Dab2IP Regulates Neuronal Positioning, Rap1 Activity and Integrin Signaling in the Developing Cortex

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Key Words

GTPase activating protein · Migration · Rap1GAP · RasGAP · Reelin

Abstract

Dab2IP (DOC-2/DAB2 interacting protein) is a GTPase-activating protein which is involved in various aspects of brain development in addition to its roles in tumor formation and apoptosis in other systems. In this study, we carefully examined the expression profile of Dab2IP and investigated its physiological role during brain development using a Dab2IP-knockdown (KD) mouse model created by retroviral insertion of a LacZ-encoding gene-trapping cassette. LacZ staining revealed that Dab2IP is expressed in the ventricular zone as well as the cortical plate and the intermediate zone. Immunohistochemical analysis showed that Dab2IP protein is localized in the leading process and proximal cytoplasmic regions of migrating neurons in the intermediate zone. Bromodeoxyuridine birth dating experiments in combination with immunohistochemical analysis using layer-specific markers showed that Dab2IP is important for proper positioning of a subset of layer II–IV neurons in the developing cortex. Notably, neuronal migration was not completely disrupted in the cerebral cortex of Dab2IP-KD mice and disruption of migration was not strictly layer specific. Previously,

we found that Dab2IP regulates multipolar transition in cortical neurons. Others have shown that Rap1 regulates the transition from multipolar to bipolar morphology in migrating postmitotic neurons through N-cadherin signaling and somal translocation in the superficial layer of the cortical plate through integrin signaling. Therefore, we examined whether Rap1 and integrin signaling were affected in Dab2IP-KD brains. We found that Dab2IP-KD resulted in higher levels of activated Rap1 and integrin in the developing cortex. Taken together, our results suggest that Dab2IP plays an important role in the migration and positioning of a subpopulation of later-born (layers II–IV) neurons, likely through the regulation of Rap1 and integrin signaling.

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Introduction

The mammalian neocortex has a highly organized six-layered structure containing different types of neurons arranged in stereotypical patterns [1]. Neuronal migration plays an essential role in the development of this laminar structure. At early stages of neocortical development, migrating neurons enter the cortical plate via glial-independent somal translocation [2, 3]. Later-born neurons migrate further distances using several modes of migra-

tion. First, postmitotic neurons move radially to the sub-ventricular zone and lower intermediate zone, where they become multipolar [3, 4]. Next, the newly born neurons become bipolar by extending a leading process and a trailing axon while retracting other neurites [5, 6]. These bipolar-shaped neurons migrate long distances along the radial glial fibers to the upper part of the cortical plate [2, 5]. Finally, migrating neurons attach their leading processes to the marginal zone and switch to a glial-independent terminal translocation mode to position themselves beneath the outermost region of the cortical plate [7, 8].

Several signaling pathways have been shown to regulate various aspects of neuronal migration in the developing mammalian cortex. Reelin is a large extracellular glycoprotein secreted by Cajal-Retzius cells in the marginal zone that controls both early and late events in cortical development. Mutations in the Reelin signaling pathway (including lipoprotein receptors ApoER2/VLDLR and Dab1 cytosolic adapter protein) disrupt early splitting of the cortical plate in addition to migration and positioning of early- and late-born neurons [9–12]. Upon Reelin-induced tyrosine phosphorylation, Dab1 binds to the p85 regulatory subunit of PI3K and CrkL, and C3G, a Rap1 guanine nucleotide exchange factor (GEF) [13, 14]. Recent reports suggest that Rap1 plays an important role in neuronal migration by regulating N-cadherin-mediated adhesion required for multipolar to bipolar transition [7, 15] and integrin-mediated adhesion required for terminal somal translocation [16, 17].

We initially found Dab2IP (DOC-2/DAB2 interacting protein) by virtue of its interaction with Dab1 in a yeast two-hybrid screen [18]. Dab2IP, also known as ASK1 (apoptosis signal-regulating kinase-interacting protein-1), is a GTPase-activating protein associated with aggressive metastatic prostate cancer [19], abdominal aortic aneurysms [20] and coronary heart disease [21]. Dab2IP was shown to inhibit Ras, JNK (c-Jun kinase) and PI3K-AKT signaling pathways and to regulate mesenchymal-to-neuroepithelial transition of stem cells [22–25]. Recently, we and others have shown that Dab2IP is expressed in a variety of neurons in the brain and plays a role in the regulation of synaptogenesis, dendritic morphogenesis, neuronal differentiation and migration [24, 26, 27]. In utero knockdown (KD) of Dab2IP by siRNA disrupted the transition of migrating neurons from multipolar to bipolar morphology in the developing neocortex [26]. In this study, we carefully examined the role of Dab2IP in the neuronal migration and lamination of the cortex using a mouse model with Dab2IP expression KD by a retroviral gene trap strategy.

Materials and Methods

Animals

All mice used in this study were maintained in certified animal facilities at the University of Memphis and all experiments were performed in accordance with the institutional guide for animal care using an animal protocol (protocol No. 0644, December 20, 2012) approved by the University of Memphis Institutional Animal Care and Use Committee. Mice were housed with unlimited access to water and food under a 12-hour-light/12-hour-dark day cycle. Throughout all experimental procedures, efforts were made to minimize the number of animals used and their suffering. Generation of Dab2IP-KD [Dab2IP^{Gt(OST348452)Lex}] mice with C57/B6 background was described previously [27].

Histology and Immunohistochemistry

Mice were perfused with 4% paraformaldehyde, and the brains were removed and postfixed for 3 h in 4% paraformaldehyde. Brains from embryonic mice were fixed in 4% paraformaldehyde without perfusion. The fixed brains were cryoprotected with 30% sucrose, embedded in tissue freezing medium (OCT), frozen in isopentane cooled with liquid nitrogen and sectioned using a Leica cryostat (CM3050).

For X-gal staining, embryos or postnatal mice which were heterozygous for the retroviral gene trap cassette in *Dab2IP* intron 5 were perfused and embedded as above. Cryosections (12 μ m) were washed 3 times in solution C (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 2 mM MgCl₂, 5 mM EGTA, 0.02% IGEPAL and 0.01% sodium desoxycholate) and incubated overnight in X-gal staining solution (solution C + 10 mM K₃Fe(CN)₆, 10 mM K₄Fe(CN)₆ and 0.5 mg/ml X-gal). After washing 3 times with 1 $\times$ PBS, sections were visualized with nuclear Fast Red counterstaining.

For immunohistochemical staining, coronal sections (12 μ m) were incubated overnight at 4°C with the following primary antibodies: anti-CuX1 (1:1,000, rabbit; Santa Cruz), anti-Tbr1 (1:500, rabbit; Abcam), anti-Brn 2 (1:500, rabbit; Abcam), anti-bromodeoxyuridine (BrdU; 1:500, mouse; Millipore), anti-Dab2IP (1:2,000–1:4,000, rabbit) and anti-CSPG (1:1,000; Sigma). After washing in 1 $\times$ PBS, sections were incubated with Alexa Fluor (Invitrogen) conjugated secondary antibodies (1:2,000), and images were collected using confocal laser scan microscopy (Nikon A1) at the Integrated Microscopy Center at the University of Memphis.

BrdU Birth Dating Analysis

Heterozygous Dab2IP^{Gt(OST348452)Lex} were mated and the pregnant mothers were injected intraperitoneally with BrdU (Sigma-Aldrich) dissolved in 0.9% NaCl, 50 mg/kg body weight at either E12.5 or E16.5. The age of embryos was calculated from noon on the day of the vaginal plug, which was set at embryonic day 0.5 (E0.5). Cryosections from postnatal day 21 (P21) pups were incubated in 2 N HCL for 30 min at room temperature to denature DNA, neutralized for 5 min with 0.1 M sodium borate buffer (pH 8.5 and subsequently stained with BrdU.

Primary Culture

E14 mouse embryonic cerebral cortices were treated with 0.25% Trypsin-EDTA for 20 min at 37°C and dissociated into single cells by gentle trituration. Cells were suspended in Neurobasal

medium (Invitrogen) supplemented with B27 (Invitrogen) and 2 mM L-glutamine, and then seeded on poly-D-lysine-coated cover slides. After a 3-hour incubation at 37°C, cells were fixed with 2% paraformaldehyde in PBS for 20 min and stained with anti-Dab2IP (1:4,000) plus rhodamine-phalloidin antibody (1:2,000; Millipore).

Immunoblotting

Brains were dissected and homogenized in lysis buffer containing 0.05% NP40, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10% glycerol, EDTA-free protease inhibitor cocktail (Roche) and a phosphatase inhibitor mixture PhosSTOP (Roche). Protein amounts were quantified using a BCA protein assay kit (Pierce), and an equal amount of protein was loaded for each sample, separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were then subjected to immunoblot analysis using anti-Dab2IP (1:5,000), anti-actin (Sigma), anti-Rap1 (1:1,000; Thermo Scientific) and anti-integrin (9EG7, 1:1,000; BD Pharmingen) antibodies. Proteins were visualized using Super Signal West Pico or Femto reagents (Thermo Scientific). For quantitative analysis of Western blotting results, X-ray films were scanned and densitometric analysis was performed using NIH ImageJ software.

Ras and Rap1 Activation Assay

Activated Ras or Rap1 levels were assayed with the active Ras or Rap1 pull-down and detection kit from Thermo Scientific following the manufacturer's protocol. In brief, brain lysates (1,000 µg total protein) from E16 wild-type (WT) and Dab2IP-KD cerebral cortices were incubated with GST-Raf1-RBD or GST-RalGDS-RBD fusion protein for 2 h at 4°C on a rotating platform. The GTP-bound Ras or Rap1 (active form) was affinity precipitated, washed and then eluted with 50 µl SDS sample buffer by boiling for 5 min. The amount of GTP-bound Ras or Rap1 was analyzed by SDS-PAGE followed by immunoblotting using the anti-Ras or -Rap1 antibody included in the kit.

Results

Expression of the Dab2IP Gene in the Cerebral Cortex during Development

Dab2IP appears to be exclusively expressed in neurons in various brain structures [27]. Previously, we found that multiple isoforms of Dab2IP protein exist in the adult cerebellum [27]. Here, we investigated the temporal and spatial expression patterns of Dab2IP in the developing cortex using different approaches. Immunoblot analysis was performed on lysates prepared from telencephalon (E12) and cortex (E16, P0 and P30) of C57/B6 mice. Dab2IP protein was observed as early as E12 in the telencephalon (fig. 1A). Multiple Dab2IP isoforms were observed at all stages, but their relative levels differed during development. At P30, three major isoforms of Dab2IP protein were detected, similar to our previous observations in the cerebellum [27]. Multiple isoforms of Dab2IP of different

molecular weights have been described [28, 29], which is consistent with our results.

To examine the spatial distribution of Dab2IP, we performed LacZ staining using Dab2IP heterozygous mice at different ages. In these mice, LacZ protein is expressed under the endogenous Dab2IP promoter, thereby providing a histological marker [β -galactosidase (gal) staining] to determine Dab2IP promoter activity. At E12 and E14, Dab2IP expression was detected in the cerebral cortex, trigeminal ganglion and cartilaginous tissues (fig. 1Ba, Bb). Dab2IP was highly expressed in various brain structures from embryonic ages to adulthood (fig. 1Bc–Bh). At E12, cells in the cortical plate appeared to express high levels of Dab2IP compared to cells in the ventricular zone (fig. 1Bi). At E14, Dab2IP was strongly detected in cells in the intermediate zone (fig. 1Bj) and at E16, and high expression of Dab2IP was observed in the intermediate zone and cortical plate. Reduced levels of Dab2IP were also present in the ventricular zone (fig. 1Bk). All cortical layers expressed Dab2IP after birth (fig. 1Bk–Bn). Notably, Dab2IP was also expressed in layer I at all postnatal ages (fig. 1Bm, Bn).

To investigate the subcellular localization of the Dab2IP protein in the developing cortex, we performed immunohistochemistry to frozen sections of E14 cerebral cortex using anti-Dab2IP antibody (fig. 2A). Consistent with the β -gal staining above, Dab2IP was highly expressed in many cells in the intermediate zone and fewer cells in the cortical plate (fig. 2A). Higher-magnification images revealed that Dab2IP protein is localized in the leading process and soma of migrating neurons (fig. 2B). Similar intercellular distribution patterns of Dab2IP were observed in cultured E14 cortical neurons (fig. 2Ca–Cf). Dab2IP protein highly accumulated in the shafts of neurites and adjacent cytoplasmic regions of neurons with processes.

Dab2IP Is Required for Migration of a Subset of Late-Born Neurons in the Developing Cortex

In the neocortex, the position and fate of a neuron are strongly correlated with its birth date and subsequent migration pattern. The onset of Dab2IP expression in postmitotic migrating neurons (fig. 1Bj) suggested that Dab2IP may play a role in the migration of cortical neurons. To test this hypothesis, we performed BrdU-labeling experiments at different developmental time points. Following BrdU injections at E12.5, WT animals exhibited the most heavily BrdU-labeled cells in the inner location of the cortex when analyzed at P21 (fig. 3A). A similar BrdU-labeling pattern was observed in Dab2IP-KD

Fig. 1. Expression of the Dab2IP gene in the developing mouse brain. **A** Western blot analysis of Dab2IP expression. **B** Sagittal sections of E12 and E14 embryos (**a, b**) or of brains from E12 to P30. **a-h** Sections were hybridized with β -gal and counterstained with nuclear Fast Red. Cx = Cerebral cortex; DRG = dorsal root gan-

glion; Cb = cerebellum; OB = olfactory bulb. Scale bars: 1.0 mm. **i-n** Higher-magnification images of **c-h** around the cerebral cortices. PP = Preplate; VZ = ventricular zone; CP = cortical plate; IZ = intermediate zone; WM = white matter; II/VI = the layer of the cerebral cortex. Scale bars = 50 μ m.

Fig. 2. Expression of Dab2IP in E14 mouse brain. **A** Dab2IP localization visualized with anti-Dab2IP antibody using E14 mouse cerebral cortex coronal sections. **B** Boxed area in **A**. Some cell shapes are highlighted with dotted lines. **C (a–f)** Primary cultured neurons derived from E14 cerebral cortex. Cells were stained with anti-Dab2IP monoclonal antibody (**a, d**) and phalloidin (**b, e**). Scale bars = 25 μ m (**A**); 10 μ m (**B**), and 10 μ m **C(a–f)**. CP = Cortical plate; IZ = intermediate zone; VZ = ventricular zone.

Fig. 3. Dab2IP controls positioning of late-born neurons in the cortex. **A, C** BrdU labeling was performed at E12 (top panels) or E16 (lower panels) followed by immunostaining for BrdU at P21. **B, D** Quantification of BrdU-positive cells in each of 10 bins span-

ning across the cortex. Graphs show the distribution of BrdU-positive cells in WT (solid lines) and Dab2IP-KD (dashed lines) brains. Data represent means $\pm$ SD using three independent animals for each genotype. Scale bars = 100 μ m

cerebral cortex. We found no quantitative differences in the position of early-born neurons between WT and Dab2IP-KD mice (fig. 3B). When BrdU was injected at E16.5 (to label late-born neurons), we found that some BrdU-labeled neurons were misplaced in Dab2IP-KD animals compared to WT controls (fig. 3C, D). These results suggest that Dab2IP regulates neuronal migration of a specific cohort of late-born neurons.

To investigate if Dab2IP deficiency disrupts migration of neurons in a layer-specific manner, we performed im-

munochemical studies (fig. 4). The transcription factor Tbr1 is strongly expressed in cortical layer VI neurons, which are found in E12–E13 in mice [30]. On the other hand, transcription factors Cux1 (layers II–IV) and Brn-2 (layers II/III and partially V) are specifically expressed in neurons born around E16 [31]. Consistent with our birth dating experiments above, we found Tbr1-positive neurons were positioned in the inner part of the cortex in both WT and Dab2IP-KD mice at P21 (fig. 4). In contrast, Cux1 staining was reduced in the upper cor-

Fig. 4. A KD of Dab2IP results in mislocalization of late-born cortical neurons. Coronal sections of P21 WT and Dab2IP-KD neocortex (at the level of the hippocampus) were stained with layer-specific markers Tbr1 (layers V/VI), Cux1 (layers II–IV) and Brn-2 (layers II/III, part V). Scale bars = 50 μ m. **B** Quantification of layer-specific marker immunoreactivity at P30. Graphs depict the percentage and SEM of positive cells in each of ten equal-sized vertical bins for WT (solid lines) and Dab2IP KD (dashed lines). Approximate positions of cortical layers are identified by alternating gray- and white-shaded areas.

tical layers and increased in putative layers V and VI of Dab2IP-KD mice. Brn-2-positive cells were also reduced in layer II/III and appeared to accumulate in the deep cortical layers (V/VI). These results indicate that subpopulations of neurons in layers II–IV of the cortex require Dab2IP and are ectopically placed when Dab2IP expression is disrupted.

Dab2IP KD Increases Activation of Rap1 and Integrin β_1

Previous studies demonstrated that early-born neurons destined to be in the deep cortical layers migrate preferentially by somal translocation. On the other hand, late-born neurons destined to be in the upper cortical layers proceed in three phases: multipolar transition, radial

Fig. 5. Dab2IP deficiency causes increased Rap1 and β_1 -integrin activation. **A** Activated Rap1 or Ras in E16 cortical lysates was pulled down using GST-RalGDS-RBD or GST-Raf1-RBD and detected by immunoblotting using anti-Rap1, anti-Ras antibody. Total Rap1 or Ras in the brain lysate was examined by immunoblotting (bottom panel). **B** Activated β_1 -integrin was examined in E16 cortical lysates using 9EG7 antibody. Actin levels were examined

in the same lysates as control. Band intensities were quantified using ImageJ software. The relative amount of activated Rap1, Ras and β_1 -integrin in Dab2IP KD was compared to WT levels, which were set to 1. Each bar represents the mean relative intensity $\pm$ SD. Each experiment was performed on multiple littermates. ** $p < 0.01$ by paired t test, $n = 3$ for Rap1, Ras activation assay and $n = 4$ for integrin assay.

translocation along glial fibers and somal translocation. Lee et al. [26] showed that siRNA-mediated KD of Dab2IP inhibited multipolar to bipolar transition. Other studies suggest that Rap1 activity is required for multipolar transition and proper cortical plate targeting [7, 15]. To investigate whether Dab2IP regulates Rap1 activity in the developing cortex, we measured activated Rap1 levels via its binding to a Rap1 binding domain, RalGDS-RBD, using E16 WT and Dab2IP-KD cortical lysates. We found a significant ($p < 0.01$, $n = 3$, paired t test) increase in activated Rap1 levels in Dab2IP-KD mice compared to WT controls (fig. 5A). Previous reports have shown that Dab2IP is an Ras-specific GTPase-activating protein in other systems [22, 28]. Therefore, we also examined Ras activation in Dab2IP-KD brains compared to WT controls. We found no significant difference in activated Ras levels in brain lysates from WT and Dab2IP-KD animals (fig. 5A). These results suggest that Dab2IP specifically regulates Rap1 activity in the developing cortex.

Rap1 plays an important role in the final somal translocation phase of migrating late-born neurons through Reelin-induced activation of $\alpha_5\beta_1$ -integrin signaling and increased adhesion to fibronectin [7, 16]. Immunoblot

analysis of cortical lysates from E16 WT and Dab2IP-KD mice revealed a significant ($p < 0.01$, $n = 4$, paired t test) increase in activated β_1 -integrin levels in Dab2IP-KD mice (fig. 5B). These results suggest that Dab2IP also regulates integrin activation, which is required for terminal translocation of late-born cortical neurons.

Discussion

In this study, we have shown that Dab2IP is abundantly expressed in developing mouse cortex and regulates migration and positioning of a subpopulation of late-born neurons, possibly through Rap1 regulation. We demonstrated that Dab2IP KD does not affect migration and positioning of early-born neurons, as Tbr1-positive cells were correctly positioned in the Dab2IP-KD mice. Similarly, our birth dating studies showed that early-born neurons migrated correctly, whereas a subset of neurons born at E16.5 did not (fig. 3). Further examination of late-born neurons using layer-specific markers revealed that Dab2IP KD causes a subset of Cux1-positive neurons (destined to form layers II–IV of the mature cortex to be

misplaced in deeper layers). Also, a subset of Brn-2-positive cells (destined for layers II/III) were misplaced in deep cortical layers in Dab2IP-KD mice (fig. 4). These data indicate that migration of late-born neurons is not completely disrupted in Dab2IP-KD mice. Many Cux1- and Brn-2-positive cells migrated normally.

Although Rap1 appears to have multiple roles during early and late cortical development, our results indicate that downregulation of Rap1 signaling by Dab2IP is important only in later stages of cortical development. The temporal and spatial regulation of Rap1 is critical for its cell signaling function. As with other small GTPase signaling molecules, Rap1 cycles between an inactive GDP-bound and an active GTP-bound conformation. Whereas GEFs such as C3G activate Rap1 signaling by stimulating replacement of GTP with GDP, multiple GTPase activating proteins (GAPs) inhibit Rap1 signaling by stimulating the rate of GTP hydrolysis. Thus, subcellular localization of GEFs and GAPs and their specific interactions with different signaling pathways play an important role in temporal and spatial regulation of Rap1 and consequently different aspects of cortical development. Although Dab2IP is expressed in preplate neurons at E12 (fig. 1Ci), CSPG staining revealed that the preplate splits normally in Dab2IP-KD mice (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000369092). This is in sharp contrast to the defects observed in mice lacking Rap1 activator C3G, which fail to split the preplate and show defects in radial glial attachment as well as neuronal migration [32].

Dab2IP was originally characterized as an Ras-specific GTPase-activating protein [22, 28]. However, our data provide the first evidence that Dab2IP also regulates Rap1 GTPase *in vivo*. We have shown that Dab2IP deficiency enhanced Rap1 activation in E16.5 brain (fig. 5A), whereas it did not affect Ras activation at this age (fig. 5A). Based on these results, we conclude that Dab2IP may be a dual-specific GAP protein. A number of different GAP proteins have been identified with specificity toward either Ras or Rap1, depending on the presence of an arginine finger in their catalytic domain [33]. However, recent studies have identified a family of RasGAPs which also have Rap1GAP activity, such as GAP1^{IP4BP}, CAPRI, Rasal and SynGAP [34–37]. These dual-specific GAPs share structural similarity with Dab2IP in that they contain conserved Pleckstrin homology and C2 domains near their catalytic GAP domain. Studies have shown the presence of these domains contribute to Ras and Rap1 specificity by regulating their subcellular localization which determines accessibility to Ras and Rap1 proteins,

as well as by directly modulating the catalytic domain of GAPs [34, 38]. For instance the C2 domain of Rasal mediates lipid binding and confers Rap1GAP specificity [38]. Deletion of the Rasal C2 domain reduced its Rap1GAP activity 1,000-fold but only reduced its RasGAP activity 6-fold [34]. It is interesting to note that different isoforms of SynGAP, varying in their PH domain and C-terminal protein interaction domains, exert opposing effects on synaptic plasticity [39]. This is consistent with the notion that Rap1 and Ras have opposing functions in different cell systems. Dab2IP is structurally very similar to SynGAP and we have shown that different protein isoforms of Dab2IP exist in the developing brain (fig. 1). Thus, we speculate that the dual specificity of Dab2IP may in part be defined by the presence of different domains. Further molecular experiments will be necessary to investigate this hypothesis.

Reelin plays an important role in multiple phases of migration in late-born cortical neurons through activation of the CrkL/C3G/Rap1 signaling pathway. Reelin-induced Rap1 signaling is required for N-cadherin-mediated bipolar orientation of migrating neurons as well as integrin-mediated fibronectin adhesion required for terminal translocation [7, 15, 16]. Several groups have shown that both inhibition and excessive activation of Rap1 disrupt migration of cortical neurons, indicating that fine-tuned regulation of Rap1 is necessary for proper migration [7, 16, 26]. Consistent with these observations, we previously found that siRNA-mediated downregulation of Dab2IP as well as overexpression of Dab2IP inhibited migration of cortical neurons using an *in utero* electroporation approach [26]. In this study, we showed that disruption of the Dab2IP gene also inhibits migration of some, but not all, late-born cortical neurons. Moreover, Dab2IP deficiency resulted in enhanced activation of Rap1 as well as β_1 -integrin signaling (fig. 5). It will be important to investigate precisely how Dab2IP may be involved in various aspects of Reelin signaling during cortical development.

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Disclosure Statement

The authors have no conflict of interest to declare.

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