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Reelin Binds α3β1 Integrin and Inhibits Neuronal Migration

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Summary

Mice that are mutant for Reelin or Dab1, or doubly mutant for the VLDL receptor (VLDLR) and ApoE receptor 2 (ApoER2), show disorders of cerebral cortical lamination. How Reelin and its receptors regulate laminar organization of cerebral cortex is unknown. We show that Reelin inhibits migration of cortical neurons and enables detachment of neurons from radial glia. Recombinant and native Reelin associate with α3β1 integrin, which regulates neuron–glia interactions and is required to achieve proper laminar organization. The effect of Reelin on cortical neuronal migration in vitro and in vivo depends on interactions between Reelin and α3β1 integrin. Absence of α3β1 leads to a reduction of Dab1, a signaling protein acting downstream of Reelin. Thus, Reelin may arrest neuronal migration and promote normal cortical lamination by binding α3β1 integrin and modulating integrin-mediated cellular adhesion.

Introduction

Normal development of the mammalian cerebral cortex requires the coordinated migration of postmitotic neurons from the proliferative ventricular zone to the outermost layer of the developing cortical plate. Migrating neurons travel hundreds to thousands of cell body lengths through varying environments, migrate past previously generated neuronal cohorts of the cortical plate, and organize themselves into precise layers. Although a number of genes have recently been implicated in this process (Komuro and Rakic, 1998; Hatten, 1999; Walsh, 1999), three mutant mice show a remarkably similar cellular phenotype, suggesting that the corresponding proteins represent a biochemical pathway that mediates proper formation of cerebral cortical lamination. In the *reeler* mouse, the disorganized cortex is approximately inverted, with early born neurons occupying abnormal superficial positions and later born neurons adopting abnormal deep positions (Caviness and Sidman, 1973). Reelin, the product of the *reeler* gene (D'Arcangelo et al., 1995), acts non cell autonomously (Miyata et al., 1997), and the protein is synthesized and secreted in the cerebral cortex predominantly by the Cajal-Retzius cell of the marginal zone, the outermost layer of the developing cortex (D'Arcangelo et al., 1995; Ogawa et al., 1995).

Mutations at a second locus, *dab1*, have been found to produce a phenotype indistinguishable from mutations in the *reeler* gene (Howell et al., 1997b; Sheldon et al., 1997; Ware et al., 1997). The *Dab1* gene encodes a cytoplasmic adaptor protein (Dab1) expressed by neurons in the developing cortical plate, suggesting that Dab1 represents a link in the signaling pathway that receives the Reelin signal. This idea is confirmed by the observation that Reelin expression is normal in the *dab1* mutant cortex (Gonzalez et al., 1997), but Dab1 protein accumulates in the *reeler* mouse brain (Rice et al., 1998), and Dab1 is phosphorylated in response to applied recombinant Reelin (Howell et al., 1999a). The phosphotyrosine-interacting/phosphotyrosine binding (PI/PTB) domain of Dab1 binds proteins that contain an NPXY motif (Howell et al., 1997a, 1999b; Trommsdorff et al., 1998), a motif that has been implicated in clathrin-mediated endocytosis (Chen et al., 1990) and integrin signaling (Law et al., 1999).

More recently, mice with compound mutations in both the VLDL receptor (*VLDLR*) and the ApoE receptor 2 (*ApoER2*) have been found to have a phenotype indistinguishable from *reeler* and *dab1* mutants (Trommsdorff et al., 1999). VLDLR and ApoER2 are members of the low-density lipoprotein (LDL) receptor superfamily, and they interact with Dab1 in two-hybrid screens through the PI/PTB domain of Dab1 and the NPXY motif of LDL superfamily members (Howell et al., 1997a; Trommsdorff et al., 1998). The NPXY motif of LDL receptor family members is essential for clathrin-mediated endocytosis (Chen et al., 1990). Recent studies have demonstrated that both recombinant ApoER2 and the VLDLR bind Reelin and that this binding leads to both the tyrosine phosphorylation of Dab1 and, in the case of VLDLR, the internalization of the receptor and Reelin (D'Arcangelo et al., 1999; Hiesberger et al., 1999). Thus, there is compelling evidence that Reelin, VLDLR, ApoER2, and Dab1 function in a common signaling pathway between Cajal-Retzius cells and cortical plate neurons.

Despite the identification of other molecules in the Reelin signaling pathway, the response of cortical neurons to the Reelin signal remains unknown. Reelin is thought to regulate cortical plate organization by initiating the splitting of preplate into the marginal zone and the subplate, by acting as an attractant of neurons to the top of the cortical plate, or by functioning as a stop signal for neuronal migration at the interface between the marginal zone and the developing cortical plate.

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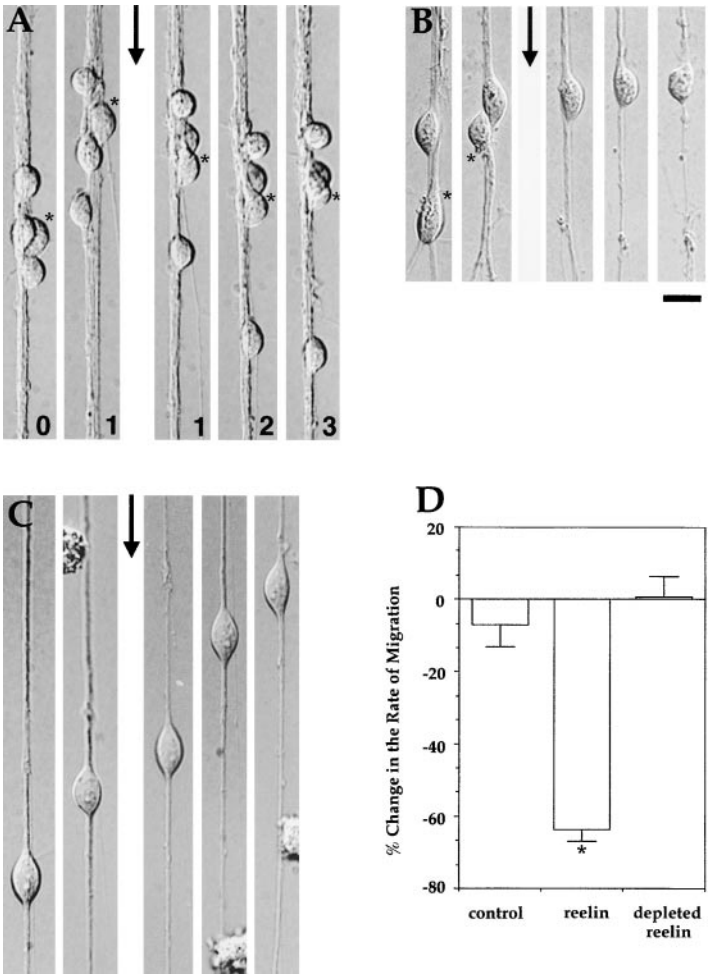

Figure 1. Reelin Inhibits Neuronal Migration In Vitro

(A) Neurons migrating on a glial cell process were monitored prior to (panels to the left of black arrow) and after (panels to the right of black arrow) addition of Reelin. Time elapsed between each panel is 1 hr. Neurons were motile prior to the addition of Reelin. After exposure to Reelin, neurons reduced their rate of migration (e.g., see neuron next to asterisk).

(B) Detachment of a migrating neuron from the glial process following Reelin exposure. Prior to exposure to Reelin, these two neurons migrated actively. However, 1 hr after exposure to Reelin, one of them detached (see neuron next to asterisk), and the other one ceased to migrate.

(C) No changes in migratory behavior were observed after exposure to control 293T supernatants.

(D) The rate of neuronal migration was measured before and after exposure to Reelin, control supernatants, or supernatants depleted of Reelin. Exposure to Reelin reduced the rate of migration by 64% $\pm$ 3%. Control 293T CM and Reelin-depleted supernatants did not alter the rate of migration significantly. Asterisk indicates significance, $p < 0.005$. Data shown are mean $\pm$ SEM for each group ($n > 75$ for each group).

Scale bar, 10 μm.

Since such Reelin function may involve the modulation of neuronal–radial glial interaction, molecules that modulate neuron–glia interactions are logical targets of Reelin's effects. The α3 integrin–deficient mouse shows abnormal cortical lamination due to an apparent deficit in neuronal migration along radial glial fibers (Anton et al., 1999). Integrins are transmembrane receptors that link the extracellular matrix (ECM) to the cytoskeleton and have demonstrated functions in cell migration and adhesion in many cell types, including neurons (Fishman and Hatten, 1993; DeFreitas et al., 1995; Andressen et al., 1998; Georges-Labouesse et al., 1998; Zhang and Galileo, 1998). In vitro studies show that blocking $\alpha3\beta1$ integrin function with function-blocking antibodies leads to deadhesion of migrating neurons from the radial glial fiber (Anton et al., 1999). Deficiency in $\alpha3\beta1$ integrin appears to switch embryonic cortical neurons from gliophilic adhesive preference to a neurophilic adhesive preference. Thus, the overly adhesive phenotype of early born neurons in the *reeler* cortex (Hoffarth et al., 1995), the persistent apposition of *reeler* mutant neurons with radial glial fibers (Pinto-Lord et al., 1982), and the structural homology of Reelin to ECM molecules are suggestive of potential $\alpha3\beta1$–Reelin interactions. We have therefore examined the function of Reelin during laminar organization of cerebral cortex and the possible connection between Reelin and integrins during this process.

We show here that Reelin arrests radial migration of neurons. Native and recombinant Reelin proteins associate with native $\alpha3\beta1$ integrin, a receptor previously shown to mediate neuronal adhesion to radial glial fibers and radial migration (Anton et al., 1999). In embryonic cortical neurons, $\alpha3\beta1$ integrin is coexpressed with Dab1, and these two proteins show overlapping subcellular localization. Consistent with a biochemical interaction between Reelin and integrins, deficiency in functional $\alpha3\beta1$ integrins leads to deficiency in Reelin function, in vitro and in vivo. In cerebral cortices of $\alpha3\beta1$ integrin–deficient mice, deficiency in functional $\alpha3\beta1$ integrins leads to a reduction of Dab1 protein levels and to elevated expression of a 180 kDa Reelin fragment. These results suggest that Reelin may regulate neuronal migration and layer formation through modulation of $\alpha3\beta1$ integrin–mediated neuronal adhesion and migration.

Results

Reelin Inhibits Neuronal Migration In Vitro

Reelin secreted by the Cajal-Retzius neurons at the top of the developing cortical plate is hypothesized to influence the migratory behavior of incoming neurons during layer formation. We therefore analyzed the function of Reelin in radial glial guided neuronal migration in vitro. In the cortical imprint migration assay, neurons migrate

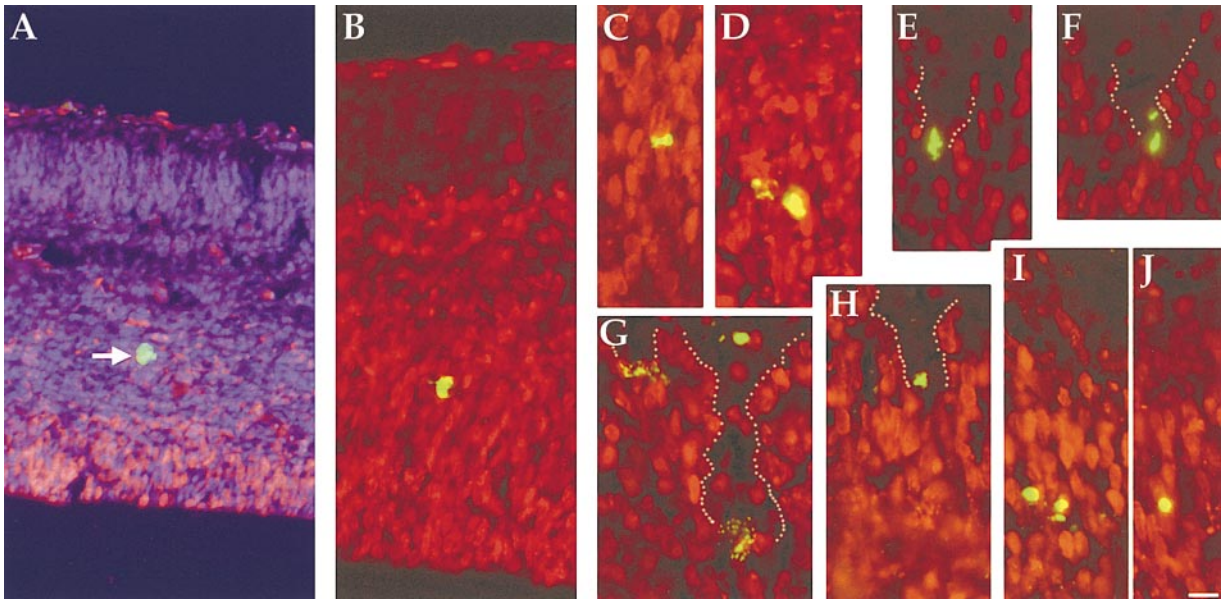

Figure 2. Reelin Inhibits Neuronal Migration In Vivo

(A) Reelin- or control CM-coated fluorescent microspheres (arrow) were deposited in the intermediate zone of the developing cerebral wall. Neurons being generated just prior to microsphere injection were labeled with BrdU (pink). Nuclei in this section were counterstained with bisbenzimide (blue/purple).

(B) Twenty-four hours later, once BrdU-labeled neurons (red) had migrated into the cerebral wall, interactions between microspheres (green) and labeled neurons were evaluated. In this panel, labeled neurons migrate past control microspheres coated with 293T CM with no apparent effect.

(C–I) Higher magnification views of neuronal distribution around Reelin-coated [E–J] or control [C and D] microspheres.

(C and D) Control microspheres injected into the rat embryonic cerebral cortex do not inhibit neuronal migration.

(G and H) Reelin microspheres injected into the rat embryonic cerebral cortex inhibit neuronal migration. Neurons either do not migrate past Reelin-coated microspheres or generally avoid them (dotted lines outline the area devoid of labeled cells).

(E and F) Reelin-coated microspheres inhibit neuronal migration in wt mouse cerebral cortex.

(I and J) $\alpha 3\beta 1$ integrin–deficient neurons, however, migrate past Reelin-coated microspheres, indicating that $\alpha 3\beta 1$ integrin is essential to produce Reelin-inhibited neuronal migration in vivo.

Scale bar, 20 μm (A); 17.5 μm (B); 10 μm (C–I).

at an average rate of 11.62 μm/hr ($n = 508$) on radial glial processes. Exposure to full-length recombinant Reelin inhibits neuronal migration in this assay. Neurons reduced their rate of migration by 64% in response to Reelin (Figures 1A, 1B, and 1D); 44.1% of the neurons observed detached from their radial glial guides following Reelin exposure. When Reelin-containing media were depleted of Reelin with an antibody to Reelin (CR50) prior to its use in our assays, the Reelin-depleted media showed no effect on neuronal migration (Figure 1D). Similarly, control 293T conditioned medium (CM) did not induce any significant changes in neuronal migration (Figures 1C and 1D). Only 1.2% and 6.3% of the neurons analyzed detached from their radial glial guides following perfusion of Reelin-depleted medium or control 293T supernatants, respectively.

Reelin Inhibits Neuronal Migration In Vivo

To further test Reelin's function, we presented Reelin to migrating neurons in vivo, midway in their migration. Fluorescent microspheres (Lumafluor) were coated with Reelin or Reelin-negative control media (293T CM), and small volumes (0.1–0.2 μl) of these beads were injected into the developing rat cerebral wall at embryonic day 16.5–17 (E16.5–E17) using a picospritzer (Figure 2A). Analysis of Reelin-coated and control beads with an anti-Reelin antibody indicated that all of the beads incubated with Reelin were coated with Reelin. Newly generated neurons were pulse labeled with bromodeoxyuridine (BrdU) 2 hr prior to the bead injection (Figure 2A). As the BrdU-labeled neurons migrate into the cerebral wall from the ventricular zone, if they come in contact with the microspheres they will encounter focal sources of Reelin. After 24 hr, brains were removed, sectioned, and analyzed for the pattern of BrdU-labeled cell distribution around Reelin-coated beads in the intermediate zone (Figure 2B). We only analyzed brain sections in which the coated microspheres were deposited in the intermediate zone, since almost all of the neurons in this division of the cerebral wall are actively migrating, and this region is far from the endogenous Reelin present in the marginal zone. This method allows for the focal ectopic presentation of Reelin to the migrating cells in vivo, thus enabling us to test whether Reelin modulates neuronal migration in vivo.

Cells that came into direct contact with Reelin beads did not migrate past the beads, and labeled cells nearby appeared to avoid contact with the beads, thus creating an area devoid of labeled cells above the beads (see area outlined by dotted lines; Figures 2G and 2H). In contrast, neurons migrated past control beads without any hindrance (Figures 2B–2D). Densitometric estimates of BrdU-labeled cells around microspheres indicate

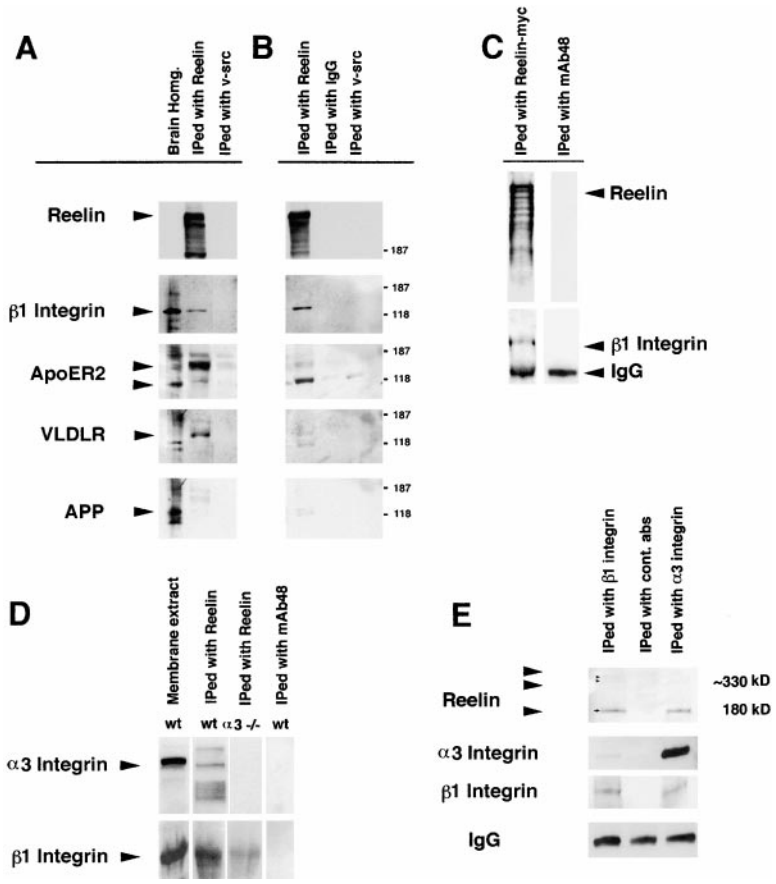

Figure 3. Reelin Binds $\alpha3\beta1$ Integrin

(A and B) Sepharose beads were linked to CR50 (an anti-Reelin antibody) or anti-v-src, as a control antibody, and then incubated with recombinant Reelin-containing supernatants. After washing, the beads were incubated with (A) octylglucoside-solubilized whole-brain homogenates or (B) Triton X-100–solubilized neuronal membrane extracts for 12 hr. Beads were then washed with PBS buffer. Retained proteins were removed from the beads by boiling SDS sample buffer and were subjected to SDS–PAGE and immunoblotting. β1 integrin, the ApoE2, and the VLDLR are detected in the retained fraction from the Reelin complexed beads but not the control beads.

(C) Myc-tagged Reelin secured to sepharose beads with the 9E10 anti-Myc antibody also immunoprecipitates β1 integrin, whereas control beads, in this case linked to mAb48, an anti–synaptic vesicle antibody, do not immunoprecipitate β1 integrin.

(D) To determine if $\alpha3\beta1$ integrin associates with Reelin, immunoprecipitations were repeated with membrane extracts prepared from α3 integrin–deficient brains ($-/-$) and compared with wt littermates ($+/+$). The α3 band is detected in the wt lane and is absent in the immunoprecipitation from the α3-deficient membrane extracts (upper panels). The lower molecular weight bands below the α3 band in lane 2 are likely due to degradation during the immunoprecipitation, whereas the higher molecular weight band is likely to be a larger α3 subunit previously reported (Dedhar et al., 1992). β1 integrin was immunoprecipitated by Reelin from both wt and $\alpha3^{-/-}$ homogenates (lower panels).

(E) The immunoprecipitation was repeated using anti–β1 integrin or anti–α3 integrin–coated beads. Reelin was coimmunoprecipitated by β1 integrin or α3 integrin–coated beads but not by the control beads (coated with rabbit anti-GFAP polyclonal antibodies), suggesting a direct in vivo association between Reelin and $\alpha3\beta1$ integrins. In immunoprecipitations with anti-integrin antibodies, the 180 kDa Reelin component (large arrow) was detected prominently. Faint bands of higher molecular weight fragments (~330 kDa) of Reelin (small arrowheads) were detected in β1 integrin immunoprecipitates.

49.5% reduction in labeled neurons around Reelin-coated microspheres, when compared with that of control (Figure 5A). Together, these in vivo observations support the inhibitory function of Reelin observed in vitro.

$\alpha3\beta1$ Integrin Is a Receptor for Reelin

To identify Reelin binding proteins in embryonic brain, an immunoprecipitation protocol was developed that exposed brain membrane extracts or total brain homogenates to Reelin-coated beads. Recombinant Reelin secreted by 293T cells was immobilized to IgG-sepharose beads with the CR50 antibody, which recognizes and binds Reelin (Ogawa et al., 1995; D'Arcangelo et al., 1997). Immunoprecipitation with Reelin-linked beads was compared with immunoprecipitations with control antibody-coated beads or IgG beads alone, which also had been incubated in 293T supernatants containing Reelin but did not bind Reelin (Figure 3). These Reelin-coated or control beads were used to immunoprecipitate Reelin binding proteins from either whole brain homogenates solubilized in 100 mM octylglucoside (Figure 3A; Busk et al., 1992) or membrane extracts solubilized in 1% Triton X-100 (Figure 3B; Kaprielian and Patterson, 1993). Beads were then washed, and proteins bound to Reelin and control beads were analyzed by SDS–PAGE and immunoblotting.

Reelin-coated beads robustly immunoprecipitated β1 integrin, while control immunoprecipitations using either an anti-v-src antibody or the anti-mouse IgG beads themselves (Zymed), which do not recognize Reelin, showed no bound β1 integrin (Figures 3A and 3B). Strong β1 integrin signal was observed in the Reelin immunoprecipitation using both solubilization protocols (Figures 3A and 3B). To test the specificity of the interaction between Reelin and β1 integrin, the immunoprecipitation was repeated from wild-type (wt) brain homogenates using Myc-tagged Reelin, and the recombinant Reelin was bound to the IgG beads by an anti-Myc antibody. Again, β1 integrin was immunoprecipitated by beads coated with Reelin but not control beads (Figure 3C), indicating that the β1 integrin association with Reelin is not dependent on the CR50 antibody used to secure Reelin to the sepharose beads. Direct immunoprecipitation of native Reelin under nonreducing conditions also coimmunoprecipitates native β1 integrin, suggesting a complex of the native proteins exists in vivo (data not shown).

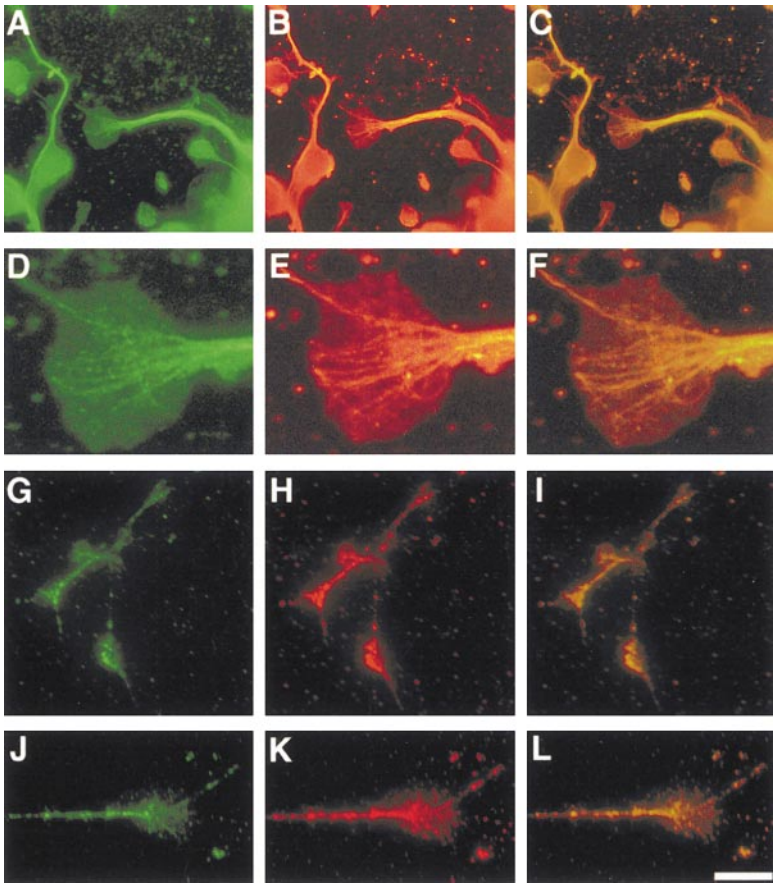

Figure 4. Overlapping Distribution of α3β1 Integrin and Dab1 Expression in Developing Cortical Neurons

Embryonic cortical cells were double labeled either with anti–β1 integrin (A and D) and Dab1 antibodies (B and E) or with anti–α3 integrin (G and J) and Dab1 antibodies (H and K). Colocalization of β1 integrin (A) and Dab1 (B), as well as α3 integrin (G) and Dab1 (H), is observed. Extensive overlap (yellow) is observed in soma and processes of the merged image (C and I).

(D–F) High magnification view of a neurite and growth cone immunolabeled with β1 integrin (D) and Dab1 (E). Extensive punctate colabeling is seen in the neurite and growth cone (F).

(J–L) Similar colocalization (L) is evident between α3 integrin (J) and Dab1 (K).

Scale bar, 15 μm (A–C); 4 μm (D–F); 25 μm (G–I); 7 μm (J–L).

As a control, we also tested whether Reelin immunoprecipitates any of the other previously characterized Reelin receptors in our assay. At least two isoforms of ApoER2 and VLDLR are also immunoprecipitated by Reelin in this assay. The 160 kDa isoform probably corresponds to the glycosylated receptor, while the 120 kDa form is most likely the unmodified protein (Figures 3A and 3B; Tolleshaug et al., 1982; Patel et al., 1997). ApoER2 immunoprecipitation appeared more robust than did VLDLR binding, likely reflecting the greater sensitivity of the detecting antibody and/or higher protein expression in the homogenate. Reprobing of the same samples with antibodies to E-cadherin, antisera to β6 integrin, or α1 integrin (data not shown) exhibited no signal and therefore serves as a negative control for the protocol. The amyloid precursor protein (APP), another transmembrane receptor identified as a Dab1-interacting protein (Homayouni et al., 1999; Howell et al., 1999b), also showed little immunoprecipitation by Reelin (Figures 3A and 3B).

To determine if the α3β1 integrin complex is associated with Reelin, the immunoprecipitation was repeated using brain membrane homogenates prepared from wt and α3β1 integrin–deficient brains. α3β1 integrin is detected in immunoprecipitations using the Reelin-coated beads, and the identity of the α3 integrin subunit is confirmed by the absence of the band in the immunoprecipitations from the α3β1-deficient membrane extracts (Figure 3D, upper panels). The SDS gel separation and immunoblotting were repeated with the remaining portion of the same immunoprecipitations and probed for β1 integrin. β1 integrin is detected in the immunoprecipitation from both the α3 integrin–deficient brains and wt littermates. However, relatively less β1 integrin is detected in the immunoprecipitation from the α3-deficient brains compared with the wt littermates (Figure 3D, lower panels), which is reflective of reduced levels of β1 integrin expression in α3-deficient mice (Hodivala-Dilke et al., 1998). To further confirm the Reelin–α3β1 integrin association, we performed immunoprecipitations with β1 integrin– or α3β1 integrin–coated sepharose beads. Both β1 and α3 integrins immunoprecipitate native Reelin from embryonic brain extracts, and Reelin was not retained by control beads (Figure 3E). Together, these results indicate that α3β1 integrin associates with Reelin in embryonic brain.

Colocalization of Dab1 and α3β1 Integrin in Cortical Neurons

To determine if individual cells coexpress both α3β1 integrin and Dab1, immunolabeling was performed on cultured cortical neurons from E16 brains at 1 day in vitro (DIV) after dissociation and plating on polylysine-coated coverslips. Cultured neurons were fixed in paraformaldehyde (4%) and were labeled with either a rat monoclonal antibody against the cytoplasmic tail of β1 integrin (Chemicon) or a mouse monoclonal antibody against Dab1 (mAbD1, generous gift of A. Goffinet) and visualized using appropriate FITC- and Cy3-linked secondary antibodies (Jackson Laboratories). Some cultures were double labeled with a polyclonal anti–α3 integrin antibody (Ab8-4, generous gift of Dr. M. DiPersio)

and the mouse monoclonal antibody against Dab1. Consistent with previous descriptions of cellular Dab1 expression detected by a polyclonal antiserum (Howell et al., 1997a, 1999b), the monoclonal antibody detected Dab1 both in the cell soma and in the processes of cultured neurons (Figure 4). As previously described, β1 integrin shows a similar expression pattern (Grabham and Goldberg, 1997), with strong staining around the perimeter of the soma and in the cell processes (Figure 4B). The merged image reveals colabeled regions in the soma, process, and growth cone (Figure 4C). Higher magnification views of the neurite and growth cones show punctate, fiber-like labeling of both Dab1 (Figure 4D) and β1 integrin (Figure 4E), with frequent double labeled punctae in the processes and both double and single labeled areas in the merged image of the growth cones (Figure 4F). α3 integrins were also found to be expressed in a punctate fashion in the cell soma and processes and, like β1 integrin, showed significant colocalization with Dab1 (Figures 4G–4I). This overlapping localization was further evident in higher magnification views of growth cone regions (Figures 4J–4L). Cells from α3 integrin–deficient cortices do not label with anti-α3 antibodies and served as control for the α3 integrin/Dab1 colocalization studies. Together, these results indicate that α3β1 integrin is coexpressed in developing cortical neurons with Dab1, a downstream component of the Reelin signaling pathway, and shows a predominantly overlapping expression within these cells.

Role of α3β1 Integrin in Reelin Function

To determine if Reelin's interaction with α3β1 integrin is functionally important, we tested the effect of Reelin on neuronal migration of cortical cells from wt and α3β1 integrin mutant embryos, in vitro and in vivo. If Reelin binding to α3β1 integrin is crucial for Reelin's effect on neuronal migration, embryonic cortical cells from normal and mutant cerebral cortices may respond differently to Reelin. Reelin significantly reduced the rate (−64%) of radial migration of wt cortical neurons (Figure 5C); in contrast, Reelin did not inhibit the migration of α3β1 integrin mutant cortical neurons (Figure 5C): 56% of wt neurons detached from their radial glial guides following Reelin exposure, whereas only 2.4% of α3β1 mutant cortical neurons detached from radial guides in response to Reelin. Control 293T CM were ineffective in modulating the radial migration of either wt or mutant neurons (Figure 5C). Furthermore, in long-term migration assays, in which we monitored the number of migrating neurons at the beginning of and 24 hr after Reelin addition, Reelin significantly reduced the number of migrating neurons from wt but not from the mutant cortices (Figure 5D). When Reelin was preabsorbed with anti-CR50 antibodies, Reelin's effect was abolished (Figure 5D).

We also tested whether one of the recently identified receptors for Reelin, CNR (cadherin-related neuronal receptor; Senzaki et al., 1999) had any influence on neuronal migration. Inhibition of the Reelin binding domain (RBD) of CNRs with an antibody to this site did not affect Reelin-induced inhibition of neuronal migration (Figure 5D), suggesting that CNRs may not influence Reelin's regulation of neuronal migration. Antibodies to the RBD of CNRs alone did not affect neuronal migration (Figure 5D). Together, these results suggest that Reelin–α3β1 integrin binding is essential for Reelin's inhibitory effect on neural migration. In the absence of functional α3β1 integrins, Reelin did not inhibit migration. Furthermore, this function of Reelin is specific to Reelin–α3β1 integrin signaling, and the Reelin–CNR signaling pathway appears not to be involved in this process.

To further assess the influence of Reelin–α3β1 integrin interactions on neuronal migration, we tested the effect of Reelin on neuronal migration in vivo with wt and α3β1 integrin–deficient cerebral cortex. Reelin-coated and control microspheres were injected into the developing cerebral cortex as described above; 2 hr prior to microsphere injection, neurons were pulse labeled with BrdU. This method allows us to evaluate the response of wt and α3β1 integrin–deficient cortical neurons to ectopically presented Reelin in vivo. Wt neurons did not migrate past and generally avoided Reelin-coated microspheres (Figures 2E and 2F), whereas they continued to migrate past control microspheres. α3β1 integrin–deficient neurons, however, did not alter their migration in response to Reelin-coated beads (Figures 2I and 2J). α3β1 integrin–deficient neurons migrated normally past both Reelin-coated and control beads. Densitometric measurements of BrdU-labeled neurons around the two types of microspheres indicate a 55% reduction in labeled neurons around Reelin-coated microspheres, when compared with that of control microspheres in wt cerebral cortex (Figure 5B). No such difference was observed between Reelin and control microspheres in α3β1 integrin–deficient cerebral cortex (Figure 5B). These results further confirm our in vitro observations that Reelin-induced inhibition of cortical neuronal migration depends on Reelin–α3β1 integrin signaling.

Expression of Dab1 and Reelin in α3 Integrin–Deficient Embryos

If α3β1 integrin is part of the Reelin signaling pathway, it would be expected that α3β1 integrin would regulate Dab1, a cytoplasmic adaptor protein downstream of Reelin. Therefore, we analyzed Dab1 protein levels in α3β1 integrin–deficient animals. Homogenates of α3-deficient brains (E16.5) were compared with brain homogenates from wt and heterozygote littermates. Proteins were separated by SDS gel and immunoblotted with an anti-Dab1, anti-Reelin, or anti-tubulin (loading control) antibody. We noticed a significant downregulation of Dab1 protein levels in α3β1 integrin–deficient brains (Figure 6A). α3β1 integrin mutant embryonic brains contained ∼50% less Dab1 protein than did the wt littermates. This observation is in marked contrast with previous studies in which the absence of Reelin or Reelin receptors (VLDL/ApoE) has led to significant Dab1 protein accumulation.

To further assess this issue, we compared the levels of Reelin in wt, heterozygous, and α3β1 integrin–deficient brains (Figure 6A). A prominent increase in the amount of the 180 kDa fragment of Reelin was observed in α3 integrin–deficient brains in comparison to brains from wt littermates (∼4- to 7-fold). The 180 kDa fragment of Reelin is produced by a zinc-dependent protease, most likely after Reelin is secreted by the Cajal-Retzius cells (Lambert de Rouvroit et al., 1999). The enhanced levels of 180 kDa Reelin and Reelin's ability to bind and activate multiple other cell surface receptors may contribute to the downregulation of Dab1 in α3β1 mutants.

We next asked if α3β1 integrin function was required for Reelin-dependent Dab1 phosphorylation (Howell et al., 1999a). Wt and α3β1 integrin–deficient cortical neurons were treated with recombinant Reelin. After Reelin

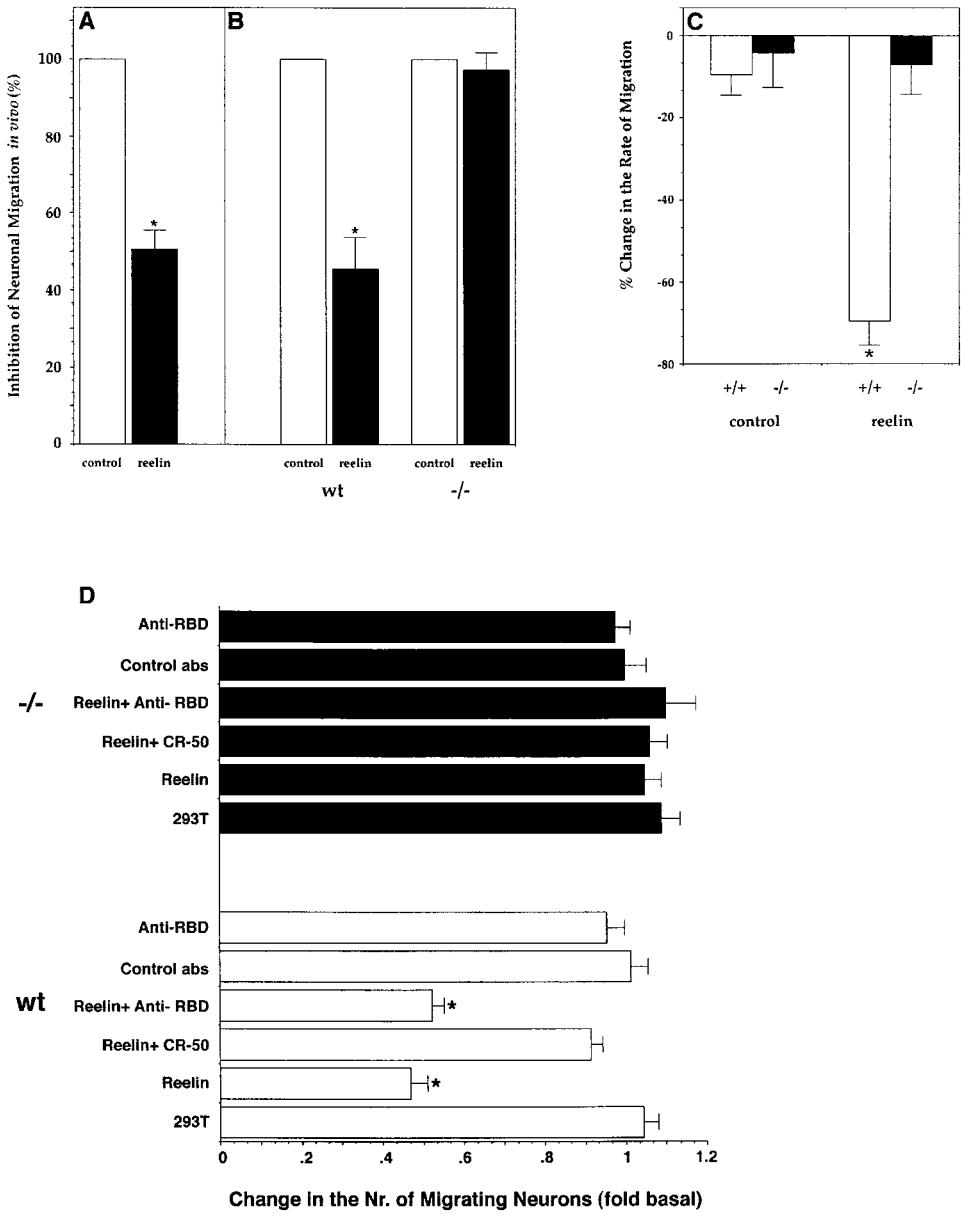

Figure 5. Reelin-Induced Inhibition of Neuronal Migration Is Mediated through $\alpha3\beta1$ Integrin

(A and B) Quantification of the effect of control or Reelin beads on neuronal migration in vivo. A 225K μm^2 grid was placed on cortical sections with the microspheres at the center of the grid. OD (minus that of the microspheres) was then measured in the grid using NIH Image 1.6. OD reflects the number of BrdU-labeled neurons around the different types of microspheres. Measurements from Reelin microsphere-containing sections were normalized to the OD of littermate control microsphere-containing sections (set to 100). Reelin inhibited neuronal migration when injected into embryonic rat cerebral wall (A) and wt mouse cerebral wall (B). However, Reelin did not affect migration when injected into the $\alpha3\beta1$-deficient ($-/-$) embryonic cerebral cortex (B). Data shown are mean $\pm$ SEM for each group. Measurements were averaged from seven different brain sections from three different experiments. Asterisk indicates significance, $p < 0.05$.

(C) Essential role of $\alpha3\beta1$ integrin in Reelin's effect. The rate of neuronal migration of wt and $\alpha3\beta1$ integrin–deficient neurons was measured before and after exposure to Reelin or control supernatants. Exposure to Reelin reduced the rate of migration of wt neurons by 69% $\pm$ 5.9%. In contrast, $\alpha3\beta1$ integrin–deficient neurons were not responsive to Reelin. Control 293T CM did not alter the rate of migration of either wt or $\alpha3\beta1$ integrin–deficient neurons significantly. Asterisk indicates significance, $p < 0.05$. Data shown are mean $\pm$ SEM for each group ($n > 35$ for each group).

(D) Reelin-induced inhibition of neuronal migration depends on $\alpha3\beta1$ integrins. In long-term migration assays, the number of migrating neurons in cultures from wt and $\alpha3\beta1$-deficient cerebral cortices was counted before and after addition of Reelin, control 293T CM, Reelin + anti-Reelin CR50 antibodies (incubated together for 1 hr prior to addition), anti-RBD antibodies + Reelin (Reelin added 60 min after anti-RBD antibodies), anti-RBD antibodies alone, or control antibodies (10 μg/ml of mouse and rat immunoglobulins). In wt cultures, Reelin reduced the number of migrating neurons, and this effect was observed even when the RBDs of CNRs were blocked with anti-RBD antibodies. This indicates that Reelin–CNR interaction is not essential for Reelin's effect on neuronal migration. However, Reelin's effect on neuronal migration was not observed in $\alpha3\beta1$-deficient cells, indicating that $\alpha3\beta1$ integrin is an essential component of Reelin signaling. Data shown are mean $\pm$ SEM for each group (number of cells analyzed is >400 for each group). Asterisk indicates significance, $p < 0.05$. Abbreviation: RBD, Reelin binding domain of cadherin-related neuronal receptors.

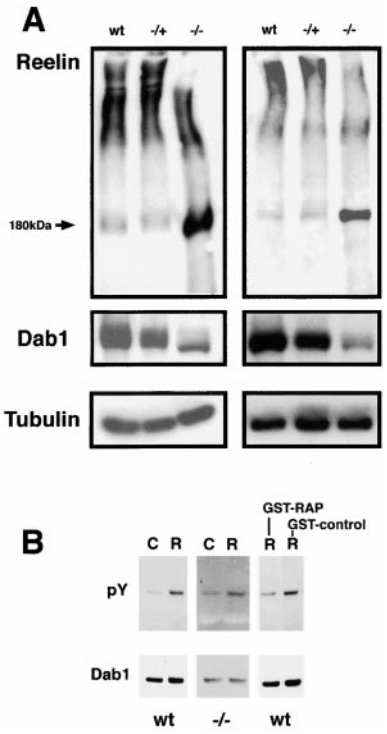

Figure 6. Downregulation of Dab1 and Accumulation of Reelin Fragment in α3 Integrin–Deficient Mice

(A) After PCR genotyping, E16.5 brains were homogenized in STM buffer, run on SDS–PAGE gel, and immunoblotted. Western blots of brain homogenates from α3 integrin–deficient mice reveal elevated levels of the 180 kDa fragment of Reelin and reduced levels of Dab1 in comparison to wt controls. The same samples were probed with anti-tubulin as control for sample loading in individual wells. Samples from two different litters are shown.

(B) Reelin–α3β1 integrin interactions do not regulate Dab1 phosphorylation. Western blots of immunoprecipitations from whole-cell lysates of cultured cortical neurons reveal that in both wt and α3β1 integrin–deficient (−/−) cortical neurons, application of Reelin (R) for 15 min causes tyrosine phosphorylation of Dab1 in comparison to CM application alone (C). In contrast, pretreatment of wt cortical cultures with 1 μg/ml GST-RAP, a competitive inhibitor of ligand binding by LDL receptor family members, reduced Dab1 phosphorylation, while GST control (1 μg/ml) had no effect. Dab1 was immunoprecipitated with a mixture of C-terminal and N-terminal anti-Dab1 antibodies, and Western blots were probed with anti-phosphotyrosine antibody (pY99, Santa Cruz) and anti-Dab1 monoclonal antibody.

incubation, cells were lysed, and Dab1 was immunoprecipitated using a mixture of N-terminal and C-terminal anti-Dab1 polyclonal antibodies. Precipitated proteins were then separated by SDS gel electrophoresis and immunoblotted with anti-phosphotyrosine antibody (pY99, Santa Cruz) followed by immunoprobing with mAbE1, an anti-Dab1 antibody (Figure 6B). In both wt and α3β1 integrin–deficient cortical neurons, application of Reelin for 15 min caused a significant increase in the amount of Dab1 phosphorylation in comparison to CM application alone (Figure 6B). In contrast, preincubation (30 min) with GST-RAP (1 μg/ml), a universal inhibitor of ligand binding to LDL receptor family members (Willnow et al., 1996), blocked this Dab1 phosphorylation (Figure 6B), as has been previously shown (Hiesberger et al., 1999). Application of control glutathione S-transferase (GST; 1 μg/ml) had no effect. Together, these results demonstrate that Reelin–α3β1 integrin interactions regulate Dab1 protein levels but not Dab1 phosphorylation.

Discussion

Laminar organization of neurons in cerebral cortex requires Reelin signaling. Reelin's effect on cortical layering is hypothesized to result from three distinctly different cellular effects. First, Reelin may regulate cortical plate organization by initiating the splitting of preplate into marginal zone and subplate. Failure of this process in *reeler* mutants leads to the accumulation of cortical neurons underneath the preplate neurons. Second, a Reelin gradient may act as an attractant of neurons to the top of the cortical plate, thus enabling newly generated neurons to migrate past earlier generated ones in the developing cortical plate. Third, Reelin may induce detachment of neurons from their radial glial guides and thus stop neuronal migration at the marginal zone–developing cortical plate interface and initiate the differentiation of neurons into distinct layers. In the present study, we provide a direct demonstration of the effect of Reelin protein upon migrating cerebral cortical neurons. We have shown that Reelin retards neuronal migration and induces neuronal detachment from radial glial guides. This particular function of Reelin depends on Reelin–α3β1 integrin interactions, since in the absence of α3β1 integrin, Reelin does not measurably affect neuronal migration in vitro and in vivo. Neurons have to stop their migration, detach from their radial glial guides, and begin elaborating neurites in order to generate the synaptic connectivity characteristic of distinct layers. By triggering the appropriate termination of neuronal migration and initiation of postmigratory neuronal differentiation, Reelin controls cortical layer formation. The functional role of Reelin in modulating integrin-mediated adhesion is reflected in the overly adhesive phenotype of early born neurons in the *reeler* cortex (Hoffarth et al., 1995) and the persistent apposition of *reeler* mutant neurons with radial glial fibers (Pinto-Lord et al., 1982).

Our data do not exclude alternate or additional functions for Reelin during layer formation, since other cell surface molecules, such as VLDLR and ApoER2, or members of the cadherin-related neuronal receptor family (CNRs) are found to bind Reelin and mediate signaling to Dab1 (Senzaki et al., 1999). In addition, APP and APLP1 bind to Dab1 (Trommsdorff et al., 1998; Homayouni et al., 1999; Howell et al., 1999b) and may be targets of Reelin as well. The endocytic compartment is increasingly recognized as a critical site at which adhesion molecules converge with LDL receptors (Pol et al., 1997), and Reelin may thus in principle regulate a number of adhesion receptors simultaneously if they are complexed in sufficiently tight proximity to the VLDL/ApoE receptor that contains the endocytic motif. Reelin concentration, like laminin concentration, may determine the surface levels of integrins by regulating the rate at which integrin receptor is removed from the cell surface (Condic and Letourneau, 1997). Ligands can also regulate polarized flow of integrins toward or away from growth cone membranes (Grabham and Goldberg, 1997). Reelin, released from the layer 1 cortical neurons, is likely to be present in a top-down gradient in the developing cortical plate. Thus, Reelin gradient-mediated modulation or endocytosis of a large adhesion

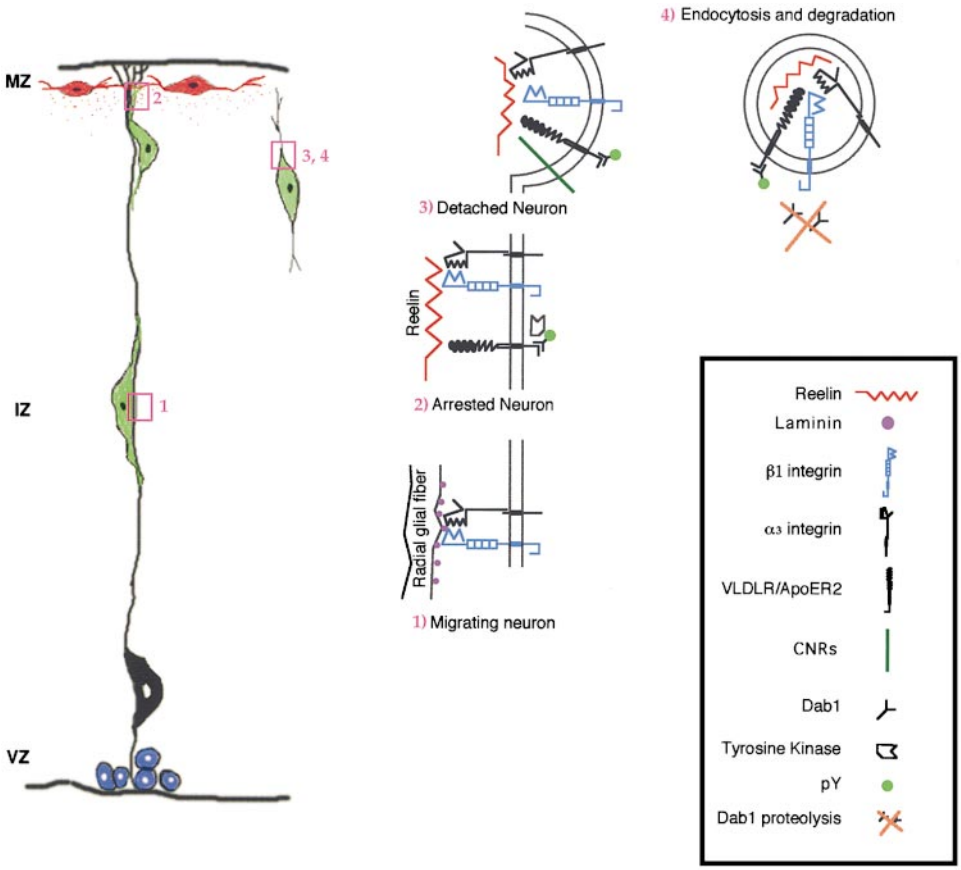

Figure 7. Model of Reelin–α3β1 Integrin Interactions

In (1), α3β1 integrin is required for neuronal migration along the radial glial fiber and may interact with glial cell surface molecules, such as laminin, during migration. In (2), at the top of the cortical plate, the ligand preference of α3β1 integrins changes from radial glial cell surface molecules to Reelin. Reelin binds to α3β1 integrin and forms a complex with VLDLR and ApoER2. This Reelin–receptor complex causes Dab1 to be phosphorylated, probably by a nonreceptor tyrosine kinase. In (3) and (4), the binding of Reelin to the complex of receptors leads to modulation of α3β1 integrin function and endocytosis. Endocytosis results in the degradation of Reelin and Dab1. Modulation of α3β1 integrin receptor function and levels leads to a switch from a gliophilic adhesion system to a neurophilic adhesion system, such that the migratory neuron detaches from the radial glial fiber and begins to differentiate in its "home" cortical layer. The absence of the Reelin signal causes early born neurons to retain their gliophilic adhesion system, preventing their appropriate detachment from the radial glial fiber. Abbreviations: MZ, marginal zone; IZ, intermediate zone; VZ, ventricular zone.

complex, including α3β1 integrins, might be necessary to "switch" migrating neurons from a gliophilic adhesive preference to a neurophilic adhesive preference.

While it is clear that other proteins in addition to α3β1 integrin bind Reelin, interactions between Reelin and α3β1 integrin appear to be crucial for the effects of Reelin upon migrating neurons. Reelin induces arrest of migrating cells and release from radial glia, and mutations in Reelin prevent normal dissociation of migratory neurons from radial glia, which has been described morphologically (Pinto-Lord et al., 1982). Inhibition of β1 integrins with blocking antibodies releases neurons from radial glia (Fishman and Hatten, 1993; Anton et al., 1999), and inhibition of β1 integrins with antisense retroviruses also blocks radial migration (Galileo et al., 1992). Similarly, targeted mutation of the α3 integrin gene causes inhibition of radial migration, possibly via premature release from radial glia. These data suggest that α3β1 integrin, either alone or in association with other partners, is necessary for radial glial guided migration and that inhibition of the α3β1 integrin complex is sufficient to induce release of neurons from radial glia.

The elevated levels of cleaved Reelin (180 kDa) observed in the absence of α3β1 integrin suggest that a normal function of α3β1 integrin is to inhibit the production or enhance the clearance of the Reelin fragment. α3β1 integrin could inhibit production of 180 kDa Reelin by modulating the activity of the zinc-dependent metalloproteinase that normally cleaves Reelin (Lambert de Rouvroit et al., 1999). Attractive candidates for the metalloproteinase would include brain-expressed members of the metalloproteinase-disintegrin family (ADAMs), which are zinc dependent and have integrin binding sites (Poindexter et al., 1999). In addition, α3β1 integrins may also bind to the 180 kDa fragment and promote its clearance. Although it is unclear at this juncture whether the 180 kDa Reelin fragment is functionally active, upregulation of this Reelin fragment may contribute to the aberrant cortical lamination observed in α3β1 integrin mutant cerebral cortex (Anton et al., 1999).

The analysis of α3β1 integrin deficiency is complicated by the fact that α3β1 can function as a transdominant inhibitor of other integrin receptor functions (Hodivala-Dilke et al., 1998). In keratinocytes, α3β1 deficiency leads to increased activity of fibronectin and collagen type IV receptors, possibly including α5β1 integrins, which can bind Reelin. Similar upregulation of other integrin receptor activity may also occur in α3β1

mutant cerebral cortex and contribute partially to the aberrant phenotype. Increased levels of cleaved Reelin, release of trans-dominant inhibition of other integrins capable of interacting with Reelin, and the availability of multiple other cell surface receptors for Reelin (Senzaki et al., 1999; Trommsdorff et al., 1999) may account for the downregulation of Dab1 protein levels in the absence of $\alpha3\beta1$ integrins.

Our data allow a tentative model of Reelin function (Figure 7). Migration along radial glial cells is mediated by $\alpha3\beta1$ integrin in addition to other adhesive or signaling molecules (Komuro and Rakic, 1998; Maeda and Noda, 1998). Neuronal $\alpha3\beta1$ integrin may interact with ligands such as laminin, distributed along the radial glial strands (Liesi, 1990), during neuronal translocation from the ventricular zone to the cortical plate. In the cortical plate, when migrating neurons encounter Reelin, the ligand preference of $\alpha3\beta1$ integrins in neurons changes from a radial glial cell surface molecule such as laminin to Reelin. Reelin binds to $\alpha3\beta1$ integrin in a complex that includes the VLDLR, the ApoER2, and $\alpha3\beta1$ integrin, and the formation of this complex leads to altered integrin-mediated adhesion, perhaps due to internalization of the integrin receptor along with the ApoER2 and VLDLR (D'Arcangelo et al., 1999). The altered ligand preference of $\alpha3\beta1$ integrins, coupled with the endocytosis-driven removal of $\alpha3\beta1$ integrins from the cell surface, may then cause rapid dissociation of migrating neurons from radial glia. Reelin undoubtedly also induces other downstream effects, exemplified by its effects on the phosphorylation of Tau (Hiesberger et al., 1999), that may also be crucial to its inhibitory effects on migration; however, regulation of availability, function, and ligand preference of integrins appears to be critical for the dissociation of neurons from radial glia. Cultured cells rapidly regulate the surface expression of integrins in response to ligand (Raub and Kuentzel, 1989), and neurons regulate integrin surface expression to alter adhesion and neurite extension (Condic and Letourneau, 1997). Regulation of ligand preference and endocytosis may be a general means for rapid, regionally specific removal of adhesive or signaling molecules from the leading edge of migrating neurons as they terminate their migration and begin their aggregation into distinct layers in cerebral cortex.

Experimental Procedures

Antibodies and Immunoblotting

Mouse monoclonal anti-Reelin antibodies CR50 and 5A5 were generously gifted by Dr. M. Ogawa (RIKEN) and Dr. A. Goffinet (University of Liege, Belgium). Affinity-purified polyclonal antibodies to Dab1 were obtained from Dr. B. Howell (National Institutes of Health). Affinity-purified monoclonal antibodies to mDab1 were obtained from Dr. A. Goffinet (University of Liege, Belgium). The RBD antibody was obtained from Dr. T. Yagi (RIKEN). Immunoblots were probed with the following antibodies diluted in blocking solution: 1:100 anti-Dab1 E1 monoclonal antibody (gift of A. Goffinet), 1:250 anti-Dab1 B3 rabbit polyclonal antibody (gift of B. Howell and J. Cooper), 1:1000 anti-ApoER2 (gift of J. Herz), 1:1000 anti-VLDLR (gift of J. Herz), 1:100 anti-VLA3 (V76720, Transduction Laboratories), 1:250 anti-phosphotyrosine pY99 (sc-7020, Santa Cruz Biotechnology, Santa Cruz, CA), 1:4000 anti-amyloid precursor protein (Ab681, gift of D. Selkoe), 1:2500 anti-E-cadherin (C20820, Transduction Laboratories), 1:500 anti–$\beta1$ integrin (Ab1952, Chemicon), and 1:500 anti–$\beta6$ integrin (sc-6632, Santa Cruz). An enhanced chemiluminescence kit (Amersham) was used to detect horseradish peroxidase–labeled secondary antibodies (Bio-Rad).

Mice

Mice were cared for according to animal protocols approved by the Institutional Animal Care and Use Committees of Children's Hospital, Beth Israel Deaconess Medical Center, and Pennsylvania State University. Genotypes of the $\alpha3$ integrin mutant embryos and *reeler* mice were determined by PCR as described earlier (D'Arcangelo et al., 1996; DiPersio et al., 1997).

Reelin-Containing Supernatants

293T cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Life Technologies). Cells were transfected with Reelin constructs encoding either full-length Reelin or Myc-tagged full-length Reelin (D'Arcangelo et al., 1997; gift of G. D'Arcangelo and T. Curran) using lipofectamine reagent as per the protocol of the manufacturer (Life Technologies). After transfection, serum-containing media were exchanged with 293 serum-free media (SFM; Life Technologies). The supernatant fraction was collected 1–3 days later. The supernatants were filtered and in most cases used directly; otherwise, supernatants were stored at 4°C.

Cortical Cultures

Cortical cultures were made from individual E16-E18 embryos using minor modifications to the previously described papain dissociation method (Murphy et al., 1989). For Dab1 phosphorylation assays, dissociated cortical cells were suspended (at 10^6 cells/ml) in α-MEM + Glutamax + 2% B-27 supplement (Life Technologies) and plated into 35 mm tissue culture dishes pretreated with a 1:60 dilution of Matrigel (Becton Dickinson).

Dab1 Phosphorylation Assay

Media (0.5 ml) were removed from each 35 mm dish of cultured 1 DIV cortical neurons and replaced with 0.5 ml of 293T Reelin-containing CM. After 15 min, cultures were placed on ice and lysed and scraped in 300 μl RIPA buffer. Dab phosphorylation determinations were performed essentially as described (Howell et al., 1999a).

Cortical Homogenates

Brains were excised, the vesicles of the telencephalon were pinched off, and the meninges were removed. The dorsal lateral telencephalon was separated from the ganglionic eminences and then homogenized in TX-IPB buffer (Howell et al., 1997a) and clarified by centrifugation at 14,000 × g. E16.5 $\alpha3$ integrin mutant and littermate brains were homogenized in STM buffer (10 mM Tris-HCL (pH 8.0), 0.25 M sucrose, 10 mM $MgCl_2$, 1 mM DTT, and protease inhibitors). Protein amounts were measured using the Bio-Rad microassay protocol, and constant amounts of protein were loaded into each lane for SDS–PAGE analysis and immunoblotting.

Immunoprecipitation of Reelin and Integrins

Equal volumes of anti-mouse IgG sepharose beads (Zymed, South San Francisco, CA) were incubated either with CR50 (gift from M. Ogawa), a monoclonal anti-Reelin antibody, or with mAb48, a monoclonal anti–synaptic vesicle antigen antibody, or anti-v-src (OP07, Calbiochem). mAb48 and anti-v-src are of the same isotype as CR50. After 12 hr at 4°C, beads were washed three times with phosphate-buffered saline (PBS) and incubated in siliconized (Sigmacote, Sigma) tubes with Reelin-containing 293T cell CM for another 12 hr. The Reelin-coated or control beads were then washed three times and incubated with either octylglucoside-solubilized E16 brain membrane homogenates (Busk et al., 1992) or Triton X-100– (1%) solubilized brain membrane extracts (Kaprielian and Patterson, 1993). Immunoprecipitations from brain membrane extracts were washed three times with PBS +1% Triton X-100, while immunoprecipitations from brain membrane homogenates were washed three times with buffer containing 25 mM octylglucoside followed by three washes in PBS + 1 mM $CaCl_2$. Proteins were eluted by boiling in SDS sample buffer and analyzed by SDS–PAGE and immunoblotting.

In some experiments, anti-rabbit IgG beads (Zymed) were incubated with either polyclonal anti–$\beta1$ integrin (Chemicon) or anti–$\alpha3$ integrin antibodies (Ab8-4, gift from Dr. M. DiPersio). Beads were then washed and incubated with brain membrane extracts to determine if native integrins associated with endogenous Reelin.

Immunochemistry

Immunocytochemistry was performed on cortical cultures plated on poly-D-lysine-treated coverslips as described previously (Fox et al., 1998; Anton et al., 1999). Primary antibodies were anti-Dab1 D1 (1:100), anti–β1 integrin (1:100, Ab1997, Chemicon), and anti–α3 integrin (1:100; DiPersio et al., 1997).

In Vitro Migration Assay

Cortical imprint assays containing intact radial glial cells with migrating neurons attached to them were made as described previously (Anton et al., 1999). Cortical neurons migrating on radial glial cells were monitored using a Zeiss Axiovert 135 microscope. After 60–120 min of baseline recording, Reelin supernatants or control 293T CM were added to the cultures, and monitoring continued for an additional 60–180 min. Changes in the rate of cell migration, morphological features of migrating neurons and glial cell substrates, and the extent of neuron–glia cell contact were monitored. The extent of cell soma movement was divided by time elapsed between observations to obtain the rate of cell migration for each neuron studied. Statistical difference between experimental groups was tested by student's t test.

In long-term migration assays, the number of migrating neurons was counted in four predefined regions in each well. Reelin, control 293T CM, anti-CR50 antibodies, anti-RBD antibodies, or control mouse/rat immunoglobulins (10 μg/ml) were then added to the cultures. After 24 hr, the number of migrating cells in the four predefined regions in each well was counted again. Change in the number of migrating neurons was determined for each group.

In Vivo Migration Assay

Fluorescent latex beads (Lumafluor) were incubated overnight at 4°C with Reelin or 293T CM. Microspheres (10 μl) were incubated with 500 μl of 5× concentrated Reelin or control solution. Microspheres were centrifuged and suspended in 7.5 μl of sterile saline.

Pregnant rats (E17) or mice (E16) were injected twice with BrdU (50 μg/g body weight) at 1 hr intervals. Animals were then anesthetized with a cocktail (2 ml/kg body weight) of 50 mg/ml ketamine, 2.6 mg/ml xylazine, and 0.5 mg/ml acepromozine. A midline incision was made through the abdominal wall under sterile conditions, embryos were exposed, and the abdominal cavity was continuously perfused with saline. After positioning the embryos with sterile cotton balls, they were illuminated with a high intensity illuminator. Microspheres (~100–200 nl) were pressure injected (PicospritzerII, Parker Instrumentation) into the cerebral wall of the telencephalon using a pulled glass micropipette with a tip diameter of ~20–40 μm (Sutter Instruments). The abdominal wall was sutured, and the animal was placed in warm recovery chamber. After 24 hr, embryos were removed, fixed in 4% paraformaldehyde, postfixed in 30% sucrose/PBS, and frozen in OCT, and 15 μm coronal sections were made in a cryostat. Sections were then labeled with anti-BrdU antibodies (Becton Dickenson), and the interaction between labeled neurons and microspheres deposited in the intermediate zone of the cerebral wall was analyzed.

Images of microsphere deposits and BrdU-labeled cells in cortical sections were acquired using a an ORCA-1 CCD camera attached to a Zeiss Axiophot microscope. A 225k square micrometer grid was placed on the images with the microspheres at the center of the grid. Optic density (OD) of the grid, an indicator of the number of labeled neurons in the area surrounding the microspheres, was measured using NIH Image 1.6. OD values from Reelin bead-containing sections were normalized to the values of littermate sections containing control beads.

To ensure Reelin coating of microspheres, aliquots of unused Reelin and control microspheres were incubated with blocking solution for 1 hr at room temperature and suspended in anti-Reelin CR50 antibodies overnight, and CR50 binding was visualized with anti-mouse antibodies conjugated to Cy3.

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References

Andressen, C., Arnhold, S., Puschmann, M., Bloch, W., Hescheler, J., Fassler, R., and Addicks, K. (1998). β1 integrin deficiency impairs migration and differentiation of mouse embryonic stem cell derived neurons. Neurosci. Lett. *251*, 165–168.

Anton, E.S., Kreidberg, J.A., and Rakic, P. (1999). Distinct functions of α3 and αV integrin receptors in neuronal migration and laminar organization of the cerebral cortex. Neuron *22*, 277–289.

Busk, M., Pytela, R., and Sheppard, D. (1992). Characterization of the integrin αVβ6 as a fibronectin-binding protein. J. Biol. Chem. *267*, 5790–5796.

Caviness, V.S., Jr., and Sidman, R.L. (1973). Time of origin or corresponding cell classes in the cerebral cortex of normal and reeler mutant mice: an autoradiographic analysis. J. Comp. Neurol. *148*, 141–151.

Chen, W.J., Goldstein, J.L., and Brown, M.S. (1990). NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. J. Biol. Chem. *265*, 3116–3123.

Condic, M.L., and Letourneau, P.C. (1997). Ligand-induced changes in integrin expression regulate neuronal adhesion and neurite outgrowth. Nature *389*, 852–856.

D'Arcangelo, G., Miao, G.G., Chen, S.C., Soares, H.D., Morgan, J.I., and Curran, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. Nature *374*, 719–723.

D'Arcangelo, G., Miao, G.G., and Curran, T. (1996). Detection of the Reelin breakpoint in reeler mice. Brain Res. Mol. Brain Res. *39*, 234–236.

D'Arcangelo, G., Nakajima, K., Miyata, T., Ogawa, M., Mikoshiba, K., and Curran, T. (1997). Reelin is a secreted glycoprotein recognized by the CR-50 monoclonal antibody. J. Neurosci. *17*, 23–31.

D'Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D., Sheldon, M., and Curran, T. (1999). Reelin is a ligand for lipoprotein receptors. Neuron *24*, 471–479.

Dedhar, S., Gray, V., Robertson, K., and Saulnier, R. (1992). Identification and characterization of a novel high-molecular-weight form of the integrin α3 subunit. Exp. Cell Res. *203*, 270–275.

DeFreitas, M.F., Yoshida, C.K., Frazier, W.A., Mendrick, D.L., Kypta, R.M., and Reichardt, L.F. (1995). Identification of integrin α3β1 as a neuronal thrombospondin receptor mediating neurite outgrowth. Neuron *15*, 333–343.

DiPersio, C.M., Hodivala-Dilke, K.M., Jaenisch, R., Kreidberg, J.A., and Hynes, R.O. (1997). α3β1 Integrin is required for normal development of the epidermal basement membrane. J. Cell Biol. *137*, 729–742.

Fishman, R.B., and Hatten, M.E. (1993). Multiple receptor systems promote CNS neural migration. J. Neurosci. *13*, 3485–3495.

Fox, J.W., Lamperti, E.D., Eksioglu, Y.Z., Hong, S.E., Feng, Y., Graham, D.A., Scheffer, I.E., Dobyns, W.B., Hirsch, B.A., Radtke, R.A., et al. (1998). Mutations in filamin 1 prevent migration of cerebral cortical neurons in human periventricular heterotopia. Neuron *21*, 1315–1325.

Galileo, D.S., Majors, J., Horwitz, A.F., and Sanes, J.R. (1992). Retrovirally introduced antisense integrin RNA inhibits neuroblast migration in vivo. Neuron *9*, 1117–1131.

Georges-Labouesse, E., Mark, M., Messaddeq, N., and Gansmüller,

A. (1998). Essential role of α6 integrins in cortical and retinal lamination. Curr. Biol. *8*, 983–986.

Gonzalez, J.L., Russo, C.J., Goldowitz, D., Sweet, H.O., Davisson, M.T., and Walsh, C.A. (1997). Birthdate and cell marker analysis of scrambler: a novel mutation affecting cortical development with a reeler-like phenotype. J. Neurosci. *17*, 9204–9211.

Grabham, P.W., and Goldberg, D.J. (1997). Nerve growth factor stimulates the accumulation of β1 integrin at the tips of filopodia in the growth cones of sympathetic neurons. J. Neurosci. *17*, 5455–5465.

Hatten, M.E. (1999). Central nervous system neuronal migration. Annu. Rev. Neurosci. *22*, 511–539.

Hiesberger, T., Trommsdorff, M., Howell, B.W., Goffinet, A., Mumby, M.C., Cooper, J.A., and Herz, J. (1999). Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. Neuron *24*, 481–489.

Hodivala-Dilke, K.M., DiPersio, C.M., Kreidberg, J.A., and Hynes, R.O. (1998). Novel roles for α3β1 integrin as a regulator of cytoskeletal assembly and as a trans-dominant inhibitor of integrin receptor function in mouse. J. Cell Biol. *142*, 1357–1369.

Hoffarth, R.M., Johnston, J.G., Krushel, L.A., and van der Kooy, D. (1995). The mouse mutation reeler causes increased adhesion within a subpopulation of early postmitotic cortical neurons. J. Neurosci. *15*, 4838–4850.

Homayouni, R., Rice, D.S., Sheldon, M., and Curran, T. (1999). Disabled-1 binds to the cytoplasmic domain of amyloid precursor-like protein 1. J. Neurosci. *19*, 7507–7515.

Howell, B.W., Gertler, F.B., and Cooper, J.A. (1997a). Mouse disabled (mDab1): a Src binding protein implicated in neuronal development. EMBO J. *16*, 121–132.

Howell, B.W., Hawkes, R., Soriano, P., and Cooper, J.A. (1997b). Neuronal position in the developing brain is regulated by mouse disabled-1. Nature *389*, 733–737.

Howell, B.W., Herrick, T.M., and Cooper, J.A. (1999a). Reelin-induced tyrosine phosphorylation of disabled 1 during neuronal positioning. Genes Dev. *13*, 643–648.

Howell, B.W., Lanier, L.M., Frank, R., Gertler, F.B., and Cooper, J.A. (1999b). The disabled 1 phosphotyrosine-binding domain binds to the internalization signals of transmembrane glycoproteins and to phospholipids. Mol. Cell. Biol. *19*, 5179–5188.

Kaprielian, Z., and Patterson, P.H. (1993). Surface and cytoskeletal markers of rostrocaudal position in the mammalian nervous system. J. Neurosci. *13*, 2495–2508.

Komuro, H., and Rakic, P. (1998). Orchestration of neuronal migration by activity of ion channels, neurotransmitter receptors, and intracellular Ca^{2+} fluctuations. J. Neurobiol. *37*, 110–130.

Lambert de Rouvroit, C., de Bergeyck, V., Cortvrindt, C., Bar, I., Eeckhout, Y., and Goffinet, A.M. (1999). Reelin, the extracellular matrix protein deficient in reeler mutant mice, is processed by a metalloproteinase. Exp. Neurol. *156*, 214–217.

Law, D.A., DeGuzman, F.R., Heiser, P., Ministri-Madrid, K., Killeen, N., and Phillips, D.R. (1999). Integrin cytoplasmic tyrosine motif is required for outside-in alphaIIbeta3 signaling and platelet function. Nature *401*, 808–811.

Liesi, P. (1990). Extracellular matrix and neuronal movement. Experientia *46*, 900–907.

Maeda, N., and Noda, M. (1998). Involvement of receptor-like protein tyrosine phosphatase zeta/RPTP beta and its ligand pleiotrophin/heparin-binding growth-associated molecule (HB-GAM) in neuronal migration. J. Cell Biol. *142*, 203–216.

Miyata, T., Nakajima, K., Mikoshiba, K., and Ogawa, M. (1997). Regulation of Purkinje cell alignment by Reelin as revealed with CR-50 antibody. J. Neurosci. *17*, 3599–3609.

Murphy, T.H., Miyamoto, M., Sastre, A., Schnaar, R.L., and Coyle, J.T. (1989). Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. Neuron *2*, 1547–1558.

Ogawa, M., Miyata, T., Nakajima, K., Yagyu, K., Seike, M., Ikenaka, K., Yamamoto, H., and Mikoshiba, K. (1995). The *reeler* gene–associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. Neuron *14*, 899–912.

Patel, D.D., Forder, R.A., Soutar, A.K., and Knight, B.L. (1997). Synthesis and properties of the very-low-density-lipoprotein receptor and a comparison with the low-density-lipoprotein receptor. Biochem. J. *324*, 371–377.

Pinto-Lord, M.C., Evrard, P., and Caviness, V.S., Jr. (1982). Obstructed neuronal migration along radial glial fibers in the neocortex of the reeler mouse: a Golgi-EM analysis. Brain Res. *256*, 379–393.

Poindexter, K., Nelson, N., DuBose, R.F., Black, R.A., and Cerretti, D.P. (1999). The identification of seven metalloproteinase-disintegrin (ADAM) genes from genomic libraries. Gene *237*, 61–70.

Pol, A., Ortega, D., and Enrich, C. (1997). Identification and distribution of proteins in isolated endosomal fractions of rat liver: involvement in endocytosis, recycling and transcytosis. Biochem. J. *323*, 435–443.

Raub, T.J., and Kuentzel, S.L. (1989). Kinetic and morphological evidence for endocytosis of mammalian cell integrin receptors by using an anti-fibronectin receptor beta subunit monoclonal antibody. Exp. Cell Res. *184*, 407–426.

Rice, D.S., Sheldon, M., D'Arcangelo, G., Nakajima, K., Goldowitz, D., and Curran, T. (1998). Disabled-1 acts downstream of Reelin in a signaling pathway that controls laminar organization in the mammalian brain. Development *125*, 3719–3729.

Senzaki, K., Ogawa, M., and Yagi, T. (1999). Proteins of the CNR family are multiple receptors for Reelin. Cell *99*, 635–647.

Sheldon, M., Rice, D.S., D'Arcangelo, G., Yoneshima, H., Nakajima, K., Mikoshiba, K., Howell, B.W., Cooper, J.A., Goldowitz, D., and Curran, T. (1997). Scrambler and yotari disrupt the disabled gene and produce a reeler-like phenotype in mice. Nature *389*, 730–733.

Tolleshaug, H., Goldstein, J.L., Schneider, W.J., and Brown, M.S. (1982). Post-translational processing of the LDL receptor and its genetic disruption in familial hypercholesterolemia. Cell *30*, 715–724.

Trommsdorff, M., Borg, J.P., Margolis, B., and Herz, J. (1998). Interaction of cytosolic adaptor proteins with neuronal apolipoproteinE receptors and the amyloid precursor protein. J. Biol. Chem. *273*, 33556–33560.

Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimpf, J., Hammer, R.E., Richardson, J.A., and Herz, J. (1999). Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. Cell *97*, 689–701.

Walsh, C.A. (1999). Genetic malformations of the human cerebral cortex. Neuron *23*, 19–29.

Ware, M.L., Fox, J.W., Gonzalez, J.L., Davis, N.M., Lambert de Rouvroit, C., Russo, C.J., Chua, S.C., Jr., Goffinet, A.M., and Walsh, C.A. (1997). Aberrant splicing of a mouse disabled homolog, mdab1, in the scrambler mouse. Neuron *19*, 239–249.

Willnow, T.E., Rohlmann, A., Horton, J., Otani, H., Braun, J.R., Hammer, R.E., and Herz, J. (1996). RAP, a specialized chaperone, prevents ligand-induced ER retention and degradation of LDL receptor–related endocytic receptors. EMBO J. *15*, 2632–2639.

Zhang, Z., and Galileo, D.S. (1998). Retroviral transfer of antisense integrin α6 and α8 sequences results in laminar redistribution or clonal cell death in developing brain. J. Neurosci. *18*, 6928–6938.