Mutant mice with scrambled brains: understanding the signaling pathways that control cell positioning in the CNS

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The characterization of mouse neurological mutants has proven to be an extremely valuable approach to the analysis of central nervous system development (CNS). In particular, over the past four years, remarkable progress has been made toward the identification and characterization of genes that are required for correct cell positioning in the developing brains of mice and humans (D’Arcangelo and Curran 1998; Walsh 1999). This work has provided an impressive collection of genes (Table 1) that play key roles in both the radial and tangential migration of neurons (Pearlman et al. 1998; Hatten 1999).

The classical ataxic mutant mouse reeler has served for many years as a prototype for the investigation of neurological mutations affecting neuronal migration and the organization of the CNS. The hallmark of this mutant is the disruption of neuronal cytoarchitecture in the cerebral cortex, cerebellum, and hippocampus (Caviness 1977, Rakic and Caviness 1995). The molecular basis of the reeler phenotype was uncovered by the fortuitous insertion of a fos transgene into the reeler locus, which led directly to the identification of the reelin (Reln) gene (D’Arcangelo et al. 1995). In short succession, the disabled-1 (Dab1) gene was found to be responsible for a reeler-like phenotype in both man-made and naturally occurring mouse mutants (Howell et al. 1997b; Sheldon et al. 1997; Ware et al. 1997), and related neuronal migration disorders were observed in mice deficient in either the cyclin dependent kinase 5 (Cdk5) gene or its neuronal specific activator p35 (Ohshima et al. 1996; Chae et al. 1997). The saga continued recently with reports that mice lacking both the very low density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor 2 (ApoER2) exhibit anatomical trademarks of the reeler and that Reln binds directly to these receptors, activating a kinase signaling cascade that leads to tyrosine phosphorylation of Dab1 (D’Arcangelo et al. 1999; Hiesberger et al. 1999; Trommsdorff et al. 1999). These new findings, together with studies on the properties of the proteins encoded by these genes, are elaborating a molecular pathway that directs cell positioning in the developing CNS.

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The neurological mutant mouse reeler

The ataxic gait of reeler mice was first described in Fal- coner (1951). Subsequent histopathological studies revealed that Purkinje cells in the reeler cerebellum, which is dramatically decreased in size and which lacks foliation, are located in ectopic clusters beneath the granule cell layer (Mariani et al. 1977). In the cerebral cortex and the hippocampus, neurons fail to align into appropriate cell layers leading to a disruption in the cytoarchitecture of these brain structures (Caviness 1973, 1982). Anatomical abnormalities in reeler are also evident in other important brain structures, such as the thalamus, midbrain, brain stem, and spinal cord (Goffinet 1984; Frost et al. 1986; Yip et al. 1998). Despite these widespread defects, most neurons are capable of making appropriate synaptic connections in the reeler mouse, suggesting that the primary role of the reeler gene product is to direct the positioning of specific neuronal classes in the developing brain (Rakic and Caviness 1995).

Reln is a secreted protein

The identification and characterization of the Reln gene have been reviewed in recent publications (Curran and D’Arcangelo 1998; D’Arcangelo and Curran 1998). This important gene is conserved in a number of vertebrate species. For example, in mice (accession no. U24703) and humans (U79716), the nucleotide sequence is 87% identical and the amino acid sequence is 94% identical (D’Arcangelo et al. 1995; DeSilva et al. 1997). In mice, the Reln locus is composed of 65 exons spread over a region of ~450 kb on chromosome 5 (Royaux et al. 1997). Reln is encoded by a very large mRNA (~12 kb) that contains an ORF of 10,383 bases encoding a protein of ~385 kD (D’Arcangelo et al. 1995). The amino terminus of Reln contains a cleavable signal peptide and a region of similarity with F-spondin, a protein secreted by floorplate cells that directs neural crest cell migration and that may function as an inhibitory signal during boundary formation (Klar et al. 1992; Burstyn-Cohen et al. 1999; Debby-Brafman et al. 1999). The main body of Reln consists of a series of eight internal repeats (Reln repeats).
of 350–390 amino acids. Each Reln repeat contains two related subdomains flanking a pattern of conserved cysteine residues related to the epidermal growth factor (EGF)-like motif. A short region of positively charged amino acids near the carboxyl terminus is required for secretion (D’Arcangelo et al. 1997). In reeler2J mice, a large deletion in the genomic locus results in loss of Reln mRNA expression (D’Arcangelo et al. 1995). Reln can be immunoprecipitated from the supernatant of primary neuronal cultures and COS cells transfected with a Reln construct, indicating that it is an extracellular protein (D’Arcangelo et al. 1997). Recently, it was suggested that Reln is proteolytically processed by a metalloproteinase into two smaller proteins of ~250 and 180 kD (de Rouvrot et al. 1999). The truncated fragments are likely generated by the activity of a protease present in the post-Golgi secretory pathway or by an extracellular protease as they are not detectable in Relnorl mice, which fail to secrete Reln, or in brefeldin-treated cell extracts.

**Reln and cortical development**

The mammalian brain comprises a highly ordered array of neurons connected by myriad projections. This complex structure assembles via a choreographed series of cell 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

| Gene | Name | Protein type | Chromosome<sup>a</sup> | References |
|------|------|--------------|-------------------------|------------|
| ApoER2 | apolipoprotein E receptor 2 | transmembrane | N.D. | Trommsdorff et al. [1999] |
| Astn | astrotactin | secreted glycoprotein | N.D. | Hatten [1999] |
| Cdk5 | cyclin-dependent kinase 5 | cyclin-dependent kinase | 7q36 | Ohshima et al. [1996] |
| Cdk5r | p35 | cyclin-dependent kinase | 11 N.D. | Chae et al. [1997] |
| Cxcr4 | chemokine receptor 4 | receptor | 1 2 | Zou et al. [1998] |
| Dab-1 | disabled 1 | signal transduction | 1q | Howell et al. [1997b]; Sheldon et al. [1997] |
| Dlx-1,2 | distal-less homeobox 1,2 | transcription factor | 2 2q32 | Anderson et al. [1997] |
| Dcx | doublecortin | microtubule-associated protein | X Xq22–23 | des Portes et al. [1998]; Gleeson et al. [1998] |
| Erbb4<sup>b</sup> | avian erythroblastsis oncogene B4 | tyrosine kinase receptor | N.D. 2q34 | Rio et al. [1997] |
| Fgf-2 | fibroblast growth factor 2 | growth factor | 3 4q25–27 | Dono et al. [1998] |
| Flna | filamin α | actin-binding protein | X Xq28 | Fox et al. [1998] |
| FCMD | fukutin | secreted glycoprotein | N.D. 9q31 | Kobayashi et al. [1998] |
| Itga3 | integrin α 3 | integrin receptor subunit | 11 N.D. | Anton et al. [1999] |
| Itga6 | integrin α 6 | integrin receptor subunit | 2 2 | Georges-Labouesse et al. [1998] |
| KAL1 | Kallmann’s syndrome | novel protein | N.D. Xp22.3 | Franco et al. [1991] |
| Lama2 | laminin α 2 | extracellular matrix | 10 6q22–23 | Helbling-Leclere et al. [1995] |
| Pafah1b1 | lissencephaly-1 | subunit of platelet activating factor acetylhydrolase | 10 17p13.3 | Reiner et al. [1993] |
| MARCKS | myristoylated alanine-rich C-kinase substrate | neural substrate for PKC | 10 6q22 | Blackshear et al. [1997] |
| Ncam | neural cell adhesion molecule 180 | adhesion molecule | 9 11q22.2 | Tomasiewicz et al. [1993] |
| Ntn-1 | netrin-1 | extracellular ligand | 11 17q12 | Bloch-Gallego et al. [1999] |
| Ntf-4/5† | neurophin 4/5 | neurotrophin | 7 19 | Brunstrom et al. [1997] |
| Hg 1<sup>b</sup> | neuregulin | growth factor | N.D. 8 | Anton et al. [1997] |
| Pax6 | paired box gene 6 | transcription factor | 2 11p13 | Caric et al. [1997] |
| Pex2 | peroxisome assembly factor 1 | peroxisome membrane | N.D. 8 | Faust and Hatten [1997] |
| Pex5 | peroxisome receptor 1 | peroxisomal import receptor | N.D. 12p13 | Baes et al. [1997] |
| Ptn | pleiotrophin | ligand for receptor-like protein | 6 7q33 | Maeda and Noda [1998] |
| Reln | reelin | extracellular protein | 5 7q22 | d’Arcangelo et al. [1995] |
| Slit-1 | Slit Drosophila homolog 1 | secreted | N.D. 10q23.3 | Wu et al. [1999] |
| Unc5h3 | UNC-5 C. elegans homolog 3 | netrin receptor | 3 N.D. | Ackerman et al. [1997]; Leonardo et al. [1997] |
| Vldlr | very low density lipoprotein receptor | transmembrane | 19 9p24 | Trommsdorff et al. [1999] |

<sup>a</sup>[N.D.] Not determined.

<sup>b</sup>Mice deficient in these genes die at E10.5; therefore, their role in neuronal migration in vivo is not known.
which they exit the cell cycle and migrate to their ultimate locations, where they adopt a definitive neuronal phenotype. For example, in the cerebral cortex, neurons that become postmitotic early in development occupy deep laminae, whereas those exiting the cell cycle at later stages are located in superficial laminae (Rakic 1974; Luskin and Shatz 1985; McConnell and Kaznowski 1991).

Reln is produced and secreted from distinct neuronal populations during development of the cerebral cortex. Immunostaining and in situ hybridization with Reln-specific probes reveal a discrete layer of cells distributed across the surface of the telencephalic neuroepithelium (D’Arcangelo et al. 1995; Ogawa et al. 1995). These cells are known as Cajal-Retzius neurons, which were named after their co-discoverers in rabbits and humans, respectively [Konig 1978; Marin-Padilla 1998]. These transient cells are among the earliest neurons to be generated in the mammalian neocortex, in which they occupy positions near the pial surface on the superficial aspect of the brain in a region known as the preplate or primordial plexiform layer (Konig et al. 1977; Meyer and Goffinet 1998). The preplate is composed of Cajal-Retzius neurons, subcortical projections, and subplate neurons [Super et al. 1998]. Neocortical development begins with the appearance of the preplate above the ventricular zone (Fig. 1). The next phase of development occurs when the first cortical plate neurons exit the cell cycle near the ventricular surface and migrate along radial glial fibers to invade the preplate. These cells 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 (Fig. 1). As new cortical plate neurons arrive on the same glial fibers, they migrate past the older neurons in the subplate and cortical plate before inserting directly beneath the Reln-rich marginal zone. The systematic migration of younger neurons past those generated at earlier times results in the classic inside-out pattern of development in the mammalian neocortex, in which the cortical plate [future layers II–VI] develops between the marginal zone [future layer I] and the subplate [Angevine and Sidman 1961; Rakic 1988].

Although the preplate forms in the embryonic reeler brain, the first cohort of migrating cortical plate neurons fails to invade this region. As a result, the preplate is not split into the marginal zone and the subplate [Caviness 1982; Hoffarth et al. 1995; Ogawa et al. 1995; Sheppard and Pearlman 1997]. As additional cohorts of neurons arrive via the radial guides, they are unable to bypass their predecessors and instead, they accumulate underneath in a disorganized cortical plate (Fig. 1). This abnormality can be recapitulated in rotating cultures of reeler neurons or by incubating normal neurons with the CR-50 antibody, which inhibits Reln function [Ogawa et al. 1995; Nakajima et al. 1997]. The mechanism responsible for this phenotype is not clear. It is possible that Reln acts as a repellent for subplate neurons to make room for the cortical plate neurons in the preplate. Alternatively, Reln could bind directly to cortical neurons and signal an end to the migration process phase or it could modify the interactions between neurons and radial fibers or interactions among cortical neurons. The observation that early generated cohorts of neurons in reeler mice are
more adhesive than their normal counterparts suggests that cell–cell interactions are disrupted in the absence of Reln (Hoffarth et al. 1995). Even though the exact mechanism has not yet been defined, it is clear that Reln in the marginal zone provides a local cue that directs the laminar organization of the cerebral cortex.

**The neurological mutant mouse scrambler**

Shortly after Reln was identified, Davisson and colleagues at the Jackson Laboratory (Bar Harbor, ME) described a reeler-like phenotype in a new spontaneous mouse mutant named scrambler (Sweet et al. 1996). These defects were associated with an autosomal recessive mutation that arose in the dancer (DC/Le) strain of mice. Genetic mapping of scrambler placed this locus on chromosome 4, implying that the gene responsible was distinct from Reln. Scrambler mice exhibit cerebellar abnormalities, namely the ectopic position of Purkinje cells and the subsequent reduction in the number of granule cells, identical to those in reeler. However, Reln is produced at normal levels in the scrambler cerebellum and it is secreted from primary cultures of scrambler granule cells (Goldowitz et al. 1997). The similarity between the reeler and scrambler phenotypes also extends to the hippocampus and the cerebral cortex. Although neurons are generated at correct times, the normal inside-out sequence of neurogenesis in the cortex is relatively inverted in scrambler (Gonzalez et al. 1997). These observations led to the suggestion that the scrambler gene acts within a signaling pathway activated by Reln.

**Identification of the scrambler gene: the cytoplasmic adapter protein Dab1**

Several groups were searching for the scrambler gene by a variety of approaches when an inroad was provided by a gene disruption study of mouse Dab1. Dab1 was originally identified as a Src-binding protein in a yeast two-hybrid screen (Howell et al. 1997a). Dab1 (Y08379) encodes a cytoplasmic protein that contains a motif known as a protein interaction/phosphotyrosine binding (PI/PTB) domain (Margolis 1996). Tyrosine phosphorylation of Dab1 promotes an interaction with several nonreceptor tyrosine kinases, including Src, Fyn, and Abl, through their SH2 domains (Table 2), implying that Dab1 functions in signal-transduction processes. Dab1 is expressed predominately in the CNS and it is phosphorylated on tyrosine residues during brain development (Howell et al. 1997a). Remarkably, mice with a targeted disruption of Dab1 exhibit ataxia and a neuroanatomical structure indistinguishable from those described in reeler. Importantly, Dab1 mutations do not alter the expression of Reln (Goldowitz et al. 1997). This implies that Dab1 is involved in the transduction of a Reln-initiated signaling pathway that controls the positioning of neurons in the CNS.

**Dab-related genes**

The Dab gene was first discovered in Drosophila as a genetic modifier of the Abl cytoplasmic tyrosine kinase (Gertler et al. 1989). Flies deficient in Abl die as adults and their eyes appear rough due to irregular spacing of retinal cells [Henkemeyer et al. 1987]. Haploinsufficiency of Dab in an Abl−/− fly results in defects in axonal pathways and flies that lack both Dab and Abl exhibit frequent breaks in axonal tracts in the CNS (Gertler et al. 1993). These results suggest that Abl and Dab are involved in a common molecular cascade that controls the formation of axonal pathways.

Dab also contributes to Drosophila eye development through its association with a signaling pathway involving the sevenless (Sev) receptor tyrosine kinase. Sev is expressed in R7 precursor cells and it is activated by the ligand, bride of sevenless (Bos), which is expressed on R8 cells and the subsequent reduction in the number of granule cells, identical to those in reeler. However, Reln is produced at normal levels in the scrambler cerebellum and it is secreted from primary cultures of scrambler granule cells (Goldowitz et al. 1997). The similarity between the reeler and scrambler phenotypes also extends to the hippocampus and the cerebral cortex. Although neurons are generated at correct times, the normal inside-out sequence of neurogenesis in the cortex is relatively inverted in scrambler (Gonzalez et al. 1997). These observations led to the suggestion that the scrambler gene acts within a signaling pathway activated by Reln.

**Table 2. Mouse Dab1 binding proteins**

| Protein* | Name | Function |
|----------|------|----------|
| Nonreceptor tyrosine kinase | Abl | signal transduction |
| | Fyn | signal transduction |
| | Src | signal transduction |
| Amyloid precursor protein family | APP | unknown |
| Lipoprotein receptor family | APLP1 | unknown |
| | APLP2 | unknown |
| | ApoER2 | neuronal migration |
| Phosphoinositide phosphatase | LDLR | cholesterol homeostasis |
| | LRP | unknown |
| | VLDLR | neuronal migration |
| | Ship | signal transduction |

*Identified by Trommsdorff et al. [1998, 1999]; Homayouni et al. [1999]; Howell et al. [1997a, 1999b].
photoreceptor cells. Following activation, the adapter protein Drk links Sev to the guanine nucleotide exchange factor known as Son of sevenless (Sos) stimulating a Ras-activated protein kinase pathway. This engages a series of downstream events that specifies the fate of R7 photoreceptor cells (Zipursky and Rubin 1994). Flies in which the Dab gene is inactivated have disorganized eye structures and a frequent loss of R7 photoreceptors. Additional domains of Dab interact with other components of the Sev pathway including Drk, the fly homolog of mammalian adapter protein Grb2 (Le and Simon 1998). These studies implicate Dab in tyrosine kinase signaling pathways that control the development of the nervous system in Drosophila.

A number of species contain Dab-related genes. The most conserved region of Dab is the protein interaction domain. A Dab-related gene is present in Caenorhabditis elegans (Z49968), although there are no reports about its function. Mammals contain at least two Dab orthologs. The first to be isolated in mice was p96 (U18869), which is now referred to as Dab2 (Xu et al. 1998). The function of Dab2 is unknown, but it is phosphorylated on serine residues in response to mitogenic growth factor stimulation. The human DOC2 gene (NM001343), the ortholog of mouse Dab2, has been implicated in ovarian tumorogenesis [Mok et al. 1998]. Recently, it was shown that Dab2 binds Grb2 and that it may act as a competitor with Sos in the Ras signaling pathway [Xu et al. 1998]. These studies suggest that some protein–protein interactions involving Dab are conserved among species.

Reln and Dab1 are expressed in adjacent cells in the developing brain

The similar neuroanatomical phenotypes in Reln−/− or Dab1−/− mice imply that these molecules function in a common signaling pathway. This hypothesis is supported by the patterns of expression of these genes during the formation of several brain regions. At the initial stages of cortical development, migrating neurons destined to form the cortical plate express Dab1 when they invade the preplate, in which Reln is expressed at high levels [Fig. 1]. This may explain the failure of cortical plate neurons to invade the preplate and split this layer into the marginal zone and subplate in scrambler mice (Rice et al. 1998). During formation of the hippocampus, pyramidal cells express Dab1 as they move in the direction of the outer molecular layer (oml). The oml contains Cajal-Retzius-like cells that produce Reln [Nakajima et al. 1997; Alcantara et al. 1998]. The positioning of neuronal cells into compact layers in the dentate gyrus arises following the migration of granule cells that express Dab1 towards cells that contain Reln. In Reln−/− and Dab1−/− mice, the organization of neurons in the dentate gyrus is disrupted.

Reln and Dab1 are also expressed in neighboring cells in the developing cerebellum. Reln is expressed by cells in the external germinal layer (EGL) located on the outer surface of the cerebellum (D’Arcangelo et al. 1995; Miyata et al. 1996; Schiffmann et al. 1997). The EGL contains precursors of the cerebellar granule cells. Purkinje cell precursors, which arise from the ventricular zone lining the fourth ventricle, lie directly beneath the EGL and express high levels of Dab1 (Howell et al. 1997b; Sheldon et al. 1997; Rice et al. 1998). In the normal cerebellum at E16, Purkinje cells have migrated away from their site of origin to form a structure known as the Purkinje cell plate, that is located directly beneath cells producing Reln [Miyata et al. 1997]. The Purkinje cell plate does not form in mice lacking Reln or Dab1 and the majority of Purkinje cells remain in clusters deep within the cerebellum [Mariani et al. 1977; Goffinet et al. 1984; Goldowitz et al. 1997; Gallagher et al. 1998]. As a consequence of this failure in cell migration, Purkinje cells do not reside close enough to granule cells to support their proliferation. This may arise from a failure of the granule cell mitogen sonic hedgehog, that is produced by Purkinje cells, to reach the cell precursors in the EGL [Dahmane and Ruiz-I-Altaba 1999; Wallace 1999; Wechsler-Reya and Scott 1999]. The net effect of the abnormal positioning of Purkinje cells is a lack of cerebellar foliation due to a paucity of granule cells and a disruption in the physiological function of this important laminated brain structure. Therefore, Reln and Dab1 are expressed in adjacent cell populations prior to the time when neuroanatomical defects become apparent in either reeler or Dab1-deficient animals, implying that neurons expressing Dab1 respond to Reln just as they stop migrating and detach from radial glia.

Dab1 levels are increased in reeler brain

Dab1 is thought to function downstream of Reln in a signaling pathway that controls cell positioning in the developing brain. Surprisingly, immunohistochemical signals obtained with Dab1-specific antibodies are more intense in reeler compared with normal brain, suggesting that there is an increase in Dab1 levels. Immunoblotting analysis shows that Dab1 accumulates in reeler brain to a level ~5- to 10-fold greater than that in normal brain (Rice et al. 1998). The peak time of overexpression of Dab1 corresponds to the period in which neuronal migration is underway and when Reln is required for normal positioning of neurons in the CNS. This increase could arise if, under normal circumstances, Dab1 is degraded after fulfilling a signaling function evoked by Reln as part of a switch mechanism that controls cell positioning. Alternatively, it is possible that the absence of Reln results in an increase in the translation rate of Dab1, leading to higher levels of protein. Regardless of the mechanism responsible for the increased levels of Dab1 in reeler, this finding establishes a biochemical link between Reln and Dab1.

Reln and Dab1 are expressed in adult brain

Interactions among neurons that express Reln and Dab1 may extend beyond the control of cell positioning during
neurons that express expression of Reln in the marginal zone, many cortical neurons that express y-aminobutyric acid as their primary neurotransmitter also express Reln (Alcantara et al. 1998; Pesold et al. 1998; Super et al. 1998). These cells function as intrinsic interneurons that modulate cortical synaptic circuitry. Interestingly, many of these cells synapse on cortical pyramidal cells, which express Dab1 (Pesold et al. 1999). Purkinje cells in the adult cerebellum express Dab1 at high levels in their cell soma and dendrites but not in their axons. Granule cells provide a primary synaptic input to Purkinje cells and they express Reln at high levels (Gallagher et al. 1998; Pesold et al. 1998). Reln and Dab1 are also expressed in adjacent cells in the olfactory bulb and in the retina, structures that do not show dramatic alterations in cell positioning in reeler or in mice deficient in Dab1. Therefore, it is possible that Reln and Dab1 fulfill signaling functions in mature neurons.

Expression of Reln and Dab1 in other tissues

Reln is also expressed in non-neuronal tissues in developing and adult mice. High levels of Reln are present in liver and kidney, and lower levels are expressed in several other organs [Ikeda and Terashima 1997]. In reeler, T-cell and macrophage functions are reported to be attenuated, suggesting that Reln may be important for cellular responses in the immune system [Green-Johnson et al. 1995]. Alternatively, defects in the immune system may be secondary to changes in the biochemical environment in the reeler brain. However, the cellular organization of the internal organs that contain Reln appears normal in reeler mice [Ikeda and Terashima 1997]. Dab1 is predominantly restricted to the CNS, but expression has been noted in several hematopoietic cell lines [Howell et al. 1997a]. As we begin to understand more about Reln and the transduction of intracellular responses by Dab1, it may be possible to define the function of these genes in other organs. An important piece of the puzzle would be to identify proteins that associate with Reln and Dab1.

Dab1 contains a protein interaction domain

Dab1 is capable of docking proteins inside the cell via its PI/PTB domain. This provides a useful approach to the identification of membrane receptors and other components of a signaling pathway linking Reln and Dab1. The PTB domain was originally identified in the adapter protein Shc because of its ability to bind the epidermal growth factor (EGF) receptor, the insulin receptor, and other tyrosine-phosphorylated proteins [Margolis 1996]. One important binding site for the PTB domains of Shc and another adapter protein named IRS-1 (insulin receptor substrate) 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 in which it is important for clathrin-mediated endocytosis [Chen et al. 1990]. Although the binding specificities of Shc and IRS-1 are slightly different, the interaction of the PTB domain with the NPxY motif is dependent on tyrosine phosphorylation [Gustafson et al. 1995; van der Geer and Pawson 1995; Farooq et al. 1999].

Other adapter proteins with PTB domains can bind to NPxY motifs in a phosphotyrosine-independent manner. This is true for the interaction between the amyloid precursor protein (APP) and the brain-enriched adapter proteins Fe65 and X11 [Fiore et al. 1995; Borg et al. 1996]. APP is a transmembrane protein that is subject to proteolytic cleavage. Recent evidence suggests that X11 and Fe65 may be involved in the processing of APP by regulating different aspects of protein trafficking. Fe65 and APP have been shown to colocalize in the endoplasmic reticulum/Golgi and in vesicles that resemble endosomes. Overexpression of Fe65 results in a substantial increase in the secretion of proteolytic fragments of APP, which may be due to increased amounts of APP at the cell surface [Sabo et al. 1999]. Binding of the X11 PTB domain to the NPxY motif in APP increases the half-life of this protein in cultured cells, resulting in decreased levels of the secreted cleavage product of APP, amyloid peptide [Borg et al. 1998; Sastre et al. 1998]. Thus, adapter proteins may have important biological functions as the aberrant processing and trafficking of APP and the generation of the amyloid β peptide are associated with the pathological progression of Alzheimer’s disease [Selkoe 1994].

The PTB domain also mediates other important docking functions. The three-dimensional structure of the PTB domain is very similar to the pleckstrin-homology (PH) domain, although the primary sequences of these peptides are different [Zhou et al. 1995]. The PH domain binds phosphoinositides and it is present in proteins that function in signal transduction and cytoskeletal organization [Bottomley et al. 1998]. The PH domain of the GTPase dynamin localizes dynamin to the plasma membrane, where it is required for clathrin-mediated endocytosis [Vallis et al. 1999]. Recently, the PI/PTB domain of Dab1 has 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]. This contrasts with the properties of the PTB domain of Shc, which binds phospholipids and targets Shc to the cell membrane allowing it to interact with phosphorylated receptors [Ravichandran et al. 1997]. Mutations in Shc that prevent association with phospholipids result in a disruption of signal transduction following receptor activation. Therefore, potentially Dab1 could interact with the plasma membrane while simultaneously docking another protein involved in signal transduction events.
Dab1 binds to cell surface proteins

Dab1 has been shown to specifically interact with several proteins expressed on the cell surface (Table 2). Protein interaction screens in yeast and coexpression experiments in vitro identified APP and the related proteins, amyloid precursor-like proteins 1 and 2 [APLP1 and APLP2] as binding partners of Dab1 ([Trommsdorff et al. 1998; Homayouni et al. 1999; Howell et al. 1999b]). The interaction of Dab1 with NPxY motifs present in the cytoplasmic domains of the APP family of proteins does not require tyrosine phosphorylation. In fact, phosphorylation of the tyrosine in the NPxY-containing peptide strongly inhibits binding of APP to the PI/PTB domain of Dab1 ([Howell et al. 1999b]). The association of APLP1 with Dab1 in cotransfected mammalian cells results in enhanced serine phosphorylation of Dab1, suggesting that Dab1 may be part of a signaling mechanism involving multiple protein kinases ([Homayouni et al. 1999]). Dab1 associates preferentially with APLP1 compared with APP and APLP2, whereas, Fe65 binds strongly to APP ([Bressler et al. 1996; Homayouni et al. 1999]. Fe65 contains two protein interaction motifs arranged in a linear fashion. The aminoterminal interaction domain binds to the low-density lipoprotein receptor-related protein [LRP] and the carboxy-terminal interaction domain binds to APP, suggesting that Fe65 may link LRP and APP at the cell membrane and affect the processing of APP ([Trommsdorff et al. 1998]. Dab1 also binds to the cytoplasmic tail of LRP and it may be recruited to a large complex at the cell surface ([Trommsdorff et al. 1999]. These results imply that different PI/PTB domain-containing proteins selectively interact with different members of the same family of proteins.

APP and apolipoprotein E [apoE] are ligands for LRP that, along with presenilins 1 and 2, have been linked to Alzheimer’s disease ([Price et al. 1998]). Primary cultures of embryonic hippocampal neurons and neurons in the adult hippocampus coexpress Dab1, APP, and APLP1 ([Homayouni et al. 1999; Howell et al. 1999b]). APP has been shown to be involved in a variety of cellular functions in the nervous system such as differentiation, attachment, and survival ([Selkoe 1994]). APP is thought to affect neurite outgrowth through its ability to bind several extracellular matrix proteins ([Qiu et al. 1995; Beher et al. 1996]). APP and APLP1 are expressed in the developing cortex, implying that they are involved in some aspect of neurodevelopment ([Lorent et al. 1995]). In transfected cells, Dab1 and APLP1 colocalize in membrane ruffles and intracellular vesicles, suggesting that interactions between these proteins may be important for vesicular trafficking within the cell ([Homayouni et al. 1999]). Mice deficient in either APP, APLP1, APLP2, or in combinations of these genes have been generated ([von Koch et al. 1997; U. Müller, S. Stahl, S. Sisodia, A. Aguzzi, and B. Lwoub, unpubl.]), but there are no reports of phenotypes similar to Rln−/− mice.

Members of the LDLR family also bind to the PI/PTB domain of Dab1, presumably through an interaction with the NPxY motif in their cytoplasmic tails ([Trommsdorff et al. 1998, 1999; Howell et al. 1999b]). There are currently five mammalian members of this receptor family that can be categorized into two groups by molecular mass. The LDLR, VLDLR, and ApoER2 are ~95–105 kD, whereas LRP and megalin [gp330] are ~600 kD ([Willnow 1999]). The low molecular weight receptors are composed of five distinct modules. On the extracellular surface of the receptors, there is a series of ligand-binding repeats comprised of ~40 amino acids. Each ligand-binding repeat is rich in cysteines and negatively charged amino acids that are important for ligand-receptor interactions. The ligand-binding repeats are typically followed by EGF precursor repeats, separated by spacer regions containing WYTD motifs. These regions are important for the dissociation of the ligand from the receptor in endosomal compartments. Each receptor has a single transmembrane domain followed by a cytoplasmic tail that contains an internalization signal with the NPxY motif. The larger receptors also contain these same five modules, but they possess different combinations of the ligand-binding repeats and EGF precursor homology domains such that their extracellular modules appear as a mosaic of the smaller receptors. The LDL-related receptors in C. elegans and Drosophila are similar in structure to mammalian LRP and Megalin, implying that the smaller receptors evolved from the large members of the LDLR family ([Willnow 1999]).

Disruption of lipoprotein receptor genes VLDLR and ApoER2 results in a reeler-like phenotype

Recently, behavioral and neuroanatomical defects similar to those observed in reeler were reported in mice in which two members of the LDL receptor family, VLDLR and ApoER2, were disrupted by gene targeting ([Trommsdorff et al. 1999]). ApoER2−/−/VLDLR−/− mice are smaller than normal and they exhibit ataxia at 2 weeks after birth. Purkinje cells are located in ectopic clusters deep in the cerebellum, which is decreased in size and lacks foliation. In the hippocampus, pyramidal cells are loosely arranged into multiple layers and granule cells fail to form distinct neuronal layers. Although neurogenesis proceeds normally, neurons fail to find their appropriate positions within the cerebral cortex. These lamination defects in ApoER2−/−/VLDLR−/− brain are highly reminiscent of those seen in Reln−/− and Dab1−/− mice, implying that these receptors are involved in a common signaling pathway.

Disruption of either ApoER2 or VLDLR alone causes neuroanatomical phenotypes that are more subtle than those observed in mice deficient in both receptors. For example, the marginal zone is discernible in the cerebral cortex of ApoER2−/− mice, despite clear alterations in the layering of neurons in laminae II–VI. In contrast, the marginal zone in ApoER2−/−/VLDLR−/− mice is not apparent and neurons occupy ectopic positions in the most superficial aspect of the cortex. In the VLDLR−/− cerebellum, Purkinje cells are not aligned in a discrete layer and the dendritic arborizations of these cells are not as extensive as those in the normal cerebellum ([Trommsdorff
et al. 1999). These findings demonstrate that one receptor can partially compensate for the loss of the other receptor, suggesting that ApoER2 and VLDLR have similar functions.

The lipoprotein receptors

The VLDLR gene [D11100] was first isolated from a rabbit heart library by low-stringency hybridization with a probe derived from the LDL receptor [Takahashi et al. 1992]. The ApoER2 gene [D50678] was isolated from human placenta with degenerate oligonucleotides corresponding to the FDNPVY internalization sequence in the cytoplasmic tail of the LDLR family [Kim et al. 1996]. The chicken homolog [X97001] of human ApoER2 and a partial mouse cDNA clone were isolated from brain mRNA with degenerate oligonucleotides corresponding to the EGF precursor-domain sequence in the extracellular portion of the receptors [Novak et al. 1996]. In these species, the receptor has been given the name LR8B because it is related to the LDL-receptor [LR], it contains eight ligand binding repeats, and it is expressed predominantly in the brain. Subsequent studies have shown that ApoER2 and LR8B are orthologs in humans and birds and that the gene products are differentially spliced [Brandes et al. 1997; Clatworthy et al. 1999].

The structural features of VLDLR and ApoER2 are similar to those of the LDLR. However, there are some differences among these receptors that could be important for their function. One difference relates to the number of ligand-binding repeats in the extracellular domain. LDLR contains seven ligand binding repeats, whereas VLDLR contains eight repeats, and ApoER2 contains seven or eight, depending on the species [Brandes et al. 1997; Nimpf and Schneider 1998; Clatworthy et al. 1999]. The number of ligand-binding repeats and the linear arrangement of the repeats are believed to be important for binding specificity [Schneider et al. 1997]. Alternative splicing of LR8B [ApoER2] in mice and humans produces a receptor containing an additional 59 residues in the cytoplasmic tail that is not present in chicken [Brandes et al. 1997]. This insert does not disrupt the NPXY internalization motif. There are only five residues that differ between the mouse and human inserts, suggesting an important function for this peptide in mammalian cells [Brandes et al. 1997]. A specific sequence downstream of the internalization motif in LDLR has been suggested to be important for basolateral targeting of the receptor in hepatocytes [Yokode et al. 1992]. Thus, it is conceivable that the ApoER2 cytoplasmic tail may be involved in targeting the receptor to specific neuronal compartments, such as the leading edge of migrating cells. The subtle differences in the neuroanatomical phenotypes in ApoER2−/− or VLDLR−/− mice compared with those deficient in both genes may be related to the divergence in sequences of the receptors. Alternatively, differences in the levels of VLDLR and ApoER2 expression in specific brain regions may relate to the observed phenotypes.

Role of lipoprotein receptors in the brain

Mice deficient in megalin die perinatally because of respiratory failure and they exhibit defects in forebrain development [Willnow et al. 1996]. This gene is expressed in the neuroepithelium, in which it may be involved in lipid and nutrient metabolism following gastrulation. Interestingly, Dab1 is also expressed at high levels in the neuroepithelium where it could interact with Megalin, which has two NPXY motifs in the cytoplasmic domain [Howell et al. 1997a; Rice et al. 1998; Willnow 1999]. LRP is expressed in many tissues and it is enriched in neurons in the CNS. The function of LRP in the developing nervous system is unclear because disruption of this gene results in early embryonic lethality [Herz et al. 1992]. However, LRP has been suggested to be important in the brain because of its high affinity for apoE, which may be involved in maintaining high lipid demands during myelination and during neurite outgrowth [Gliemann 1998]. In humans, three isoforms of apoE (apoE2, apoE3, and apoE4) are encoded by different alleles of ApoE [Mahley 1988]. Inheritance of the apoE4 allele is a risk factor for late-onset Alzheimer's disease [Rubinshtein 1997]. Another ligand of LRP is the secreted form of APP [Kounnas et al. 1995]. ApoE, APP, and LRP have been found in senile plaques and polymorphisms in LRP have been linked to Alzheimer's disease [Rebeck et al. 1995; Hollenbach et al. 1998]. LRP is also believed to mediate the differential neurite outgrowth responses of cultured neurons in response to the various isoforms of ApoE [Gliemann 1998; Sun et al. 1998]. APP has also been shown to stimulate neurite outgrowth, however, LRP may not be directly involved in this response, at least in cultures of chick sympathetic neurons [Kounnas et al. 1995; Qiu et al. 1995; Postuma et al. 1998]. Thus, lipoproteins and their receptors may play several roles in brain development and function.

VLDLR, ApoER2, and Dab1 are expressed in similar populations of neurons

VLDLR and ApoER2 are expressed in several tissues, but they are particularly enriched in brain. In fact, ApoER2 has also been referred to as LR7/8B to emphasize its high level of expression in the brain and that it contains either seven or eight ligand-binding repeats [Takahashi et al. 1992; Kim et al. 1996; Schneider et al. 1997]. Both receptors are expressed in neuronal cells that contain Dab1 during development of the cerebral cortex, cerebellum, and hippocampus. These cells include the major populations of neurons that are responsive to Reln such as the cortical plate neurons, Purkinje cell precursors, and granule and pyramidal cells in the hippocampal formation [Trommsdorff et al. 1999]. In the adult brain, ApoER2 and VLDLR continue to be expressed in cells that contain Dab1, implying that these proteins interact in mature neurons [Kim et al. 1996; Clatworthy et al. 1999].

In reeler brain, Dab1 levels are increased substantially compared with normal embryonic brain [Rice et al. 1998]. The biochemical interaction between the PI/PTB
domain of Dab1 and the cytoplasmic tails of VLDLR and ApoER2 suggests that these receptors relay positional information from Reln across the plasma membrane of migrating neurons where Dab1 is engaged in a signaling function. This notion is supported by the observation that there is a substantial increase in Dab1 levels in embryonic ApoER2−/−/VLDLR−/− brains (Trommsdorff et al. 1999). Therefore, in the absence of the lipoprotein receptors or a Reln-evoked signaling pathway, Dab1 levels are increased. This suggests that ApoER2 and VLDLR are involved in a signaling pathway with Reln and Dab1.

**Reln is a ligand for lipoprotein receptors**

LDLR transports cholesterol-containing lipoproteins into the cell and its functions in cholesterol homeostasis. The importance of this receptor is underscored by the effects of numerous mutations in the LDLR gene that result in familial hypocholesterolemia [Schneider et al. 1982; Yamamoto et al. 1984; Brown and Goldstein 1986; Russell et al. 1986]. The LDLR receptor binds both apoB-100 containing low-density lipoproteins and apoE-containing lipoproteins. In contrast, VLDLR and ApoER2 bind only apoE-containing lipoproteins. The affinity of VLDLR for very low-density lipoprotein-containing particles is low compared with LDLR, suggesting that other ligands may bind to this receptor with higher affinities [Nimpf and Schneider 1998].

Recently, it was established that Reln is a high affinity ligand for ApoER2 and VLDLR (D’Arcangelo et al. 1999; Hiesberger et al. 1999). Reln binds to these receptors with an apparent affinity in the 0.5 nM range. After binding to lipoprotein receptors on the cell surface, Reln is internalized into vesicles. Binding of Reln to the receptors is inhibited by mAb CR-50, which blocks Reln function in vivo and in vitro (D’Arcangelo et al. 1995, 1999; Ogawa et al. 1995; Del Rio et al. 1997; Nakajima et al. 1997). These findings imply that Reln is a physiological ligand for lipoprotein receptors in the brain.

Several lipoprotein receptors interact with a diverse array of ligands including lipoprotein lipase, vitamin transport proteins, peptide hormones, thrombospondin and various proteinases [Krieger and Herz 1994; Gliemann 1998]. LRP, Megalin, and VLDLR bind the urokinase-type plasminogen activator type-1 and the plasminogen activator inhibitor, which is involved in cell migration and extracellular matrix remodeling during metastasis [Andreasen et al. 1994, 1997; Heegaard et al. 1995]. Internalization of proteolytic enzymes following receptor activation might modulate cell movement by regulating the function of proteins involved in restructuring the extracellular matrix. This mechanism may be particularly important in the nervous system in which interactions between neurons and the extracellular environment are important during migration, synaptic plasticity, and disease [Sappino et al. 1993; Romancic and Madri 1994; Vaillant et al. 1999; Yoshida and Shiosaka 1999].

**The Reln-signaling pathway**

Several lines of evidence place Reln, VLDLR, ApoER2, and Dab1 in a common signaling pathway that controls cell positioning in the developing CNS. First, mutations in these genes result in similar disruptions of laminated brain regions. Second, Reln binds to the ectodomain of the lipoprotein receptors and Dab1 interacts with the cytoplasmic tails of VLDLR and ApoER2 [D’Arcangelo et al. 1999; Hiesberger et al. 1999; Trommsdorff et al. 1999]. Third, Dab1 levels are increased severalfold in embryonic Reln−/− and VLDLR−/−/ApoER2−/− neurons [Rice et al. 1998; Trommsdorff et al. 1999]. Fourth, Dab1 phosphorylation is decreased in embryonic reeler brain compared to normal brain [Howell et al. 1999a]. Fifth, application of Reln to neuronal cultures causes an increase in tyrosine phosphorylation of Dab1 [Howell et al. 1999a]. Sixth, Reln-induced tyrosine phosphorylation of Dab1 is inhibited by the lipoprotein receptor ligand apoE and the lipoprotein receptor-associated protein [D’Arcangelo et al. 1999; Hiesberger et al. 1999]. Finally, Reln is produced by cells located adjacent to the region in which neurons expressing Dab1, VLDLR, and ApoER2 organize into defined layers. One possible function of Reln is to mark the end of the line for neuronal migration. Upon binding to specific receptors, Reln could signal neurons as they arrive along radial glia to detach from their migratory guide and insert into the appropriate position (Fig. 2a). Intracellular responses to this signal may modulate surface properties of neurons, allowing them to segregate into defined layers. In the absence of either Reln, the lipoprotein receptor(s), or Dab1, normal cell–cell interactions do not occur and neurons are unable to move to their final location. Collectively, these data support the hypothesis that lipoprotein receptors are involved in transmitting information from Reln across the plasma membrane to Dab1, where recruitment of a tyrosine kinase is associated with the activation of a signaling pathway in migrating neurons.

Lipoprotein receptors do not possess intrinsic tyrosine kinase activity. Therefore, because Reln involves phosphorylation of Dab1, ligand binding could result in association of a tyrosine kinases [such as Abl] with Dab1 at the cell membrane. Once phosphorylated, phosphotyrosines on Dab1 may serve as docking sites for the recruitment of various SH2 domain-containing signaling molecules. Phosphorylated Dab1 may resemble the cytoplasmic tail of autophosphorylated growth factor receptors, providing a mechanism to activate downstream signaling cascades that alter gene expression (Fig. 2b). Another possibility is that ligand binding results in internalization of the receptor complex and recruitment of a tyrosine kinase to Dab1 in endocytotic vesicles. Alternatively, Dab1 might specifically regulate intracellular sorting of lipoprotein receptors in neurons through the secretory pathway to the plasma membrane, where they bind to Reln and become internalized. In this model, neurons would overproduce Dab1 to compensate for the loss of the lipoprotein receptor complex in VLDLR−/−/ApoER2−/− mice. In reeler mice, the normal turnover of
the receptor complex would be compromised and Dab1 may not be targeted for degradation following activation by Reln.

Reln may also bind to other transmembrane proteins and recruit lipoprotein receptors into a large complex at the cell surface. The β1 integrin subunit contains two NPxY motifs in its cytoplasmic tail, but unlike the situation with LDLR, these motifs are not involved in internalization (Vignoud et al. 1994). At least one NPxY-containing peptide has been suggested to function in migration by targeting integrin to focal adhesions (Vignoud et al. 1997; Buttery et al. 1999). Integrins are known to trigger activation of tyrosine kinases such as Fak and Syc, which are involved in signaling transduction pathways that affect cytoskeletal organization (Clark and Brugge 1995). Interestingly, migrating cortical neurons express α3β1 integrin and disruption of the α3 integrin subunit results in a reeler-like phenotype in the cerebral cortex (Table 1; Anton et al. 1999). Other signaling mechanisms such as the stimulation of voltage- and ligand-activated Ca2+ channels are important for neuronal migration by mediating arrangements of the cytoskeletal network (Komuro and Rakic 1992, 1993). Dab1 could be a component of a pathway that links adhesion complexes to the actin and microtubule network by interacting with molecules such as Cdk5 that are known to regulate cytoskeletal proteins (Lew and Wang 1995; Nikolic et al. 1998). Indeed, mice deficient in Cdk5 or its neuronal specific activator p35 exhibit some similarities to Reln−/− and Dab1−/− mice (Gilmore et al. 1998; Kwon and Tsai 1998).

The analysis of spontaneous and man-made mutant mice has clearly demonstrated a critical role for the Reln signaling pathway in normal brain development. However, all of the known components of the Reln pathway are expressed in adult brain cells, and it is likely that these proteins also contribute to the physiology of the mature nervous system. Indeed, it is striking to note that the Reln pathway is influenced by numerous factors that have been linked to the pathogenesis of neurodegenerative disorders. For example, the binding of Reln to lipoprotein receptors is differentially inhibited by various isoforms of apoE, which is a susceptibility gene for Alzheimer’s disease (Rubinsztein 1997). ApoC3 and apoE4 are potent inhibitors of Reln binding, whereas, apoE2, which does not bind efficiently to lipoprotein receptors, has no effect (D’Arcangelo et al. 1999). Furthermore, tau phosphorylation is markedly enhanced in mice deficient in

Figure 2. Model of the potential signaling pathway involving Reln, surface proteins, and Dab1. (a) Reln (blue), which is produced and secreted by pioneer neurons, may signal directly to migrating cells containing Dab1 (yellow) by binding to receptors expressed on the cell surface. This results in a translocation of the migrating cell to its final position and detachment from the radial glia. (b) The possible intracellular changes in response to Reln are shown. Reln binds (solid line) directly to the lipoprotein receptors ApoER2 and VLDLR. Reln may affect the amyloid precursor protein family (APPs) and the α3β1 integrin receptor. Each of these surface proteins contain one or more NPxY motifs (stars) in their cytoplasmic tails. Dab1 binds (solid black line) to the cytoplasmic tails of ApoER2, VLDLR, APP, APLP1, and APLP2. The interaction between Dab1 and the β1 subunit of the integrin subunit is speculative (broken line). However, integrins are involved in the focal adhesion complex, which may activate tyrosine kinases that phosphorylate Dab1. On receptor activation by Reln, Dab1 is phosphorylated on tyrosine residues and it may serve as a docking site for recruitment of various SH2-domain-containing proteins. Phosphorylated Dab1 may activate downstream signaling kinase cascades resulting in alterations in gene expression. Activation of Cdk5 may stimulate serine phosphorylation of Dab1 and affect its localization within the cell. In addition, Cdk5 phosphorylates cytoskeletal components such as tau and neurofilaments, which affect the organization of the cytoskeleton. Ultimately, this signaling cascade alters the surface properties of migrating neurons.
Reln and in VLDLR−/−/ApoER2−/− mice (Hiesberger et al. 1999). Hyperphosphorylation of tau, which leads to dissociation of microtubules, has been suggested to cause neurofibrillary tangles characteristic of Alzheimer’s disease and mutations in tau are associated with dementia [Hardy et al. 1998]. Cdk5 is present in early stage tangles and it has been suggested as a candidate kinase both for tau and Dab1 [Imahori and Uchida 1997; D’Arcangelo et al. 1999]. Thus, it is possible that the Reln signaling pathway, which modulates cell migration, cell shape changes, and branching of neuronal fibers during development may also participate in communicating extracellular signals to alterations in cell shape and physiology in the adult brain. Conceivably, the transmission of a signal from Reln through lipoprotein receptors to tyrosine phosphorylation of Dab1 may act antagonistically with a pathway involving apoE, lipoprotein receptors, and tau phosphorylation (Fig. 2b). Therefore, it may be interesting to investigate the potential role of the Reln pathway in neuropathological disorders such as Alzheimer’s disease.

In summary, the formation of laminated brain structures involves a complex series of interactions among migrating cells, their substrates, and the extracellular environment. These interactions ultimately impinge on intracellular signaling mechanisms that direct key components of the migratory machinery. The identification of Reln put to rest some of the hypotheses concerning the reeler phenotype. However, other questions were raised regarding the mechanisms whereby this large extracellular protein signals migrating neurons. Identification of reeler-like phenotypes in several lines of mutant mice provides an important clue to the signal transduction pathway that may be activated by Reln. The finding that Reln and Dab1 bind lipoprotein receptors links two seemingly unrelated fields of study. The task at hand is to elucidate the role of these receptors in the Reln signaling pathway and to understand how migrating neurons acquire appropriate positional information.

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Mutant mice with scrambled brains: understanding the signaling pathways that control cell positioning in the CNS

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