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ROLE OF THE REELIN SIGNALING PATHWAY IN CENTRAL NERVOUS SYSTEM DEVELOPMENT

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■ **Abstract** The neurological mutant mouse *reeler* has played a critical role in the evolution of our understanding of normal brain development. From the earliest neuroanatomic studies of *reeler*, it was anticipated that the characterization of the gene responsible would elucidate important molecular and cellular principles governing cell positioning and the formation of synaptic circuits in the developing brain. Indeed, the identification of *reelin* has challenged many of our previous notions and has led to a new vision of the events involved in the migration of neurons. Several neuronal populations throughout the brain secrete Reelin, which binds to transmembrane receptors located on adjacent cells triggering a tyrosine kinase cascade. This allows neurons to complete migration and adopt their ultimate positions in laminar structures in the central nervous system. Recent studies have also suggested a role for the Reelin pathway in axonal branching, synaptogenesis, and pathology underlying neurodegeneration.

INTRODUCTION

One of the fundamental issues facing the field of developmental neurobiology is to understand the mechanisms that control the deployment of neurons with similar properties into specific layers. This is a daunting question given the fact that there are so many different types of neurons that must be precisely connected for proper brain function. Neuronal positioning is critical for the formation of cytoarchitecturely distinct brain regions such as the cerebral cortex, hippocampus, and cerebellum. During development of laminated brain structures a series of coordinated migrations takes neurons from their site of origin to their final destinations, where they adopt definitive morphological phenotypes by elaborating dendritic and axonal processes (Ramón y Cajal 1955).

The analysis of neurological mutant mice has led to dramatic progress in the identification and characterization of genes important for neuronal migration in the developing brain (Rice & Curran 1999). Over a relatively short period of time, genetic and biochemical studies have identified a new signaling pathway that controls neuronal cell positioning. The gene disrupted in *reeler* mice encodes a

TABLE 1 Mouse genes implicated, genetically or biochemically, in the Reelin signaling pathway

Description	Gene	Name	Reference
Extracellular	*ApoE*	ApolipoproteinE	D'Arcangelo et al 1999
	Bdnf	Brain-derived neurotrophic factor	Ringstedt et al 1998
	Reln	Reelin	D'Arcangelo et al 1995
Transmembrane	*ApoER2*	Apolipoprotein E receptor 2	Trommsdorff et al 1999
	CNR	Cadherin-related neuronal receptor	Senzaki et al 1999
	itga3	α3 integrin	Dulabon et al 2000
	PS-1	Presenilin-1	Hartmann et al 1999
	Vldlr	Very low–density lipoprotein receptor	Trommsdorff et al 1999
Cytoplasmic	*Abl*	Abelson proto-oncogene	Howell et al 1997a
	CASK	Membrane-associated guanylate kinase	Hsueh et al 2000
	Cdk5	Cyclin-dependent kinase 5	Ohshima et al 1996
	Cdk5r	p35	Chae et al 1997
	Dab-1	Disabled 1	Sheldon et al 1997
	Src	Src proto-oncogene	Howell et al 1997a
Nuclear	*Emx2*	Related to *empty spiracles*	Mallamaci et al 2000
	P73	Transformation-related protein 73	Yang et al 2000
	Tbr-1	T-box brain-1	Bulfone et al 1995

large extracellular protein, Reelin, that is produced by discrete populations of cells in the brain (D'Arcangelo et al 1995). Reelin binds to transmembrane receptors, the very low–density lipoprotein receptor (Vldlr), and the apolipoprotein E receptor 2 (ApoER2) present on migrating neurons (D'Arcangelo et al 1999, Hiesberger et al 1999). The cytoplasmic domains of these receptors bind to disabled-1 (Dab1), an intracellular adapter protein (Trommsdorff et al 1999). Binding of Reelin to lipoprotein receptors induces tyrosine phosphorylation of Dab1 (Howell et al 1999a), triggering an intracellular signaling cascade that instructs neurons to occupy their proper locations in the developing central nervous system (CNS). Other genes have been implicated in the Reelin signaling pathway, although their role is not fully understood (Table 1). Collectively, these genes provide a molecular starting point for biological, biochemical, and cellular studies that are providing important and exciting insights into the molecular mechanisms that control brain development and, potentially, the pathogenesis of neurodegenerative disorders.

HISTORICAL PERSPECTIVE

The study of heritable mutations in mice can be traced back to 2000 BC in the Middle East and the Orient. While most of the world viewed mice as pests and carriers of disease, China and Japan embraced the mouse as a symbol of prosperity

(Keeler 1931). So-called white mice were known to breed true and other coat-color variations, and behavioral mutant mice were collected or "fancied" as long ago as 80 BC. Many of these fancy mice made their way from Japan to Europe through the hands of British traders. Breeding studies on the propagation of colorful traits, such as white English sables and creamy buffs, were performed on mice before the rediscovery of Mendel's Law of Heredity (Davenport 1900). Therefore, modern mouse genetics in the United States was built on a foundation of pet mice through the studies of William E Castle and his students (Morse 1981). The practice of inbreeding mouse strains to study cancer was implemented in the early twentieth century. Shortly thereafter, the first scientific report of an inheritable defect in the CNS of mice was published in the *Proceeding of the National Acadamy of Sciences* (Keeler 1924). The *rodless* phenotype, which is now known to result from a mutation in the gene encoding the β-subunit of cGMP phosphodiesterase, was identified by analysis of histological sections of mouse retinas (Pittler et al 1993). Inbreeding of mouse stocks propagated this mutation in many of the standard lines of mice used today. Inbreeding also increased the likelihood of recessive characteristics appearing in offspring, some of which produced noticeable behavioral phenotypes. Over time, more "deviant" mice were reported, and the first database of mutations affecting the development of the CNS, produced in 1965, contained over 100 records (Sidman et al 1965). *Reeler* mice represented one of the founding members of the behavioral mutants reported in the first record, and they were recognized to exhibit widespread neuroanatomic defects in the cerebral cortex, cerebellum, and hippocampus. Additional anatomical abnormalities are also evident in other important brain structures, such as the thalamus, midbrain, brain stem, and spinal cord (Yip et al 2000, Caviness et al 1988). The neuroanatomical disruptions in the *reeler* brain suggested that the mutation affected a gene that is critical for controlling cell positioning in the developing CNS.

THE NEUROLOGICAL MUTANT MOUSE *reeler*

The *reeler* mutation arose spontaneously in a stock of "snowy-bellied" mice at the Institute of Animal Genetics in Edinburgh, Scotland. Falconer (1951) described the locomoter abnormality in *reeler* and in another neurological mutant named *trembler*. In the first description of the *reeler* cerebellum Hamburgh (1960:460) reported, "The typical appearance of the folia is missing. The arrangement of Purkinje cells which normally surround the granular layer is severely disturbed. The granular layer is much reduced and the area of white matter contains large numbers of cells, which resemble Purkinje cells." There are several known alleles of *reeler*, all of which exhibit identical phenotypes (D'Arcangelo & Curran 1998). Recently, mutations in *reelin* were also reported in humans with autosomal recessive lissencephaly with cerebellar hypoplasia (Hong et al 2000). Lissencephaly is a condition of severe neuronal migration disorders that result in a smooth appearance of the brain surface in humans. Although mice are normally lissencephalic,

migration disorders result in similar misplacements of neurons in laminated brain regions in both mice and humans.

THE *reeler* MUTATION DISRUPTS CELL POSITIONING IN THE BRAIN

In mammals the organization of the cerebral cortex follows a stereotypic plan during development. Neurons with similar morphologies and connections are positioned in the same layer. This cellular organization reflects the patterning of neuronal processing that occurs in the cerebral cortex, where certain layers receive input and connect to other layers, allowing multiple levels of information integration, modulation, and extraction. One of the most revealing techniques used to characterize the *reeler* brain was neuronal "birthdating" analysis. This approach employed autoradiography of brain slices after administration of tritiated thymidine to mice, which is permanently incorporated into DNA during synthesis. Cells that undergo their final division and adopt a postmitotic phenotype are strongly marked by radioactivity, whereas those that re-enter the cell cycle further dilute the label through successive rounds of cell division. Therefore, the temporal and spatial assembly of cells can be revealed by autoradiographic analysis of brains that received tritiated thymidine at specific stages during development. The relative position of neurons with respect to their birthdate in the adult cortex is shown schematically in Figure 1. Using this approach, it was found that cortical neurons in the mouse are generated between embryonic days (E) 10 to 18 and that the 6 layers of the cerebral cortex assemble in an inside-out sequence (Angevine & Sidman 1961). These studies demonstrated that, in addition to morphological and physiological characteristics, neurons positioned within specific cortical layers also share a common birthdate.

The *reeler* cerebral cortex violates this fundamental plan of cortical assembly (Caviness & Sidman 1973a). Layer I is not discernible in the mutant, and the position of cells comprising other layers is relatively inverted (Figure 1). Importantly, all major morphological cell classes are present in the *reeler* cortex, and cohorts of neurons comprising specific layers are generated on schedule (Caviness & Sidman 1973b). The neuronal classes in *reeler* cortex fail to align in an inside-out fashion like their counterparts in the normal cortex. In *reeler*, neurons born relatively late during corticogenesis reside in deep layers beneath the older neurons (Caviness & Sidman 1973b). These comparative studies demonstrated that the *reeler* mutation affects the positioning of neurons within specific layers. Thus, the molecular mechanisms controlling cell positioning are distinct from those that determine the number and proportion of cell types constituting various layers of the cerebral cortex (McConnell & Kaznowski 1991, Herrup 1987).

Disorganization of fiber patterns representing the major afferent and efferent systems accompanies cellular ectopia in the mutant cerebral cortex (Caviness et al 1988). Therefore, *reeler* mice provide a model system in which to address the

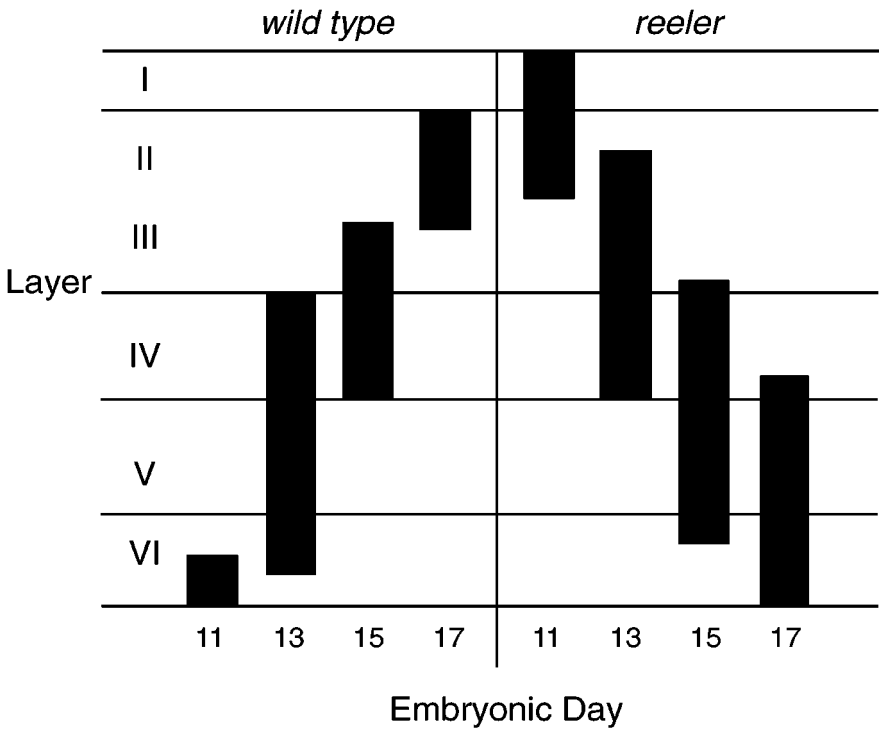


Figure 1 The position of cells born at specific developmental times is relatively inverted in the adult *reeler* cerebral cortex. In this schematic the pial surface is at the top and the white matter is at the bottom. Injections of [^{3}H] thymidine were administered at 48-hour intervals throughout corticogenesis. Cells generated on E11 are located in Layer VI in the wild-type cortex, whereas cells with similar birthdates and morphologies are located near the surface of the *reeler* brain. Other neuronal classes are generated on schedule in *reeler*, but they obtain an incorrect location in the adult cerebral cortex. A similar situation is observed in the *scrambler* cortex. This data was taken from Caviness 1982.

relationship between cell positioning and synaptic circuit formation during development of the nervous system. Careful studies of *reeler* provided conclusive evidence that the molecular mechanisms controlling cell positioning are distinct from those that control axonal guidance, which is a key first step in the formation of synaptic circuitry (Caviness & Rakic 1978). Afferent projections to the visual, olfactory, somatosensory, and motor cortices rearrange their trajectories and actually find their correct target cells even though they are located in ectopic positions (Terashima et al 1983, Simmons et al 1982, Caviness 1976). Some of the axonal projections take circuitous routes to their postsynaptic targets in the *reeler* cortex. Thalamocortical projections that are normally restricted to deep cortical layers, namely Layer IV, ascend to the superficial aspect of the *reeler* cerebral cortex

before "looping" down to their correct target cells (Caviness 1976, Steindler & Colwell 1976). Studies of *reeler* demonstrated that the overall organization of the major systems and the physiological responses of individual neurons are comparable to those in normal brain (Silva et al 1991, Lemmon & Pearlman 1981, Dräger 1981). The conclusion from these studies is that the general wiring pattern of the brain is independent of cell position, implying that other genes control axon guidance. However, changes in synaptic density, distribution, and topology are present in a number of brain structures in *reeler* including the cerebellum, hippocampus, and piriform cortex (Borrell et al 1999a, Caviness 1977, Mariani et al 1977). Thus, while the overall mechanisms of axon guidance appear unaffected in *reeler*, other attributes of synaptic circuit formation are abnormal.

DEVELOPMENT OF CELL LAMINATION IN THE CEREBRAL CORTEX

The mammalian brain is assembled through a choreographed series of far-ranging migrations that results in the segregation of neurons with similar properties into discrete layers. The initial alignment of neurons in the CNS relates to the time at which they exit the cell cycle and migrate to their ultimate locations where they adopt a definitive neuronal phenotype (McConnell & Kaznowski 1991, Luskin & Shatz 1985, Rakic 1974). Two modes of migration, tangential and radial, are recognized in the developing brain. Radial migration is the most common form of migration in the developing cortex and it relies on a specialized scaffold of cells that span the cerebral wall (Hatten 1999, Rakic 1972). This scaffold is comprised of bipolar cells, known as radial glia, that were first described using the Golgi technique, but they can also be visualized with immunohistochemical markers (Bentivoglio & Mazzarello 1999). The descriptive name accurately depicts the morphology of radial glia, which send a short process from the cell soma to the ventricular surface and a long process that spans the width of the neural axis. The ascending process splits into several branches near the pial surface. Migrating cortical neurons physically associate with radial glia during their ascent to the top of the cortex, and their migratory paths are likely guided by molecules expressed on the plasma membrane (Anton et al 1996, Cameron & Rakic 1994). Radial glia are present only during neuronal development. Subsequently, they transform into astrocytes in the cerebral cortex and Bergmann fibers in the cerebellum (Hatten 1999).

Neocortical development begins with the appearance of the preplate or primordial plexiform layer above the ventricular zone (Figure 2*A*). The preplate is composed of subcortical afferents and two populations of postmitotic neurons, the Cajal-Retzius cells and the subplate neurons (Marin-Padilla 1998, Super et al 1998). Cajal-Retzius cells are among the earliest neurons to be generated in the mammalian neocortex, where they occupy positions near the pial surface on the superficial aspect of the brain (Meyer & Goffinet 1998, König et al 1977).

Subplate neurons are born slightly later than Cajal-Retzius cells and they serve as transient synaptic targets for thalamocortical projections (Allendoerfer & Shatz 1994). The next phase of development occurs when the cortical plate neurons exit the cell cycle near the ventricular surface and invade the preplate. Migrating neurons move past the subplate, displacing this layer away from the Cajal-Retzius cells, which remain adjacent to the pial surface in a cell-sparse area known as the marginal zone (Figure 2*A*). As new cortical plate neurons arrive on the radial glial, they migrate past the older subplate and cortical plate neurons before inserting directly beneath Cajal-Retzius cells. The systematic migration of younger neurons past their predecessors results in the "inside-out" pattern of development, in which the cortical plate (future Layers II-VI) develops between the marginal zone (future Layer I) and the subplate (Marin-Padilla 1998, Angevine & Sidman 1961).

Histological and ultrastructural studies on the embryonic *reeler* cortex demonstrated that cell position defects arise during the formation of the cortical plate. The preplate develops normally and migration of cortical plate neurons commences on schedule. Shortly thereafter, disorganization of the cortical plate becomes apparent when the first cohort of migrating neurons fails to invade the preplate (Figure 2*B*). As a result, the preplate is not split into the marginal zone and the subplate (Sheppard & Pearlman 1997, Hoffarth et al 1995, Ogawa et al 1995, Caviness et al 1988, Caviness 1982, Pinto Lord & Caviness 1979, Goffinet 1979). As additional cohorts of neurons arrive on the radial glia guides, they are unable to bypass their predecessors. This leads to formation of a disorganized cortical plate underneath the superplate. Figure 2*A* shows the histotypical appearance of a normal cerebral cortex compared to a *reeler* cerebral cortex at E16.5. The marginal zone in normal mice contains fibers and Cajal-Retzius cells. However, the marginal zone in *reeler* is replaced by a relatively cell-dense region known as the superplate, which contains Cajal-Retzius cells, subplate neurons and a few cortical plate neurons (Derer 1985, Caviness 1982). Cells in the *reeler* cortical plate are frequently displaced by fibers that run obliquely through the cerebral wall (Goffinet 1979). This gives the appearance of cell-free rifts in the *reeler* cortex on histological examination (Figure 2*A*). It is important to note that the cortical plate does form in *reeler* and that migrating cells reach this level. Ultrastructure analysis of the *reeler* cerebral cortex at E17 revealed that several postmigratory neurons remain closely associated with the radial glia (Pinto-Lord et al 1982). Leading processes of migrating cells in *reeler* are apparently unable to breach these inappropriate contacts and their ascent to the marginal zone is impeded. Moreover, the characteristic appearance of the radial glia scaffold is disturbed during later stages of corticogenesis in the *reeler* cortex, and radial fibers are deployed at oblique angles (Hunter-Schaedle 1997, Mikoshiba et al 1983). These studies led to the suggestion that the *reeler* mutation affects molecular interactions between migrating neurons and the glial guides.

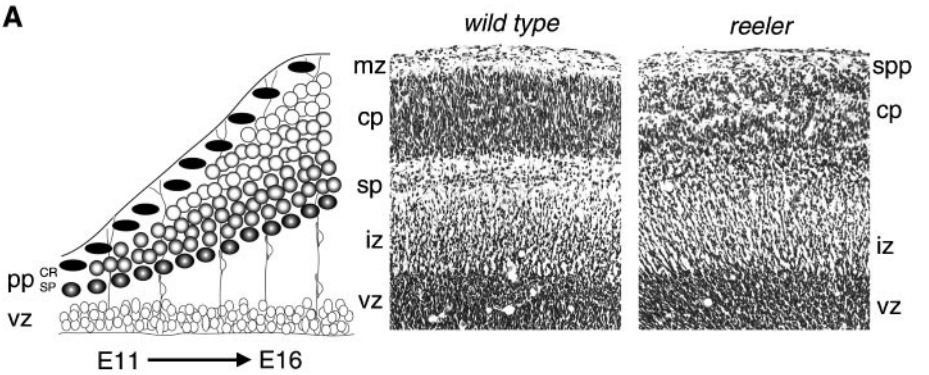
An alternative proposal suggested that the *reeler* mutation affects interactions among cortical neurons destined for particular layers. This idea is based on studies

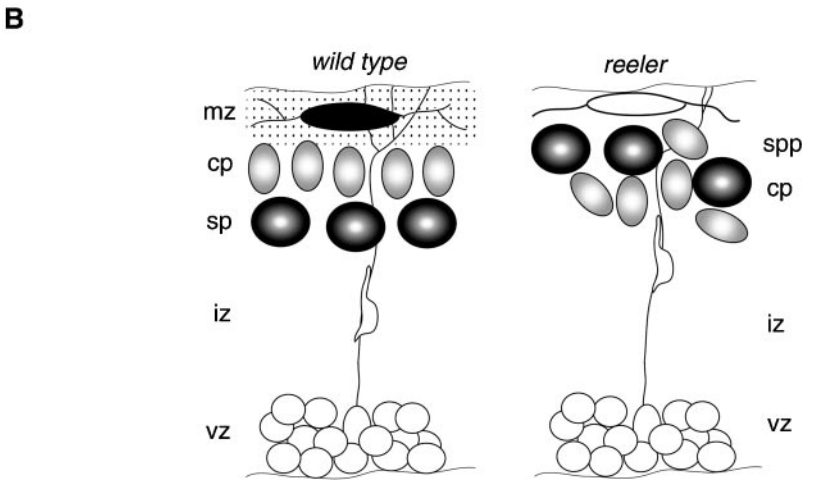
conducted in vitro by De Long & Sidman (1970), in which neurons isolated from *reeler* cortex fail to show the typical patterns of cell aggregation. This observation was confirmed and extended by Hoffarth et al (1995) using a similar in vitro assay. Analysis of *reeler* neurons generated during the earliest stages of corticogenesis (ca E11–12) revealed that they clump together more frequently than their normal counterparts. Moreover, retrograde labeling of different neuronal populations revealed that the *reeler* mutation preferentially targeted the earliest born cortical neurons. These studies suggested that the mutant gene affected neuron-neuron interactions. In addition, chimeric mouse studies indicated that the gene functioned extrinsically (Terashima et al 1986, Mullen 1984). The two prevailing models at the time proposed disruptions of cellular interactions either between migrating neurons and radial fibers or among migrating neurons themselves. Whereas these models could explain certain aspects of the *reeler* phenotype, it was hoped that identification of the *reeler* gene would resolve the cellular target and clarify the molecular events underlying the neuroanatomic disruptions.

Figure 2 A simplified view of corticogenesis and the histological appearance of the developing cerebral wall. (*A*) Corticogenesis in mammals begins with the appearance of the preplate (pp), which is located directly above the proliferating cells in the ventricular zone (vz). The preplate forms around embryonic days (E) 11–12 in mice and it contains Cajal-Retzius neurons (CR) and subplate (SP) neurons, among others. Cells destined for the cortical plate are generated over the next week. The first cohort of cortical plate cells (gray) migrates past the subplate and stops beneath the Cajal-Retzius cells in the marginal zone (mz). Successively generated waves (gray to white) of cells migrate past their predecessors and stop beneath the Cajal-Retzius cells. Therefore, by E16.5 in the mouse young cortical plate neurons are positioned above the older neurons, with the exception of the Cajal-Retzius cells. Cortical plate neurons mature into adult neurons in the cerebral cortex by the elaboration of dendritic and axonal fibers. A disruption in cortical development in *reeler* is obvious at E16. The marginal zone in the wild-type is relatively cell-free, except for Cajal-Retzius and several other neurons. The cortical plate (cp) contains tightly packed cells with a radial alignment. The subplate is beneath the cortical plate. Many neurons destined for superficial layers migrate along radial glia in the intermediate zone (iz). Cells continue to divide in the ventricular zone (vz). Although the overall divisions of the cerebral wall are obvious in *reeler*, the cortical plate is disorganized and cells are displaced by fibers that run obliquely in the cerebral wall. Many subplate cells are located with the Cajal-Retzius cells in a relatively cell-dense region known as the superplate (spp). (*B*) The defect in the *reeler* cortex is apparent at the onset of cortical plate formation. In wild-type cortex the first cohort of cortical plate neurons (gray) positions itself between the Cajal-Retzius cells (black) that produce Reelin (*stipples*) and the subplate layer. In *reeler* the first cohort of cortical plate neurons fails to migrate past the subplate and instead accumulates in a disorganized fashion in the superplate. Subsequently, many cortical neurons lose their radial alignment and deploy dendritic and axonal projections at oblique angles.

REELIN IS A SECRETED PROTEIN

The identification and characterization of the *reelin* gene (*Reln*) have been reviewed recently (D'Arcangelo & Curran 1998). Briefly, a transgene insertion into the *reeler* locus led directly to the isolation of a large mRNA (>12 kb) that contains an open reading frame of 10,383 bases encoding a protein of approximately 385 kDa (D'Arcangelo et al 1995). Several isoforms of Reelin are present in brain extracts and in the supernatant of primary neuronal cultures. These isoforms arise via cleavage of full-length Reelin into two smaller proteins of approximately 250 and 180 kDa (D'Arcangelo et al 1999, de Rouvroit et al 1999). The N-terminus of Reelin contains a cleavable signal peptide and a region of similarity with





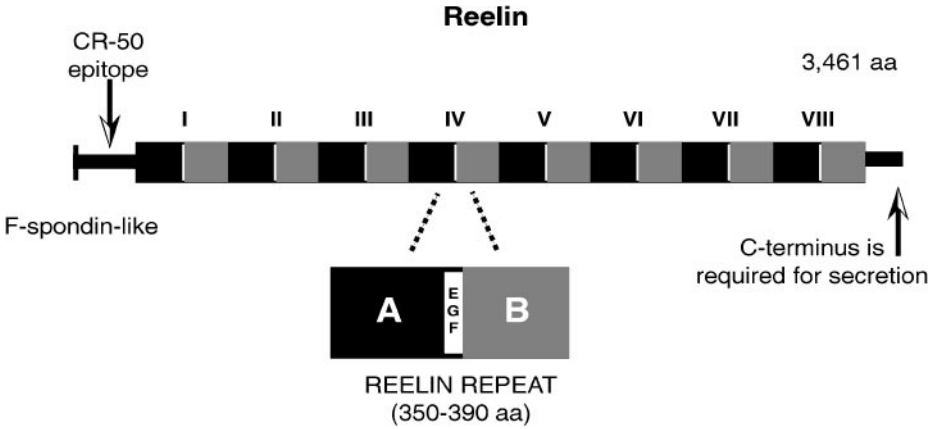


Figure 3 The *reelin* open reading frame predicts a novel protein of 3461 amino acids (aa) at a molecular mass of approximately 385 kDa. Reelin contains a cleavable signal peptide at the N-terminus, followed by a region of similarity to F-spondin, a secreted protein produced by floor plate cells that controls cell migration and neurite outgrowth. The most striking feature of Reelin is the presence of a series of eight internal repeats comprising 350–390 aa. These so-called Reelin repeats contain two related subdomains, A and B, separated by a stretch of 30 aa harboring an epidermal growth factor–like motif. A region rich in arginine residues at the C-terminus of Reelin is required for secretion. A monoclonal antibody named CR-50 recognizes an epitope defined to aa 230–346, which is C-terminal to the F-spondin region but before the first Reelin repeat. This antibody blocks Reelin function in vivo and in vitro.

F-spondin (Figure 3), a protein secreted by floor plate cells that directs neural crest cell migration and neurite outgrowth (Klar et al 1992). The main body of Reelin consists of a series of eight internal repeats (Reelin repeats) of 350–390 amino acids. Each Reelin repeat contains two related subdomains flanking a pattern of conserved cysteine residues related to epidermal growth factor–like motifs. These motifs are related to those in other extracellular proteins such as tenascin and restrictin and in the integrin family of receptors. Deletion analyses demonstrated that a short region of positively charged amino acids near the C-terminus is required for secretion of Reelin (D'Arcangelo et al 1997). In the *reeler* Orleans mutant (rl^{orl}) Reelin is made but not secreted due to the insertion of a L1 transposable element that alters the amino acid sequence of the C-terminus (de Bergeyck et al 1997).

One of the most surprising findings uncovered by the identification of *reelin* was the elucidation of its expression pattern in the developing brain. By using in situ hybridization, *reelin* was found to be present in a relatively small population of cells located in the marginal zone of the cerebral cortex (Figure 2*B*). These cells were identified as Cajal-Retzius neurons by their position and morphology

(D'Arcangelo et al 1995). At the same time *reelin* was described, Ogawa et al (1995) reported the generation of a monoclonal antibody by immunizing *reeler* mice with brain extracts from normal mice. Immunohistochemical studies in wild-type mice revealed that this antibody labeled Cajal-Retzius cells, which also express the calcium-binding protein calretinin. Importantly, the cerebral cortex of *reeler* mice did not react with this antibody, although Cajal-Retzius neurons are present (Ogawa et al 1995, Derer 1985). Subsequent studies demonstrated that the CR-50 antibody recognizes Reelin (D'Arcangelo et al 1997). Importantly, several experimental approaches revealed that CR-50 functions as a blocking antibody both in vivo and in vitro (Borrell et al 1999a, Miyata et al 1997, Nakajima et al 1997, Ogawa et al 1995). In an elegant series of experiments, Ogawa et al (1995) employed a cortical cell aggregation assay to address the physiological effects of the CR-50 antibody. Neurons dissociated from normal cortices distributed into a radial pattern as viewed with antibodies specific for microtubule-associated protein-2. In parallel cultures dissociated neurons obtained from the *reeler* cortex formed large clumps of microtubule-associated protein-2–positive neurons that lacked radial organization. When the CR-50 antibody was added to cultures of normal neurons at the time of cultivation the resulting cellular organization of the aggregates appeared very similar to those obtained with *reeler* neurons. The histotypic conversion of wild-type neurons to *reeler* neurons was abolished when CR-50 was preabsorbed against normal cortices but not *reeler* cortices. Recently, Reelin was shown to form multimeric complexes through the CR-50 epitope region (Utsunomiya-Tate et al 2000). These complexes were disrupted in the presence of CR-50, suggesting that multimerization of Reelin is required for its function.

Several hypotheses were proposed to explain the *reeler* phenotype and the function of Reelin in the developing cerebral cortex. The spatial and temporal expression pattern of Reelin suggested that it was important during the final phases of migration. Reelin is expressed by Cajal-Retzius before the first wave of cortical plate neurons reaches the preplate and it is secreted precisely at the location where these neurons stop migrating and detach from radial glia (Figure 2*B*). Therefore, Reelin was suggested to provide positional information to migrating neurons that instructs them to stop and detach from their guides. Reelin expression persists in the marginal zone throughout corticogenesis, implying that all migrating neurons encounter Reelin after their ascent to the top of the radial glia (Alcantara et al 1998). Reelin may act as an attractant molecule that enables each wave of cortical neurons to bypass their predecessors. Alternatively, Reelin could repel subplate neurons, thereby facilitating the invasion of the cortical plate (D'Arcangelo & Curran 1998). Although these ideas have merit in explaining the cortical defect in *reeler*, they rely on the assumption that Reelin binds cortical neurons directly. After identification of Reelin, the major question facing the field was, "How is the extracellular signal received and interpreted by migrating neurons?"

DISRUPTION OF THE *Disabled-1* GENE CAUSES *reeler*-LIKE PHENOTYPES

Davisson and colleagues at the Jackson Laboratory reported *reeler*-like phenotypes in a new neurological mutant named *scrambler* (Sweet et al 1996). *Scrambler* arose spontaneously and the mutants were ataxic by 2 weeks of age. This behavior was attributed to the abnormal cerebellum, which was small and lacked foliation. Closer examination revealed a reduction in the number of granule cells and Purkinje cell ectopia that were identical to that in *reeler* (Goldowitz et al 1997). Similarities between *reeler* and *scrambler* extended to the hippocampus and the cerebral cortex. Detailed studies of the histology and sequence of corticogenesis in *scrambler* demonstrated that Layer I was absent and the normal "inside-out" patterning of neurons was relatively inverted (Gonzalez et al 1997). These neuroanatomical defects were associated with an autosomal recessive mutation, and genetic mapping studies proved that the gene responsible was distinct from *reelin* (Sweet et al 1996). The striking similarities between *scrambler* and *reeler* suggested that the *scrambler* gene controls Reelin expression, either directly or indirectly by affecting cells that normally produce Reelin. However, immunohistochemical studies failed to reveal alterations in Reelin expression, and primary neuronal cultures obtained from *scrambler* mice produced and secreted Reelin (Gonzalez et al 1997, Goldowitz et al 1997). This genetic evidence suggested that *scrambler* functions downstream of Reelin in a common signaling pathway.

The *scrambler* locus was mapped near the *disabled-1* (*Dab1*) gene on chromosome 4, and mice with a targeted disruption of *Dab1* were found to exhibit ataxia and neuroanatomical defects indistinguishable from those in *scrambler* and *reeler* (Howell et al 1997b). Immunoblotting studies with Dab1-specific antibodies revealed a substantial decrease in Dab1 in *scrambler* brain (Sheldon et al 1997). Northern blot analysis demonstrated that the levels of the normal 5.5 kb Dab1 mRNA in *scrambler* were dramatically decreased. Furthermore, an additional mRNA species of approximately 7 kb was present that arose as a consequence of splicing defects in *Dab1* (Sheldon et al 1997, Ware et al 1997). Splicing defects at the same nucleotide in *Dab1* are also responsible for *reeler*-like traits observed in *yotari* mice, which appeared in a colony of mice carrying a disruption of the gene encoding the receptor for inositol-1,4,5-trisphosphate (Sheldon et al 1997, Yoneshima et al 1997). These studies provided convincing evidence that disruption of *Dab1*, either by spontaneous or targeted mutation, results in behavioral and anatomical abnormalities that are indistinguishable from those described in *reeler*.

Dab1 encodes a cytoplasmic protein containing a motif known as a protein interaction/phosphotyrosine binding (PI/PTB) domain (Figure 4). This domain was originally identified in the adapter protein Shc as a region required for binding to the epidermal growth factor receptor, the insulin receptor, and other tyrosine-phosphorylated proteins (Margolis 1996). Mammalian Dab1 was originally identified as a Src-binding protein in a yeast two-hybrid screen (Howell et al 1997a). It is expressed at high levels in the developing CNS and it is phosphorylated on

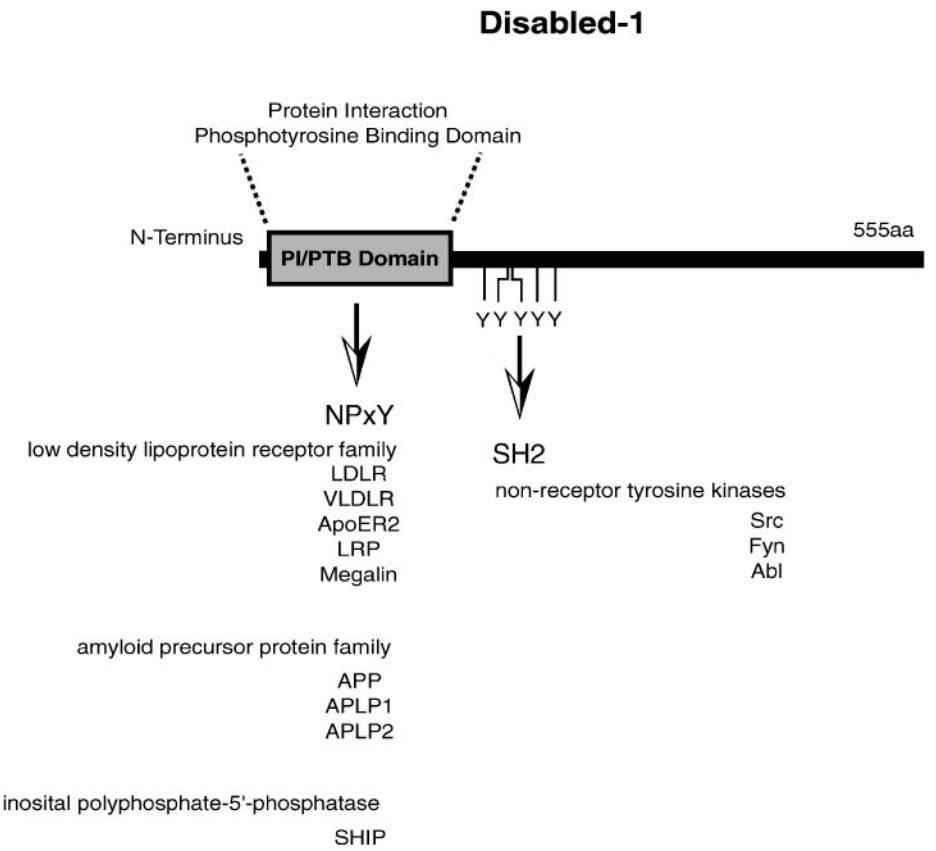


Figure 4 The *disabled-1* open reading frame predicts a protein of 555 amino acids that exhibits properties of adapter proteins. Near the N-terminus of Disabled-1 (Dab1) is a region of approximately 150 amino acids known as a protein interaction/phosphotyrosine binding domain (PI/PTB). The PI/PTB domain of Dab1 is most closely related to that in p96 (Dab2) and *Drosophila* Disabled. The PI/PTB domain binds to NPxY-containing motifs present in several proteins such as members of the low-density lipoprotein receptor family and the amyloid precursor protein family. Dab1 also binds to the NPxY motif in SHIP, an inositol polyphosphate-5′-phosphatase. A cluster of tyrosine residues (Y) downstream of the PI/PTB domain serve as docking sites for SH2 domain–containing proteins, such as the nonreceptor tyrosine kinases Src, Fyn, and Abl. LDLR, low-density lipoprotein receptor; VLDLR, very low–density lipoprotein receptor; ApoER2, Apolipoprotein E receptor 2; LRP, low-density lipoprotein receptor–related protein; APP, amyloid precursor protein; APLP1 and APLP2, amyloid precursor–like protein 1 and 2, respectively.

tyrosine residues during brain development. Tyrosine phosphorylation of Dab1 promotes an interaction with several nonreceptor tyrosine kinases, including Src, Fyn, and Abl through their SH2 domains, implying Dab1 functions in kinase signaling cascades during development (Howell et al 1997a).

Dab1-related genes in other species are involved in kinase signaling pathways that function during brain development. The *Dab* gene in *Drosophila* was first discovered as a genetic modifier of the Abl tyrosine kinase (Gertler et al 1989). Flies deficient in Abl die as adults and their eyes appear rough owing to irregular spacing of retinal cells (Henkemeyer et al 1987). Haploinsufficiency of *Dab* in an Abl-deficient fly results in axonal pathway defects, and flies that lack both Dab and Abl exhibit frequent breaks in axonal tracts in the CNS (Gertler et al 1993). Dab also physically associates with the sevenless (Sev) receptor tyrosine kinase and contributes to *Drosophila* eye development (Le & Simon 1998). Flies in which the *Dab* gene is inactivated have disorganized eye structures and a frequent loss of R7 photoreceptors. These results demonstrate that Dab is an important adapter protein in both receptor and nonreceptor tyrosine kinase signaling pathways that control formation of the nervous system in flies.

One important binding site for the PTB domain of Dab1 is the peptide sequence Asn-Pro-x-Tyr (NPxY). The NPxY motif was first recognized in members of the low-density lipoprotein receptor (LDLR) family and it is present in several other transmembrane proteins where it functions in clathrin-mediated endocytosis (Chen et al 1990). Interactions between the PTB domains in several adapter proteins and the NPxY motif are dependent on tyrosine phosphorylation (Margolis 1996). However, the PTB domain of Dab1 can bind to NPxY motifs in a phosphotyrosine-independent manner. Biochemical protein interaction assays identified F/YxNPxY motifs in the cytoplasmic domains of the LDLR family and the amyloid precursor protein (APP) family as binding partners of Dab1 (Homayouni et al 1999, Howell et al 1999b, Trommsdorff et al 1998). The PI/PTB domain of Dab1 has also been shown to bind to phosphoinositides present in phospholipid bilayers. The association between Dab1 and phosphoinositides does not interfere with its ability to bind to peptides, suggesting that these two ligands do not compete for the PI/PTB domain (Howell et al 1999b). Therefore, Dab1 could interact with the plasma membrane while simultaneously docking another protein involved in signal transduction events.

The similar neuroanatomical phenotypes observed in mice deficient in *reelin* or *Dab1* imply that these genes function in a signaling pathway that controls cell positioning in the CNS. The spatial and temporal patterns of Reelin and Dab1 expression in several brain regions support this notion (Figure 5). For example, in the cerebellum, Reelin is expressed as early as E13.5 by cells in the nuclear transitory zone (ntz) and the external germinal layer (egl), which contains granule cell precursors (Schiffmann et al 1997, Miyata et al 1996, D'Arcangelo et al 1995). Purkinje cells arise between E11 and E13 from the proliferative zone lining the fourth ventricle and they migrate along glial fibers in the direction of the cells producing Reelin (Goldowitz et al 1997). In situ hybridization and

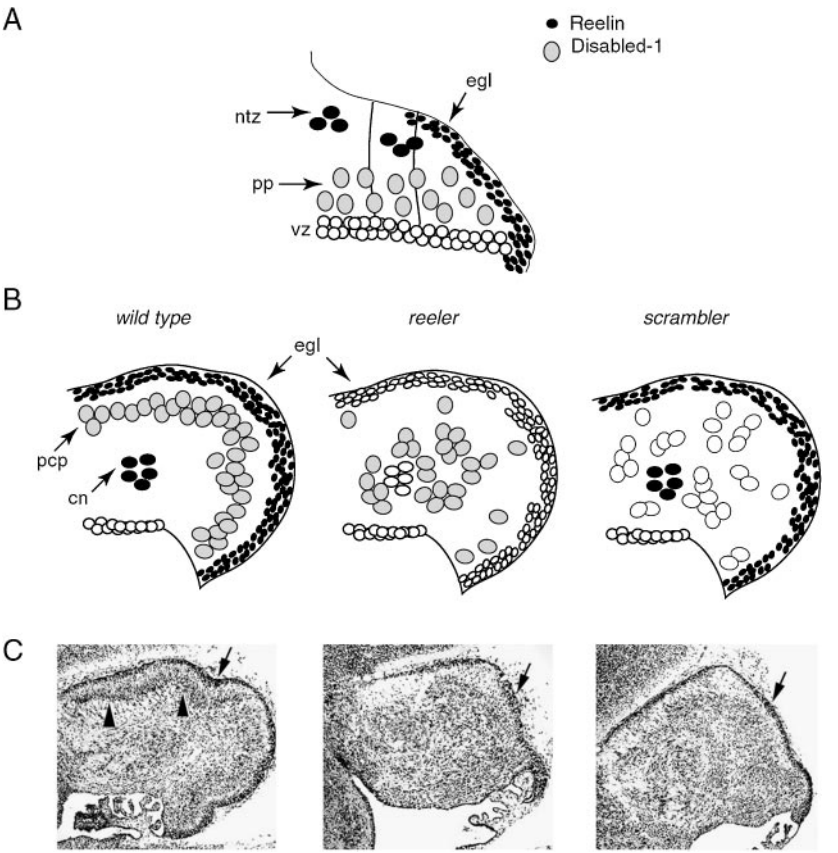


Figure 5 A simplified view of cerebellar development at embryonic days 13.5 and 16.5 and the histological appearance of the cerebellum at embryonic day 16.5 in wild-type, *reeler*, and *scrambler* mice. The IV ventricle is at the bottom of each panel. (*A*) Schematic of a sagittal view of the developing cerebellum at approximately embryonic day 13.5. Reelin (black) is present in the nuclear transitory zone (ntz) and in the external germinal layer (egl) during the initial phase of cerebellar development. The Purkinje cell precursors (pp), which express high levels of Dab1, arise from the ventricular zone (vz) located beneath these Reelin-rich areas and move towards the surface of the cerebellum. (*B*) Several days later in the wild-type, Purkinje cells form a rudimentary Purkinje cell plate (pcp) that is located beneath the egl. Reelin is produced by the granule cell precursors in the egl, which continues to increase in thickness owing to the proliferation of precursors. The cerebellar nuclei (cn), which are likely comprised of cells that moved through the ntz, are located beneath the pcp. These cells eventually lose expression of Reelin. In both *reeler* and *scrambler* Purkinje cells migrate away from the vz, but many fail to form the Purkinje cell plate beneath the egl. Instead, clusters of Purkinje cells are present in the cerebellum. (*C*) Sagittal view of the cerebellum stained with cresyl violet from wild-type, *reeler*, and *scrambler* mice. Rostral is to the left in these micrographs. The Purkinje cell plate (arrowheads) is obvious in the wild-type beneath the egl (arrow). In both *reeler* and *scrambler* the Purkinje cell plate does not form beneath the egl (arrows).

immunohistochemical studies revealed that Purkinje cells express high levels of Dab1 mRNA and protein during the migratory phase of Purkinje cell development (Rice et al 1998, Sheldon et al 1997, Howell et al 1997b). In the normal cerebellum at E16.5 Purkinje cells have already migrated away from their site of origin to form a structure known as the Purkinje cell plate located directly beneath cells that produce Reelin (Miyata et al 1996). However, the Purkinje cell plate does not form in mice lacking *reelin* or *Dab1* (Gallagher et al 1998, Goffinet 1984, Mariani et al 1977).

Two sources of Reelin are present near the Purkinje cell precursors that express Dab1 (Figure 5). Reelin is produced by cells in the ntz and egl, implying that one or both sources could provide a signal to Purkinje cells. Recent genetic evidence suggests that both sources of Reelin are required for the proper positioning of Purkinje cells. Mice with a targeted disruption of *Math-1* fail to form an egl, and consequently the cerebellum lacks granule cells (Ben-Arie et al 1997). Nevertheless, the majority of Purkinje cells migrate to the surface of the cerebellum, implying that transient expression of Reelin by cells in the ntz is sufficient for the migration of many Purkinje cells (Jensen et al 2000). The fact that some Purkinje cells fail to migrate implies that Reelin produced by granule cells in the egl is also required for proper migration. Multiple sources of Reelin are present in other structures affected by the *reeler* mutation. For example, Reelin is expressed initially in the marginal zone of developing cortex, followed by a population of deep cortical plate cells around E17 (Alcantara et al 1998). This additional source of Reelin may contribute to the proper migration of the last cohort of cortical plate neurons.

The close proximity of Reelin-producing cells to the Purkinje cells that express Dab1 is critical for the subsequent organization of the cerebellar cortex. In *reelin*- or *Dab1*-deficient mice the cerebellum lacks characteristic foliation, and the majority of Purkinje cells are found in several clusters beneath the cortex (Figure 5). The lack of cerebellar foliation in the mutants arises as a consequence of a decrease in the number of granule cells. Purkinje cells are known to produce the mitogenic factor sonic hedgehog that acts on granule cell precursors. Presumably, the overall levels of sonic hedgehog that reach the egl are reduced in the mutants owing to the misplacement of Purkinje cells (Wechsler-Reya & Scott 1999). This does not affect the remaining granule cells, which are located in an internal granule cell layer (Goldowitz et al 1997, Mariani et al 1977). Therefore, Reelin and Dab1 are required for the proper placement of Purkinje cells, but not granule cells, in the developing cerebellum.

The expression patterns of *reelin* and *Dab1* in the developing cerebral cortex are consistent with the notion that these genes function during the onset of corticogenesis. *Dab1* is expressed in the forebrain at relatively low and uniform levels throughout the ventricular zone as early as E11.5, whereas *reelin* is present in the Cajal-Retzius cells in the preplate (Rice et al 1998). A dramatic increase in *Dab1* occurs at the onset of cortical plate formation. Migrating neurons destined to form the cortical plate express *Dab1* when they invade the preplate. The first cohort of

cortical plate neurons is unable to invade the preplate in *Dab1*-deficient mice, even though Reelin is present in the marginal zone. This disturbance is similar to that in *reeler* mice, in which the preplate fails to split into the marginal zone and subplate (Figure 2). Immunohistochemical studies using Dab1-specific antibodies revealed more intense staining in the *reeler* cerebral cortex at E16.5 compared with normal, suggesting that there is an increase in Dab1 levels. Immunoblotting analysis confirmed that Dab1 accumulates in *reeler* brain to a level approximately 5 to 10-fold greater than that in normal brain (Rice et al 1998). The peak time of over-expression of Dab1 corresponds to the period in which neuronal migration is underway and when Reelin is required for normal positioning of neurons in the CNS.

Recent biochemical and genetic data suggest that intracellular transduction of the Reelin signal involves activation of a tyrosine kinase cascade involving Dab1 (Howell et al 1999a). Dab1 is phosphorylated in both wild-type and *reeler* neurons, as revealed by immunoprecipitation with Dab1-specific antibodies followed by immunoblotting with antiphosphotyrosine antibodies. In the *reeler* brain the amount of phosphotyrosine in Dab1 is lower than that in normal brain, despite the presence of elevated levels of Dab1. Primary neurons exposed to Reelin-conditioned media display an increased level of phosphorylated Dab1 (Howell et al 1999a). This response is dependent on divalent cations and is not mimicked by a phosphatase inhibitor, suggesting that the Reelin action is mediated through specific receptors expressed on target neurons. The biochemical data imply that Reelin binding to receptors stimulates a kinase that phosphorylates Dab1, leading to the activation of an intracellular signaling cascade.

There are five potential sites for Reelin-induced tyrosine phosphorylation of Dab1 immediately C-terminal to the PI/PTB domain (Figure 4). Analysis of the predicted amino acid sequence indicated that this region is a good substrate for both receptor and nonreceptor tyrosine kinases, such as Src and Abl (Howell et al 2000). To determine if these sites are important during Reelin-induced signaling cascades, mice were generated that express a mutant form of Dab1, in which the five tyrosines were substituted with phenylalanine. Remarkably, these mutant mice display ataxia and disruptions of cell positioning in the cerebral cortex, cerebellum, and hippocampus that closely resemble those in *reelin*- and *Dab1*-deficient mice (Howell et al 2000). Immunoblotting studies revealed that Dab1 is present and that it lacks detectable tyrosine-phosphorylated residues. Moreover, Dab1 levels are slightly elevated in the phosphotyrosine Dab1–deficient brain, implying that Reelin signaling affects turnover of Dab1. Alterations in Dab1 levels likely represent a secondary consequence of Reelin-induced signaling and they are not responsible for the cell positioning defects observed in *reeler, scrambler*, or *yotari* mice. Dab1 is phosphorylated on tyrosine residues in both *reeler* and wild-type brain, suggesting that phosphorylation of Dab1 at basal levels is independent of Reelin. It will be interesting to determine the kinase(s) involved and the specific residues that are phosphorylated in response to Reelin. These sites of Reelin-induced phosphorylation are likely to represent docking areas for protein-protein interactions that are involved in downstream signaling events.

GENETIC EVIDENCE IMPLICATES LIPOPROTEIN RECEPTORS IN REELIN SIGNALING

The PI/PTB in Dab1 binds to the cytoplasmic tails of the five members of the LDLR family and the three members of the APP family of proteins (Homayouni et al 1999; Trommsdorff et al 1998, 1999; Howell et al 1999b). The first indication that lipoprotein receptors function in the Reelin pathway came from a gene disruption study (Trommsdorff et al 1999). Mice deficient in both the very low–density lipoprotein receptor (*Vldlr*) and the apolipoprotein E receptor-2 (*ApoER2*) exhibit behavioral and neuratomical defects that are identical to those in *reeler*. The double mutants are smaller and they exhibit ataxia at two weeks after birth. The cerebellum is decreased in size and lacks foliation. In situ hybridization with a riboprobe recognizing *calbindin*, a gene expressed specifically in cerebellar Purkinje neurons, revealed large clusters of cells beneath the cerebellar cortex. In the hippocampus pyramidal cells are loosely arranged in multiple layers, and granule cells fail to form the histotypical layers in the dentate gyrus. Lamination defects in the cerebral cortex of *ApoER2* and *Vldlr* double mutants are also highly reminiscent of those seen in *reelin* or *Dab1* mutants, in which Layer I is not discernible, and there is an apparent increase in cell density in this area. This observation suggests that both receptors are capable of transmitting the Reelin signal to Dab1 and proper splitting of the preplate. Moreover, Dab1 levels are elevated in mice that lack both *Vldlr* and *ApoER2*, implying that signaling through Reelin and the lipoprotein receptors results in increased turnover of Dab1.

In contrast, mice deficient in only *Vldlr* or *ApoER2* exhibit subtle neuroanatomical defects that do not resemble *reeler*. For example, Layer I in the cerebral cortex appears normal in either *Vldlr* or *ApoER2* knock-out mice, implying that the early events of corticogenesis occur properly. However, cell positioning defects are obvious in other cortical layers in mice lacking *ApoER2*, as revealed by birth-dating experiments (Trommsdorff et al 1999). In mice that lack *vldlr*, a small subset of Purkinje cells is inappropriately positioned in the cerebellum, which appears slightly reduced in size. The CA1 region of the hippocampus proper is thinner, and several pyramidal cells fail to align in the appropriate layer in mice lacking *ApoER2*. Abnormalities in the hippocampus are less dramatic in the *Vldlr*-deficient mice. These comparative studies suggest that each receptor is important during neuronal migration, but that they may exhibit both overlapping and distinct functions in transmission of a signal from Reelin in different brain regions.

The intracellular domains of LDLR family members bind, at least in vitro, to a variety of proteins implicated in kinase signaling, cell adhesion, cytoskelatal organization, vesicle transport, and synaptic transmission (Gotthardt et al 2000). ApoER2 contains a domain in its cytoplasmic tail that associates with the JNK family of interacting proteins, JIP-1 and JIP-2, which are highly expressed in the developing CNS (Stockinger et al 2000). JIPs are kinase scaffolding proteins important in the JNK kinase signaling pathway, and JIP-1 associates with the RhoA

GTPase exchange factor, rhoGEF (Meyer et al 1999). Therefore, extracellular guidance cues could bind to lipoprotein receptors and initiate different responses depending on the adapter molecules and signaling proteins associated with the receptor complex. This may explain the different phenotypes of mice deficient in *Vldlr* compared to those that lack *ApoER2*.

REELIN IS A LIGAND FOR LIPOPROTEIN RECEPTORS

Both *Vldlr* and *ApoER2* are expressed in the target cells of Reelin in several different brain regions during the critical period in which Reelin directs cell positioning (Trommsdorff et al 1999). For example, Vldlr and ApoER2 are expressed in the developing cortical plate and the intermediate zone. *ApoER2* is more widely expressed compared with *Vldlr*, and both receptors are present in similar cell populations that contain *Dab1* (Trommsdorff et al 1999). Although genetic evidence suggested that lipoprotein receptors functioned in the Reelin pathway, it was necessary to obtain direct biochemical evidence to support their role as Reelin receptors. Two different experimental strategies demonstrated that Reelin binds to both Vldlr and ApoER2 with an apparent affinity in the 0.5 nM range (D'Arcangelo et al 1999, Hiesberger et al 1999). The binding was inhibited by the CR-50 antibody, which binds to the N-terminus of Reelin and blocks its function in vivo and in vitro (D'Arcangelo et al 1999, Nakajima et al 1997, Ogawa et al 1995). CR-50 also decreased Reelin-induced phosphorylation of Dab1 in primary neurons, suggesting that Reelin binding to these receptors is critical for activation of a downstream kinase (Senzaki et al 1999). The association of Reelin with the lipoprotein receptors is also blocked by the receptor-associated protein, which acts as a molecular chaperon for the LDLR family (Willnow 1998). The presence of receptor-associated protein or fragments of the VLDLR receptor also blocked Reelin-induced tyrosine phosphorylation of Dab1 (Hiesberger et al 1999). These biochemical studies demonstrate that Reelin binds to Vldlr and ApoER2, resulting in internalization of Reelin and activation of a tyrosine kinase signaling cascade that results in phosphorylation of Dab1 (D'Arcangelo et al 1999). Thus, both genetic and biochemical evidence suggest that Reelin is a ligand for lipoprotein receptors.

Dab1 binds to the cytoplasmic domains of VLDLR, ApoER2, and other LDL-receptor family members, including LDLR, LRP, and Megalin (Howell et al 1999b, Trommsdorff et al 1998). The LDLR family of receptors does not possess intrinsic tyrosine kinase activity, and they have not been shown to physically associate with tyrosine kinases or phosphatases. This raises a question regarding the mechanism whereby Reelin binding to the receptors activates a tyrosine kinase that phosphorylates Dab1. The PI/PTB domain in Dab1 interacts with the receptor tails via an association with the NPxY motif, which is important in clathrin-mediated endocytosis and receptor cycling (Chen et al 1990). Reelin is internalized after binding lipoprotein receptors, suggesting that endocytosis may recruit a tyrosine kinase to Dab1 in vesicles and further activate additional signaling molecules (D'Arcangelo

et al 1999). Alternatively, Reelin may also bind to coreceptors expressed on migrating neurons that have an associated kinase activity.

REELIN BINDS TO OTHER TRANSMEMBRANE PROTEINS EXPRESSED ON NEURONS

Recently, it was reported that Reelin binds to a novel class of proteins (Senzaki et al 1999). This family of transmembrane proteins, known as cadherin-related neuronal receptors (CNRs), contains six ectodomains that are similar to those found in cadherins. CNRs diverge from cadherins in the sequence of their cytoplasmic domains (Kohmura et al 1998). CNR proteins were discovered through their ability to interact specifically with the nonreceptor tyrosine kinase Fyn in yeast two-hybrid assays. Expression studies demonstrated that CNRs are present in synapses throughout the adult brain (Kohmura et al 1998). The function of CNRs is unclear at present, although the similarity of the extracellular domains to cadherins implies that CNRs are involved in homophilic or heterophilic cell-cell interactions.

To investigate the possibility that Reelin binds to CNRs, alkaline phosphatase fusion proteins containing a partial Reelin N-terminal region (amino acids 28–911) were coprecipitated with an Fc fusion protein corresponding to the extracellular domain of CNR1 (Senzaki et al 1999). Using these truncated proteins, it was found that the B domain in the first Reln repeat (Figure 3) bound to CNR1 in vitro. The binding region was mapped to a domain in CNR1 that contains an RGD motif, which functions in ligand receptor interactions (Pierschbacher & Ruoslahti 1984). Mutation of the arginine residue in this motif abolished Reelin binding to CNR1. Rotation cultures, in which cortical neurons were dissociated and allowed to self-aggregate in the presence of an antibody recognizing the RGD region of CNR1, were used to examine the function of CNRs in corticogenesis. In normal aggregates, microtubule-associated protein-2–positive neurons were located on the surface of the aggregate. However, in the presence of the CNR1 antibody many neurons were clustered in the central region, and the aggregate was smaller than controls (Senzaki et al 1999). As noted by the investigators, the histotypic appearance of aggregates obtained following treatment with CNR1 antibodies was quite distinct from that obtained in the presence of CR-50 antibody or in aggregates prepared using *reeler* neurons (Ogawa et al 1995, De Long & Sidman 1970). It is possible that CNRs participate in neurophilic interactions based on the aggregation results. The fact that the cytoplasmic domains of CNRs bind to a kinase that phosphorylates Dab1 in vitro raises the possibility that CNRs participate in a complex with lipoprotein receptors on the surface of migrating neurons. Unlike the situation with the lipoprotein receptors, genetic evidence has not been presented that supports a role for CNR genes in Reelin signaling.

Other transmembrane receptor classes function to direct cell migration in the brain. Gene disruption analysis has shown that $\alpha 3$ integrin plays a role in cell migration during corticogenesis. The migratory patterns of neurons in the cortex

lacking $\alpha 3$ integrin are somewhat distinct from those of *reeler*. For example, neuronal heterotopias are present in the intermediate zone, implying that neurons failed to migrate properly. Neurons born on E13.5 are normally located in the deep layers of the cortical plate, but in $\alpha 3$ integrin–deficient mice these cells are scattered throughout the cortical plate and the intermediate zone (Anton et al 1999). The $\alpha 3$ integrin protein is highly expressed in ventricular zone, migrating neurons in the intermediate zone, and at low levels in the cortical plate (Anton et al 1999). Studies carried out using in vitro migration assays suggested that the primary function of $\alpha 3\beta 1$ integrin is to promote association between migrating neurons and their radial glia guides. In the absence of $\alpha 3$ integrin, neurons alter their preference for radial glia and exhibit an increased propensity to associate with other neurons (Anton et al 1999). Thus, ectopic cells in the cortex and neuronal heterotopias in mice lacking the $\alpha 3$ integrin may arise because of a failure in cell-cell interactions between migrating neurons and the radial glia.

Integrin receptors bind a variety of ligands associated with the extracellular matrix and they mediate cell-cell interactions in many tissues. Functional receptors consist of heterodimers between two subunits encoded by different genes. There are over 20 integrin receptors comprising different combinations of α and β subunits, with differing ligand-binding specificity and associated intracellular signal transduction cascades (Hynes 1992). Recently, Reelin was suggested to bind $\alpha 3\beta 1$ integrin, resulting in an alteration in neuronal migration in vitro (Dulabon et al 2000). Incubation with Reelin-conditioned medium reduced the rate of migration and stimulated neurons to detach from the radial glia in cortical imprint assays. Importantly, these effects were abolished when Reelin was inhibited by treatment with the CR-50 antibody. Moreover, neurons obtained from mice lacking the $\alpha 3$ integrin did not respond to Reelin-conditioned medium in the imprint assay. To determine if Reelin binding to $\alpha 3$ integrin is relevant in vivo, Reelin-coated beads were injected into the intermediate zone of the developing cerebral cortex. This treatment prevented the subsequent migration of BrdU-labeled neurons, whereas neurons that failed to express the $\alpha 3$ integrin ignored the Reelin-coated beads. These observations led to the proposal that Reelin acts as a negative regulator of neuronal migration by inducing changes in cell-cell interactions and detachment from radial glia. Protein association assays carried out using the CR-50 antibody and antibodies against $\beta 1$ integrin, $\alpha 3$ integrin, and a myc epitope tag on Reelin suggested that Reelin bound to $\beta 1$ and, to a lesser degree, $\alpha 3$ integrin (Dulabon et al 2000). Surprisingly, in contrast to the interaction of Reelin with lipoprotein receptors and in vivo and in vitro biological assays of Reelin function, CR-50 did not inhibit the interaction of Reelin with integrin. This implies that multimerization of Reelin is not required for this activity.

The cytoplasmic tail of $\beta 1$ integrin contains two NPxY motifs, one of which has been suggested to function in cell migration by targeting integrin to focal adhesions (Vignoud et al 1994, 1997). Integrins are known to trigger activation of tyrosine kinases such as Fak and Syk, which are involved in signaling transduction pathways that affect cytoskeletal organization (Clark & Brugge 1995).

Interestingly, $\alpha 3$ integrin does not appear to be required for the Reelin-induced tyrosine phosphorylation of Dab1 in primary neurons (Dulabon et al 2000). This implies that the kinase activity associated with Reelin binding to the lipoprotein receptors on neurons is independent of integrin signaling. Moreover, Dab1 levels are decreased in $\alpha 3$ integrin–deficient mice. This is in stark contrast to the situation in *reeler* brains or those that lack both the *Vldlr* and *ApoER2* genes, in which Dab1 accumulates in neurons that go astray (Trommsdorff et al 1999, Rice et al 1998).

GENES THAT MODULATE THE REELIN SIGNALING PATHWAY IN BRAIN

A number of genes have now been suggested to play a role in the Reelin signaling pathway that controls neuronal positioning during development (Table 1). Although many of these have been directly implicated in the Reelin pathway, others appear to influence Reelin signaling through effects on the generation or survival of Cajal-Retzius cells. Targeted disruption of either cyclin-dependent kinase 5 (Cdk5) or its regulatory subunit p35 produce migration defects in brain regions affected in *reeler*. Cdk5 is a member of the cyclin family of serine/threonine kinases that is widely expressed in postmitotic neurons. Cdk5 phosphorylates a variety of substrates associated with the cytoskeleton (Tsai et al 1993). Cdk5 is activated by association with its regulatory subunits p35 or p39 that are expressed in postmitotic neurons (Cai et al 1997, Tsai et al 1994). *Cdk5*-null mice die shortly after birth with failed migrations in the cerebral cortex, cerebellum, and hippocampus (Ohshima et al 1996). The cerebellar phenotype in the $Cdk5^{-/-}$ mice is characterized as a complete block in Purkinje cell migration and a failure in the inward migration of some granule cells towards the internal granule cell layer (Ohshima et al 1999). Less severe, but similar defects are present in the p35-deficient cerebellum (Chae et al 1997). This contrasts with *reeler*, in which granule cell migration is apparently normal, and a small subset of Purkinje cells is capable of migrating to the cerebellar cortex (Mariani et al 1977).

During corticogenesis the preplate splits into the marginal zone and subplate in both Cdk5- and p35-deficient mice. This implies that the migration of the first cohort of cortical plate neurons is independent of Cdk5/p35 (Kwon & Tsai 1998, Gilmore et al 1998). Similar to the situation in the embryonic $\alpha 3$ integrin–deficient cortex, there is an increase in the apparent cell density in the intermediate zone in mice that lack either Cdk5 or p35. Recently, Li et al (2000) demonstrated that $\alpha 1\beta 1$ integrin stimulated Cdk5 kinase activity. It is conceivable that $\alpha 3\beta 1$ integrin activates Cdk5 in the later cohorts of migrating neurons, allowing them to traverse the intermediate zone. Indeed, Cdk5 has been shown to negatively regulate N-cadherin-mediated adhesion via an association between p35 and β-catenin (Kwon et al 2000). This led to the proposal that Cdk5 kinase activity functions to

silence N-cadherin-mediated adhesion in migrating neurons during corticogenesis (Kwon et al 2000). In this model integrin stimulation of Cdk5 would suppress N-cadherin-mediated adhesion among neurons migrating through the intermediate zone and cortical plate, which contains high levels of N-cadherin (Redies & Takeichi 1993). This may explain the failed migrations in later cohorts of neurons in mice that lack either $\alpha 3$ integrin or Cdk5 (Anton et al 1999, Kwon & Tsai 1998, Gilmore et al 1998).

It is interesting to compare the cortical defects observed in mice lacking *Cdk5* to those in *reeler*. In contrast to *reeler*, the preplate splits in $Cdk5^{-/-}$ mice, suggesting that the Reelin/lipoprotein receptor/Dab1 pathway is distinct from that affected by Cdk5. However, it remains possible that Reelin has different effects on early- and late-born cortical neurons. In the later phases of cortical neuron migration Reelin could influence Cdk5 kinase activity, resulting in abrogation of N-cadherin-mediating adhesion (Homayouni & Curran 2000). Thus, Reelin binding to lipoprotein receptors may simultaneously suppress neuronal-glia interactions by internalization of integrin receptors, which may stimulate neurophilic interactions by reducing Cdk5 kinase activity.

ADDITIONAL FUNCTIONS OF THE REELIN SIGNALING PATHWAY IN THE BRAIN

Recent evidence suggests that the Reelin pathway is involved in other aspects of neurodevelopment and in the mature CNS. Soriano and colleagues have shown that Reelin and Cajal-Retzius cells are important for the correct formation of synaptic circuits in the hippocampus. *Reeler* mice display several alterations in the entorhino-hippocampal projections, including reduced axonal branching, decreased number of synapses, and abnormal topography of synapses (Borrell et al 1999a, Del Rio et al 1997). Others have shown colocalization of Reelin and integrins in synaptic clefts, implicating a role for Reelin in synaptogenesis (Rodriguez et al 2000). Application of the Reelin-blocking antibody CR-50 to organotypic cultures obtained from normal entorhino-hippocampal slices recapitulated the fiber pathway abnormalities observed in *reeler*. Moreover, the length of axonal collaterals in the stratum lacunosum-moleculare is decreased in *reeler*, suggesting that Reelin promotes neurite outgrowth (Borrell et al 1999a). In addition, misrouting of commissural afferents is a feature of the *reeler* hippocampus (Borrell et al 1999b). This was attributed to loss of some Cajal-Retzius cells in hippocampus, particularly in the CA3 region, of *reeler* Orleans. These studies show that the overall layer specificity and targeting of afferent projections to the hippocampus are normal in *reeler*, which implies that Reelin may not function as a long-range attractant molecule. This conclusion is consistent with other observations on the major fiber pathways in the *reeler* mutant (Caviness and Rakic 1988). Rather, Reelin functions locally to promote synaptogenesis, axonal branching and collateral outgrowth in

the developing hippocampus. Therefore, Reelin acts in conjunction with other short- and long-range chemoattractive and chemorepulsive cues during formation of synaptic circuitry (Chedotal et al 1998).

Several components of the Reelin signaling pathway have also been reported to be expressed in neurons after completion of cell positioning. In the neural retina *reelin* is expressed by retinal ganglion cells, amacrine cells, and cone bipolar cells (Rice & Curran 2000b). Reelin is present at high levels in the outer and inner synaptic layers. Dab1 becomes apparent during the first postnatal week in a subpopulation of cells in the inner nuclear layer (Rice & Curran 2000a). Interestingly, at postnatal day 6 Dab1 was found to accumulate in type AII amacrine cells of *reeler* mice, suggesting that the Reelin pathway is active in the retina during the period of synaptogenesis. Indeed, the synaptic layering of type AII amacrine cells and rod bipolar cells is defective in mice deficient in Reelin and Dab1. These findings suggest that the Reelin pathway modulates the formation of the scotopic synaptic circuitry in mammalian retina.

The complementary patterns of Reelin and Dab1 expression in other brain regions suggest that they may have functions in adults. For example, in the mature cerebellum, Reelin is expressed in the granule cell layer and the molecular layer, which contains the parallel fibers that synapse on Purkinje cell dendrites expressing Dab1 (Pesold et al 1998, Ikeda & Terashima 1997). Lipoprotein receptors are also present in adult neurons, where they could potentially contribute to a signal transduction pathway affecting the stability of the cytoskeleton (Trommsdorff et al 1999). Many neurons in the adult cerebral cortex that produce γ-aminobutyric acid (GABA) as their primary neurotransmitter also express *reelin* (Alcantara et al 1998). These inhibitory interneurons modulate cortical synaptic circuitry and they synapse on cortical pyramidal cells expressing Dab1 (Rodriguez et al 2000, Pesold et al 1999). Mitral cells and a subpopulation of periglomerular neurons express very high levels of *reelin* in the adult olfactory bulb (Alcantara et al 1998, Ikeda & Terashima 1997). The olfactory bulb actively remodels synaptic connections, suggesting that Reelin may participate in synaptic maintenance or plasticity. In addition, Reelin may be a component of the extracellular matrix that is important for the localization of neurotrophic factors, which contribute to the survival of neurons (Celio & Blumcke 1994). This may relate to the modest loss of certain subpopulations of neurons in the *reeler* brain (Wyss et al 1980, Stanfield & Cowan 1979, Mariani et al 1977).

IS THE REELIN SIGNALING PATHWAY INVOLVED IN NEUROPATHOLOGY?

The interaction of Reelin with lipoprotein receptors is inhibited in the presence of another ligand of the LDL receptor family, apolipoprotein E (ApoE), which is a major constituent of very low–density lipoproteins (D'Arcangelo et al 1999, Mahley

1988). Three alleles of *ApoE* are present in humans, and epidemiological studies have linked *ApoE4* with late-onset and sporadic Alzheimer's disease (Strittmatter & Roses 1996). ApoE3 and ApoE4, but not ApoE2, inhibit Reelin binding to the lipoprotein receptors in vitro (D'Arcangelo et al 1999). Furthermore, whereas the basal level of tyrosine phosphorylation of Dab1 is not altered by treatment of primary neurons with ApoE3 alone, Reelin-induced tyrosine phosphorylation of Dab1 is inhibited. Transgenic mice that express human ApoE4 in neurons display progressive motor abnormalities, wasting, and premature death (Tesseur et al 2000). These mice also exhibit elevated levels of hyperphosphorylated tau, a microtubule-associated protein important in the organization of the cytoskeleton. Hyperphosphorylation of tau, which leads to dissociation of microtubules, has been associated with Alzheimer's disease, and mutations in *tau* are linked to dementia (Hardy et al 1998, Hong et al 1998). A recent study has indicated that increased levels of tau phosphorylation can be detected in mice deficient in *reelin* or both *Vldlr* and *ApoER2* (Hiesberger et al 1999).

Several protein kinases have been implicated in tau hyperphosphorylation, including Cdk5. Recently, p25, a truncated isoform of p35, the regulatory subunit of Cdk5, was found to accumulate in Alzheimer's disease. This isoform constitutively activates Cdk5, resulting in elevated levels of tau phosphorylation (Patrick et al 1999). This may lead to disruption of the cytoskeletal network and formation of paired helical filaments, which are characteristic of Alzheimer's and other neurodegenerative diseases. Dab1 was reported to be an in vitro substrate of Cdk5, and it is tempting to speculate that Cdk5 activity may be modulated by the Reelin signaling pathway (Homayouni et al 1999). Dab1 may affect Cdk5 kinase activity directly or indirectly by interacting with its binding partners such as Abl (Zukerberg et al 2000). Alternatively, Dab1 could influence Cdk5 function by binding to its substrates such as APP (Iijima et al 2000, Homayouni et al 1999, Howell et al 1999b). Thus, it is possible that the Reelin signaling pathway, which controls cell positioning and branching of neuronal fibers during development, may also participate in communicating extracellular signals to alterations in the cytoskeleton, which affect cell shape and physiology in the adult brain. Conceivably, the transmission of a signal from Reelin through lipoprotein receptors to tyrosine phosphorylation of Dab1 may act antagonistically with a pathway involving ApoE, protein kinases, and tau phosphorylation.

Dab1 interacts with the cytoplasmic tails of all three members of the APP family (Homayouni et al 1999, Howell et al 1999b, Trommsdorff et al 1998). A proteolytic product of APP, amyloid β-protein, accumulates in amyloid plaques, and mutations in APP are associated with familial Alzheimer's disease (Selkoe 1998). Dab1 may mediate the intracellular sorting of the cytoplasmic cleavage product of APP, which has recently been associated with neuronal death (Lu et al 2000). Alternatively, Dab1 could affect the production or localization of the mature form of APP by interacting with its cytoplasmic NPxY motif. It is also plausible that Dab1 modulates the endocytotic pathway by competing with other adapter

proteins involved in APP trafficking (Guenette et al 1999). Therefore, it will be interesting to determine if Reelin signaling through Dab1 affects generation of the amyloid β-protein peptide and other APP peptides associated with the pathological progression of Alzheimer's disease (Selkoe 1998).

CONCLUSIONS

According to legend, there is an ancient Chinese curse that states, "May you live in interesting times!" The Reelin field has now entered an interesting period. Over the past few years, a number of proteins have been shown to function in the Reelin signaling pathway. We are now faced with the challenging task of integrating almost 50 years of research on the anatomy and biology of the *reeler* mouse with knowledge of the biochemical properties and expression patterns of these proteins in the developing brain. Although the earliest descriptions of *reeler* defined the mutation as a disorder of cell migration, it is now clear that the genetic lesion compromises a signaling pathway whose biological role is context dependent. For example, in the cerebral cortex migration per se is not affected; rather, there is a failure in cell positioning in the final phase of cortical plate formation. In the cerebellum Purkinje cells complete the first phase of migration, but they fail to form a Purkinje cell plate. Hippocampal pyramidal neurons do not align appropriately, and granule cells of the dentate gyrus are dispersed. Furthermore, the Reelin pathway does not function exclusively in cell positioning, because entorhino-hippocampal projections and retinal synaptic layering are disrupted in the mutant mice. Thus, in discussing the function of Reelin it may not be appropriate to ascribe biological properties such as "attractant" or "stop signal" to an intercellular signal that depends on additional cues in each specific context to elicit an appropriate response.

The biochemical role of Reelin is emerging from studies of the molecular properties of components of the pathway. Reelin is an extracellular protein that is present at specific locations throughout the developing brain. It binds to lipoprotein receptors and is internalized, while activating a tyrosine kinase cascade that leads to phosphorylation of Dab1 (Figure 6). In the developing cerebral cortex the *reeler* phenotype is apparent when the first cohort of cortical plate neurons encounters the preplate (Caviness 1982, Goffinet 1979). This implies that Reelin alters the surface properties of migrating neurons and causes them to insert into the preplate. It is difficult to present a model that accounts for all of the events that have been associated with the Reelin pathway. It is possible that the interaction of Reelin with lipoprotein receptors changes the properties of leading processes on migrating cells or synaptic structures by redistributing and reorganizing cell surface molecules. This could be accomplished by downregulation of adhesion molecules such as integrin, which are thought to mediate the cell-cell contacts among migrating neurons and radial glia, or changes in molecules such as CNRs that could influence homotypic interactions among neurons. Alternatively, Reelin

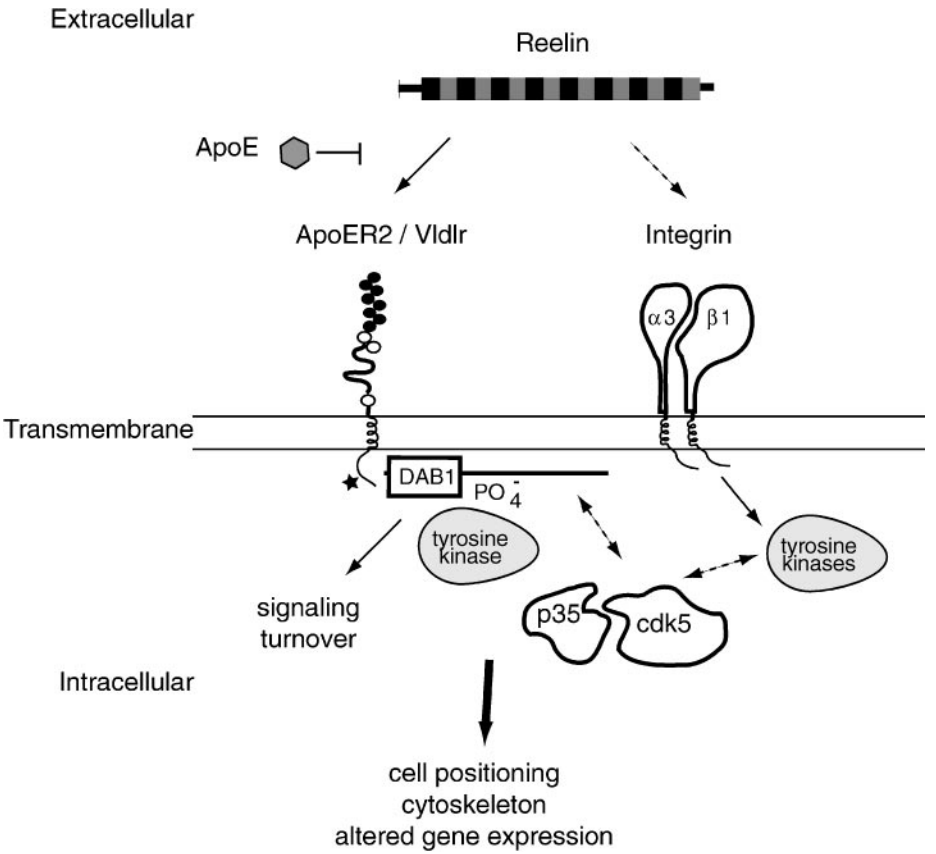


Figure 6 Model of the Reelin signaling pathway based on both genetic and biochemical data. Reelin is an extracellular protein that binds to lipoprotein receptors, Vldlr and ApoER2, expressed on neurons. Dab1 is an adapter protein that associates with an NPxY motif (star) in the cytoplasmic domain of these receptors. Reelin binding to lipoprotein receptors activates a tyrosine kinase, resulting in phosphorylation of Dab1. This effect is inhibited by ApoE. Reelin is internalized following binding to lipoprotein receptors, and Dab1 is degraded. The Reelin signaling pathway activates a complex array of biological responses. Mutations in Cdk5, p35, and integrin elicit similar responses that may be mediated through interactions with the Reelin pathway.

could provoke a more global response through effects on endocytosis, changes in the distribution of intracellular signal transduction molecules such as protein kinases, or through targeting of other proteins to the cell surface. In addition to activating second-messenger cascades, the Reelin pathway may also provoke changes in gene expression that lead to long-lasting alterations in neuronal properties. We hope clues about the molecular events initiated in the target cells of Reelin will emerge in the coming years.

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