2008; Kamiya et al., 2005; Mao et al., 2009). IUE-mediated knockdown of DISC1 demonstrated that the loss of DISC1 by RNAi leads to the accumulation of cortical neurons at the IZ/SVZ boundary (Kamiya et al., 2005).

Others

The best-characterized neuronal MAPs are Map2, Map1b and Tau, which are localized specifically to dendrites or axons (Liu, 2011). These proteins exist as several isoforms that are produced by alternative splicing, and their expression is developmentally regulated (Crandall and Fischer, 1989; Goedert et al., 1989; Menezes and Luskin, 1994; Riederer and Matus, 1985). The functions of Map2 and Tau are thought to be, at least in part, redundant with Map1b during early brain development. For example, knockdown of either Map2 or Tau does not cause a significant defect in cortical development, including layer formation and neurite outgrowth (Takei et al., 2000; Teng et al., 2001). However, both Map2/Map1b and Map1b/Tau double knockout mice exhibit a neuronal migration defect and layer malformation as well as inhibited neurite growth. These deficits are more severe than the phenotype of single Map1b knockout mice (Takei et al., 1997; Takei et al., 2000; Teng et al., 2001). Moreover, the combined downregulation of these MAPs has been shown to affect neuronal polarity (Gonzalez-Billault et al., 2002).
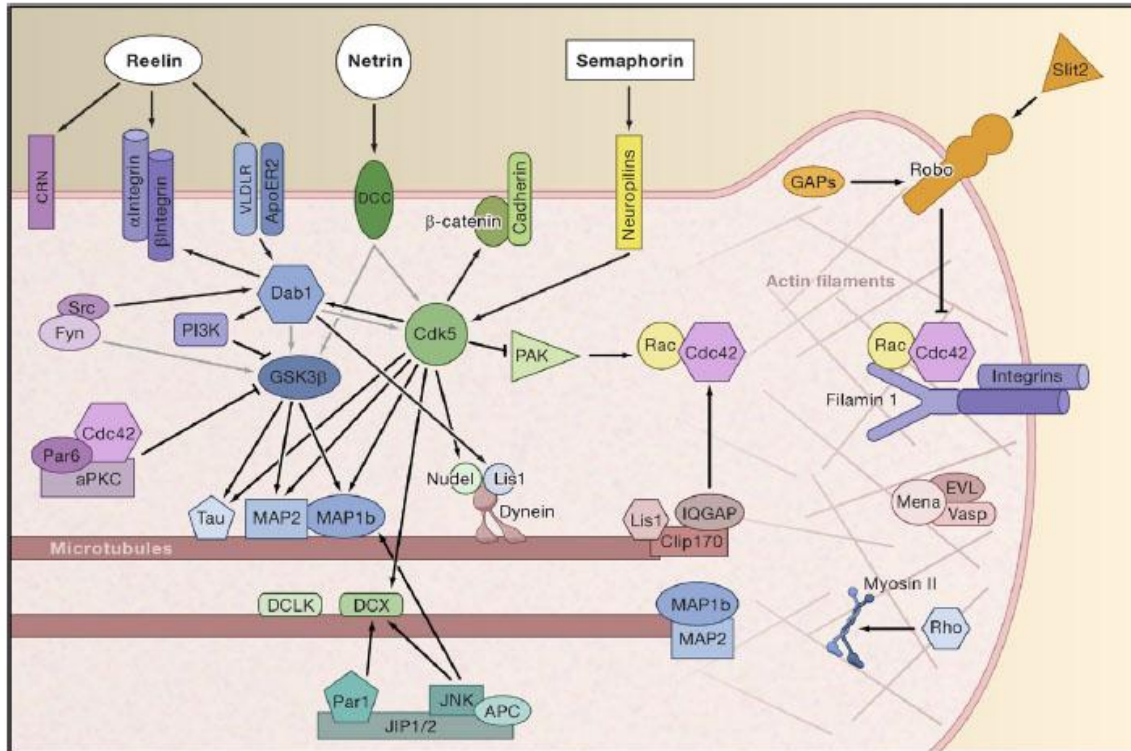
Figure 3. Molecular networks regulating cytoskeletal dynamics in migrating cortical neurons.

Extrinsic guidance cues including extracellular matrix proteins, growth factors, neurotrophic factors, and adhesion molecules evoke a wide range of intracellular signaling cascades to control the cytoskeletal dynamic during the neuronal migration. Black arrows represent direct interactions between molecules, and gray arrows indicate activation of the pathway without direct protein-protein interaction.

1-3. Generation and tangential migration of inhibitory interneurons

The cortical interneurons are generated mostly from the medial and the lateral ganglionic eminences (MGE and LGE, respectively) in the ventral telencephalon

(Kriegstein and Noctor, 2004; Marin and Rubenstein, 2001). Like pyramidal neurons, the migration of interneurons is also spatially and temporally regulated. During the early embryonic corticogenesis (E11.5 in the mouse), interneurons originated from the MGE and the anterior entopeduncular area (AEP) pass the developing striatum superficially and travel toward the dorsal forebrain tangentially to enter the developing neocortex along the subplate and marginal zone. During the mid-embryonic phase (E12.5-E14.5), interneurons generated from the MGE invade the SVZ, the lower IZ, and the subplate before entering the cortical plate. At a late embryonic phase (E14.5-E16.5), both the MGE and the LGE contribute to the generation of cortical interneurons. The interneurons originating from the MGE migrate into the cortical plate, while the neurons originated in the LGE head toward the proliferative SVZ, where they can undergo mitotic cell division (Ayala et al., 2007; Marin and Rubenstein, 2001). Genetic manipulations have revealed molecular mechanisms that regulate the differentiation or the regionalization of cortical interneurons in the basal telencephalon (Ayala et al., 2007). For example, in mice lacking the homeobox proteins Dlx-1 and Dlx-2, the tangential migration of cortical interneurons is significantly disrupted, whereas the Mash1 mutant mouse has revealed that this transcription factor is required for neurogenesis in the MGE (Anderson et al., 1997; Casarosa et al., 1999).

2. Dab2ip

2-1. Functions

Dab2ip (DOC-2/DAB2 interacting protein, ASK1 interacting protein) is a member of the Ras GTPase-activating protein (GAP) family, which was identified as a protein that interacts with the Dab1 and Dab2 adapter proteins through their PTB (phosphotyrosine-binding domain) domain (Chen et al., 2002; Homayouni et al., 2003; Wang et al., 2002). Dab2ip possesses growth inhibitory activity in prostate cancer cells and it has been shown to inhibit multiple members of the Ras family, such as H-RAS, R-RAS, K-Ras, and TC21, which are critical for the regulation of the Raf-Erk pathway (Wang et al., 2002). This inhibitory function is due to the Ras GAP activity of Dab2ip and its loss has been suggested to be critical for the increased proliferation of cancer cells in which Dab2ip expression is downregulated.

Dab2ip is also involved in the tumor necrosis factor α (TNF-α)-mediated cell apoptosis through the activation of apoptosis signal-regulating kinase 1 (ASK1)/JNK (Zhang et al., 2003). Dab2ip participates in a complex with tumor necrosis factor receptor 1 (TNFR1), TNFR associated death-domain protein (TRADD), receptor interacting protein 1 (RIP1), and TNFR associated factor (TRAF2) in the plasma membrane (Zhang et al., 2004). Upon binding of TNF-α to the receptor, Dab2ip is released from the TNFR1 and translocates to the cytoplasm along with the complex proteins (Zhang et al., 2004). RIP1 in the complex phosphorylates Dab2ip, which leads to a conformational change of Dab2ip. This conformational change causes ASK1 to be released from 14-3-3, and this in turn results in the activation of the ASK1-JNK apoptosis pathway (Zhang et al., 2007b; Zhang et al., 2003). Cooperating with the ASK1 apoptosis pathway, Dab2ip also

modulates the phosphatidylinositol 3-kinase (PI3K)-Akt pathway to balance cell survival and apoptosis (Xie et al., 2009).

Recently, it has been reported that Dab2ip suppression promotes metastasis in a model of prostate cancer progression (Min et al., 2010; Xie et al., 2010). The metastasis is modulated by a small Ras GTPase and NF-κB, which is activated by Dab2ip-downregulation in prostate cancer cells (Min et al., 2010). Another group also reported that epithelial-to-mesenchymal transition (EMT) is initiated by the activation of GSK 3β/β-catenin, followed by the reduction of E-cadherin and the upregulation of vimentin (Xie et al., 2010).

2-2. Expression

The human Dab2ip gene is located on chromosomal region 9q33.1-q33.3 spanning almost 96 kb (Chen et al., 2002). Several transcript variants are expressed that contain either 14 or 15 exons and share exons 2-13 (Chen et al., 2002). The transcript variants are generated by alternative splicing starting from different first exons that are generated from multiple transcription start sites, due to the lack of TATA sequence in the promoter region of the Dab2ip gene (Chen et al., 2006; Chen et al., 2002). It has been reported that the 5' region of human and mouse exon1A of Dab2ip gene is rich in CpG, which suggests that transcription might be regulated epigenetically (Chen et al., 2006; Chen et al., 2002). In fact, Dab2ip expression has been found to be lower in several cancers, due to histone deacetylation by decreased histone acetyltransferase 1 (HDAC1) and DNA hypermethylation by N-methyltransferase EZH2 at the promoter region of the

human Dab2ip gene (Chen et al., 2003; Chen et al., 2005). It is interesting to note that

HDAC1 inhibitors and DNA methyltransferase inhibitors reverse the expression of

Dab2ip to the level of normal prostate cells (Chen et al., 2003; Qiu et al., 2007).

Under normal conditions, the expression pattern of the several mRNA isoforms varies depending on tissues and ages (Chen et al., 2006; Homayouni et al., 2003; Qiu et al., 2007; Wang et al., 2002). The tissues that exhibit the abundant Dab2ip expression are the brain, salivary gland, testis, and kidney (Chen et al., 2006). In the brain, Dab2ip is expressed throughout many regions including the neocortex, hippocampus, and cerebellum from embryonic to postnatal ages (Homayouni et al., 2003).
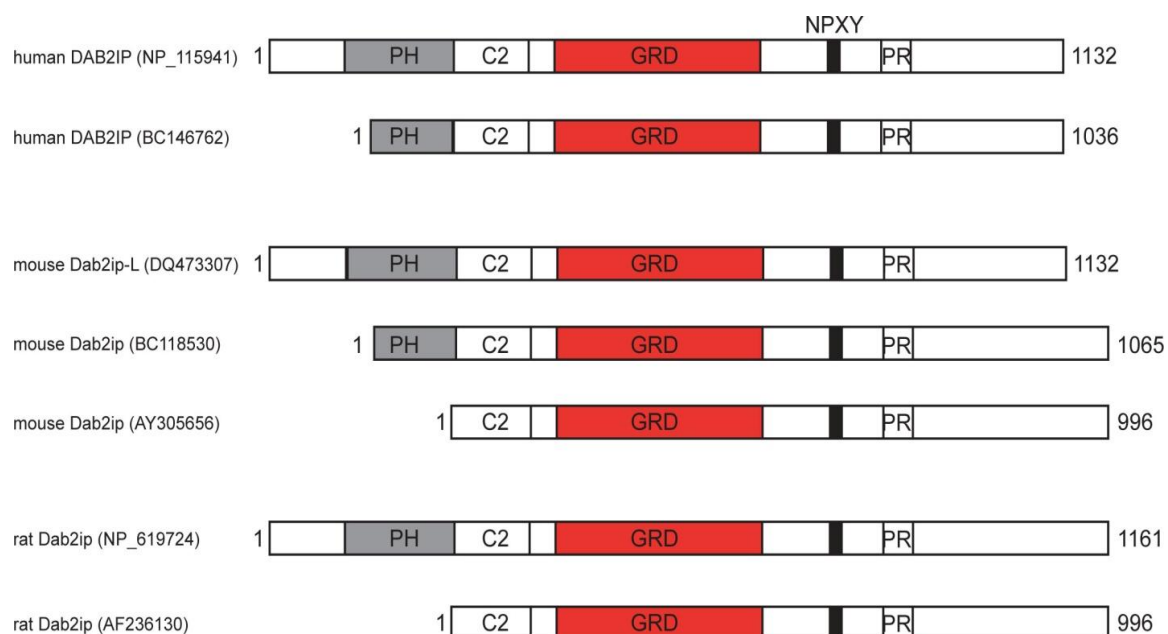
Figure 4. Diagram of Dab2ip isoforms.

The structure of predicted Dab2ip protein is fairly conserved among species. Common protein domains include the C2, GRD, and PR domain. The PH domain is present in most, but not all, isoforms. The N-terminal region of Dab2ip is the most divergent among

isoforms. Dab2ip isoforms are expressed from transcripts that are generated by alternative splicing from different first exons and multiple transcription start sites.

2-3. Structural features

Two protein isoforms of Dab2ip have been described, with molecular weights of 110kDa and 135kDa, which are differentially expressed in multiple tissues and ages (Wang et al., 2002) (S. Qiao, unpublished). Both isoforms consists of several functional domains: a pleckstrin homology (PH), a PKC-conserved 2 (C2), a GTPase activating protein (GAP) homology, a PERIOD-like (PER), a proline-rich (PR), and a leucine zipper (LZ) domain that is required for protein dimerization (Homayouni et al., 2003; Wang et al., 2002). Intramolecular interaction between the N-terminus and the C-terminus of Dab2ip has been suggested to mediate a conformational change that is induced by TNF-α signaling or ER stress (Zhang et al., 2003). Dab2ip is considered a scaffolding protein, whose functional domains recruit different kinds of interacting proteins for post-translational modification and downstream signaling (Homayouni et al., 2003; Xie et al., 2009; Zhang et al., 2004; Zhang et al., 2003).

The PH domain usually mediates membrane localization, and is found in many signaling molecules (Rebecchi and Scarlata, 1998). In acute myeloid leukemia (AML) patients, DAB2IP has been identified as a MLL fusion partner because of the chromosome translocation between the intron 9 of MLL gene and the exon 2 of Dab2ip gene (von Bergh et al., 2004). This translocation disrupts the PH domain, resulting in modification of Dab2ip function and enabling leukemia transformation (von Bergh et al., 2004). The PH domain is also critical for inositol-requiring enzyme-1 (IRE1) interaction during ER stress-induced ASK1-JNK activation and apoptosis (Luo et al., 2008; Zhang et al., 2004).

The C2 domain of Dab2ip contains two lysine-rich clusters that form a phosphatidyl-binding motif (Zhang et al., 2003). This domain is reported to be critical for ASK1 interaction (Zhang et al., 2004; Zhang et al., 2003). The GAP domain is a catalytic domain that mediates the function of Dab2ip as a Ras GTPase-activating protein (Wang et al., 2002). It also cooperates with the C2 domain for interaction with ASK1, and associates with RIP1 to promote Dab2ip phosphorylation (Zhang et al., 2004; Zhang et al., 2003). The PER domain interacts with TRAF2 in the cytoplasmic complex II that is involved in the activation of ASK1 and inhibition of TNF-α-induced NF-κB (Zhang et al., 2004). The phosphorylation of a serine residue in the PER domain of Dab2ip by RIP1 is critical for the conformational change to an open form, which then allows Dab2ip to associate with ASK1 inhibitor, 14-3-3 and Akt (Xie et al., 2009; Zhang et al., 2007b). The PR domain of Dab2ip presumably interacts with proteins containing a Src homology 3 (SH3) domain; thus, the p85 regulatory subunit of PI3K, which contains a SH3 domain, interacts with the PR domain of Dab2ip suppressing a PI3K signaling pathway (Xie et al., 2009). The PR domain also functions to maintain the closed form of Dab2ip, which can interact with TNFR1 near the plasma membrane (Zhang et al., 2004). The NPXY motif in Dab2ip is necessary for the interaction with PTB domain in Dab1 and Dab2 (Homayouni et al., 2003; Wang et al., 2002).

2-4. Genetic studies

Genome-wide association studies in prostate cancer patients have shown that a single nucleotide polymorphism (SNP) located in human *DAB2IP* gene is significantly

associated with aggressive prostate cancer (Duggan et al., 2007). Recently, it was also pointed out that *DAB2IP* exhibits a risk allele in an association study for abdominal aortic aneurysms and coronary heart disease (Gretarsdottir et al., 2010).

In genetic studies related to the central nervous system, *DAB2IP* has been identified as an Autism candidate gene from a genome-wide association study in family-based autism; in this study, a role in neurite regulation was suggested based on the Ras GAP activity of Dab2ip (Hussman et al., 2011). Another study identified autism-associated single nucleotide polymorphisms (SNPs) in clock genes, including *npas2*, *per1*, and *en2*, which may generate possible microRNAs targeting Dab2ip (Nicholas et al., 2008 Nature proceedings). Also, in a genomic convergence analysis of schizophrenia, mRNA sequencing showed that a Dab2ip transcript was decreased significantly by 11% (transcript alignment) and 15% (genomic alignment) in schizophrenia patients compared to a control cohort (Mudge et al., 2008).

Chapter 2. Dab2ip regulates neuronal migration and neurite development in the neocortex.

1. Introduction

In this chapter I investigated the expression and the function of Dab2ip in the embryonic mouse cerebral cortex using *in vivo* and *ex vivo* knock down and overexpression approaches. I discovered that Dab2ip is important for the migration of late-born principal neurons and for neurite development, affecting the transition to a bipolar morphology in the intermediate zone. The downregulation of Dab2ip was accompanied by a marked reduction in the expression of several microtubule-associated proteins (MAPs), thus providing a likely mechanism by which Dab2ip exerts its function in neuronal migration and maturation. The data presented in this chapter is included in the manuscript: (Gum Hwa Lee, Sun Hong Kim, Ramin Homayouni, Gabriella D'Arcangelo, 2012 Dab2ip regulates neuronal migration and neurite outgrowth in the developing neocortex, PLoS ONE). As of August 14, 2012, this manuscript has been tentatively accepted for publication after minor revisions.

2. Materials and Methods

Animals handling and in utero electroporation (IUE)

All animals used in this study were handled in accordance with a protocol approved by the Association for Assessment and Accreditation of Laboratory Animal Care committee at Rutgers, the State University of New Jersey. Wild type mice (ICR mice, Taconic Farms) were used for *in utero* electroporation (IUE) experiments as described previously (Yabut et al., 2010). Approximately 2 µl of a plasmid mix (3 µg) containing Fast Green dye (Sigma) was injected into the lateral ventricle of embryonic day (E) 12.5 and 14.5 embryos, and electroporated using the ElectroSquarePorator ECM 830 (BTX) set at five 50 ms pulses of 40 V with 950 ms intervals. Embryos were allowed to develop *in utero* for 2-4 days after electroporation. Brains were then dissected, fixed overnight in 4% paraformaldehyde (PFA) at 4ºC and then placed in 30% sucrose/1x PBS mix for cryoprotection. Brains were frozen in OCT (Tissue-Tek) and sectioned coronally at 30 µm for confocal analysis. All experiments were conducted at least in triplicates. Mutant mouse colonies were: *Reeler* mice (B6C3Fe-*ala-Relnrl*/+) (Jackson Laboratories, Bar Harbor, ME, USA), *Dab1* knock-out mice (a gift of J. A. Cooper, Fred Hutchinson Cancer Research Center, Seattle, WA), and conditional *Pten* knockout mice (NEX-*Pten*) (Kazdoba et al., 2012).

Expression constructs

Three different shRNA constructs targeting the Dab2ip coding sequence were generated using the mU6pro vector (a gift from Dr. David Turner, University of Michigan). The hairpin shRNA sequences are: 5'-GAGCACTTTGAGTTCCATAAC-3',

shDab2ip 1; 5'-GACCTCTCTGGTCTGATAGAT-3', shDab2ip 2; 5'-GATATCAGTGAACGGCTCATC-3', shDab2ip 3. As a non-targeting shRNA control, we used pGE-2-hrGFP II (Stratagene). pEGFP and pmCherry expression plasmids in which fluorescent proteins are encoded from the CAG promoter were described previously (Saito and Nakatsuji, 2001; Yabut et al., 2010). Full-length Dab2ip cDNA (L isoform, GenBank accession number Q473307) was cloned by RT-PCR from mouse (C57B/6 and 129SvEv mixed genetic background) brain cDNA using BioXact polymerase (Biolase, UK) and primers with nested restriction enzyme sites (EcoRI forward primer: 5'-TAGAATTCGCCACCATGGAGCCCGACTCCCTCCTGGAC and NotI reverse primer: 5'-TAGCGGCCGCCTAATGCATACTCTCTTTCAGCTGTGT). The resulting PCR fragment was cloned into pcDNA3 mammalian expression vector and fully sequenced by primer walking strategy. The pcDNA3-Dab2ip-L plasmid was used as a template for generation of the full-length or truncated forms of Dab2ip into pCMV-Tag3B vector (Stratagene, La Jolla, CA, USA) and then the construct were moved to pCAG –IRES-EGFP vector (a gift from Mikio Hoshino, Kyoto University, Kyoto, Japan) for *in vivo* expression of Dab2ip constructs: pCAG-Myc-Dab2IP-L (aa 1-1132), pCAG-Myc-Dab2IP-LΔPH (aa 171-1132), pCAG-Myc-Dab2IP-LΔGRD (1-307/567-1132) was used. To generate point mutations Dab2ip GRD*, Dab2ip-L-R385L; Dab2ip NP/AA; Dab2ip-L m, a transcript resistant to Dab2ip shRNA degradation and a Dab2ip-LΔGRD construct, site-directed mutagenesis was performed using the QuickChange® XL kit (Stratagene).

HEK 293T, COS-7, and primary cortical neuron culture and transfection

HEK 293T, COS-7 cells were grown and maintained in DMEM (high glucose; Invitrogen) with 10% fetal bovine serum (Invitrogen) at 37°C in 5% CO_2. Expression and knock down constructs were delivered to these cells using Fugene 6 (Roche) according to the manufacturer's protocol. For primary cortical neuron cultures, cerebral cortices were dissected from E15.5 mouse ICR embryos. Neurons were dissociated using a Papain Dissociation Kit (BioWorthington, cat# LK003150). The cortical neuron cultures were maintained for 2-8 days *in vitro* (DIV) in Neurobasal medium supplemented with 0.5 mM L-glutamine, 2% B-27 supplement, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen), replacing half the medium after the first 3 days. To test knock down efficiency by Western blot analysis, approximately 5×10^6 dissociated cells were transfected with shRNA constructs using an Amaxa nucleofector kit, and plated in a 6-well plate coated with poly-L-lysine. For Dab2ip protein localization experiments, dissociated cortical neurons were transfected by the calcium phosphate method at 5 DIV. For immunofluorescence analysis of neurons transfected by IUE at E14.5, cerebral cortices were dissected from E16.5 embryos, and the neurons were dissociated and cultured for 2 DIV. Approximately 0.2×10^6 cells were placed on a glass coverslip in a 24-well plate coated with poly-L-lysine.

Fluorescence analysis

Auto- and immunofluorescence analysis of brain tissue and dissociated cells was performed essentially as previously described (Yabut et al., 2010). Tissue sections were imaged by confocal microscopy using a Yokogawa CSU-10 spinning disk attached to an inverted fluorescence microscope (Olympus IX50). The dissociated cortical neurons

transfected *in vivo* by IUE as described above were fixed in 4% paraform aldehyde (PFA) after 2-5 DIV. For Dab2ip immunofluorescence, E16.5 mouse brains were isolated, frozen on dry ice in OCT, and sectioned at 25 µm. The fixed samples were permeabilized with the 0.1% Triton X-100/phosphate buffer solution (PBS) for 10 minutes and incubated with blocking solution (10% donkey serum or 10% goat serum in 0.1% Triton X-100/PBS) for 1 hour at room temperature. The samples were incubated with the indicated primary antibodies (Table 1) at 4°C for overnight and then with the indicated primary antibodies (Table 2) for 1 hour at room temperature. After 3 times of washing with PBS, the samples were applied with mounting medium, VECTASHIELD (Vector) to be investigated under the microscope.

Western blot analysis

Primary cortical neurons, HEK 293T cells, and the cerebral cortex of wild type or mutant mice were lysed in RIPA buffer (50mM Tris pH 7.4, 1% NP40, 0.25% deoxycholate, 150mM NaCl, 1mM EGTA) and cleared by centrifugation at 3,000 x *g* for 3 min at 4°C. 5 µg of HEK 293T lysate and 15 µg of cortical neuron lysate were loaded onto 8% or 12% SDS-PAGE gels, run at 130 V for 2 h, and transferred to 0.22 µm nitrocellulose membranes. The membranes were blocked with 3% milk in 1x TBS-T (Tris-buffered saline with 0.1% Tween 20) for 1 h at room temperature, followed by incubation with the indicated primary antibodies overnight at 4°C, and incubation with the indicated secondary antibodies for 1 h at room temperature. After several washes with TBS-T, membranes were developed with ECL-Plus Western Blotting Detection System (GE Healthcare) and exposed to autoradiographic film (Denville).

Antibodies

Information of the primary and secondary antibodies used for this study is described in the tables below (Table 1 and Table 2).

Table 1. Information of the primary antibodies applied in this study.

Antigen	Species	Supplier	Titer	Application
Dab2ip	rabbit	Abcam	1:5,000 1:100	WB IF
Dab1	mouse	A gift from Dr. Goffinet (Université Catholique de Louvain , Belgium)	1:1,000	WB
Phospho Akt (S473)	rabbit	Cell Signaling Technology	1:5,000	WB
Akt	rabbit	Cell Signaling Technology	1:5,000	WB
Actin	mouse	Millipore	1:5,000	WB
Tbr1	rabbit	Millipore	1:250	IF
GFP	rabbit	Abcam	1:500	IF
GFP	mouse	Sigma	1:500	IF
Myc	rabbit	Sigma	1:5000 1:250	WB IF
NeuN	mouse	Millipore	1:250	IF
Map2	rabbit	Millipore	1:2000 1:500	WB IF
Map1b	goat	Santa Cruz	1:2000 1:500	WB IF

Tau	goat	Santa Cruz	1:100	IF
Tuj1	mouse	Covance	1:500	IF
Phopho Erk1/2 (phosphor p42/44)	rabbit	Cell Signaling Technology	1:2000	WB
Erk1/2 (p42/44)	rabbit	Cell Signaling Technology	1:2000	WB

Table 2. Information of the secondary antibodies applied in this study.

Conjugant	Specificity	Supplier	Titer	Application
Horseradish peroxidase (HRP)	mouse IgG	Sigma	1:10,000	WB
HRP	rabbit IgG	Sigma	1:10,000	WB
HRP	Goat IgG	Biorad	1:20,000	WB
Alexa Fluor 647	mouse IgG	Invitrogen	1:500	IF
Alexa Fluor 488	mouse IgG	Invitrogen	1:500	IF
Alexa Fluor 488	rabbit IgG	Invitrogen	1:500	IF
Cy5	rabbit IgG	Invitrogen	1:500	IF
Alexa Fluor 660	Goat IgG	Invitrogen	1:500	IF

Statistical analysis

Data in the plots are shown as the mean +/- s.e.m., and analyzed by Student's *t* test or one-sample *t*-test as indicated in the figure legends. To count GFP+ cells in brain sections after IUE, median sections were chosen in a series of GFP+ sections, and the results were averaged from multiple sections per embryos. Statistical significance was determined when $p<0.05$.

3. Results

Dab2ip expression in the developing neocortex of wild type and mutant mice

To investigate whether Dab2ip is involved in brain development, I began by analyzing its expression in the developing mouse neocortex by immunofluorescence and Western blot analysis. Confocal analysis of brain sections obtained from embryonic day (E) 16.5 mice revealed that Dab2ip protein is expressed throughout the developing neocortex, at particularly higher levels in the cortical plate, where principal cortical neurons migrate and mature (Figure 5A and B). This expression pattern is consistent with previous studies that described the expression of Dab2ip mRNA in the developing mouse neocortex (Ayoub et al., 2011; Homayouni et al., 2003). Western blot analysis further demonstrated that Dab2ip protein is abundantly expressed in this region from E14.5 to at least postnatal day (P) 7 (Figure 5C). Even though multiple isoforms of Dab2ip of different molecular weights have been previously described (Chen et al., 2006; Qiu et al., 2007; Wang et al., 2002), in our experiments Dab2ip appeared as a single band of approximately 110 kDa (Figure 5C), suggesting that this protein isoform is predominant in the embryonic and early postnatal neocortex. Since Dab2ip has been shown to interact with Dab1, a critical adaptor molecule in the Reelin signaling pathway that controls neuronal migration and maturation, I investigated Dab2ip expression in several mutant mice carrying mutations in this pathway. Reelin induces the phosphorylation of Dab1 on tyrosine residues, and event that is coupled to the ubiquitination and degradation of Dab1 (Arnaud et al., 2003a; Arnaud et al., 2003b; Howell et al., 1999; Keshvara et al., 2001). Thus, Dab1 protein levels are increased in homozygous *reeler* mice lacking Reelin

(Howell et al., 1999) (Figure 4D). I found that Dab2ip levels, however, did not change in the brain of neither *reeler* mice (Figure 5D) nor Dab1 knockout mice (Figure 5E). Because Reelin is known to stimulate the activity of the phosphatidylinositol 3-kinase (PI3K) pathway through a mechanism that requires Dab1 (Beffert et al., 2002), and Dab1 levels are increased in mouse mutants that lack the upstream phosphatase Pten (Kazdoba et al., 2012), I also examined Dab2ip expression in the neocortex of Pten knockout mice. I found that, although levels of Dab1 and phosphoAkt were noticeably increased in homozygous Pten mutants, levels of Dab2ip were not affected (Figure 5F). Together, these data indicate that Dab2ip protein expression and stability are not modulated by the Reelin pathway.

Figure 5. Dab2ip expression in the developing neocortex of wild type and mutant mice
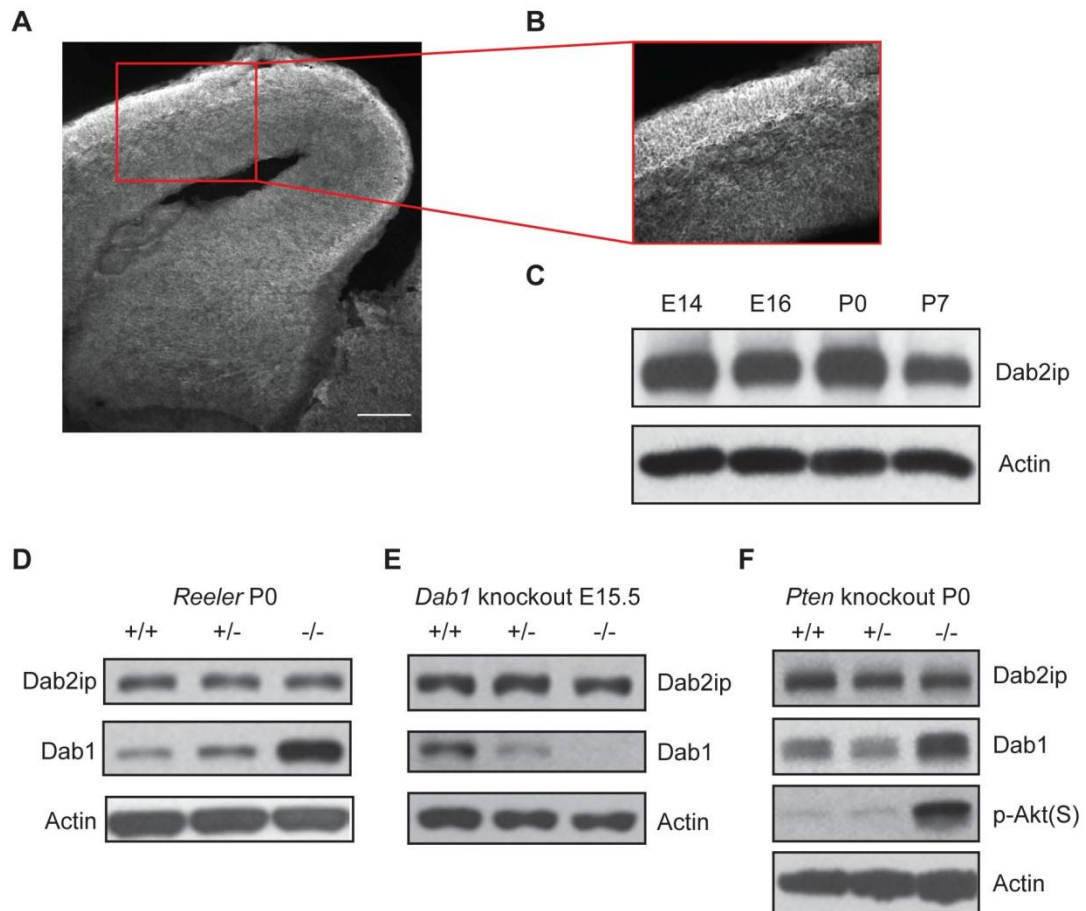
Figure 5. Dab2ip expression in the developing neocortex of wild type and mutant mice

(A, B) Brain sections obtained from E16.5 mice were immunostained with anti-Dab2ip antibodies. Dab2ip signal was distributed throughout the neocortex and appeared more strongly in the cortical plate. The image in (B) is a magnification of the inset shown in (A). (C) Western blot analysis indicates that Dab2ip is abundantly expressed in the neocortex from E14.5 to postnatal day P7. The same blot was first probed with Dab2ip antibodies, and then was reprobed with antibodies against actin as an internal loading control. Dab2ip was expressed at all ages throughout the neocortex. The neocortex of newborn wild type (+/+), heterozygous (+/-) and homozygous (-/-) *reeler* littermates (D), embryonic *Dab1* knockout mice (E), and newborn *Pten* conditional knockout mice (F), was analyzed. Western blots were sequentially probed with Dab2ip, Dab1, phospho- Akt Serine 473 (p-Akt(S)) and actin antibodies. Levels of Dab2ip, unlike Dab1 and pAkt(S), did not differ among genotypes. Scale bars: 100 μm (A), 50 μm (B)

Generation and validation of Dab2ip shRNA constructs

To explore the function of Dab2ip in cortical development, I first isolated complementary RNA from the embryonic mouse cerebral cortex. Partial sequencing of the 5' end of cloned PCR products indicated that embryonic cortical Dab2ip transcripts contained, in addition to the GAP-related domain (GRD) and the phospholipid/Ca^{++} binding motif (C2) found in all known isoforms of Dab2ip, a pleckstrin homology (PH) domain that is present in most, but not all, cloned isoforms. The cortical isoform lacked additional N terminal regions present in the long Dab2ip (isoform L) (GenBank DQ473307). To knock down Dab2ip expression during brain development, I used short hairpin RNAs (shRNAs) generated by S. Kim (Johns Hopkins University, MD, USA). The target regions of these shRNAs, referred as shDab2ip 1, shDab2ip 2, and shDab2ip 3, are shown relative to the Dab2ip protein diagram (Figure 6A). To test the efficacy of these constructs, HEK 293T cells were transfected with a plasmid encoding mouse Dab2ip (isoform L), or GFP as a negative control. Dab2ip-transfected cells were co-transfected with a non-targeting shRNA (control), shDab2ip 1, or shDab2ip 2. The data, quantified from triplicate experiments, indicate that both, shDab2ip 1 and shDab2ip 2 dramatically reduce exogenous Dab2ip expression (Figure 6B and D). To examine the effectiveness of these constructs on endogenous Dab2ip expression, primary cortical neurons were transfected with plasmids encoding control shRNA or shDab2ip 1 using the Amaxa nucleofector system. Levels of Dab2ip expression were analyzed after 2 or 4 days *in vitro* (DIV). Quantification of the data indicates that Dab2ip protein expression was dramatically reduced by shDab2ip 1 compared to control shRNA at both time points analyzed, and that the knock down efficiency reached approximately 78% at 2 DIV

(Figure 6C and E). This represents a dramatic down-regulation of Dab2ip since in parallel experiments the transfection efficiency of cortical neurons using a control GFP plasmid was estimated to be approximately 80% (not shown). Quantitative RT-PCR analysis also confirmed that Dab2ip mRNA was dramatically reduced by shDab2ip 1 (data not shown).

Figure 6. Knock down of Dab2ip by shRNA in transfected and primary cells.
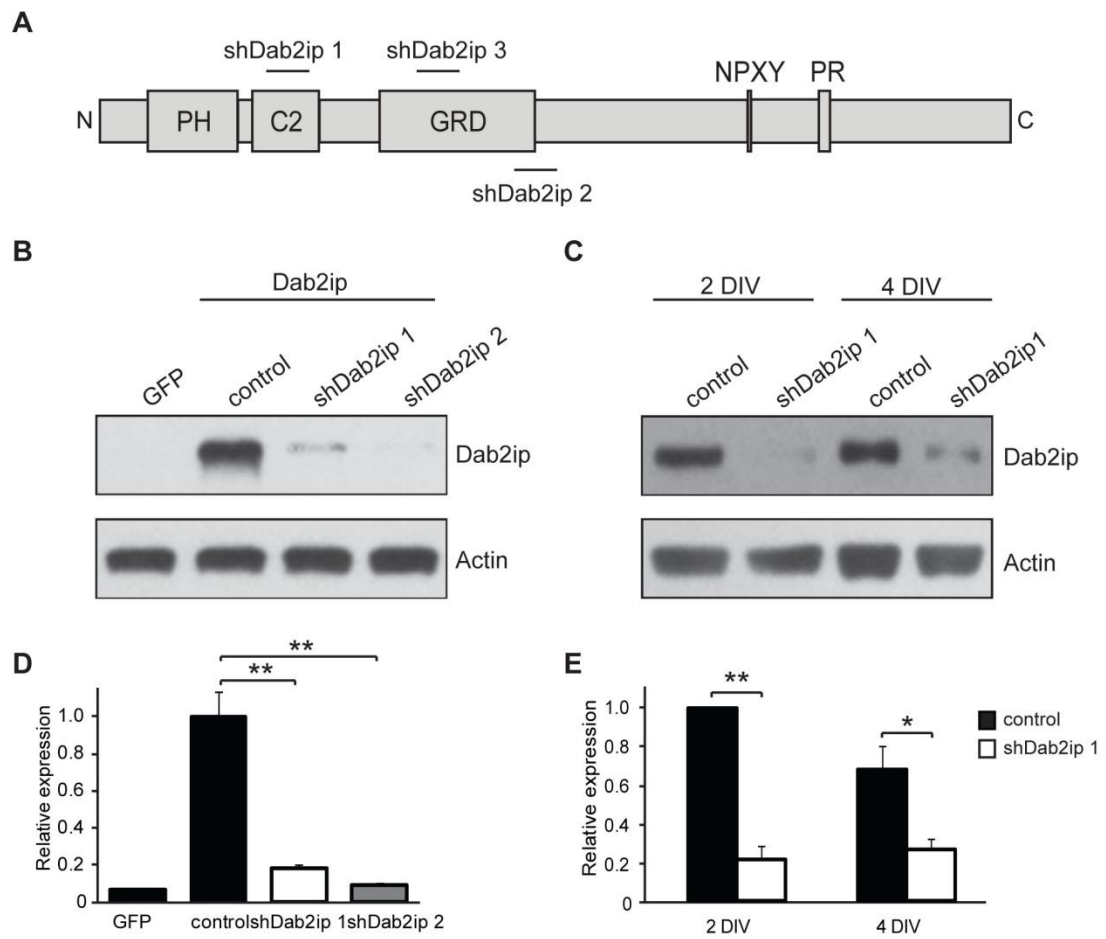
Figure 6. Knock down of Dab2ip by shRNA in transfected and primary cells.

(A) Diagram of Dab2ip, indicating protein functional domains and the targeted regions of Dab2ip shRNAs shDab2ip 1, shDab2ip 2, and shDab2ip 3. PH: Pleckstrin homology, C2: phospholipid/Ca2+ binding motif, GRD: GAP-related domain, NPxY: internalization and Dab1/Dab2 binding motif, PR: proline-rich domain. (B, D) Knock down of exogenous Dab2ip by shDab2ip 1 and shDab2ip 2 in transfected HEK 293T cells. Cells were transfected with a GFP plasmid or co-transfected with a plasmid encoding Dab2ip (isoform L) and a non-targeting shRNA (control) or Dab2ip shRNAs 1 or 2. Both specific shRNAs strongly reduced Dab2ip expression. The plot in (D) shows the results from triplicate experiments. Dab2ip levels were normalized to actin and expressed relative to the control shRNA. Statistical significance was determined by the Student's t-test. (C, E) Knock down of endogenous Dab2ip by shDab2ip 1 in primary cortical neurons. Neurons were transfected with non-targeting shRNA (control) or shDab2ip 1, and analyzed at 2 DIV and 4 DIV. Dab2ip shRNA dramatically reduced the expression of endogenous Dab2ip The plot in (E) shows the results from triplicate experiments. The data was analyzed as above, except that statistical significance was determined by a one-tailed t-test. *, $p<0.05$; **, $p<0.01$.

Dab2ip is required for cortical neuron migration

To investigate the function of Dab2ip in corticogenesis, I performed *in utero* electroporation (IUE) experiments to introduce the Dab2ip shRNAs or a control shRNA into neural progenitor cells of the embryonic mouse neocortex. Plasmids encoding shRNAs were co-transfected with a GFP expression construct to facilitate detection of the transfected cells. In embryos electroporated with control shRNA at E14.5, the great majority of neurons generated from GFP+ progenitor cells entered the subventricular zone (SVZ) and the intermediate zone (IZ) 2 days after electroporation (Figure 8 A and B), invaded the cortical plate (CP) 3 days (Figure 8 C and D) and reached the upper CP 4 days (Figure 7A) after electroporation. In embryos electroporated with shDab2ip 1 at E14.5, most GFP+ neurons also entered the SVZ and the IZ 2 days later (Figure 8A), but many failed to invade the CP 3 days (Figure 8B) and 4 days after electroporation (Figure 7A). Many knock down GFP+ neurons appeared to be arrested below the CP, although a few were able to migrate fairly normally. Similar results were obtained using shDab2ip 2 (Figure 7A) and shDab2ip 3 (not shown). In order to quantify our IUE results, I double-labeled E18.5 embryonic sections by immunofluorescence using antibodies against the early neuronal marker β-III tubulin (Tuj1), which highlights different regions of the developing cortex. Confocal images of the lateral neocortex were divided into five equal-interval bins, and the percentage of GFP$^+$ cells distributed in each bin was determined from multiple comparable sections obtained from *n*=3 embryos per construct. With this method, the lowest bin (A) roughly corresponds to the Tuj1-negative SVZ/VZ, the second lowest bin (B) corresponds to the IZ containing many Tuj1-labeled processes, and the other bins (C-E) correspond to increasingly superficial regions of the CP, which were

heavily Tuj1-positive (Figure 7B). I found that the majority of control neurons (84.6%) migrated into the CP in the E14.5-18.5 time frame, and approximately 50% of these neurons reached the most superficial CP bin E (Figure 7C). On the other hand, neurons in which Dab2ip expression had been knocked down remained mostly confined to the IZ (shDab2ip 1: 64.5% in bin B; shDab2ip 2: 67.6%; versus control shRNA: 9.5%) (Figure 7C). Some knock down neurons were able to reach the CP, but very few of these neurons were found in superficial areas (shDab2ip 1: 6.8% in bin E; shDab2ip 2: 3.4%; versus control shRNA: 49.5%). The difference between control and either, shDab2ip 1 or shDab2ip 2 was statistically significant in bins E and B ($p< 0.05$ and $p< 0.01$, respectively). To determine whether Dab2ip knock down caused a permanent migration arrest or a transient delay in migration, I also electroporated embryos at E14.4 and analyzed brains at postnatal day 8. At this age, virtually 100% of control transfected neurons were appropriately localized to the upper half of the CP, corresponding to cellular layers 2-4 (Figure 7D and E). Instead, only approximately half of the neurons transfected with Dab2ip shRNA reached the proper destination in the upper CP, whereas many remained localized underneath the CP (40.4%) or in the lower half of the CP (13.6%) (Figure 7D and E). These data indicate that Dab2ip knock down in progenitor cells destined to give rise to upper layer cortical neurons causes a persistent radial migration defect.

Figure 7. Dab2ip is required for cortical neuron migration.
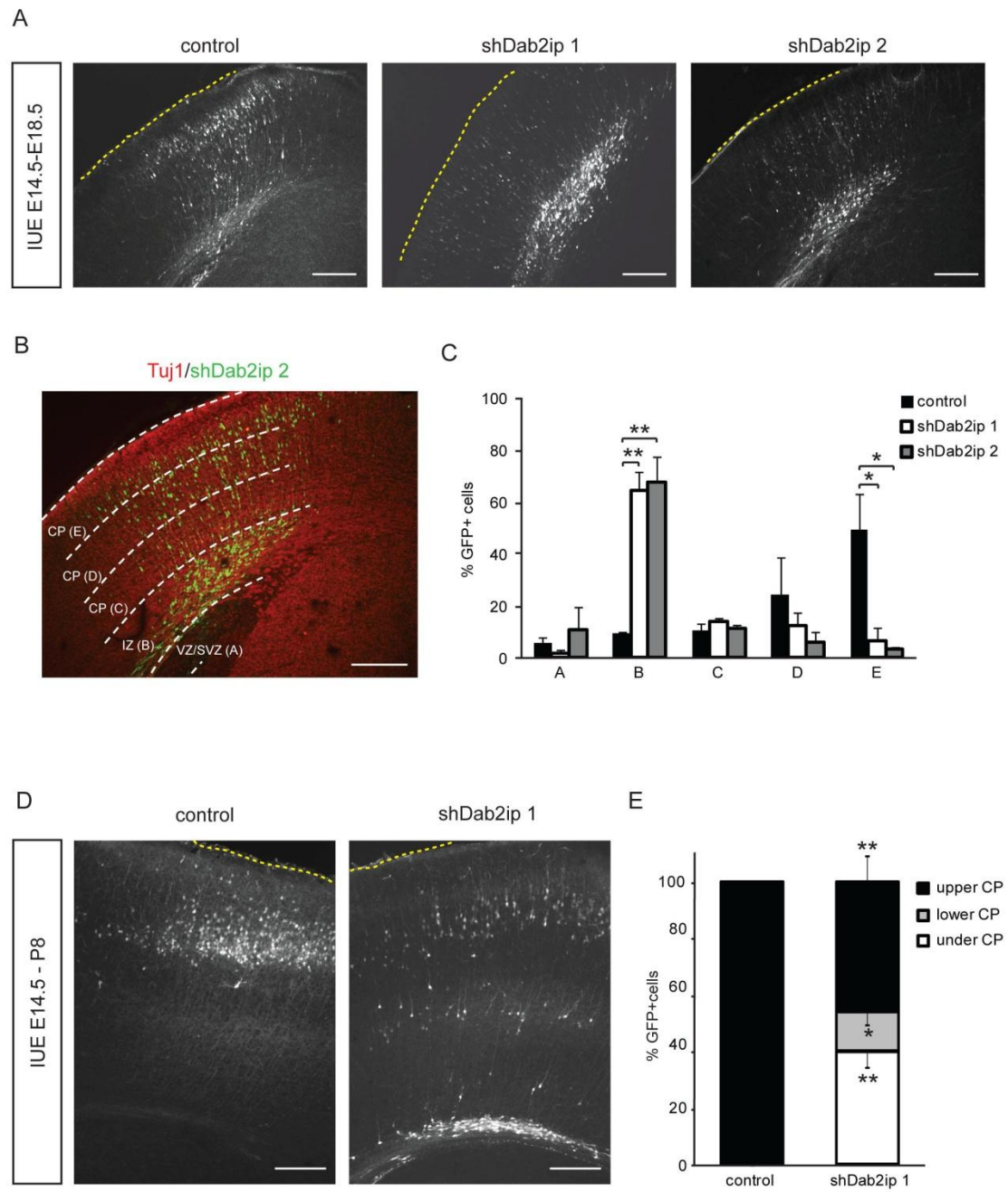
Figure 7. Dab2ip is required for cortical neuron migration.

Control shRNA or shDab2ip 1 and 2 were trasnfected into neural progenitor cells of the mouse embryonic neocortex at E14.5 by IUE. The migrating cortical neurons were visualized by co-transfection with a GFP-expressing plasmid. (A) Representative confocal images of the lateral neocortex 4 days after IUE with the indicated shRNA constructs show the distribution of GFP+ cells. The dotted lines indicate the pial surface. Dab2ip knock down neurons failed to migrate, whereas control neurons migrated efficiently toward the surface of the neocortex. (B) GFP autofluorescence (green) and Tuj1 immunofluorescence (red) were used to quantify neuronal migration. Images of the electroporated neocortex were divided into five equal-interval bins. The lowest bin A corresponds to the Tuj1- VZ/SVZ region; bin B corresponds to the IZ and contains many Tuj1+ cells; bins C, D, and E correspond to increasing superficial regions of CP, containing heavily Tuj1+ cells. (C) The percentage of GFP+ neurons in each bin was calculated from multiple electroporated embryos. In the embryos transfected with shDab2ips, most neurons were confined in the IZ (control, 9.5%, $n=3$; shDab2ip 1, 64.5%, $n=3$; shDab2ip 2, 67.6%, $n=3$), and failed to migrate into the CP (control, 49.5%; shDab2ip 1, 6.8%; shDab2ip 2, 3.4%). (D) Representative confocal images of the lateral neocortex of mice transfected at E14.5 and analyzed at postnatal day 8. Many GFP+ neurons transfected with shDab2ip 1, unlike control, remained localized underneath the CP (control, 0%, $n=3$; shDab2ip 1, 40.8%, $n=3$) or in the lower half of the CP. CP= cortical plate; IZ= intermediate zone; VZ= ventricular zone; SVZ=subventricular zone. Scale bars: 200 μm *, $p<0.05$; **, $p<0.01$.

Figure 8. Migration of neurons in the lateral neocortex following IUE.
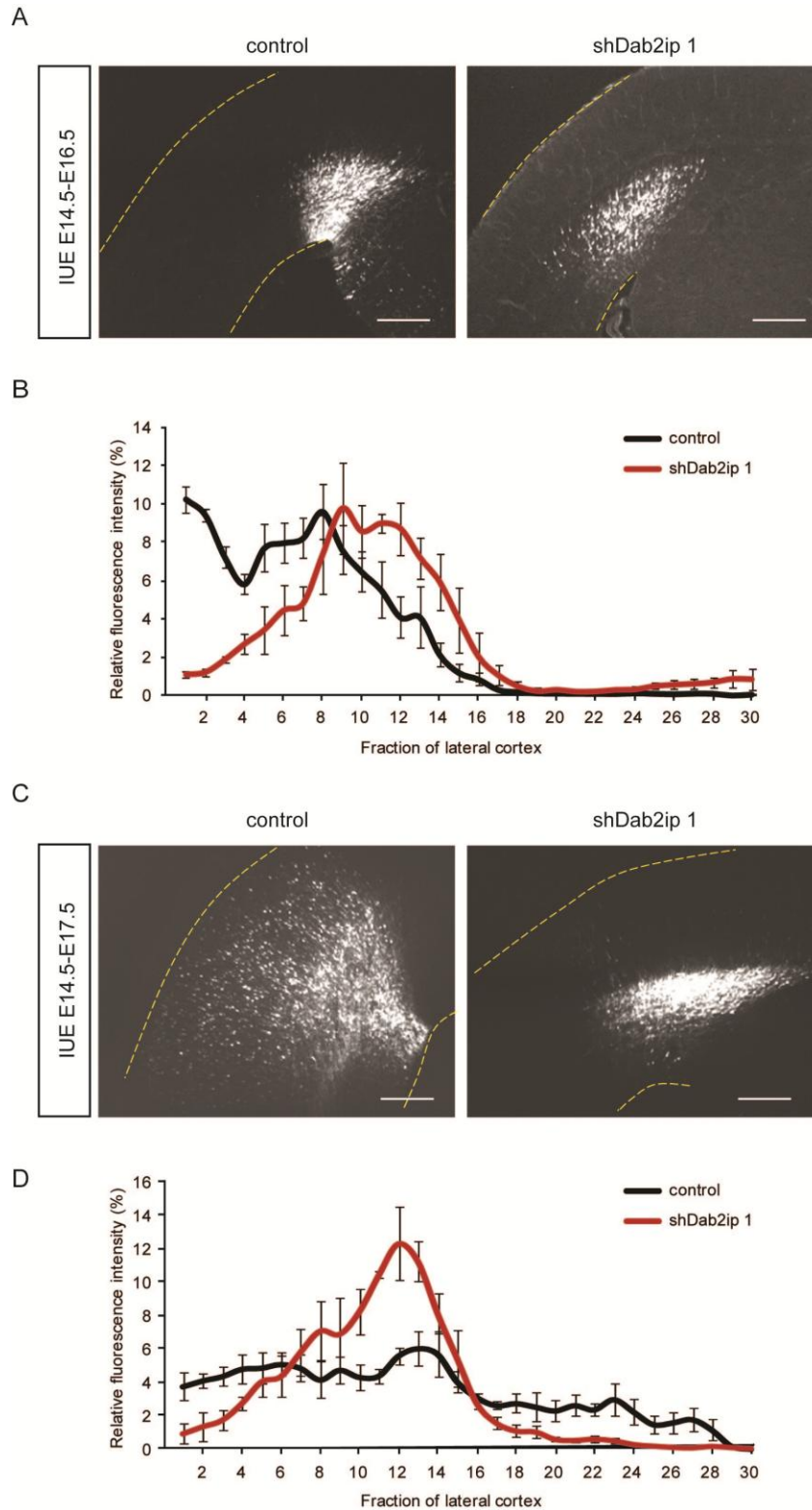
Figure 8. Migration of neurons in the lateral neocortex following IUE.

Embryos were coelectroporated with GFP and control or shDab2ip 1 shRNA at E14.5. (A, B) Both, control and knock down neurons invaded the SVZ and the IZ 2 days after IUE (E16.5). For the quantification, "plot profile" in Image J program was used to measure the intensity of GFP fluorescence from the VZ to the marginal zone with the images taken by confocal microscope. (C, D) shDab2ip 1 knock down neurons failed to invade the CP, whereas control neurons migrated efficiently into the CP 3 days after IUE. Scale bars: 200 μm, Control, *n=3*; shDab2ip 1, *n=3*.

To determine whether Dab2ip also affects the migration of early-born neurons destined for deep cortical layers, I electroporated embryos at E12.5 with a GFP expression construct and either control or Dab2ip shRNA, and examined the distribution of GFP+ cells in the neocortex at E17.5. Electroporation at this age causes GFP expression in both, deep layer neurons born at E12-13 from the first round of progenitor cell division, as well as upper layer neurons born E14-17 from subsequent cell division events. To facilitate the identification of deep cortical layers I stained brain sections with Tbr1 antibodies and examined caudal regions of the neocortex where Tbr1+ cells are predominantly confined to deep layers. In embryos electroporated with control shRNA, GFP+ neurons that co-labeled with Tbr1 (early-born neurons) were appropriately located in deep layers, whereas Tbr1− neurons (late-born neurons) invaded the upper layers (Figure 8A and B). In contrast, GFP+ neurons transfected with shDab2ip 1 were localized exclusively either in deep cortical layers (mostly Tbr1+ neurons) or underneath the CP (Tbr1− neurons) (Figure 9A and B). These data strongly suggest that Dab2ip knock down preferentially affects the migration of upper cortical neurons.

Figure 9. Dab2ip does not affect to the migration of early-born cortical neurons.
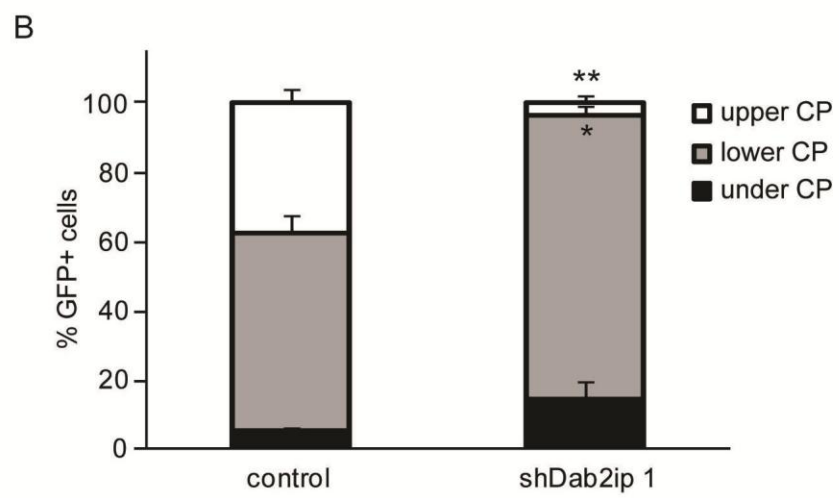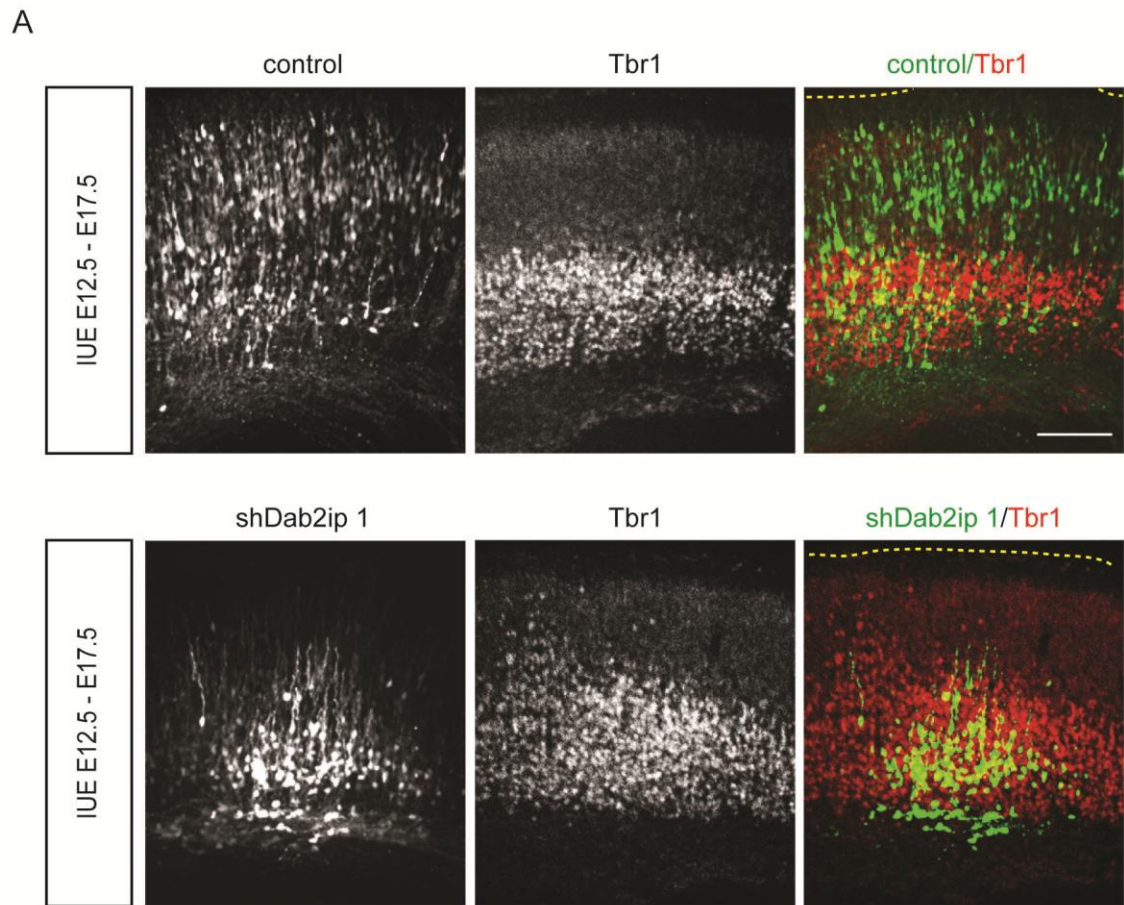
Figure 9. Dab2ip does not affect to the migration of early-born cortical neurons.

Mouse embryos were electroporated at E12.5 with a GFP-expressing plasmid and control shRNA or shDab2ip 1. Sections of the caudal neocortex were analyzed at E17.5 by GFP autofluorescence (green) and immunofluorescence with Tbr1 antibodies (red). (A) Representative confocal images of the lateral neocortex show that Tbr1+ early-born knock down neurons localize appropriately to the lower CP, whereas Tbr1- late-born neurons failed to migrate to the upper CP. (B) Quantification of the data from multiple experiments. Scale bars: 100 μm *, $p<0.05$; **, $p<0.01$.

Dab2ip overexpression and structure-function analysis *in vivo*

To determine whether increased Dab2ip expression also affects neuronal migration in the neocortex, I electroporated a construct encoding full-length mouse Dab2ip (L isoform) and several deletion or mutation constructs of Dab2ip into the brain of mouse embryos. The constructs include: Dab2ip GRD*, a point mutation in the GRD that abolishes that GTPase activity; Dab2ip ΔGRD, a deletion of GRD; Dab2ip ΔPH, a deletion of the PH domain; Dab2ip NP/AA, a double point mutation of the NPxY motif that is predicted to mediate Dab1 interaction (Homayouni et al., 2003; Wang et al., 2002). Constructs were co-electroporated with a GFP expression construct at E14.5 and neuronal migration was analyzed 4 days later. At this age, the great majority of control GFP+ electroporated neurons were found in the upper CP (Figure 10A and B). Instead, more than half of the neurons transfected with the intact Dab2ip construct were unable to reach the upper CP and many stalled around the SVZ/IZ boundary (Figure 10A and B). This migration defect was completely abolished by the deletion of the PH domain, and was also partially reduced by the GRD deletion or mutation (Figure 10A). On the other hand, mutation in the NPxY motif resulted in a migration defect that was similar or even more dramatic than that of intact Dab2ip (Figure 10A), suggesting that it did not result from interference with Dab1 signaling. Since the PH domain is known to bind phospholipids (Toker and Cantley, 1997), I examined the cellular localization of Dab2ip ΔPH compared to intact Dab2ip in COS-7 cells and primary cortical neurons. The data show that Dab2ip ΔPH was localized preferentially around the nucleus whereas intact Dab2ip was dispersed evenly throughout the cytoplasm and the plasma membrane in both cell types (Figure 10C). These findings suggest that the cellular localization of Dab2ip, mediated by

its PH domain, is crucial for the function of this protein in neuronal migration, and that the GTPase activity also plays an important role in this activity.

Figure 10. Dab2ip overexpression causes migration defects that require intact PH domain and GRD, but not the NPxY motif.
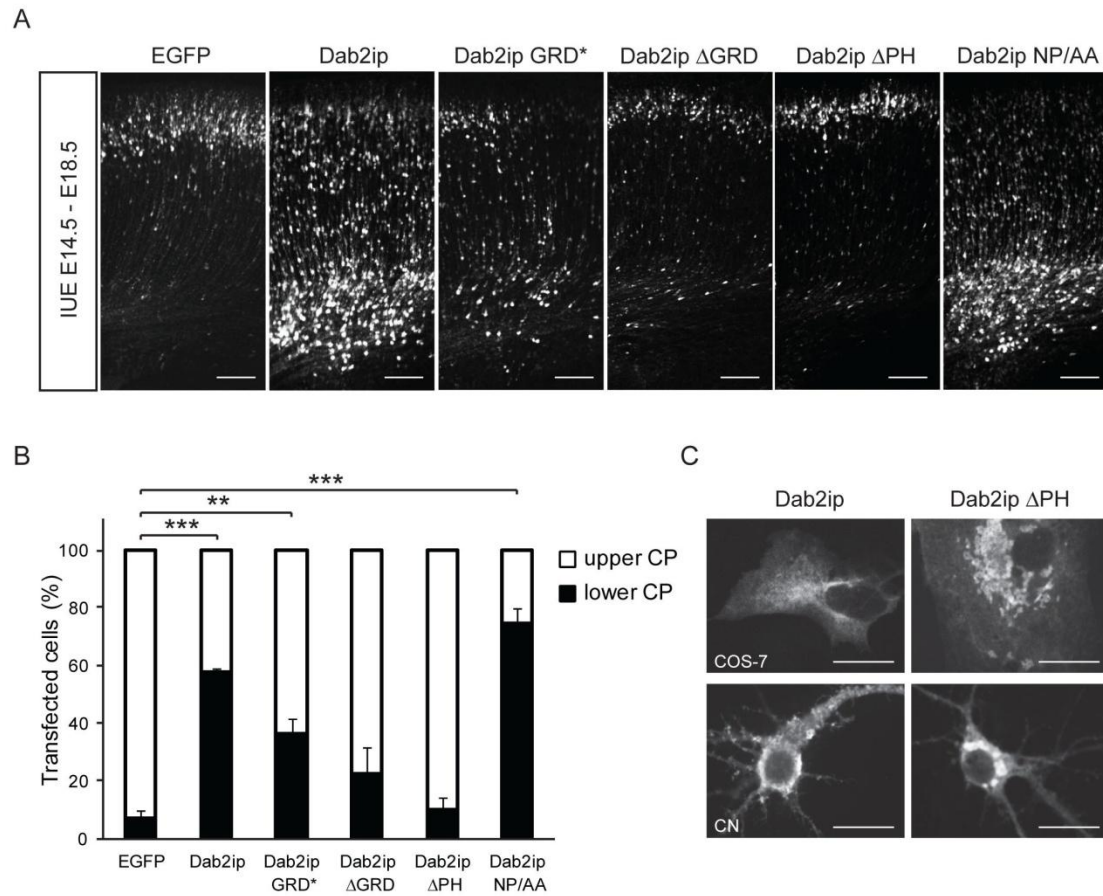
Figure 10. Dab2ip overexpression causes migration defects that require intact PH domain and GRD, but not the NPxY motif.

(A) Mouse embryos were electroporated at E14.5 with the indicated Dab2ip expression constructs. Representative confocal images of GFP+ neurons in the developing cortex at E18.5 show that exogenous expression of intact Dab2ip or a construct in which the NPxY motif was mutated cause obvious migration defects. The phenotype was attenuated when constructs carrying a mutation or deletions in the PH domain or GRD were used. (B) Quantification of the data from multiple experiments. (C) COS-7 cells and primary cortical neurons were transfected with Myc-tagged Dab2ip of Dab2ipΔPH constructs and processed by immunofluorescence of with anti-Myc antibody. COS-7 cells were analyzed 2 days after transfection and primary cortical neurons were analyzed 3 after transfection. Scale bars: 100 μm (A), 20 μm (C) **, $p<0.01$; ***, $p<0.001$.

Dab2ip levels affect neuronal morphology *in vivo*

The majority of principal cortical neurons destined for upper layers initially move radially from the VZ/SVZ into the IZ where they pause and acquire a multipolar morphology (Noctor et al., 2004). These cells then convert to a bipolar morphology and undergo glia-guided locomotion to enter the CP (Figure 11B). To investigate the effect of Dab2ip up- or down-regulation on neuronal morphology in the developing neocortex, I electroporated Dab2ip expression or knock down plasmids constructs *in utero* at E14.5. Neurons destined for upper cortical layers were identified by GFP co-transfection, and analyzed by confocal microscopy 4 days after electroporation. At this time, most GFP+ control neurons had reached the upper CP. The few control neurons still located in the IZ displayed predominantly a bipolar morphology in the upper IZ and a multipolar morphology in the lower IZ (Figure 11A). GFP+ Dab2ip knock down neurons were mostly confined in the IZ, where they exhibited a multipolar morphology in both, the upper as well as the lower IZ (Figure 11A). The few knock down neurons that were able to reach the CP exhibited neuritic processes that were slightly coiled and thinner than control neurons (Figure 11A). To quantify the morphological defect due to Dab2ip knock down, I repeated these experiments and calculated the percentage of multipolar and uni/bipolar neurons in the IZ 3 days after IUE with shDab2ip 1 or control shRNA. At this time point, approximately half of the GFP+ control neurons displayed uni/bipolar morphology in the upper IZ, whereas the other half exhibited a multipolar morphology and was mostly confined to the lower IZ (Figure 11C). In contrast, the great majority of GFP+ knock down neurons in the IZ exhibited a multipolar morphology (94.5%, *n*=3 embryos) (Figure 11C). GFP+ neurons overexpressing Dab2ip also displayed an

abnormal morphology, which was different than that produced by either control or knock down neurons. Overexpressing neurons exhibited a round soma, and often a single neuritic process (Figure 11A). Multipolar neurons could not be identified even in the lower IZ. Together, these observations suggest that normal levels of Dab2ip promote the transition from multipolar to bipolar morphology, which is necessary for efficient glial-guided migration of cortical neurons destined to upper cortical layers.

Figure 11. Dab2ip plays a role in the transition from multipolar to bipolar morphology during radial migration.
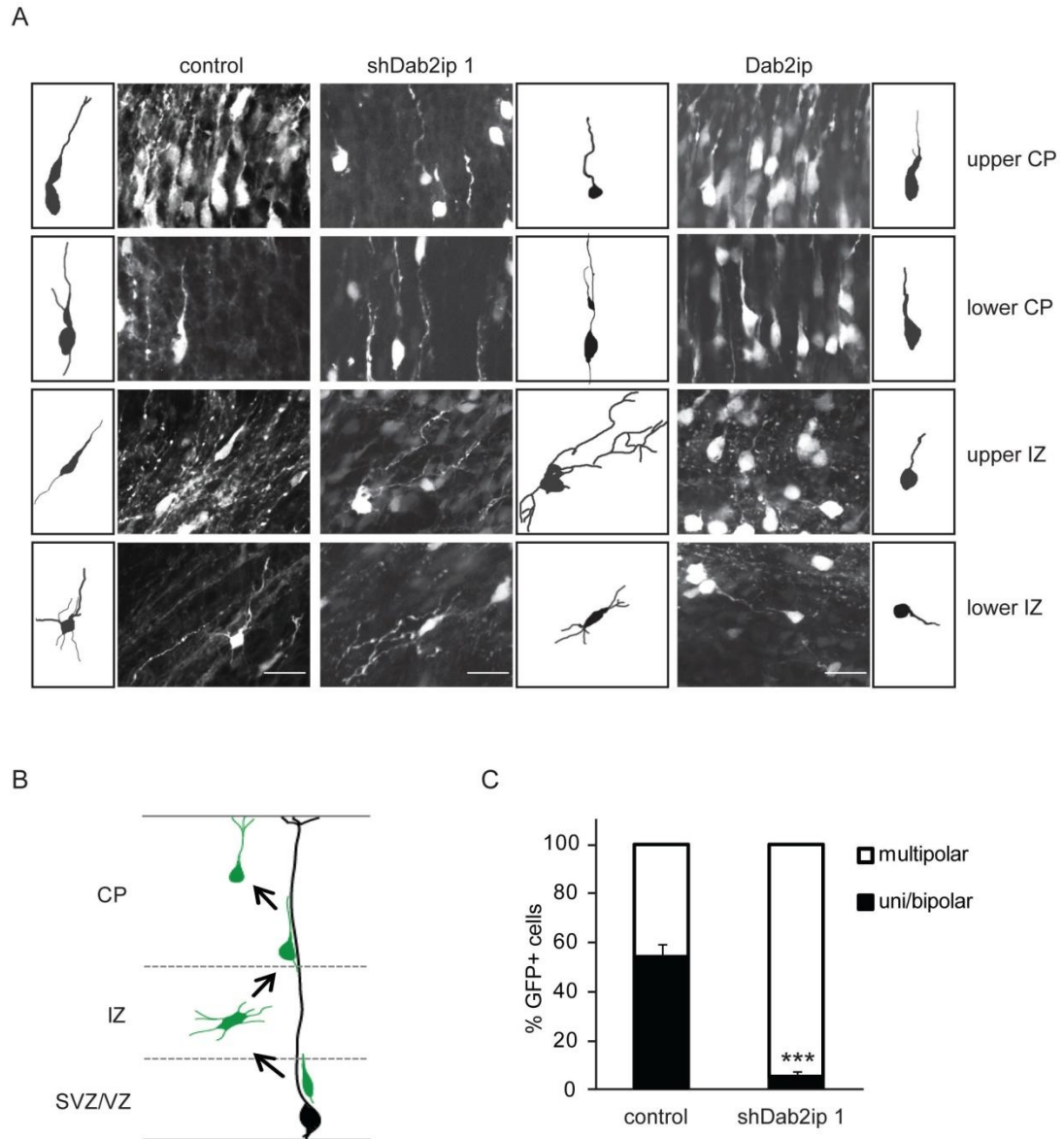
Figure 11. Dab2ip plays a role in the transition from multipolar to bipolar morphology during radial migration.

(A) E14.5 embryos were electroporated *in utero* with control shRNA, shDab2ip 1 or a Dab2ip expression plasmid, and analyzed at E18.5. High magnification confocal images and tracing of individual, representative GFP+ neurons are shown in different regions of the developing neocortex, as indicated in each row. (B) Diagram of the morphological changes expected in each cortical region during the migration of late-born neurons. Neurons born in the VZ or SVZ move radially into the IZ where they acquire a multipolar morphology. They then convert to a bipolar morphology and undergo glial-guided locomotion to enter the CP. (C) Quantification of the percentage of multipolar neurons in the IZ. The data were obtained from confocal images of multiple sections as in (A). A significantly higher percentage of knock down neurons in the IZ exhibited a multipolar morphology compared to control. Scale bars: 25 μm ***, *p*<0.001.

Dab2ip knock down disrupts neurite development in cultured neurons

To further examine the role of Dab2ip in neurite development, I isolated cortical neurons 2 days after *in utero* electroporation (IUE E14.5-E16.5) with either shDab2ip 1 or control shRNA, and co-cultured them for up to 5 DIV. To distinguish knock down from control neurons, embryos were co-transfected either with plasmids encoding shDab2ip 1 and GFP alone, or with plasmids encoding control shRNA and GFP plus mCherry. After 2, 3, or 5 DIV, I imaged knock down (GFP+) and control (GFP+ and mCherry+) neurons by confocal microscopy, and analyzed their morphology in detail. At 2 DIV, the complexity and the total length of neurites did not differ between knock down and control neurons (Figure 12B). However, by 3 DIV, Dab2ip knock down neurons showed a significant decrease in the total length of neurites compared to control (Figure 12B). This difference became more pronounced at 5 DIV, at which time the processes of Dab2ip knock down neurons also appeared thinner than control (Figure 12A and B). To further examine neurite complexity, I conducted Sholl analysis of transfected neurons at 5 DIV. This analysis indicated that the neurites of Dab2ip knock down neurons were slightly more numerous, but significantly shorter than control (Figure 12C). These *in vitro* results are consistent with our *in vivo* observations and confirm the role of Dab2ip in neurite elongation.

Figure 12. Dab2ip knock down disrupts neurite outgrowth in cultured neurons.
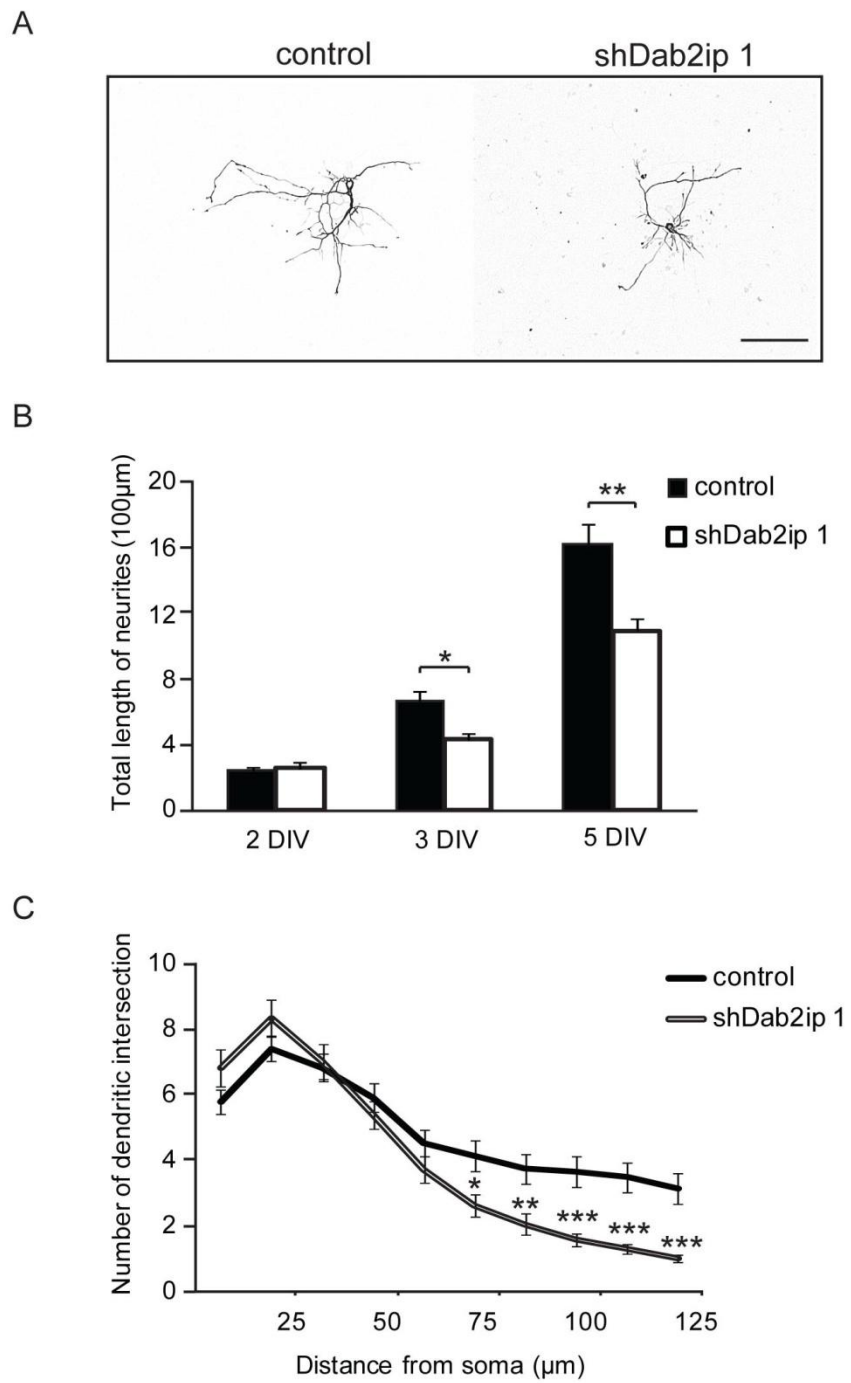
Figure 12. Dab2ip knock down disrupts neurite outgrowth in cultured neurons.

Cortical neurons were isolated 2 days after *in utero* electroporation and cultured up to 5 days *in vitro*. (A) Representative images of transfected neurons at 5 DIV using 'cost' application in ImageJ. (B) The total length of neurites of Dab2ip knock down neurons and control neurons at 2 DIV, 3 DIV, and 5 DIV was measured using the NeuronJ application in ImageJ. The length of knock down neurites was significantly reduced than control at 3 and 5 DIV. (C) Sholl analysis of transfected neurons at 5 DIV indicates that knock down neurites were significantly shorter than control. Scale bars: 100 μm *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

Dab2ip is required for the expression of microtubule–associated proteins

Microtubules are structural components necessary for the acquisition of neuronal morphology, and microtubule-associated proteins (MAPs) are important for microtubule assembly, stabilization, and interaction with other cytoskeleton proteins (Baas and Heidemann, 1986; Hirokawa, 1994). To investigate the molecular mechanisms underlying the effects of Dab2ip knock down on neurite development, I examined the expression of several MAPs by immunofluorescence in cultured neurons isolated from *in utero* electroporated embryos (IUE E14.5-E16.5). Immunofluorescence assays in cultured neurons revealed that Map2, a mature dendritic marker, was highly expressed in control (GFP+/mCherry+ double-labeled) neurons at 2 DIV, whereas it was expressed at very low levels in Dab2ip knock down (GFP+ only) neurons (Figure 13A, E). The downregulation of Map2 in Dab2ip knock down neurons was apparent compared to either, adjacent untransfected neurons in the same culture wells, as well as compared to neurons transfected with control shRNA in parallel cultures. I also examined the expression of Map1b and Tau, two MAPs that *in vivo* are predominantly restricted to dendritic and axonal compartments, respectively. Our data indicate that the expression of Map1b and Tau is also strongly reduced in Dab2ip knock down neurons at 2 DIV (Figure 13B, C, and E). The effect of Dab2ip on MAPs expression was rather specific, since levels of neuronal markers such as Tuj1 (Figure 13D), Lis1, Dcx, and NeuN (data not shown) did not differ. The data suggests that Dab2ip regulates the expression of several MAPs during neuronal development and maturation.

Figure 13. Dab2ip regulates the expression of microtubule-associated proteins.
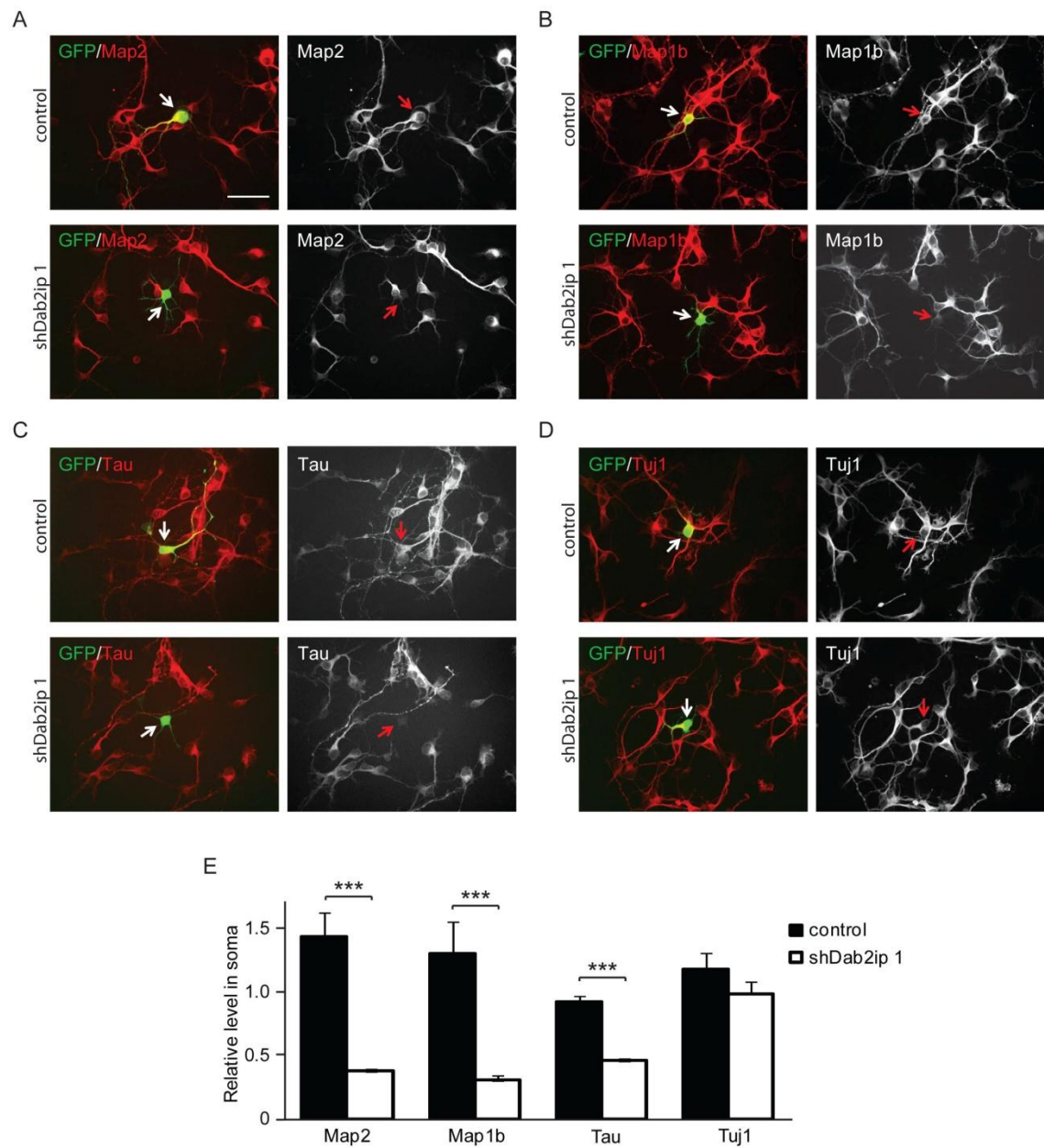
Figure 13. Dab2ip regulates the expression of microtubule-associated proteins.

(A-D) Embryos were electroporated at E14.5 with control shRNA together with GFP and mCherry (double-labeled), or with shDab2ip 1 and GFP alone. Cortical neurons were isolated from transfected embryos at E16.5, cultured for 2 DIV, and subjected to immunofluorescence using Map2 (A), Map1b (B), Tau (C), and Tuj1 (D) specific antibodies, followed by Cy5-labeled secondary antibodies. Confocal images of fields containing neurons positive for GFP/mCherry or GFP alone were acquired. For simplicity, only the GFP and Cy5 channel are shown in the overlay images. Knock down neurons expressed undetectable or low levels of Map2, Map1b and Tau, but normal levels of Tuj1 compared to adjacent or control shRNA-transfected neurons. (E) Quantification of the data collected as in (A-D) from multiple experiments. The fluorescence signal of the soma of transfected neurons was measured relative to untransfected cells in the same field. Ten untransfected cells and 1 transfected cell were analyzed in each field from multiple fields. Map2, Map1b and Tau levels were significantly decreased in knock down cells compared to control, whereas Tuj1 levels were not changed. The data was pooled from > 4 independent experiments. Scale bars: 50 μm ***, $p<0.001$.

4. Discussion

In this study, I used *in utero* electroporation (IUE) to knock down or overexpress Dab2ip in the developing mouse neocortex and examined the role of this protein on neuronal migration *in vivo*. I also used cultured dissociated neurons *ex vivo* to further examine the effects of Dab2ip knock down on neurite extension. Our *in vivo* data suggest that normal levels of Dab2ip are necessary for the migration of late-born principal cortical neurons destined for upper cortical layers, which use glial-guided locomotion as their predominant modality of migration. Both reduced and increased Dab2ip levels *in vivo* resulted in defective migration, but the effects of the two manipulations on neuronal morphology were opposite: too little Dab2ip caused neurons to stall in the IZ with a multipolar morphology characteristic of neurons in this region, whereas too much Dab2ip caused neurons to become abnormally rounded and unable to acquire a multipolar morphology. I reasoned that Dab2ip plays a role in the transition from the multipolar to the bipolar stage of glial-dependent locomotion. This conclusion is further supported by *ex vivo* observations of the altered morphology of knock down neurons. For the conversion to a bipolar morphology in the upper intermediate zone, neurons likely require specific signaling events and cytoskeleton rearrangements. Indeed, proteins that affect microtubule dynamics have also been shown to be required in this step. For example, downregulation of Lis1 and doublecortin (Dcx) by IUE produced a phenotype quite similar to that observed here for Dab2ip (Bai et al., 2003; Koizumi et al., 2006; Tsai et al., 2005). However, in our hands re-expression of Dcx, albeit able to suppress the Dcx knockdown phenotype, did not rescue the Dab2ip knock down defect (unpublished observations). Furthermore, Dab2ip knock down did not affect Lis1 or Dcx expression

(unpublished observations). Thus, it is likely that these proteins do not functionally interact with Dab2ip.

To control for potential non-targeting effects of our knock down constructs, I used a non-targeting shRNA control construct and 3 different Dab2ip shRNA constructs, which target different regions of the Dab2ip mRNA. All shDab2ip constructs resulted in similar migration defects, suggesting that the effects are specific. The phenotype resulting from Dab2ip knock down was dramatic and highly reproducible among several electroporated embryos from different litters. Thus, I conclude that Dab2ip expression is essential for proper cortical development. I also attempted to rescue the shDab1 migration defect by co-expressing mutagenized, non-degradable Dab2ip isoforms. However, these attempts were unsuccessful even when different plasmid amounts were used in the electroporation. I reasoned that the overexpression plasmids I used for the rescue either do not encode the proper isoform required for migration, or produce dominant-negative effects resulting from inappropriate levels or distribution of the exogenous Dab2ip protein. Indeed I found that overexpression of Dab2ip itself results in a migration defect, and that this phenotype is highly dependent on the presence of the PH domain, which promotes protein localization to the cell membrane. Since the intact GRD region of Dab2ip was also required for the migration phenotype in our overexpression experiments *in vivo*, the data suggest that Dab2ip normally functions at the membrane where it regulates the activity of GTP-binding proteins involved in cytoskeletal dynamics.

Radial migration in the developing neocortex is crucially regulated by Reelin, which is secreted by Cajal-Retzius cells in the marginal zone (Alcantara et al., 1998;

D'Arcangelo et al., 1995; Ogawa et al., 1995). This protein functions by recruiting the adapter protein Dab1 in migrating principal neurons, which in turn engages downstream signaling molecules such as the PI3K (Beffert et al., 2002; Bock et al., 2003; Howell et al., 1997; Jossin and Goffinet, 2007; Sheldon et al., 1997). Dab1 protein levels are tightly regulated by Reelin activity through a mechanism that involves phosphorylation, ubiquitination and degradation (Arnaud et al., 2003b; Feng et al., 2007; Howell et al., 1999; Keshvara et al., 2001). Even though Dab2ip interacts with Dab1 (Homayouni et al., 2003; Wang et al., 2002), here I show that mutations in the Reelin pathway do not affect Dab2ip expression levels. Furthermore I showed that the NPxY Dab1-interacting motif is not necessary for Dab2ip to induce a migration defect *in vivo*. Together with the observation that Dab2ip knock down affect preferentially late-born neurons, which enter the CP by glial-guided locomotion, whereas Dab1 is required in early-born neurons which migrate mostly by somal translocation, and in late-born neurons for terminal migration to the upper CP (Franco et al., 2011; Sekine et al., 2011), these findings suggest that Dab2ip is not a positive mediator of Reelin activity. However, it would be premature at this point to conclude that Dab2ip is not involved at all in Reelin signaling. Dab2ip has been shown to possess a Ras GAP activity (Wang et al., 2002) and possibly Rap GAP activity depending on the cell type (S.H.K. and R.H. unpublished results). Interestingly, molecules with a similar biochemical activity, such as Rap1GAP, have been shown to affect the migration and orientation of neurons in the intermediate zone (Franco et al., 2011; Jossin and Cooper, 2011). Reelin has been shown to induce Rap1 activity through the Dab1-dependent recruitment of Crk/CrkL adapter proteins and C3G, which functions as a GTP-exchange factor (GEF) for Rap1 (Ballif et al., 2004; Jossin and

Cooper, 2011). It is conceivable that Reelin may suppress the Rap1/Ras GAP activity of Dab2ip through Dab1 interactions, thus shifting the balance toward mechanisms that promote somal translocation at the expense of locomotion. Further biochemical and genetic studies will be necessary to investigate this possibility.

Microtubule associated proteins (MAPs) are important for microtubule assembly, stabilization, and interaction with other cytoskeleton proteins (Baas and Heidemann, 1986; Hirokawa, 1994). The best-studied neuronal MAPs are Map2, Map1b and Tau. These proteins exist as several isoforms that are produced by alternative splicing, and their expression is developmentally regulated (Crandall and Fischer, 1989; Goedert et al., 1989; Menezes and Luskin, 1994; Riederer and Matus, 1985). The function of Map2 and Tau are thought to be, at least in part, functionally redundant with Map1b during the early brain development. For example, knock down of either Map2 or Tau does not cause a significant defect in cortical development, including layer formation and neurite outgrowth (Takei et al., 2000; Teng et al., 2001). However, both Map2/Map1b and Map1b/Tau double knockout mice exhibit a neuronal migration defect and layer malformation as well as inhibited neurite growth. These deficits are more severe than the phenotype of single Map1b knockout mice (Takei et al., 1997; Takei et al., 2000; Teng et al., 2001). Moreover, the combined down-regulation of these MAPs has been shown to affect neuronal polarity (Gonzalez-Billault et al., 2002). Our immunostaining results from cultured Dab2ip knock down neurons show that Dab2ip affects the expression levels of Map2, Map1b, and Tau, suggesting that the migration defect and morphological abnormalities seen in knock down neurons may be caused by the concomitant loss of these MAPs. Although the reduced expression of MAPs appeared to be transient, and

these proteins gradually reappeared after 3 DIV (G.H.L. unpublished results), neurite development remained significantly altered in cultured Dab2ip knock down neurons, suggesting that even transient down-regulation of MAPs may have long-lasting consequences on neuronal maturation. The inhibition of MAPs expression also potentially explains the observed defects in glia-dependent locomotion, a process that is strongly dependent on the integrity of the leading edge. Further studies will be required to elucidate the exact molecular mechanism by which Dab2ip affects the expression of multiple MAPs.

In summary, I have demonstrated for the first time that Dab2ip plays a role in neuronal migration and neurite extension in the developing mammalian neocortex. These findings lay the foundation for further studies aimed at elucidating the molecular mechanisms of Dab2ip activity in the developing brain. The recent identification of Dab2ip in a set of genes that may contribute to the risk of autism and schizophrenia by affecting neurite outgrowth and guidance (Hussman et al., 2011; Mudge et al., 2008) suggests that this protein could play surprisingly important roles in normal brain development as well as in neurodevelopmental brain disorders.

Chapter 3. Dab2ip controls the expression of Dab1 in the embryonic mouse brain; analysis of *Dab2ip*-knockout brain lysates.

1. Introduction

Dab2ip has been studied mainly as a tumor suppressor protein that controls cell proliferation, apoptosis, and cell survival through inhibition of the Ras-Erk pathway, activation of the Ask1-Jnk pathway, and inhibition of the PI3K-Akt pathway respectively in several types of cancer (Wang et al., 2002; Xie et al., 2009; Zhang et al., 2004). Recently, it has been shown that Dab2ip down regulation by RNAi directly increased the levels of phospho-Erk and phosphor-Akt in mouse embryonic fibroblasts (Min et al., 2010). However, the signaling mechanisms that are regulated by Dab2ip in the brain are largely unknown.

Dab2ip is most abundantly expressed in the developing and adult brain (Chen et al., 2006; Qiu et al., 2007) (Figure 5). Previous studies have shown that Dab2ip interacts with Dab1, a critical mediator of the Reelin pathway affecting neuronal migration in the developing cortex and neuronal differentiation at postnatal days (D'Arcangelo et al., 1999; Homayouni et al., 2003; Niu et al., 2004; Qiu et al., 2007; Wang et al., 2002). In the previous chapter I have also found that mutations in the Reelin pathway do not affect Dab2ip protein expression. However, a functional interaction of Dab2ip with the Reelin/Dab1 signaling network remained to be elucidated.

Dab1 is phosphorylated at several tyrosine residues by Fyn and Src, two non-receptor tyrosine kinases that are activated when Reelin binds to its receptors, VLDLR and ApoER2 (Arnaud et al., 2003b; D'Arcangelo et al., 1999; Hiesberger et al., 1999; Howell

et al., 1999; Keshvara et al., 2001). While phosphorylated Dab1 recruits specific intracellular signaling proteins for downstream signal transduction, it also undergoes ubiquitin-dependent protein degradation (Arnaud et al., 2003a; D'Arcangelo, 2006; Feng et al., 2007; Howell et al., 1999). Dab1 is elevated in the Reeler mouse presumably because of the lack of Reelin signaling cascade results in the accumulation of unphosphorylated Dab1. Thus, post transcriptional regulation of Dab1 has been considered as an important factor in controlling intracellular Dab1 levels (Feng et al., 2007; Howell et al., 1999; Rice et al., 1998). Recently, my co-workers and I discovered that Dab1 protein, not mRNA, is increased in the PTEN-deleted mouse neocortex, suggesting that the intracellular level of Dab1 might also be regulated by serine phosphorylation via the activation of the PI3K-Akt signaling pathway (Kazdoba reference) (Kazdoba et al., 2012). Also, the findings that Dab1 can be phosphorylated at serine residues, which promotes the degradation of Dab1, are consistent with this view (Gao and Godbout, 2011; Keshvara et al., 2002; Ohshima et al., 2007b). In addition, it has been reported that Dab1 expression can be altered at the transcriptional level by showing both Dab1 protein and mRNA are decreased in the cAMP response element-binding (CREB) mutant mouse brain (Diaz-Ruiz et al., 2008). However, how Dab1 expression is regulated at a transcriptional level is still unclear.

Here I investigated the activity of candidate signaling proteins using a novel Dab2ip knockout mouse that was generated by a collaborator, Dr. Ramin Homayouni, at the University of Memphis, TN. I examined control and mutant brains by western blot analysis and quantitative real time PCR (RT-PCR). I found that, in the embryonic brain, Dab2ip deficiency resulted in a modest and transient increase in the phosphorylation of

Erk1/2 and Akt. This effect correlated with a decrease in the expression of Map2 and Map1b, which I demonstrated in the Chapter 2 to be decreased by Dab2ip knockdown. Finally, I discovered a dramatic, but transient deficit of Dab1 expression in Dab2ip mutant brains, suggesting that Dab2ip may also affect early brain development through the regulation of Dab1 and thus the activity of the Reelin pathway.

2. Materials and Methods

Animals handling and in utero **electroporation (IUE)**

Dab2ip knockout mouse were generated with a retroviral gene-trap insertion in intron 5, upstream of the second predicted promoter to disrupt the expression of Dab2ip (Qiao et al., SFN, 2010; Salami et al., SFN, 2010). Refer to the IUE method described in the Materials and Methods section in the Chapter 2.

Expression constructs

Refer to the *expression constructs* described in the Materials and Methods section in the Chapter 2. Map1b construct promoted by CAG promoter was kindly provided by Dr. Kawauchi (Keio University School of Medicine, Japan) (Kawauchi et al., 2005). Dab1 cDNA was subcloned into the CAG vector by Cheng-Chiu Huang, a former member of the D'Arcangelo's laboratory.

Preparation of total protein lysate and Western blot analysis

Brain tissue of Dab2ip wildtype (WT) and knockout (KO) mice were shipped from the laboratory of Dr. Homayouni (University of Memphis, TN, USA) on dry ice. Embryonic samples consisted of the whole brain, whereas postnatal samples included the dissected neocortex only. The samples were homogenized and lysed in RIPA buffer (50mM Tris pH 7.4, 1% NP40, 0.25% deoxycholate, 150mM NaCl, 1mM EGTA) and cleared by centrifugation at 3,000 x g for 3 min at 4°C. Approximately, 15 μg of lysate were loaded onto 8% or 12% SDS-PAGE gels, run at 120 V for 2 h, and transferred to

0.22 µm nitrocellulose membranes. The membranes were blocked with 3% milk in 1x TBS-T (Tris-buffered saline with 0.1% Tween 20) for 1 h at room temperature, followed by incubation with the indicated primary antibody overnight at 4°C, and incubation with the indicated secondary antibody for 1 h at room temperature. After several washes with TBS-T, membranes were developed with ECL-Plus Western Blotting Detection System (GE Healthcare) and exposed to autoradiographic film (Denville).

Antibodies

Refer to the table 1 and 2 in Materials and Methods section in Chapter 2.

Isolation of total RNA and RT-PCR

Total RNA from the mouse brains of Dab2ip WT or KO was purified using the Qiagen RNA purification system (RNeasy). cDNA was synthesized using the SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instruction. Genes were analyzed by SYBR green real time quantitative RT-PCR, which was conducted on the Applied Biosystem real-time PCR instrument using Power SYBR Green master mix (Applied Biosystems). The expression of specific genes was analyzed using the Pfaffl method, after normalizing to the amount of Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) in order to calculate relative levels of transcripts (Pfaffl, 2001). The sequence information of the primers used in this experiment is shown in Table 3.

Table 3. Information of the primers used in this study.

Gene	Sequence	Reference
Dab2ip	F 5'-CTTCACCCGTCAGTCCCAATC-3' R 5'-CCTTACTCCTTAGCCTATCCCTG-3'	PrimerBank 33622389a1*
Map2	F 5'-GCAGCCGAAGAAACAGCTAA-3' R 5'-CTCAGGGAATTCCATCTTCG-3'	(Casalbore et al., 2010)
Map1b	F 5'-TCGCACCGCTTCCTAGACA-3' R 5'-CTGGTCCAAGTTGCACTCAAT-3'	PrimerBank 6678946a1*
Dab1	F 5'-AAACCAGCGCCAAGAAAGACT-3' R 5'-CGGACACTTCATCAATCCCAA-3'	PrimerBank 70909360b1*
Gapdh	F 5'- AGGTCGGTGTGAACGGATTTG-3' R 5'- TGTAGACCATGTAGTTGAGGTCA-3'	PrimerBank 6679937a1*

*PrimerBank (http://pga.mgh.harvard.edu/primerbank/) (Spandidos et al., 2008; Spandidos et al., 2010; Wang and Seed, 2003)

Statistical analysis

Data in the plots are shown as the mean +/- s.e.m., and analyzed by Student's *t* test or one-sample *t*-test as indicated in the figure legends. Statistical significance was determined when *p*<0.05.

3. Results

Signal transduction in Dab2ip knockout mouse brain

As a Ras GTPase-activating protein, Dab2ip is involved in the Ras-Raf-MAPK and PI3K-Akt signaling pathways. Recently, it has been reported that the levels of phosphorylation of both Erk1/2 and Akt increased in the Dab2ip-downregulated cells using RNA interference (RNAi) system. To investigate if the signaling transduction shown in the prostate cancer cells is also observed in the mouse Dab2ip knockout (KO) brain, I performed western blot analysis. The brain lysates were prepared from two embryonic days and two postnatal days. The experiments were conducted in triplicated with different sets of samples. As expected, Dab2ip was almost undetectable in Dab2ip KO mouse brain samples (Figure 13A). In the early embryonic days (E14.5), I found a tendency for phospho-Erk1/2 and phospho-Akt to be increased in KO compared to wild type (WT) mice (phospho-Erk1/2, 33% increase; phospho-Akt, 30% increase; Figure 14A, B, C, and D). However, this tendency was limited to the samples of embryonic brains because a change was not observed in postnatal samples (Figure 14A, B, C, and D). Although some sets of samples showed some difference between the WT and KO samples at E16.5 (Figure 14C), the effect was not statistically significant due to the large variance between the sets.

Figure 14. Signal transduction in Dab2ip knockout mice using western blot analysis.
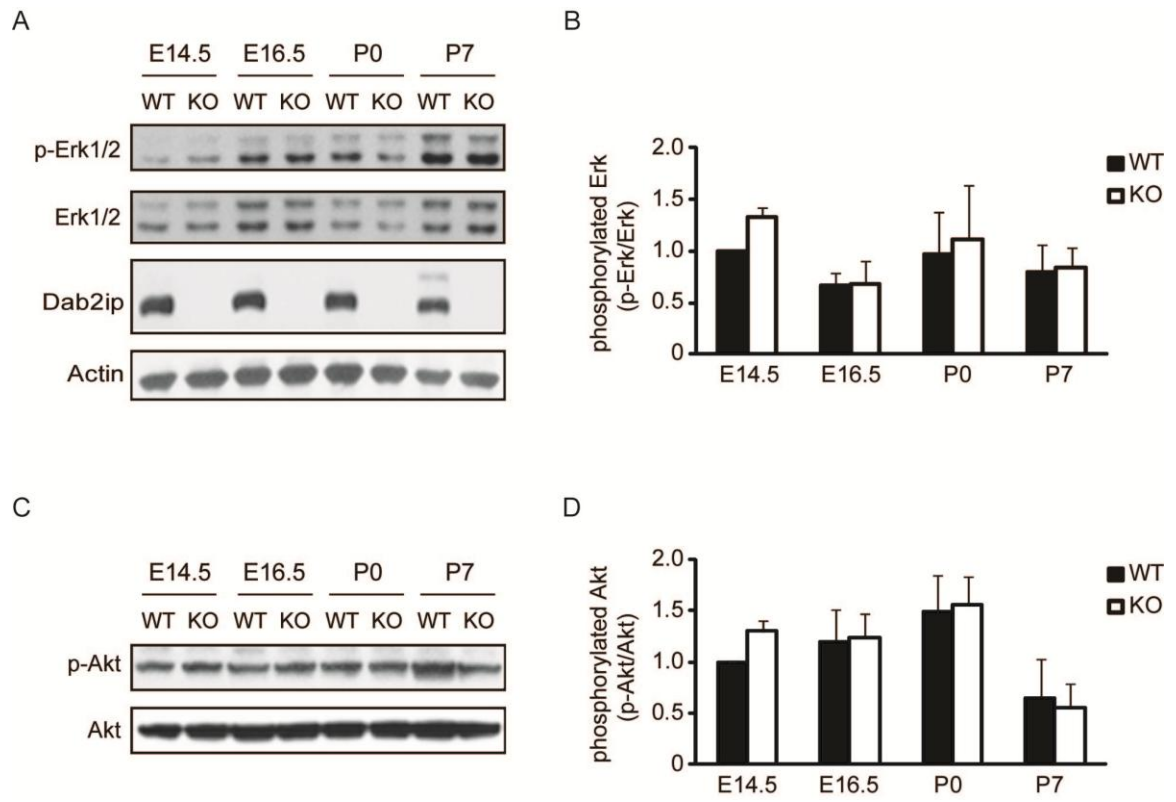
Figure 14. Signal transduction in Dab2ip knockout mice using western blot analysis.

A tendency of activation of Erk and Akt pathways was present in Dab2ip KO brain lysates. (A, B) phospho-Erk1/2 was slightly increased by 33% in E14.5 Dab2ip KO samples. Also, Dab2ip was not detected in the KO samples throughout the ages. (B) Quantification of phospho-Erk1/2 versus total Erk1/2 was shown from embryonic days to the early postnatal days. 3 sets were analyzed. (C, D) phospho-Akt also showed an increase by 30% in the KO samples in the early embryonic day E14.5. (D) Quantification of phospho-Akt versus total Akt was shown from embryonic days to the early postnatal days. 3 independent sets of mice were analyzed.

The level of MAPs in the Dab2ip KO brains

In the previous Chapter 2, my knockdown study indicated a role for Dab2ip in radial neuronal migration (Figure 7). I also demonstrated that the loss of Dab2ip affected the expression of MAPs including Map2 and Map1b in cultured neurons, which were electroporated with Dab2ip shRNA *in utero* before isolation of cortical neurons (Figure 13). Next, I checked the expression of the MAPs by western blot using Dab2ip KO brain lysates to examine how a long-term and complete loss of Dab2ip affects the level of MAPs expression in the developing brain. Again, I noticed a tendency for Map2 and Map1b to be decreased in the KO samples compared to the WT during the embryonic days (Map2, 73% in KO; Map1b, 87% in KO; Figure 15A, B, and C), however the difference was not statistically significant when triplicated experiments were quantified. The large variance among sets of samples may be due to degradation of the samples during delivery or dissection of the brains (Figure 15B, C). When the samples were compared only at the E16.5 in each set, the Map2 and Map1b levels appeared to be considerably different between WT and KO samples (Map2, 44% in KO, p=0.0581 in one way t-test; Map1b, 72% in KO, p=0.00680 in one way t-test; Figure 15D).

To see if the reduced levels of Map2 and Map1b are caused by post-transcriptional modification and degradation, I measured the relative mRNA contents of the MAPs in the E16.5 of developing brains using quantitative RT-PCR. The results revealed that Map2 mRNA levels in KO appeared to be increased compared to WT, whereas Map1b mRNA levels were not changed, suggesting that loss of Dab2ip induces a down-regulation of the levels of Map2 and Map1b proteins through post-transcriptional

mechanisms (Figure 15E). However, the RT-PCR experiment was done only with one set of samples, and the results will need to be confirmed using additional sets before a definite conclusion can be drawn.

Figure15. Expression of microtubule-associated proteins (MAPs) in the Dab2ip knockout brains.
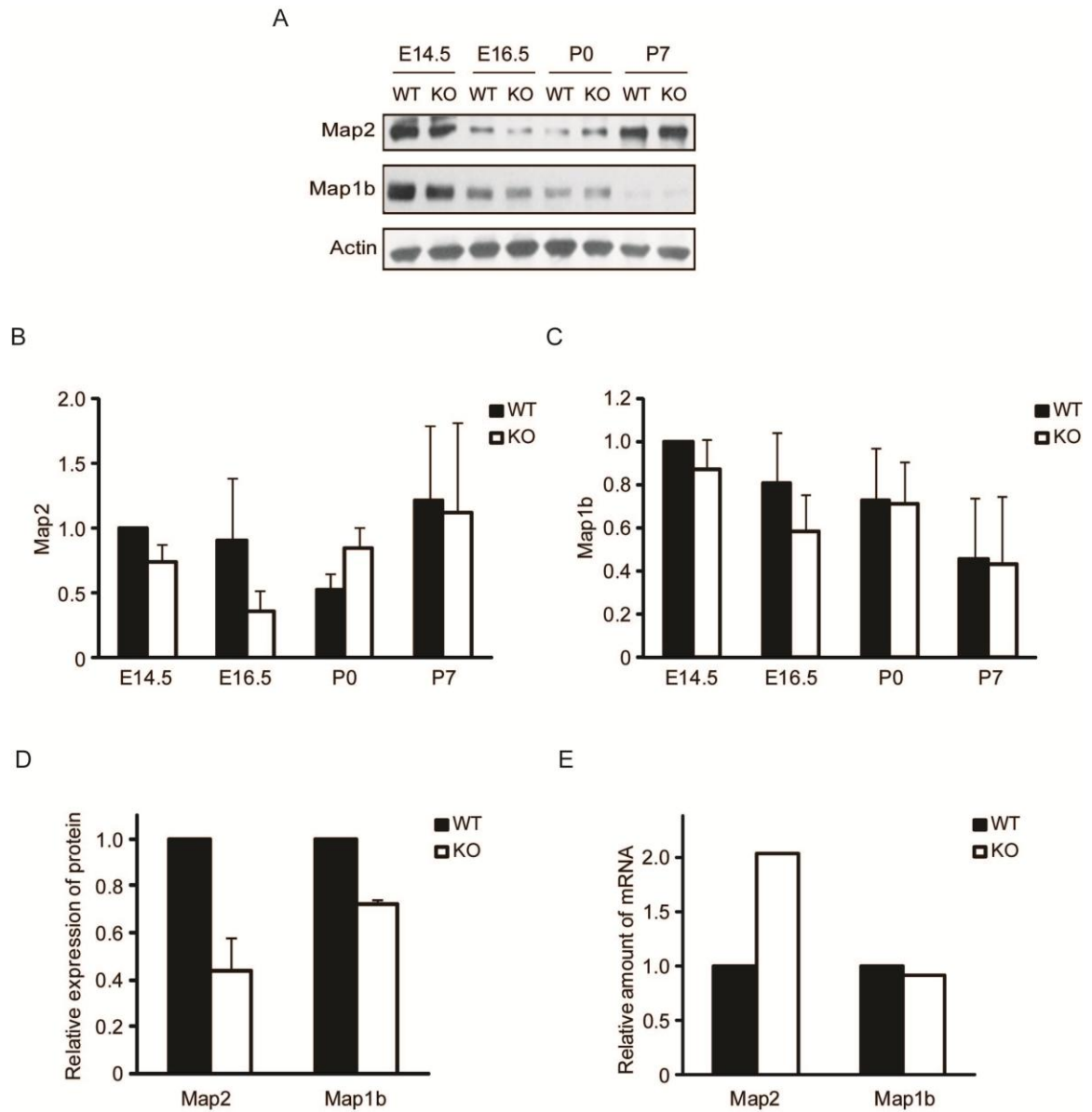
Figure 15. Expression of microtubule-associated proteins (MAPs) in the Dab2ip knockout brains.

 (A) Western blot showed that Map2 and Map1b protein expression was slightly decreased in the KO samples during embryonic days. (B) Quantification of the Map2 expression in 3 sets of samples. Map2 expression was decreased to 73% in KO compared to WT brain lysate at E14.5. (C) Quantification of the Map1b expression in 3 sets of samples. Map2 expression was decreased to 87% in KO compared to WT brain lysates at E14.5. (D) Quantification of Map2 and Map1b expression at E16.5. Map2 and Map1b significantly decreased to 44% and 72%, respectively in the KO samples compared to controls. (E) Quantitative RT-PCR showed that the mRNA amounts of both Map2 and Map1b were not decreased at E16.5 brain samples. One set of samples was used for this preliminary analysis.

Dab2ip regulates Dab1 expression during the embryonic days.

Dab1 is a key adaptor protein that mediates the activity of the Reelin-VLDLR/ApoER2 pathway (D'Arcangelo et al., 1999; Hiesberger et al., 1999). Dab2ip has been reported to interact with Dab1 through the NPxY motif, but the downstream consequences of this interaction have not been examined in the developing brain (Homayouni et al., 2003; Wang et al., 2002). Thus, I tested whether long-term loss of Dab2ip in the brain of KO mice affects the level of Dab1 expression at embryonic and early postnatal days. Surprisingly, western blot analysis revealed that Dab1 protein levels were noticeably decreased at embryonic days, but not at perinatal (P0) or early postnatal day (P7) (Figure 16A). Quantification of the data indicated that the difference between KO and WT was significant at E14.5 (p=0.93 in one way t-test) and at E16.5 (p=0.083, t-test), but not statistically valid due to sample variance (Figure 16B). Quantitative RT-PCR revealed that Dab2ip mRNA was also reduced in the Dab2ip KO brain at E16.5, suggesting that the expression of Dab1 may be governed through transcriptional regulation (Figure 16C).

To investigate whether the migration defect that I previously identified resulting from Dab2ip knockdown was induced by the transient Dab1 or MAPs down-regulation, I attempted to rescue the phenotype by overexpressing Dab1 or Map1b using *in utero* electroporation. In addition, I also co-injected Map1b and Dab1 constructs into the developing mouse neocortex in an attempt to alleviate the migration phenotype induced Dab2ip RNAi. However, the phenotype was not rescued, suggesting that Dab1 and Map1b overexpression alone are not sufficient to recover Dab2ip function (Figure 17).

Figure 16. Dab2ip controls the expression of Dab1 only in the embryonic brain.
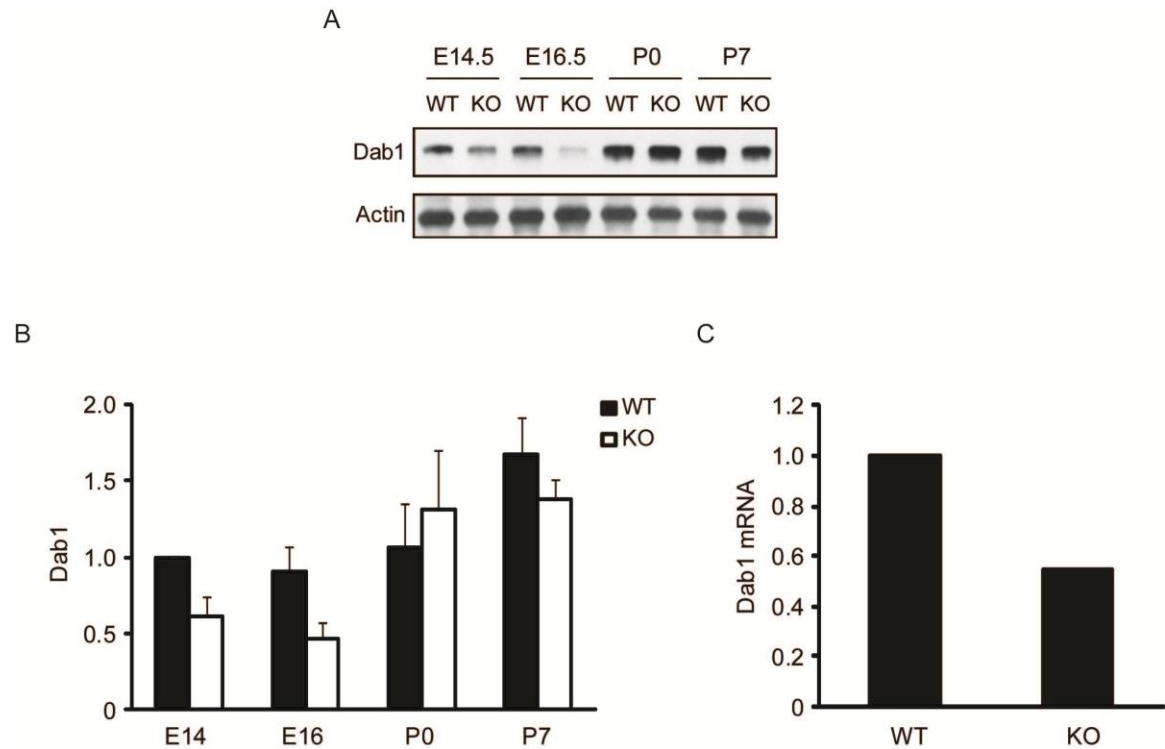
Figure 16. Dab2ip controls the expression of Dab1 only in the embryonic brain.

(A) Western blot analysis showed that Dab1 expression was reduced only during embryonic days' samples. (B) Quantification of Dab1 expression from the embryonic days to the early postnatal days. Dab1 is decreased by 61% and 55% at E14.5 and E16.6, respectively in Dab2ip KO brain (control was set as 100% in each age). (C) Quantitative RT-PCR showed that the Dab1 mRNA was decreased in the brain lysates at E16.5, which means that the reduction of Dab1 expression might be due to the transcriptional regulation by Dab2ip. One set of samples was used for this analysis.

Figure 17. Migration defect by Dab2ip RNAi was not rescued by overexpression of Map1b, Dab1, or both Map1 and Dab1 constructs.
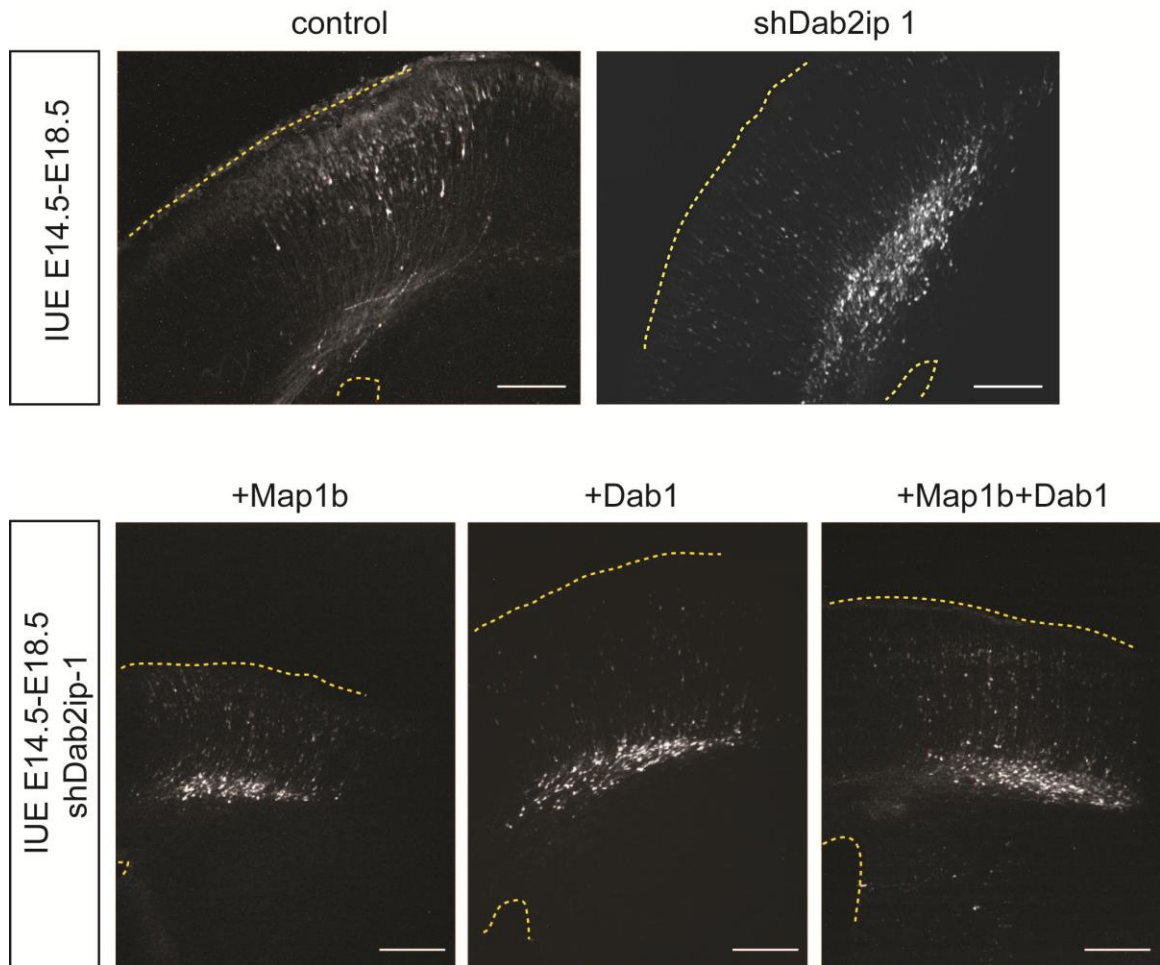
Figure 17. Migration defect by Dab2ip RNAi was not rescued by overexpression of Map1b, Dab1, or both Map1 and Dab1 constructs.

Control shRNA or Dab2ip shRNA 1 (shDab2ip 1) was injected into neuroblast cells in the E14.5 developing neocortex using *in utero* electroporation (IUE). For the rescue trials, the indicated overexpression constructs were co-injected with shDab2ip 1. 4 days after IUE the embryonic brains were dissected and processed to examine the migration phenotype of the electroporated neurons. As shown in the Chapter 2, knocking down of the level of Dab2ip by RNAi in migrating neurons caused migration defect under the cortical plate. The majority of the GFP+ cortical neurons co-injected with overexpression constructs, showed similar migration defects as shDab2ip 1 alone. Thus, attempts to rescue the migration deficit of Dab2ip RNAi were not successful.

4. Discussion

In this study, I used KO brains to analyze the signal transduction and protein expression affected by loss of Dab2ip. When altered protein expression was detected, I also determined whether it was regulated at a transcriptional level using quantitative RT-PCR. This study revealed a tendency toward the activation of Erk1/2 and Akt signaling in the Dab2ip KO brain at early embryonic days (E14.5), which suggests that those signaling pathway may contribute to the function of Dab2ip in neuronal migration, as demonstrated in Chapter 2, or other possibly functions in early brain development. However, those changes were not statistically significant, possibly due to a large sample variation, and therefore, will need to be confirmed using additional sets of fresh samples.

In Chapter 2 I showed that the expression of MAPs, including Map2 and Map1b, was decreased in the primary cortical neurons after *in utero* electroporation with Dab2ip shRNA. In accordance with this result, protein analysis with Dab2ip KO mouse brains indicated that Map2 and Map1b expression tended to be reduced during embryonic days, due to post-transcription mechanisms (Figure 15). Since Map2 and Map1b are involved in aspects of neocortical development, including neuronal migration and dendrite growth (Takei et al., 1997; Teng et al., 2001), their reduced levels may contribute to the abnormal development of Dab2ip knockdown (chapter 2) or KO neurons. Consistently, it has been reported that Map2 levels are reduced in Cdk5 knockout and Reeler mice, which also show abnormal brain development, including layering defects in the neocortex (Niu et al., 2004; Ohshima et al., 2007a). Also, the Map2 reduction could explain the developmental deficit in dendrite growth in the developing neocortex of these mutant

mice (Niu et al., 2004; Ohshima et al., 2007a). However, the exact mechanims that lead to MAPs expression abnormalities in Dab2ip deficient neurons remain to be elucidated.

Reelin and its adaptor molecule, Dab1 have been studied as critical regulator for brain development during embryonic days and postnatal days, such as neuronal migration, dendrite development, and synapse formation (D'Arcangelo et al., 1999; D'Arcangelo et al., 1995; Niu et al., 2004; Niu et al., 2008; Ventruti et al., 2011). Since neurogenesis and neuronal migration and positioning are predominant during embryonic brain development, my data indicating that Dab2ip affects the levels of Dab1 expression at this time is consistent with the notion that Dab2ip controls these events by affecting Reelin signaling. Since Dab2ip levels are not changed in Dab1 KO mice or Reeler mice (as shown in Chapter 2, Figure 5D, E), Dab2ip may function upstream of Dab1. However, my attempts to rescue the Dab2ip RNAi-induced migration defect with Dab1 or Map1b constructs were not successful, suggesting that Dab2ip controls neuronal migration through multiple molecules. Indeed, while Dab1 is required mainly for somal translocation during radial neuronal migration, it might also function in other aspects of migration such as orientation of migrating late-born neurons in the intermediate zone, where Dab2ip is required for transition from multipolar to bipolar morphology (Franco et al., 2011; Jossin and Cooper, 2011; Sekine et al., 2011). Additionally, Dab2ip may regulate Dab1 expression to control other aspects of neuronal development, such as dendrite growth.

The decrease of Dab1 mRNA levels noted in Dab2ip KO at embryonic ages (E16.5) suggested that Dab1 expression might be controlled by transcriptional activation, but the mechanism by which Dab2ip regulates the transcription of Dab1 needs to be further

investigated. A previous study has shown that Dab1 expression is decreased in the CREB mutant mice by a transcriptional mechanism (Diaz-Ruiz et al., 2008). Taking into account the fact that the Ras-Raf-Erk pathway is well known to affect transcription through CREB phosphorylation, it is conceivable that the activation of the Erk pathway in Dab2ip KO brain may result in decreased Dab1 transcription (Wang et al., 2007).

In summary, in these preliminary studies I have found that Dab2ip may be involved in the activation of Erk1/2 and Akt signaling in the embryonic mouse brain. Also, I found that Dab2ip transiently affects the expression of MAPs such as Map2 and Map1b, a finding that is consistent with the results of my Dab2ip-knockdown experiments in Chapter 2. Finally, I found that Dab2ip regulates the expression of Dab1, possibly occurring at the mRNA level, suggesting a functional interaction with the Reelin pathway.

Chapter 4. Discussion and Future Directions

1. Dab2ip is required for radial neuronal migration.

It has been a decade since Dab2ip was first reported to interact with Dab1, a critical adaptor protein in the Reelin signaling pathway that regulates the lamination of developing cortex in the mammalian brain (D'Arcangelo et al., 1995; Homayouni et al., 2003; Rice et al., 1998; Wang et al., 2002). Dab2ip is abundantly expressed in the developing and adult brain (Chen et al., 2006; Homayouni et al., 2003; Qiu et al., 2007; Wang et al., 2002). Several genetic studies has presented that Dab2ip is associated with deveopmental brain disorders, such as autism, and schizophrenia (Hussman et al., 2011; Mudge et al., 2008). However, functional studies of Dab2ip in the brain had not been conducted. During the course of my doctoral studies, I studied the function of Dab2ip in the developing mouse brain by down-regulating Dab2ip levels *in vivo* using RNAi and *in utero* electroporation. Also, I analyzed the protein expression in Dab2ip KO mouse brain to elucidate molecular mechanisms affected by Dab2ip loss. My studies revealed that Dab2ip is required for radial neuronal migration of late-born cortical neurons, especially morphological transition from multipolar to bipolar in the IZ. The morphology of knockdown neurons appeared to be opposite to that of Dab2ip overexpressing neurons, which were fatty and round. The morphgical deficit resulting from Dab2ip down-regulation correlated with the abnormal neurite development of cortical neurons cultured *in vitro* after IUE. The major neuronal MAPs including Map2, Map1b, and Tau were decreased in the Dab2ip knockdown cortical neurons. Likewise, in the Dab2ip KO lysates, these neuronal MAPs tended to be reduced. The activation of Erk and Akt pathways and

the reduction of Dab1, noted in my preliminary analysis of the Dab2ip KO brain, suggests that these pathways may contribute to the neuronal migration defect and MAPs reduction induced by Dab2ip RNAi. Furthermore, regulation of Dab1 levels by Dab2ip during the embryonic brain development raises the possibility of a fucntional relationship between Dab2ip and the Reelin signaling pathway to control brain development, which needs to be investigated further in the future.

I found that overexpression of Dab2ip itself resulted in a migration defect, which means that adequate amounts of Dab2ip expression are important for neuronal migration in the developing neocortex. This phenotype allowed me to conduct an analysis of structure-function of Dab2ip. Since different isoforms of Dab2ip exist (Chen et al., 2006; Chen et al., 2002), I obtained corresponding Dab2ip cDNAs (mouse Dab2ip-L (DQ473307), human Dab2ip (BC146762), and rat DIP1/2 (AF236130)), and I subcloned them into a vector containing the CAG promoter. I then overexpressed these constructs in vivo using IUE. Overexpression of mouse Dab2ip-L and human Dab2ip, two isoforms that contain the PH domain, produced a similar migration defect in the developing neocortex (IUE E14.5-E18.5; Dab2ip-L, Figure 10). However, overexpression of rat DIP1/2, which lacks the PH domain, did not show any migration defect at the same ages (IUE E14.5-18.5). This is consistent with the finding that the migration arrest by Dab2ip overexpression was completely abolished by the deletion of the PH domain from the Dab2ip-L construct, and strongly suggests that the regulation of neuronal migration by Dab2ip is highly dependent on the presence of the PH domain. This domain is known for promoting protein localization to the cell membrane, and also participates in intramolecular interaction with other regions such as the GRD domain (Zhang et al.,

2003). As shown in the Figure 9C, the deletion of the PH domain in the Dab2ip-L construct forced the protein to become localized around the nucleus, whereas wild type Dab2ip was distributed evenly throughout the cell body. Since the intact GRD region of Dab2ip was also required for the migration phenotype in my overexpression experiments *in vivo*, these results suggest that Dab2ip normally functions at the membrane where it regulates the activity of GTP-binding proteins involved in cytoskeletal dynamics.

2. Rescue trials for the migration defect that is induced by Dab2ip RNAi

In this study, I used a combination of *in utero* electroporation (IUE) and RNA interference techniques to knock down Dab2ip in the developing mouse neocortex. To control potential non-targeting effects, I used 3 different shRNA constructs to Dab2ip, which target different regions of the Dab2ip mRNA. All shDab2ip constructs resulted in similar migration defects after IUE, which were dramatic compared to a non-targeting shRNA contructs (control) and were highly reproducible in several electroporated embryos obtained from different litters. Thus, I concluded that Dab2ip expression is essential for proper cortical development. I also attempted to rescue the shDab1 migration defect by co-expressing mutagenized, non-degradable Dab2ip isoforms (mouse Dab2ip-L, human Dab2ip, rat DIP1/2). However, these attempts were unsuccessful. The reason for this outcome is not presently clear, but several possibilities can be envisioned. For example, the Dab2ip constructs that I used for the rescue experiment may not encode the correct isoform that functions in migrating neurons. Dab2ip is expressed from various transcripts, which are generated by alternative splicing from different first exons and also from multiple transcription start sites (Chen et al., 2002; Wang et al., 2002). In addition, the expression of Dab2ip from the several mRNA isoforms varies depending on tissues and ages (Chen et al., 2006; Qiu et al., 2007). Dab2ip-L, which contains a long N-terminus with a complete PH domain, has been reported to interact with Dab1 (Homayouni et al., 2003), but its mRNA levels, as detected by RTq-PCR, was very low compared to total Dab2ip mRNA at embryonic days (data not shown). Using primers that amplify the PH domain of Dab2ip cDNA at E16.5, my quantitative RT-PR revealed that most of Dab2ip isoforms expressed in the embryonic cortex indeed contain a PH domain

(data not shown). However, it is hard to know the exact composition of the Dab2ip isoform expressed in the embryonic neocortex because multiple isoforms can exhibit similar molecular weights,, and I was unable to identify primers which could differentiate among isoforms using quantitative RT-PCR. For the rescue experiments, 3 different isoforms of Dab2ip construct were used. However, the constructs were also from different species (mouse Dab2ip-L, human Dab2ip isoform 1, and rat DIP1/2), which might affect their ability to rescue migration in the mouse neocortex.

3. **Future Direction**

3-1. IUE with Dab2ip KO embryos to confirm the morphological phenotype.

I have shown the protein analysis of Dab2ip KO brain lysates in the Chapter 3. The Dab2ip KO mouse has been generated by Dr. Homayouni's group, who is analyzing in detail the migration phenotype in the brain using layer markers and BrdU incorporation. If these mice are made available to me, I could conduct further IUE experiments and determine how Dab2ip KO neurons migrate or what morphology they possess during migration. Also, I could compare the consequences of the transient loss of Dab2ip by RNAi to the long-term loss of Dab2ip by gene deletion. Since the germ line deletion of the gene (KO) can be compensated by sequence-related or functionally redundant proteins during development, the phenotype of KO mice could be less severe than that resulting from experiments using RNAi. For example, genetic deletion of *Dcx* in a mouse model did not show any defect in neocortical lamination, due to genetic compensation by the Doublecortin-like kinase (Dclk) (Corbo et al., 2002; Deuel et al., 2006; Koizumi et al., 2006). However, knockdown of Dcx or Dclk in migrating neurons by *in utero* electroporation caused a migration arrest in the IZ as multipolar cells (Bai et al., 2003; Koizumi et al., 2006).

3-2. Potential effects of Dab2ip on the proliferation and differentiation of neural progenitors.

Dab2ip is expressed throughout the neocortex from the early embryonic day E14.5 when neurons are generated in large numbers (Figure 5A, B, and C). In Chapter 2,

I have shown that electroporated neurons (control and shDab2ip 1) migrated at 2 days and 3 days after IUE (Figure 8). However, neurons transfected with shDab2ip 1 migrated faster than control at 2 days after IUE, although they still settled under the CP at 3 days after IUE. This finding raised the possibility that Dab2ip affects migration speed of neurons to the IZ. However, knockdown cells also appeared to leave the VZ earlier than control because the intensity of the fluorescence (GFP+ neurons) in the VZ in shDab2ip 1 sample was reduced. This might reflect reduced proliferation of neuronal progenitors or earlier differentiation of the progenitors due to the loss of Dab2ip.

To further investigate the potential effect on migration speed, time lapse microscopy can be used for tracking GFP positive migrating neurons. Cortical slice cultures would be prepared *ex vivo* after IUE. To examine proliferation and differentiation, the electroporated cortex could be stained with Tuj1 antibody to differentiate the VZ/SVZ/IZ and the number of the electroporated neurons that are left in the VZ/SVZ could be monitored in 2 days after IUE. Also, BrdU can be injected in pregnant mice to label proliferating cells. 4 hours after BrdU injection, embryonic brains can be dissected and processed for immunofluorescence to count BrdU-positive cells. Furthermore, several differentiation markers such as Tbr2, NeuN, and Tuj1 can be used to label progenitors and differentiated neurons in the electroporated cortex (IUE E14.5-E16.5) to monitor the process of neuronal differentiation.

3-3. The function of Dab2ip in dendritic spine development.

Since Dab2ip has been to interact with Dab1, which affects dendritic spine development, it might be interesting to study whether Dab2ip also plays a role in spine development. Although Dab2ip is not preferentially localized to dendritic spines, it might function at the plasma membrane to affect filopodia formation or spine dynamics. In fact, I noticed that Dab2ip knockdown neurons isolated from electroporated embryos (IUE E14.5-E18.5, the same isolation method described in Chapter 2) developed dendritic filopodia at a later time point compared to control neurons (Figure 18). In this culture condition, control neurons usually develop filopodia starting from 9 DIV. At 11 DIV control neurons clearly exhibited dendritic filopodia, whereas Dab2ip knockdown neurons did not. These neurons eventually formed filopodia and developed spines at 13 DIV and 15 DIV. Future studies should be conducted to determine whether Dab2ip downregulation also affects dendritic spine density, size and morphology. Potential spine abnormalities may or may not be secondary to abnormal dendritic development (Figure 11), and delay of filopodia formation. In addition, as a Ras GTPase-activating protein, Dab2ip might be involved in the synaptic plasticity. Similar proteins, such as synaptic Ras GTPase-activating protein 1 (SYNGAP 1), have been shown to play a role in synaptic transmission through the activation of the MAP kinase signaling pathway (Kim et al., 1998; Rumbaugh et al., 2006), and gene mutations can cause intellectual disability in humans (Hamdan et al., 2009).

In summary, Dab2ip might have multiple roles in the developing cortex including proliferation, neuronal differentiation, migration, or maturation. I have demonstrated that

Dab2ip is required for radial neuronal migration and neurite development using the IUE technique. Therefore, Dab2ip may be critical for normal mammalian brain development.

Figure 18. Filopodia and spine formation in Dab2ip knock down neurons.
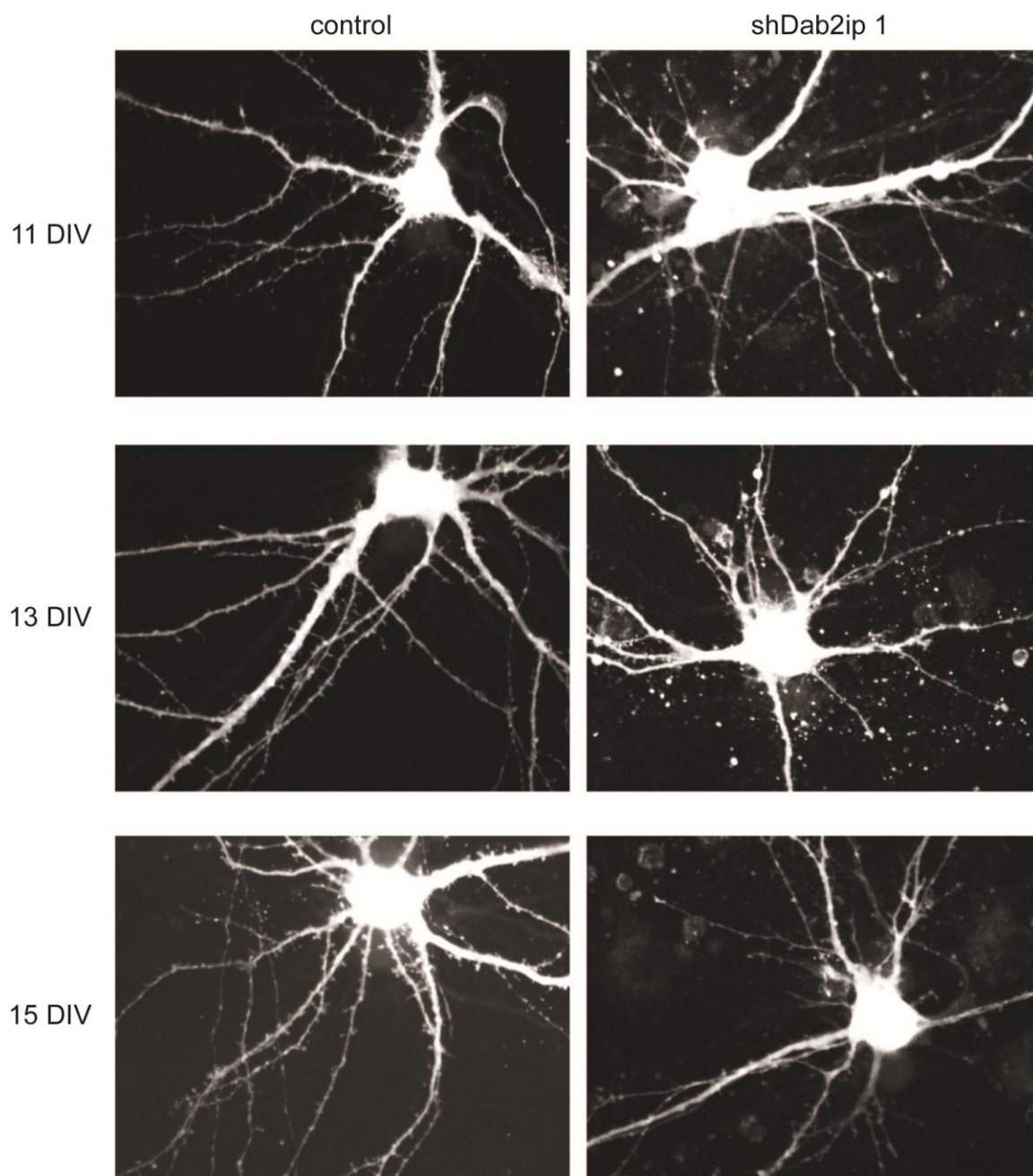
Figure 18. Filopodia formation in Dab2ip down-regulated neurons.

Co-cultured neurons, isolated from embryos electroporated with control shRNA or Dab2ip shRNA 1 using IUE, were fixed and processed for confocal imaging to examine filopodia formation and spine development. At 11 DIV, control neurons exhibited many dendritic filopodia with a thin and hair-like morphology, whereas most of Dab2ip RNAi neurons did not. At 13 DIV and 15 DIV, these knockdown neurons exhibited filopodia and spines that were similar to control neurons.

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