Chapter 5
Crystal Structure of Reelin Repeats

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Contents

1 Reelin Primary Structure

1.1 Domain Architecture of Reelin

Reelin is a large glycoprotein with a repetitive modular structure (D'Arcangelo and Curran, 1999). It consists of a signal sequence, an F-spondin-like region, another region containing at least eight "reelin repeats," and a C-terminal basic peptide of ~30 residues (Fig. 5.1A). Each complete reelin repeat contains a central EGF module flanked by two subrepeats of 150–190 amino acids (Fig. 5.1A, inset). The central EGF module is relatively short in length (~30 residues), but nevertheless has consensus signatures, including the spacings between cysteines (Campbell and Bork, 1993). Although the homology between subrepeat A and subrepeat B was first

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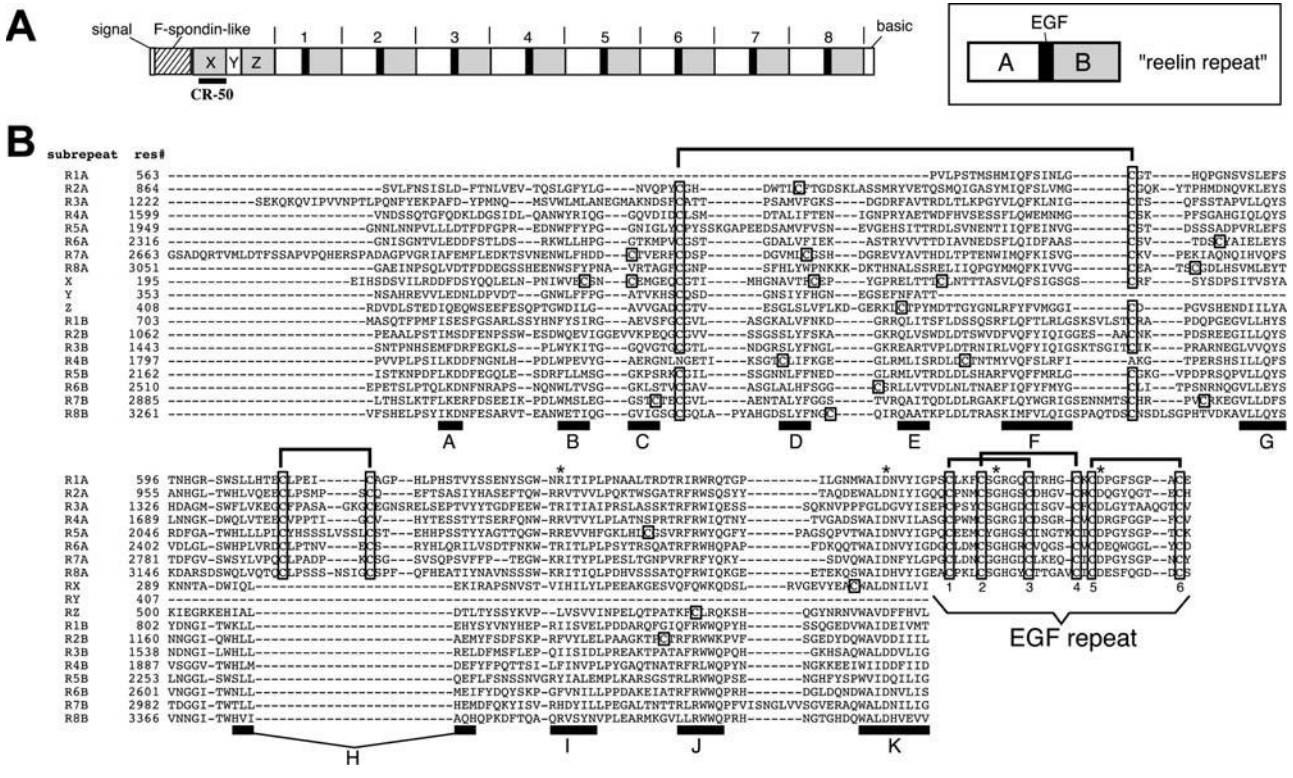

Fig. 5.1 Reelin primary structure. **(A)** Schematic representation of the domain arrangement in reelin primary structure. Location of the epitope for the antibody CR50 is indicated by a black bar. (**B)** Multiple sequence alignment of reelin repeats incorporating the structural information. From top to bottom, repeats are arranged in the order of A-type subrepeats (including the EGF module), "irregular" subrepeats, and the B-type subrepeats. Eleven β-strand segments are denoted by horizontal bars at the bottom of the alignment. Cysteines are boxed, with the conserved disulfide pairings denoted by brackets at the top. The conserved residues discussed in the text are also indicated at the top by asterisks

noted during the cloning of the reelin gene (D'Arcangelo *et al*., 1995), these repeats have failed to show any sequence homology to known protein domains.

1.2 Definition of a "Reelin Repeat"

Sequence alignment (Fig. 5.1B) clearly shows that the A and B subrepeats are related, with the critical difference being the insertion of a disulfide-bonded loop in the A subrepeat. Because of the regularity of the "A-EGF-B" concatenation, it became common to regard this as a unit. However, reexamination of the sequence identified additional reelin repeat-like sequences before the first repeat (Ichihara *et al*., 2001). This segment contains three stretches of sequences (denoted repeats X, Y, and Z) related to the reelin subrepeat, although without the intervening EGF module. Among these, repeats X and Z belong to the "B" type subrepeat, while repeat Y contains only a portion (~50 amino acids) of a subrepeat (Fig. 5.1B). The presence of such "incomplete" or "irregular" repeat segment argues against the idea that the "A-EGF-B" repeating unit corresponds to an inseparable single folding unit. In fact, it seemed entirely possible that the whole reelin molecule was merely a concatenation of independent A, B, and EGF modules. To determine the molecular architecture of reelin protein, we clearly needed to resolve the three-dimensional structure of the reelin repeat or subrepeats.

2 Crystal Structure of a Single Reelin Repeat

2.1 Overall Structure

Thus far, structures of three reelin repeats have been determined using X-ray crystallography. First, the crystal structure of reelin repeat 3 (R3) was solved at a resolution of 2.05 Å (Nogi *et al*., 2006). The crystals had one R3 molecule per asymmetric unit, and 303 out of 387 residues of the R3 construct were visible in the electron density map derived from X-ray diffraction. The electron density corresponding to the N-terminal segment (1222–1293) was poor, indicating that this segment may be mobile with regard to the rest of the domain. The most striking feature of the structure is that the three subdomains (i.e., subrepeat A, EGF, and subrepeat B) are arranged in a horseshoe-like manner, making intimate contact with one another (Fig. 5.2A). Subrepeats A and B lie distal to each other at the ends of the central EGF module, although an abrupt bend at the subdomain junction places two subrepeats in a close proximity, creating a direct A–B contact. Structures for R5 and R6 have also been determined (to be published elsewhere), revealing identical overall subdomain arrangements (Fig. 5.2A). In particular, the inter-subrepeat contact within R6 was further stabilized by a disulfide bridge whereby Cys2393 and Cys2559 directly connected subrepeats A and B at the "bottom," thus encircling the

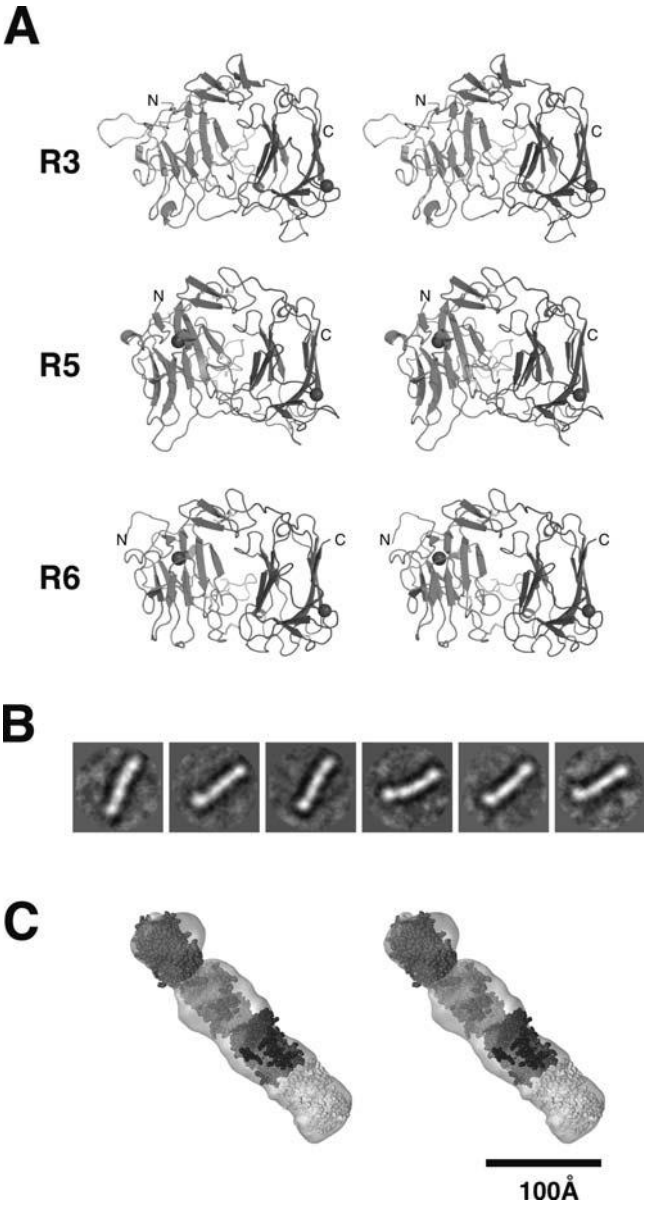

Fig. 5.2 Reelin repeat structure. (**A)** Crystal structures of single reelin repeat domains. Each panel shows a stereo presentation of R3 (top), R5 (middle), and R6 (bottom) structures. Subdomains are differently colored; subrepeat A (cyan), EGF (green), subrepeat B (magenta), and N- and C-termini are labeled. Bound calcium ions and disulfide bridges are shown as red spheres and yellow stick models, respectively. In R3, segments missing in the crystal structure are modeled and shown in gray. (**B)** Two-dimensional averages from representative particle classes obtained from the untilted electron micrographs of the R3–6 fragment. The width of each panel corresponds to 376 Å. (**C)** Three-dimensional volume map of an R3–6 fragment derived from single-particle tomography (gray) in a stereo representation. Four complete space-filling models for reelin repeats (R3, red; R4, green; R5, blue; and R6, yellow) are fitted into the envelope (*See Color Plates*)

subdomain loop. It is evident from these structures that a reelin repeat, represented by A-EGF-B set, does indeed constitute an independent structural unit, making a single globular domain with dimensions of $65 \times 45 \times 35$ Å, as opposed to an elongated domain with a linear subdomain concatenation. An important question then arises as to whether the individual submodule can exist on its own. Expression experiments using numerous truncation fragments favor the notion that, at least in R3 through R6, the three submodules are inseparable and form part of a single structural domain.

2.2 Subdomain Structure

2.2.1 Subrepeats A and B

Both subrepeats are composed of 11 β strands that form two antiparallel five-stranded β sheets in a jelly-roll fold. The two β sheets are approximately parallel to each other and curved, producing concave and convex sides. The first and the last strands are next to each other in the outer (i.e., convex) sheet, bringing the N- and C-termini close together. The presence of an "Asp-box" motif, a short structural element that has a unique β-hairpin configuration in many different structural contexts, was previously noted in a reelin repeat (Copley *et al*., 2001). The motif present in the loop connecting the G- and the H-strands (G-H loop) does indeed assume its backbone configuration identical to the other Asp-box, creating a curled protrusion that serves as an upper ridge of the concave surface. A single structural Ca^{2+} is found at the middle of the convex surface, such that it "sews together" the discontinuous sheet between the A-B and C-D loops. It is important to note that the presence of this calcium site could not be predicted by sequence analysis, and its identification was made possible by direct structural determination. Examination of the sequence alignment revealed that the Asp residue in the last (K) strand, which provides the most important bidentate Ca^{2+} ligand, was conserved in all reelin subrepeats including repeats X and Z (Fig. 5.1B). Therefore, a reelin monomer would contain a total of 18 calcium ions. The calcium is buried in the protein's interior and appears to play an important structural role in stabilizing the domain. Indeed, the expression level of R3 diminishes when this calcium site is disrupted by mutation. It was also reported that the full-length reelin becomes more susceptible to protease digestion in the presence of EDTA (Lugli *et al*., 2003). It is not known, however, whether there is any physiological function for these calcium ions other than domain stabilization.

As predicted from the sequence alignment, the only structural difference between subrepeats A and B was the insertion of a disulfide-bonded loop. The loops make long excursions such that they "sprout out" from the middle of strand H. Furthermore, this segment plays a major role in the interaction with subrepeat B (see below). Other than this loop insertion, subrepeats A and B are very similar and

can be superimposed onto each other with root mean square (RMS) deviations of 1.57–1.99 Å for matched alpha carbon atoms.

Three-dimensional comparison revealed that the reelin subrepeat shows an unexpected similarity to the carbohydrate-binding domains of numerous enzymes and nonenzymes. The top hit in a DALI search is a noncatalytic domain of xylanase from *Clostridium thermocellum* (PDB ID; 1dyo, Z-score = 12.5) (Charnock *et al*., 2000), followed by numerous jelly-roll modules all classified as "galactose-binding domain-like" in the SCOP database. When superimposed, the xylanase structure shows remarkable similarity to subrepeat B with RMS deviations of 2.6–3.0 Å for ~120 residues. Moreover, xylanase has one Ca^{2+} site that is structurally equivalent to that of the reelin repeats. Nevertheless, it shows only ~10% identity with reelin repeats at the amino acid level, making it difficult to evaluate any evolutionary relationship. As reelin is not known to bind any carbohydrates, the physiological significance of its resemblance to carbohydrate-binding domains remains unclear. It is important to note, however, that the concave side of these β jelly rolls is, in many cases, implicated in molecular recognition (Weis and Drickamer, 1996).

2.2.2 Central EGF Module

The EGF or EGF-like modules, which are extremely abundant and widespread among extracellular proteins in multicellular organisms, are frequently found in receptors and matrix proteins (Bork *et al*., 1996). Their three-dimensional structure, which has been determined for many different proteins, generally exhibits a small ellipsoid with its N- and C-termini located at the opposite ends of its major axis (Campbell and Bork, 1993). They appear in widely different structural contexts and in varying numbers. In contrast, the EGF modules in reelin are unique in that they are invariably positioned between subrepeats A and B. The EGF modules within the reelin repeat assume a typical EGF-like fold with the disulfide-bonding pattern of 1–3, 2–4, 5–6. The uniqueness of the EGF modules in reelin compared with regular EGF modules stems from their involvement in compact subdomain packing. For example, an Asp residue immediately following the fifth Cys is completely conserved among all reelin EGF modules, although this position is often occupied by hydrophobic residues in typical EGF modules. This residue forms a bidentate hydrogen bond to an invariable Arg residue present at strand I of subrepeat A, forcing the ~90° bend at the subrepeat A–EGF interface. Another unique feature conserved across the reelin EGF is the presence of a Gly residue in the +2 position from the second Cys, which is obligated to accommodate contact with the Asp-box turn in subrepeat B.

2.2.3 Inter-subrepeat Interaction

Within a single reelin repeat structure, the concave side of subrepeat B approaches from the "side" of subrepeat A and grabs the disulfide-bonded loop, causing the axes of the β-sheets to become significantly twisted toward each other. In effect,

the A-specific loop functions as a joint that enables a horseshoe-like arrangement. Because of inter-subrepeat interaction, the concave side of subrepeat B is occluded within the domain, while that of subrepeat A is freely accessible to the solvent. This interface, however, does not seem to be particularly stable since it is discontinuous and mainly hydrophilic in nature. It may therefore be possible that the repeat relaxes to assume an "open" conformation under certain conditions. Nevertheless, the compact subdomain packing is maintained in solution when multiple repeats are concatenated (see below).

3 Structure of a Four-Repeat Fragment

3.1 Two-Dimensional Electron Microscopy

Structures of single reelin repeats alone provide little information regarding the higher-order structure of a full-length reelin promoter, i.e., how each domain is organized in the context of the larger protein. It would be highly desirable to resolve the structure of larger reelin fragments or even the entire (i.e., full-length) molecule. However, protein production and crystallization on such a scale remains extremely difficult, if not impossible. Electron microscopy (EM) offers an excellent alternative to X-ray crystallography, as it requires relatively small amounts of protein and can be applied to proteins that assume multiple conformations. Negative staining EM of a recombinant fragment containing reelin repeats 3–6 (R3–6) revealed a very homogeneous molecular shape, i.e., an elongated rod with an average length of ~25 nm (Fig. 5.2B). Although a partial segment, this fragment covers about 40% of the total molecule and most importantly was capable of binding to the receptor and transducing the signal, as confirmed by both receptor binding and Dab1 phosphorylation assays (Jossin *et al*., 2004; Nogi *et al*., 2006). In these two-dimensional averaged images, particles showed largely straight, sometimes bent rod-like shapes, with four densities separated by segmentations. The most important feature of the EM images is the size of the fragment. The length of the rod was roughly the same as, or even shorter than, the longest dimension of the crystal structure of the single repeat multiplied by 4, strongly arguing against the possibility that the reelin repeat assumes a more extended conformation in the native protein. Three segmentations divide the rod into four parts, which most likely correspond to each reelin repeat.

3.2 Three-Dimensional Structure as Revealed by Electron Tomography

The conventional two-dimensionally averaged images can derive an overall molecular shape that is "projected" onto a plane. In order to examine the spatial arrangements of domains within a molecule, however, 3D information must be

obtained. In fact, a methodology called "single-particle tomography" can extract 3D information from the same specimen prepared for conventional EM (Iwasaki *et al*., 2005). It collects the image data at different tilt angles and reconstructs a 3D volume map for individual particles. From a pool of the representative tomograms, an averaged 3D volume map was constructed (Fig. 5.2C). It showed a flattened rod having dimensions of $240 \times 50 \times 30$ Å. Segmentation is no longer visible, probably due to the slight axial offset that occurred among different particles during the averaging process. Having the 3D structures for a single reelin repeat domain in hand, one can now interpret these EM-derived structures at atomic resolution.

Because the first and last strands in a subrepeat are adjacent, the N- and C-termini (of a subrepeat) are in close proximity. As a result, the N- and C-termini of the entire repeat are located at the "upper" corners of the domain, roughly aligned with the axis of the EGF module (Fig. 5.2A). As the "linker" segments connecting the two consecutive repeats are usually only several residues long, there would not be much available space at the repeat–repeat junction. The EM images proved to be in complete agreement with these predictions; they do not show the "beads-on-a-string" conformation; instead, each repeat is stacked close together.

Atomic coordinates for R3, R5, R6, and modeled R4 can be very successfully fitted in the density (Fig. 5.2C). Four repeats are arbitrarily placed in the main body of the density such that they are related by a translational movement, with their longest dimension parallel to the rod axis. This arrangement of the domains is plausible, since both the 2D and 3D EM images did not show any signs of twists or meandering. It is, therefore, very likely that reelin repeats are related by translation without significant rotation, as shown in the model in Fig. 5.2C.

4 Structural Model for the Full-Length Reelin Molecule

4.1 Arrangement of Reelin Repeats

Using the experimentally derived structural information, the 3D architecture of reelin fragment R3–6 was deduced. However, this still leaves unresolved the rest of the molecule. We are in a good position to begin building a realistic structural model of the full-length reelin protein. The sequence alignment shown in Fig. 5.1B was modified from the alignment made by Ichihara *et al*. (2001) by incorporating structural information from the crystal structures. This "structural alignment" takes into account the alignment of the secondary structural elements rather than just maximizing the residue-wise matches. By using this alignment, we can define the (sub)repeat boundary and predict the 3D structure of the unknown parts more accurately.

It is obvious from the alignment that repeats 3 and 7 have unusually long N-terminal extensions (Fig. 5.1B). The polypeptide lengths between the last strand of subrepeat B and the first strand of the following subrepeat A are 7–9 residues for most

repeats, except for repeat 3 (23 residues) and repeat 7 (34 residues). This indicates that there are long linker regions present before repeat 3 and after repeat 6. This configuration is in perfect agreement with the fact that the full-length reelin protein is cleaved at sites roughly located between repeats 2 and 3, as well as between repeats 6 and 7 by metalloproteinases *in vivo* (Jossin *et al*., 2004; Lugli *et al*., 2003). The EM images of the R3–6 fragment indicate that this segment behaves as a relatively rigid rod, with little interdomain flexibility. Therefore, it is natural to speculate that the reelin repeat region can be separated into three rods, R1–2, R3–6, and R7–8, joined by flexible and protease-susceptible linkers (Fig. 5.3A).

Another valuable piece of information extracted from the sequence alignment is the ~50-residue truncation of repeat 1A. This is not caused by a misalignment, since the segment immediately preceding the R1A (i.e., repeat Z) aligns fully to the "B-type" subrepeats, and, hence, cannot be offset. The missing part would contain five β strands participating in both the inner and outer sheets, making it difficult to imagine subrepeat 1A folding correctly without this piece. Curiously, the irregular "repeat Y" corresponds exactly to this segment (Fig. 5.1B). In fact, only "Y" and "1A" are imperfect among the 19 subrepeat segments in reelin, and they are complementary to each other. This points to the possibility that repeat Y is part of repeat 1 in the 3D structure, although they are separated by repeat Z in the primary structure (Fig. 5.3A).

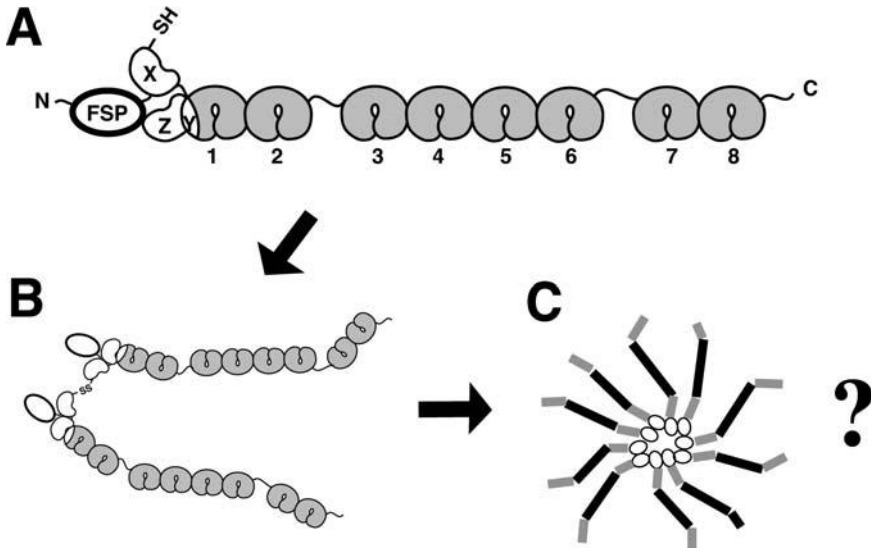

Fig. 5.3 Hypothetical model of native reelin protein. Predicted domain arrangement in a reelin protomer (**A**) suggests the presence of a segmented rod region (gray) and a dimerizing region at the N-terminus (white). The monomer may undergo dimerization using the free Cys residue in repeat X (**B**), and finally into higher-order multimerization (**C**) with radially presented rod regions containing receptor-binding sites (black)

4.2 A Model for the Reelin Monomer and Its Higher-Order Assembly

If we accept the above hypothesis that repeat Y constitutes the N-terminal portion of repeat 1, the primary sequence connectivity would place not only repeat Z but also repeat X adjacent to the A-side of repeat 1. What would then be the roles for these "lone" subrepeats?

Reelin is known to exist as a disulfide-bonded homodimer, and the Cys residue responsible for dimerization is thought to be located in the first 368-residue portion (Kubo *et al*., 2002). This region is comprised of an F-spondin domain and the repeat X, which contain 4 and 7 cysteines, respectively. Sequence consideration and model building predict that Cys256 in repeat X is likely to be missing a bonding partner. It is therefore likely that the repeat X portion serves as a dimerizing point in the native protein (Fig. 5.3B).

In addition to covalent homodimerization, secreted reelin is also known to exist as noncovalently associated multimers (Utsunomiya-Tate *et al*., 2000). In this regard, two additional B-type subrepeats with unoccupied concave sides (i.e., repeats X and Z) may provide a platform for multimerization. It is noteworthy that repeat X contains the epitope for the function-blocking antibody CR50, which has been shown to inhibit higher-order multimerization of reelin (D'Arcangelo *et al*., 1997; Utsunomiya-Tate *et al*., 2000). A multimerizing point located at the beginning of the linear reelin repeat segment, away from the receptor binding site (i.e., R3–6), could facilitate the assembly of a radially arranged reelin multimer (Fig. 5.3C), which would be ideal for transducing signals to neurons via receptor clustering (Strasser *et al*., 2004).

5 Concluding Remarks

Despite the major advances in our understanding of the genetic, physiological, and cell biological aspects of reelin made in recent years, structural and biochemical analyses have proven slow to develop. Some of the reasons for this difficulty include the unusually large size of the protein, the lack of a panel of anti-reelin monoclonal antibodies encompassing a wide range of epitope regions, the presence of multiple protease-processed products, and the instability of the full-length protein after isolation. The structural determination of representative reelin repeat fragments has laid the foundation for discussion of the true molecular architecture of this protein. A single reelin repeat domain consists of a central EGF module flanked by two related subrepeats that assume an 11-strand β jelly-roll fold. Moreover, direct contact between the subrepeats ensures that this unit maintains a compact overall structure. Repeats 3 to 6 are linearly arranged and stacked together with no twists or meandering, making a stiff rod. Combining these structural data with a detailed sequence analysis, a picture of the full-length reelin structure has emerged in which the long eight-repeat region, subdivided into three segments

(R1–2, R3–6, and R7–8), are assembled into a disulfide-bonded homodimer and additional noncovalent multimers (Fig. 5.3). The validity of this model should be tested by further ultrastructural and biochemical analyses.

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Chapter 6
Comparative Anatomy and Evolutionary Roles of Reelin

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Contents

1 The *Reelin* Gene Is Evolutionarily Preserved

The *reelin* gene maps to mouse chromosome 5 and human chromosome 7q22 (DeSilva *et al*., 1997; Royaux *et al*., 1997). The mouse *reelin* gene has a large size, about 450 kb, principally due to the presence of some very large introns. It is composed of 65 exons, 51 of which encode the eight reelin repeats. At the 3´-terminal portion of the gene, alternative splicing involves the inclusion of a hexanucleotide AGTAAG encoding amino acids Val-Ser, which create a potential phosphorylation site. This sequence is flanked by two introns and considered a *bona fide* exon (exon 64) (Royaux *et al*., 1997). The hexanucleotide sequence is evolutionarily conserved, because it is observed in the same relative location in the turtle and lizard cDNA, while the similar sequence AATAAG is present in chick (Lambert de Rouvroit *et al*., 1999). An alternative, polyadenylated product corresponds to the alternative exon 63a, expressed in the embryonic mouse brain, that codes for a truncated protein lacking the C-terminal region. This alternative mRNA represents between 10 and 25% of total reelin message

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in the embryonic mouse brain and is most abundant in Cajal-Retzius neurons of the cerebral cortex and hippocampus and in granule cells of the cerebellum; highly similar sequences are also found in human and rat. While reelin mRNA containing the microexon 64 is the major form in the brain of mouse, rat, man, turtle, and lizard, reelin transcripts in liver and kidney lack the hexanucleotide (Lambert de Rouvroit *et al*., 1999).

The human *RELN* 5'-untranslated region (UTR) has been related to genetic susceptibility to autism (Persico *et al*., 2001); the proximal *RELN* promoter is CG-rich, and it has been proposed that hypermethylation of the promoter is associated with decreased expression of reelin in psychiatric patients (M. L. Chen *et al*., 2002; Y. Chen et al., 2002).

The reelin protein is a large (3461 amino acids long), secreted glycoprotein (D'Arcangelo *et al*., 1995, 1997). N-terminal antibodies reveal two fragments of about 320 and 180 kDa, whereas C-terminal antibodies show fragments of about 240 and 100 kDa. The antibody most widely used in comparative neuroanatomical studies, which reacts with reelin in almost all vertebrates, is the N-terminal monoclonal antibody 142 (de Bergeyck *et al*., 1998), which has also been used for the figures of this review. In most brain extracts and body fluids, full-length reelin is rarely detected. The central portion of reelin (Jossin *et al*., 2004) is involved in binding of VLDLR (very-low-density lipoprotein receptor) and ApoER2 (apolipoprotein E receptor type 2) and signal activation, eliciting phosphorylation of the cytoplasmic adapter protein disabled 1 (Dab1), the target of the Reelin signal (Rice *et al*., 1998; Hiesberger *et al*., 1999; Howell *et al*., 1999a; Trommsdorff *et al*., 1999; Rice and Curran, 2001). Comparison of the *Dab1* gene of zebrafish, mouse, and human also shows an overall conservation of the genomic organization, although this very complex gene has shorter introns and some variations in the exonic sequences in the zebrafish compared to the mammalian *Dab1* gene (Bar *et al*., 2003; Costagli *et al*., 2006). The lipoprotein receptors are highly conserved in vertebrates and invertebrates (Willnow *et al*., 1999), and the *disabled* gene is present in *Drosophila melanogaster* (Gertler *et al*., 1989). By contrast, *reelin*-related cDNA sequences have been described in turtles, lizards, and chicks, but were not detected in *Caenorhabditis elegans* or *Drosophila* (Bar *et al*., 2000), and may be absent in invertebrate genomes.

2 Conserved Reelin Expression Pattern in the Central Nervous System

2.1 Reelin in the Adult CNS

Reelin is present in the nervous system of all vertebrates, from amphioxus (C. G. Pérez-García and G. Meyer, unpublished), lamprey, and fish to amphibians, reptiles, birds, and mammals. Reelin is widely expressed throughout the central nervous system (CNS), and the overall expression pattern is surprisingly conserved.

The highest divergence from the common expression pattern is observed in the dorsal telencephalon of the zebrafish, which probably corresponds to the pallium of other vertebrates (Nieuwenhuys and Meek, 1990) and will be discussed separately. In the adult lamprey (*Petromyzon marinus* L.), the most ancient representative of living vertebrates, reelin-positive neurons are present in the olfactory bulb, in pallial and subpallial regions of the telencephalon, in some hypothalamic nuclei and habenula, in nerve brain stem motor nuclei and neurons of the reticular formation, as well as in the rostral spinal cord (Pérez-Costas *et al*., 2004). In the adult zebrafish (*Danio rerio*), reelin is strongly expressed in various areas of the dorsal and ventral telencephalon, but not in the olfactory bulb. Reelin mRNA is also present in several nuclei of the dorsal thalamus and in most hypothalamic regions, in the pretectum, optic tectum, tegmentum, and throughout the meso-rhomboencephalic reticular formation. In the spinal cord, reelin expression is confined to a subpopulation of interneurons. Strong *reelin* mRNA signal is in granule cells of the cerebellum and, outside the CNS, in the retina (Costagli *et al*., 2002).

Remarkably, many of the expression sites of reelin are common to nonmammalian and mammalian vertebrates. The presence of reelin in the adult mammalian brain has been examined in mouse and rat (Alcantara *et al*., 1998; Ramos-Moreno *et al*., 2006). Reelin transcripts outside the cerebral cortex are prominent in the olfactory bulb, mainly in mitral cells but also in some periglomerular neurons. In general, expression seems to decline during the postnatal period and is rather weak in adult medial septum/diagonal band, amygdaloid area and hypothalamus, as well as in the pretectum (Alcantara *et al*., 1998).

In general, the expression of reelin is highly conserved in laminated structures [e.g., olfactory bulb (Fig. 6.1C), retina (Fig. 6.1A), cerebral cortex (Fig. 6.3B), cerebellum, and optic tectum/superior colliculus], where the expression of reelin is usually complementary to that of the reelin receptors and the effector protein Dab1 (Rice and Curran, 2001). Lamination of many of these centers, in particular the cerebral cortex and cerebellum, is disturbed in *reelin*, *Dab1*, and double receptor-deficient mice, indicating the important developmental role of the reelin signaling pathway (Lambert de Rouvroit and Goffinet, 1998; Rice *et al*., 1999; Trommsdorff *et al*., 1999). However, reelin expression is also conserved in nonlaminated structures, such as in the hypothalamus, where we were able to detect the protein in widely disparate species, such as adult lizards (Fig. 6.1B), adult mice, and young adult cats.

A wider distribution of reelin in cortical and subcortical structures, including long-projecting fiber tracts, has been reported in the ferret, along with the suggestion that reelin may be anterogradely transported by axons and secreted at their terminal arborizations (Martinez-Cerdeño *et al*., 2003). A similarly widespread, almost generalized presence of reelin has been described in the macaque brain (Martinez-Cerdeño *et al*., 2002). In contrast to previous studies that emphasized the presence of reelin in interneurons and the negativity of most long-projecting neurons such as cortical pyramidal cells and cerebellar Purkinje cells, Lambert de Rouvroit and Goffinet (1998) reported reelin immunoreactivity in the great majority of brain neurons and in their axonal projections. Further *in situ* hybridization studies are needed to confirm such abundancy of reelin in the adult mammalian brain.

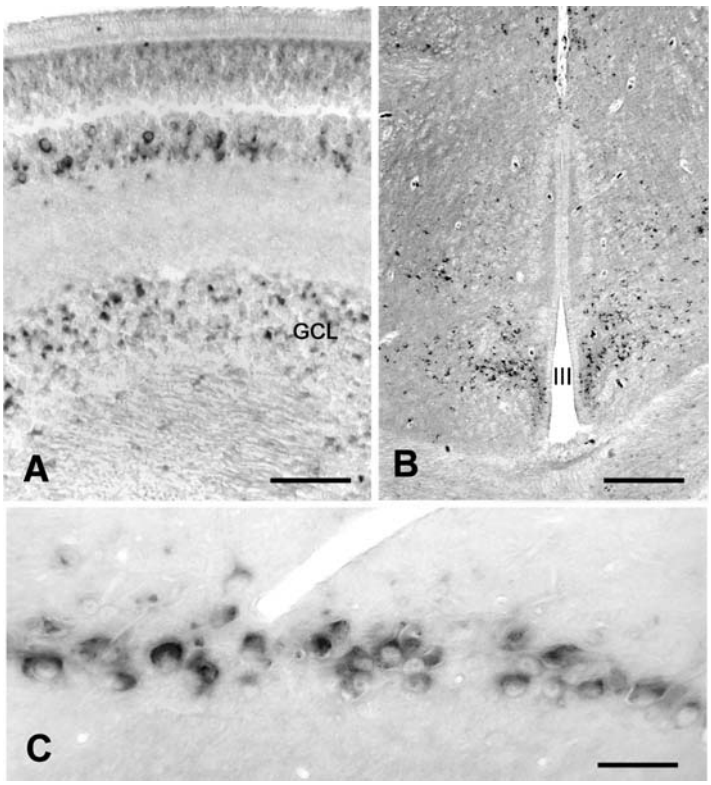

Fig. 6.1 Conserved expression pattern of reelin in the nervous system of amniotes. (**A**) Reelin-immunoreactive (ir) cells in the retina of the lizard *Lacerta galloti*. GCL, ganglion cell layer. (**B**) Reelin-ir neurons in the lizard hypothalamus. III, third ventricle. (**C**) Mitral cells in the olfactory bulb of the cat. Bars: 30 µm (**A**), 50 µm (**B**), 20 µm (**C**)

2.2 Reelin in the Developing CNS

As mentioned above, reelin is widely expressed throughout the CNS. Comparative studies in a variety of developing vertebrate species show that the reelin signal is usually higher during development than in the adult, and that more brain centers are reelin-positive. Among the centers that display *reelin* mRNA or protein expression during development of the crocodile are the septum, dorsal ventricular ridge, the subventricular telencephalic zone, lateral geniculate nucleus, cochleovestibular, and sensory trigeminal nuclei (Tissir *et al*., 2003). Similar distributions are noted in the embryonic mouse (Schiffmann *et al*., 1997; Alcantara *et al*., 1998), where reelin is also expressed in the preoptic area, striatum, zona incerta, habenula, lateral geniculate nucleus, and superior colliculus. In the zebrafish, reelin expression is particularly dynamic during the first 24–72 hours of development, with early and intense

expression in the telencephalon, ventral areas of the diencephalon, mesencephalon, hindbrain, and spinal cord. Later on, the pattern of reelin becomes restricted to specific CNS regions and cell populations where expression persists unchanged to 1–3 months of age (Costagli *et al*., 2002). Similarly, in the larval stages of the sea lamprey, reelin is expressed in the olfactory bulb, pallium, habenula, hypothalamus, and optic tectum (Pérez-Costas *et al*., 2002). On the whole, the early and widespread expression of reelin is in keeping with important roles of the reelin-–Dab1 signaling pathway in the migration and final positioning of newly born neurons and in the formation of laminated structures (Tissir *et al*., 2002; Tissir and Goffinet, 2003).

In addition to abundant reelin expression in many brain structures, reelin is highly and specifically expressed in the Cajal-Retzius cells of the developing cerebral cortex (D'Arcangelo *et al*., 1995; Ogawa *et al*., 1995; Meyer *et al*., 1999). Since these cells play a key role in the development and evolution of the cortex, they will be discussed in detail in Section 3.3.

3 The Evolution of Reelin Expression from the Submammalian Pallium to the Mammalian Neocortex

3.1 Reelin Expression in the Adult Nonmammalian Pallium

In the course of evolution, the cortical mantle of the telencephalon undergoes significant changes in architectonic organization, and reelin expression reflects these morphological and functional differences. The most divergent arrangement of pallial neurons is found in teleosts. The teleostean pallium develops through a unique process of eversion, during which the cerebral hemispheres evert and the dorsal and internal regions bend outwards. In the zebrafish pallium, no laminar arrangement can be recognized, and many neurons are reelin-positive (Pérez-García *et al*., 2001; Costagli *et al*., 2002). Fig. 6.2A shows reelin-immunoreactive neurons in the dorsolateral pallium of an adult zebrafish, where groups of reelin-positive neurons lie side by side with groups of reelin-negative neurons. Since most neurons in the dorsal telencephalon are small and their cytoplasm is sparse, the reelin signal is usually not very pronounced. The most intense reelin immunoreactivity is present along the everted ventricular layer (Pérez-García *et al*., 1991). Costagli *et al*. (2002) described *reelin* mRNA in medial, lateral, central, and dorsal regions of the dorsal telencephalon, whereas in the ventral telencephalon only a few cells showed weak positivity.

The amphibian pallium forms through evagination of the cerebral hemispheres from the prosencephalic vesicle, which is the developmental mechanism common to all vertebrates except teleosts, and comprises several subregions. The dorsal and lateral pallium consists of several rows of neurons close to the ventricle, whereas the wide molecular layer is almost cell free. In adult *Hyla meridionalis* (Mediterranean Treefrog), reelin-expressing neurons are located at the periphery of the periventricular cell layer, whereas the molecular layer is basically reelin-negative (Fig. 6.2B).

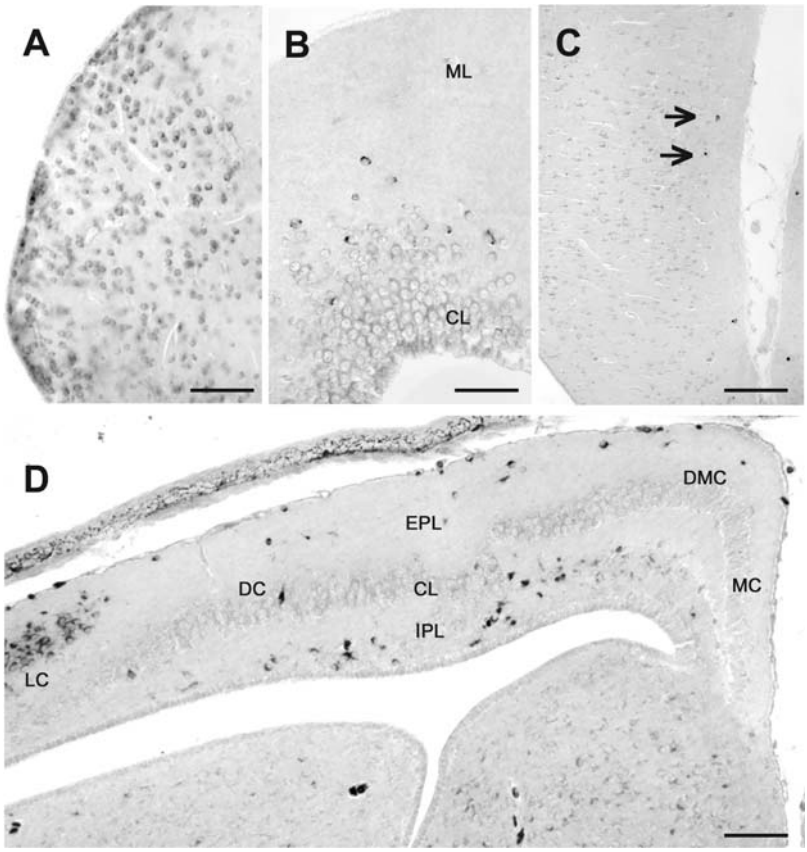

Fig. 6.2 Variable expression patterns of reelin in the adult nonmammalian pallium. (**A**) Reelin in the dorsal telencephalon of the zebrafish *Danio rerio*. Many diffusely distributed neurons are reelin-positive. (**B**) In the dorsal pallium of the Mediterranean Treefrog (*Hyla meridionalis*), few neurons outside the main cell layer (CL) show faint reelin-immunoreactivity. The molecular layer (ML) is almost cell-free. (**C**) The medial pallium of the pigeon *Columba livia* displays very few small reelin-ir neurons in the cell-free outer layer. (**D**) The pallium of the lizard *Lacerta galloti* is subdivided into a medial cortex (MC), dorsomedial cortex (DMC), dorsal cortex (DC), and lateral cortex (LC). The main cell layer is reelin-negative and sandwiched between the external plexiform and internal plexiform layers (EPL, IPL), both populated by reelin-positive neurons. In the lateral cortex, neurons in the main cell layer express reelin. Bars: 20 μm (**A**), 25 μm (**B**, **C**), 50 μm (**D**)

Although the amphibian pallium has an extremely rudimentary architecture, it already displays a basic feature of the vertebrate cortex: the principal, long-projecting neurons are usually reelin-negative, whereas reelin is confined to a subpopulation of interneurons. In the pigeon pallium (*Columba livia)* (Fig. 6.2C), reelin-positive neurons are rare, with only a few in the molecular layer of the medial cortex.

The adult lizard displays a clearly laminated cortex composed of an external plexiform or molecular layer, an intermediate cellular layer formed by densely

packed cell somata, and an internal plexiform layer (Fig. 6.2D). The plexiform layers contain the dendrites of the bitufted pyramidal cells, and a number of interneurons which are usually reelin-positive. Architectonic differences allow a subdivision into four zones, namely, the medial, dorsomedial, dorsal, and lateral cortex (Ulinski, 1990), but the three-layered organization is a common feature. Examination of three squamate reptiles (*Lacerta galloti, Tarentola delalandii,* and *Chalcides ocellatus*) showed a very similar architecture and distribution of reelin-positive neurons. The compact cell layer is reelin-negative but Dab1-positive, and sandwiched between the two plexiform layers containing small numbers of reelin-positive neurons (Pérez-García *et al*., 2001). The exception is the lateral cortex where even in adult animals the main cell layer is reelin-positive. We will discuss this peculiar feature of the lateral cortex in Section 4, in the context of reelin expression in the entorhinal cortex and hippocampus.

In the turtle *Clemmys caspica*, lamination is less pronounced than in the lizard, and the principal neurons form a loosely organized cell layer close to the ventricle. Very few reelin-positive cells lie in the wide external plexiform layer, while the internal plexiform layer is devoid of reelin (Pérez-García *et al*., 2001).

In sum, the examples presented here —teleosts, amphibians, birds, and reptiles— demonstrate that reelin expression in the adult pallium is extremely variable and ranges from abundant expression in the everted pallium of the zebrafish to an extremely sparse presence of the protein in the amphibian, bird, and turtle pallium. The fact that in the evaginated amniote pallium the main projection neurons are usually reelin-negative seems to be the most conserved trait.

3.2 Reelin Expression in the Adult Mammalian Cortex

In most mammalian species examined, the presence of reelin in the cerebral neocortex is confined to GABAergic interneurons (Alcantara *et al*., 1998; Pesold *et al*., 1998; Fatemi *et al*., 2000; Martinez-Cerdeño and Clascá, 2002), while the main projection neurons, the pyramidal cells, express Dab1 (Rice *et al*., 1998; Rice and Curran, 2001). The highest density of reelin-positive interneurons is consistently observed in the molecular layer (layer I), but they are also common in layers II–VI (Fig. 6.3B). In the molecular layer, reelin-expressing neurons are usually small and rounded, although sometimes they are larger and horizontally oriented, resembling the Cajal-Retzius cells of the fetal stage (Fig. 6.3A; see Section 3.3). Cortical layer I has a variable width in the different mammalian species, and also the reelin-positive cell populations in this layer are to some extent variable. For instance, in the wide odontocete (*Tursiops truncatus* and *Ziphius cavirostris*) molecular layer, reelin-positive neurons display a large variety of shapes and sizes and are particularly numerous in the depths of the numerous sulci and cortical folds. The opposite pattern is found in the hedgehog cortex, also characterized by a wide molecular layer, but which contains few small reelin-positive neurons located close to the pial surface (Pérez-García *et al*., 2001). In human, small reelin-positive neurons are very

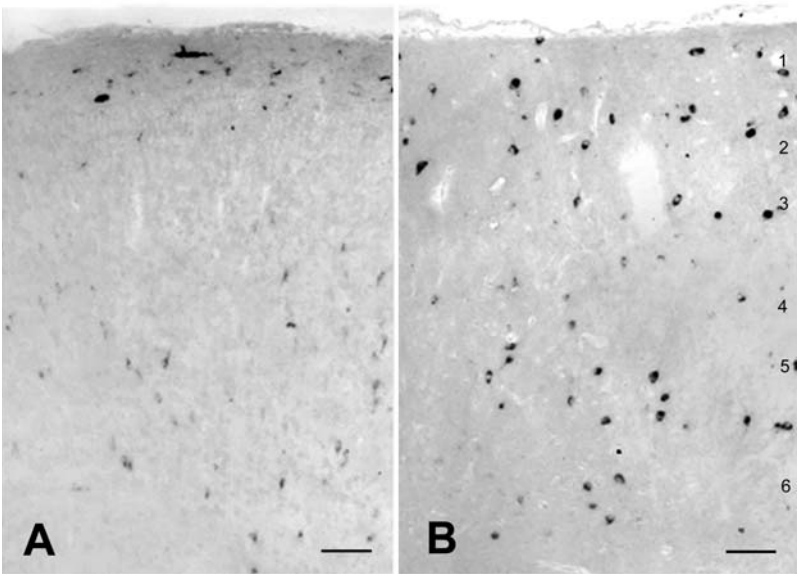

Fig. 6.3 Cajal-Retzius cells and interneurons in the cerebral cortex of the cat. (**A**) Reelin-immunoreactive (ir) neurons in a late prenatal stage. Interneurons are still faintly stained, and a Cajal-Retzius cell is still prominent in the marginal zone (layer 1). (**B**) At 1 postnatal month, interneurons in all layers are strongly reelin-ir, and Cajal-Retzius cells have disappeared. Bars: 30 µm

numerous in the postnatal layer I, but decrease in number during the first years of life. The adult human molecular layer (Fatemi *et al*., 2000) is populated by few rounded interneurons, similar to those present in the adult rodent and carnivore cortex (Fig. 6.4E).

The reelin-positive interneurons of cortical layers II–VI were examined in detail in mouse (Alcantara *et al*., 1998; Fatemi *et al*., 1999), rat (Pesold *et al*., 1998), and monkey (Rodriguez *et al*., 2000, 2002). In mice, they appear at birth, first in layers V and VI of the neocortex and later on also in layers II–IV. After a peak during the first postnatal week, *reelin* mRNA expression gradually decreases, although reelin-positive interneurons persist throughout adult life. In the developing cat cortex, reelin-positive interneurons are faintly stained before birth, and acquire intense expression only postnatally (Fig. 6.3A,B). Coexpression experiments showed that reelin is expressed in subsets of GABAergic interneurons, some of which express also the interneuron markers calbindin, calretinin, somatostatin, and NPY, indicating that *reelin* transcripts are expressed in a heterogeneous population of nonpyramidal neurons (Alcantara *et al*., 1998; Pesold *et al*., 1999). Similarly, in the adult rat, reelin-positive neurons are scattered throughout all cortical layers, and a majority are GABAergic (Pesold *et al*., 1998). Combined reelin immunolabeling and electron microscopy revealed that reelin is also present in the extracellular space surrounding dendritic

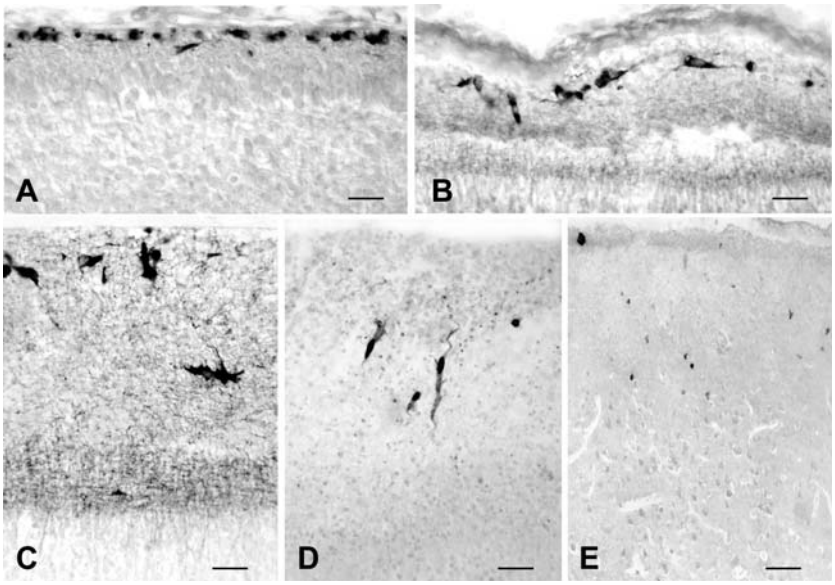

Fig. 6.4 The unique differentiation of human Cajal-Retzius cells. Human fetuses at (**A**) 9 gestational weeks (GW), (**B**) 14 GW, (**C**) 20 GW, (**D**) 25 GW, stained with anti-reelin antibody 142; in **D** doubly stained for p73 (in the nucleus) and reelin (in the cytoplasm). Cajal-Retzius are initially horizontally oriented and attached to the pial surface. They change morphology and descend to deeper levels of the marginal zone, extending a reelin-ir axonal plexus (PL in **C**). They degenerate and die around 25–30 GW. In the adult (**E**), they have disappeared, and layer 1 contains few small reelin-ir interneurons. Bars: 30 µm

shafts and dendritic spines in layers I and II of wild-type mice cortex in a discontinuous pattern; in the hippocampus, the neuropil is heavily and uniformly reelin-immunoreactive (Pappas *et al*., 2001). In the patas monkey, *reelin* mRNA is expressed in all cortical areas and layers, with the highest numbers in layer II. GABAergic interneurons have been classified according to their ability to express reelin, which is secreted into the extracellular matrix where it may interact with dendritic shafts, dendritic spines, and spine postsynaptic densities (Rodriguez *et al*., 2002).

There are significant discrepancies regarding reelin expression in adult human cortex. Whereas some authors observe protein/mRNA only in nonpyramidal neurons (Fatemi et al., 2000; Pérez-García *et al*., 2001; Martinez-Cerdeño and Clascá, 2002; Eastwood and Harrison, 2006), others describe the presence of reelin also in pyramidal cells, as well as in the neuropil, dendritic spines, small axon terminals, and postsynaptic densities (Deguchi *et al*., 2003; Roberts *et al*., 2005). The latter distribution is in line with a study of the adult macaque cortex (Martinez-Cerdeño *et al*., 2002) which reports that the majority of cortical neurons express reelin, suggesting that reelin can influence most brain circuits of the adult primate brain. It would be important to solve these discrepancies, because recent studies have involved the

reelin–dab1 signaling pathway in cortical and hippocampal synaptic plasticity (Weeber *et al*., 2002). Reelin enhances long-term potentiation (LTP), and this function is abolished if either VLDLR or ApoER2 is absent. The impairment of ApoE receptor-dependent neuromodulation may contribute to cognitive impairment and synaptic loss in Alzheimer's disease. Furthermore, synaptic strength and activity-dependent synaptic plasticity depend to a large extent on NMDA receptors on the postsynaptic side of excitatory synapses. Reelin potentiates calcium influx through NMDA receptors and may thus modulate learning and memory (Beffert *et al*., 2005, 2006; Chen *et al*., 2005). The activities of the reelin pathway in cognitive functions are of utmost importance for understanding the possible relationship between reelin and Alzheimer's disease (Saez-Valero *et al*., 2003; Botella-Lopez *et al*., 2006).

3.3 Evolutionary Aspects of the Cajal-Retzius Cells

The Cajal-Retzius cells populate the marginal zone of the developing cerebral cortex and hippocampus and are intimately related to the reelin–Dab1 signaling pathway. First described by Retzius and Cajal more than one century ago, they have attracted the interest of many researchers, sometimes with controversial opinions (reviewed by Meyer *et al*., 1999; Meyer, 2001). Today, there is an overall consensus that Cajal-Retzius cells are a heterogeneous group of neurons that appear from the earliest stages of cortical development, derive from several birth places, most notably the cortical hem, and invade the neocortical marginal zone by subpial tangential migration (Meyer and Goffinet, 1998; Meyer and Wahle, 1999; Meyer *et al*., 2002; Bielle *et al*., 2005; Yoshida *et al*., 2006; Meyer, 2007).

The most interesting feature of Cajal-Retzius cells is that they secrete high levels of reelin, which binds to the reelin receptors expressed in the upper part of the developing cortical plate (Pérez-García *et al*., 2004). In turn, Dab1 is present in radially migrating neurons and in the radial glia cells in the ventricular zone (Luque *et al*., 2003; Meyer *et al*., 2003); it docks to an NPxY sequence in the intracellular domain of VLDLR and ApoER2 (Howell *et al*., 1999b) and becomes phosphorylated on key Tyr residues when reelin binds to its receptors (Keshvara *et al*., 2001). Cajal-Retzius cells are crucial for cortical lamination and cell positioning, and degenerate and die once they have completed their developmental function (Derer and Derer, 1990; Meyer *et al*., 2002).

Comparative studies of the developing pallium of chick (Bernier *et al*., 2000), turtle (Bernier *et al*., 1999), lizard (Goffinet *et al*., 1999), and crocodile (Tissir *et al*., 2003) have shown that reelin-positive neurons are a consistent feature of the outer, cell-sparse layer of the developing cortex of all amniotes and may have evolved from an ancestral cell type present in stem amniotes (Bar *et al*., 2000). In reptiles and rodents, Cajal-Retzius cells have a horizontal shape and lie in a subpial position. In rats and particularly in humans, Cajal-Retzius cells undergo complex morphological changes during development, and settle at progressively deeper levels of the marginal zone (Fig. 6.4A–D) (Meyer and González-Hernández, 1993; Meyer *et al*.,

1998). In turtles and apparently all mammals, Cajal-Retzius cells coexpress transcription factors such as p73 (Fig. 6.4D, 6.5) (Yang *et al*., 2000; Meyer *et al*., 2002; Tissir *et al*., 2003) and Tbr1 (Hevner *et al*., 2003). Furthermore, the intensity of the reelin signal is much higher in mammals than in nonmammalian species, suggesting that the reelin pathway played a driving role in cortical evolution in mammalian and squamate lineages (Bar *et al*., 2000). In keeping with this hypothesis is the observation that in the human cortex Cajal-Retzius cells are more numerous than in other mammalian species and display highly differentiated morphological features (Meyer and González-Hernández, 1993; Meyer and Goffinet, 1998; Meyer *et al*., 2002). Most importantly, they extend a huge axonal fiber plexus at the boundary between the cortical plate and the marginal zone (Meyer and González-Hernández, 1993; Cabrera-Socorro *et al*., 2007). In the rat, the axons of Cajal-Retzius cells show secretory activity (Derer *et al*., 2001). Likewise, the human Cajal-Retzius plexus is reelin-immunoreactive (Fig. 6.4C), suggesting that it may also secrete reelin and lead to a further diffusion and amplification of the reelin signal (Cabrera-Socorro *et al*., 2007).

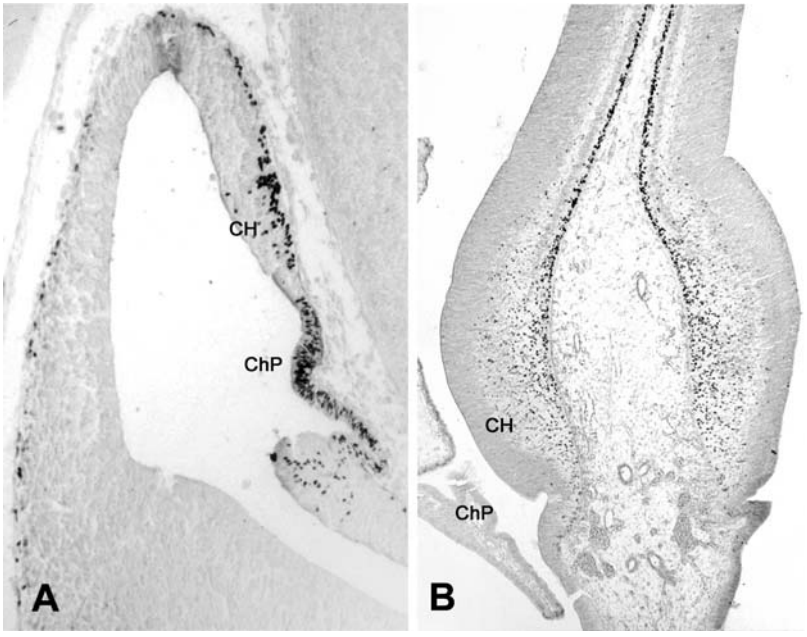

Fig. 6.5 The evolution of the cortical hem, the main source of Cajal-Retzius cells. (**A**) The cortical hem in a mouse at embryonic day 12, and (**B**) in a human embryo at 9 GW.The transcription factor p73 is highly expressed in Cajal-Retzius cells and may determine their survival and death. Note the size increase of the human hem, and the large numbers of human Cajal-Retzius cells, which lead to a high reelin signal in the human cortex. Ch, choroid plexus; CH, cortical hem. Bars: 50 μm (**A**), 100 μm (**B**)

The unique differentiation of human Cajal-Retzius cells may be related to the presence of p73 in their nucleus. Cajal-Retzius cells express Delta Np73, a truncated p73 isoform with anti-apoptotic activities (Pozniak *et al*., 2000; Yang *et al*., 2002; Meyer *et al*., 2004), which may allow Cajal-Retzius cells to survive during the protracted period of human cortical migration and to acquire their complex morphology. The majority of p73-expressing Cajal-Retzius cells have their origin in the cortical hem (Fig. 6.5A,B), a signaling center at the interface of the choroid plexus and the hippocampus (Meyer *et al*., 2002; Yoshida *et al*., 2006), although there are additional sources at the boundaries of the telencephalon (Bielle *et al*., 2005). Interestingly enough, the cortical hem undergoes a significant increase in size during evolution (Fig. 6.5), in parallel with an increase in number of p73/reelin-expressing Cajal-Retzius cells in the cortex (Cabrera-Socorro *et al*., 2007). Altogether, these findings support the view that the increasing intensity of the reelin signal in Cajal-Retzius cells of the marginal zone during phylogenesis may correlate with the increasing complexity of cortical architecture and cognitive functions in the course of evolution. The evolutionary significance of Cajal-Retzius cells is also reflected by the novel "human accelerated regions" RNA gene (HAR1F) that is expressed specifically in human Cajal-Retzius neurons (Pollard *et al*., 2006).

4 Reelin in Hippocampus and Entorhinal Cortex

The expression pattern of reelin in the hippocampal formation and entorhinal cortex is particularly interesting, because both are core structures of the explicit memory system, which is crucial for learning and cognitive abilities (Qiu and Weeber, 2007). Reelin is, in fact, highly expressed in the developing and adult hippocampus and entorhinal cortex. In the adult human entorhinal cortex, reelin is present in large polygonal cells that form clusters in layer II (Fig. 6.6C) (layer Pre-α of Braak and Braak, 1992). These neurons are important insofar as they give rise to the perforant path connecting the entorhinal cortex with the hippocampus (Steward and Scoville, 1976; Witter and Groenewegen, 1984; van Groen *et al*., 2003), and are particularly vulnerable for degeneration in Alzheimer's disease (Chin *et al*., 2007; Van Hoesen and Hyman, 1990; Jellinger *et al*., 1991; Gomez-Isla *et al*., 1996; Chin *et al*., 2007). Neurons in adult human layer Pre-α coexpress reelin and Dab1 (Meyer *et al*., 2003). In human fetuses, neurons of entorhinal layer II begin to express low levels of reelin already around 20 gestational weeks, and some pyramidal neurons in deeper layers also become positive (G. Meyer, unpublished). Reelin immunoreactivity is high after birth and in young adults and declines in intensity in the course of adult life (Pérez-García *et al*., 2001). Layer II cells lose reelin and/or die in Alzheimer's disease (Chin *et al*., 2007), suggesting a possible correlation between reelin expression and Alzheimer-related degenerative processes. This hypothesis is supported by the finding that the numbers of reelin-expressing neurons in layer II are reduced in human amyloid precursor protein (hAPP) transgenic mice, whereas reelin levels in entorhinal interneurons are not affected (Chin *et al*., 2007).

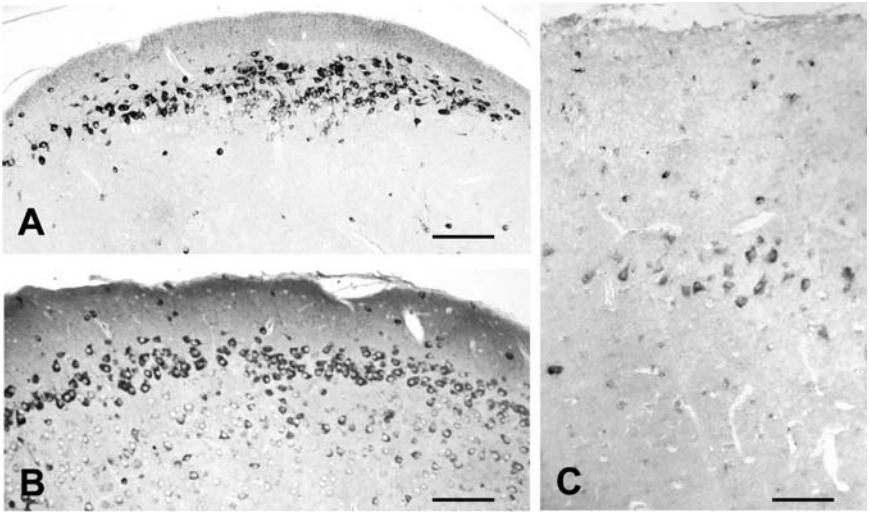

Fig. 6.6 The evolution of reelin expression in the entorhinal cortex. (**A**) The lateral cortex of the lizard *Lacerta galloti*, where the principal cells are intensely reelin-immunoreactive. (**B**) Reelin in layer 2 of the cat entorhinal cortex. There are also reelin-positive pyramidal neurons in deeper layers. (**C**) Adult human (49 years) entorhinal cortex. Reelin is expressed in clusters of large modified pyramidal neurons in layer 2; the intensity of expression declines during adult life and is moderate at 49 years. Bars: 50 μm (**A**), 60 μm (**B**), 30 μm (**C**)

The positivity of entorhinal layer II neurons is remarkably conserved in phylogenesis. All mammalian species examined so far display a usually strong reelin signal in this specific cell type, i.e., mouse (Alcantara *et al*., 1998), rat (Drakew *et al*., 1998; Pesold *et al*., 1998; Ramos-Moreno *et al*., 2006), ferret (Martinez-Cerdeño *et al*., 2003), cat (Fig. 6.6B), and gerbil (Pérez-García *et al*., 2001). Even in the lizard *Lacerta galloti*, the lateral cortex is strongly reelin-immunoreactive and closely resembles the mammalian entorhinal cortex (Fig. 6.6A). Also, the outer fiber layer of the pallium connecting the lateral with the medial cortex, the homologue of the hippocampus, shows faint reelin-positivity (Cabrera-Socorro *et al*., 2007), and may be the lacertilian equivalent of the perforant path. In mammals, reelin may be transported by axons in the perforant path and released in the target layers in the dentate gyrus and Ammon's horn, where it may contribute to the synaptic plasticity of the hippocampal circuits.

In the hippocampal subregions, some layers display high levels of reelin even in adulthood, especially the stratum lacunosum-moleculare of Ammon's horn, and the molecular layer of the dentate gyrus, which are populated by numerous small interneurons (Fatemi *et al*., 2000; Pappas *et al*., 2001; Abraham and Meyer, 2003; Abraham *et al*., 2004; Ramos-Moreno *et al*., 2006). This enrichment of reelin is again in accord with its proposed activity in modulating synaptic plasticity underlying long-term hippocampus-dependent learning.

As discussed in this chapter, reelin has multiple functions in the brain, and the adult activities are not necessarily the same as during development. In keeping with the importance of the reelin–Dab1 signaling pathway for neuronal positioning, reelin in Cajal-Retzius cells of the hippocampal fissure is crucial for the correct development of the hippocampus. Mice defective for the transcription factor p73 lack Cajal-Retzius cells in cortex and hippocampus (Yang *et al*., 2000; Meyer *et al*., 2002). While neocortical lamination is not disturbed in these mutant mice, the hippocampus is severely disorganized, and the hippocampal fissure does not form, suggesting that hippocampal architectonic development depends strongly on the presence of Cajal-Retzius cells (Meyer *et al*., 2004). Furthermore, early generated Cajal-Retzius cells of the hippocampus provide a template for entorhinal fibers to find their target layers in the hippocampus (Ceranik *et al*., 1999). In the *reeler* mouse, the granule cells fail to form a compact cell layer (Lambert de Rouvroit and Goffinet, 1998), perhaps due to a twofold function of reelin which may act as a differentiation factor for radial glia cells and also as a positional cue for radial fiber orientation and granule cell migration (Zhao *et al*., 2004). In the human hippocampus, there are several sources for Cajal-Retzius cells, which are particularly numerous in the head of the hippocampus, which is highly differentiated in man, in accordance with the cognitive functions of the human hippocampus (Abraham *et al*., 2004). The hippocampus is thus another example for the variety of reelin-related mechanisms involved in the development and evolution of the telencephalon, and demonstrates the importance of this protein in the formation and maintenance of the CNS.

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Chapter 7
Reelin/Dab1 Signaling in the Developing Cerebral Cortex

Eric C. Olson and Christopher A. Walsh

Contents

Abstract Mice lacking the cytoplasmic adapter protein Dab1 (Disabled homolog-1) display histological defects in the central nervous system (CNS) that are essentially indistinguishable from those observed in the *reeler* mouse. Dab1 is expressed in virtually all Reelin-responsive cells and is rapidly phosphorylated in response to

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Reelin application. The finding of a near identity in phenotype, coupled with a direct biochemical response to Reelin, has raised great interest in understanding Dab1 function, both as an exemplar of an adapter protein with a profound phenotypic contribution, and as a means of decoding mechanisms of Reelin signaling. What has emerged from these studies is a surprisingly complex picture of Dab1 at the genomic, mRNA, protein, and functional levels. This chapter will summarize some of the key features of Dab1, and its role as a transducer of the Reelin signal in the developing cerebral cortex.

1 Historical Context

The *reeler* mouse is one of the most fascinating and comprehensively studied neurological mutants, with a literature extending back over 50 years (Falconer, 1951). *Reeler* mice, which lack Reelin, show disordered cellular positioning in the major laminated structures of the brain, including the cerebral cortex, hippocampus, and cerebellum. In the *reeler* cortex, migrating neurons fail to split the preplate (Sheppard and Pearlman, 1997), and subsequently generated neurons "pile up" behind previously generated neurons, forming an approximate inversion of the normal cellular layering of the six-layered cortex (Caviness and Sidman, 1973). It was therefore of considerable interest when a new spontaneous mutant mouse, termed *scrambler*, was identified in 1996, which was phenotypically indistinguishable from *reeler,* but for which a recessive mutation mapped to chromosome 4 and not to chromosome 5, the location of the *reeler* mutation (Sweet *et al*., 1996; Goldowitz *et al*., 1997; Gonzalez *et al*., 1997). The characterization and mapping of the *scrambler* locus followed soon after the identification and cloning of *Reln*, the novel gene mutated in the *reeler* mouse that encodes the secreted protein Reelin (D'Arcangelo *et al*., 1995). The close phenotypic match between *scrambler* and *reeler* mice raised hope that the chromosome 4 defect would involve a gene encoding the Reelin receptor, and an effort in several labs was initiated to identify the genetic disruption in the *scrambler* mouse. About this time, a gene then called mDab1 (Howell *et al*., 1997a), now called Dab1, was "knocked out" on chromosome 4 and the resultant mouse displayed a *reeler*-like phenotype (Howell *et al*., 1997b). This observation facilitated the rapid identification of mutations in Dab1 that were within the mapped intervals in the *scrambler* and related *yotari* mice (Sheldon *et al*., 1997; Ware *et al*., 1997).

2 Mutations in S*crambler* and Y*otari* Mice

The mutations identified in *scrambler* and *yotari* mice are both associated with aberrant *dab1* mRNA message and little or no Dab1 protein. In wild-type animals, *dab1* message is expressed as a primary transcript of 5.5 kb (Howell *et al*., 1997a). In *scrambler,* very small amounts of this 5.5-kb message are detected, along with

the presence of a larger, aberrant transcript of ~7 kb. The *scm* mutation causes the splicing of an intracisternal-A particle (IAP) retrotransposon element into the Dab1 message. This leads to the insertion of an ~1.5-kb noncoding insert into the Dab1 mRNA and a near absence of Dab1 protein (Sheldon *et al*., 1997; Ware *et al*., 1997). To date, the exact mutation in *scrambler* that promotes the splicing of the IAP into the Dab1 message has not been identified. In contrast, the *yotari* mouse mutation is caused by a 962-base-pair insertion of an L1 retrotransposon fragment into chromosome 4 that disrupts three exons of the *Dab1* gene (Sheldon *et al*., 1997). In *yotari* mice, there is no detectable Dab1 protein. To date, there are no identified human mutations within the *DAB1* locus, although three distinct *RELN* mutations have been identified in humans (Chang *et al*., 2006), along with one mutation in the Reelin receptor *VLDLR* (Boycott *et al*., 2005).

The identified gene *dab1* encodes a cytoplasmic adapter protein that lacks enzymatic activity *per se* and appears instead to act as a scaffold for the assembly of a multiprotein signaling complex (Howell *et al*., 1997a). Dab1 contains an ~150-amino-acid PTB/PID (phosphotyrosine binding/phosphotyrosine interacting domain) (Howell *et al*., 1997a) that preferentially binds NPXY motifs in target proteins. Near this PTB/PID domain on the C-terminal side are a series of five tyrosine residues that are potential target sites for the Src family of tyrosine kinases (Howell *et al*., 1997a). The Dab1 PTB/PID preferentially binds F/YXNPXY amino acid sequences found in the cytoplasmic domains of a number of transmembrane proteins (Trommsdorff *et al*., 1998; Howell *et al*., 1999b), including the Reelin receptors.

3 Dab1 and the Reelin Receptor Complex

Confirmation of a biochemical relationship between Reelin and Dab1 *in vivo* is found in the expression pattern of Dab1 protein in *reeler* mice. The absolute level of Dab1 protein is 5- to 10-fold higher in the embryonic *reeler* neocortex compared to wild-type littermate controls (Rice *et al*., 1998). Elevated expression of Dab1 protein is now thought to be a diagnostic feature of Reelin signaling abnormalities. In addition, Dab1 protein displays reduced levels of tyrosine phosphorylation in the *reeler* neocortex, indicating that one consequence of Reelin deficiency is reduction in tyrosine phosphorylation of Dab1. Surprisingly, Dab1 mRNA levels are similar in mutant and control brains, suggesting that that Dab1 misregulation is posttranscriptional (Rice *et al*., 1998). In contrast, when the reciprocal experiment is performed—namely, examination of Reelin expression in *scrambler* mice—approximately normal Reelin expression is observed (Gonzalez *et al*., 1997), with the interpretation being that Dab1 is not required for the expression or secretion of Reelin. The expression studies of Dab1, particularly those performed in *reeler* mice, position Dab1 on the receptive side of the Reelin signaling pathway.

Dramatic evidence for the identity of the Reelin receptors followed from the *reeler*-like phenotype of mice doubly deficient in VLDLR (very-low-density lipoprotein receptor) and ApoER2 (ApoE receptor 2) (Trommsdorff *et al*., 1999). Mice singly

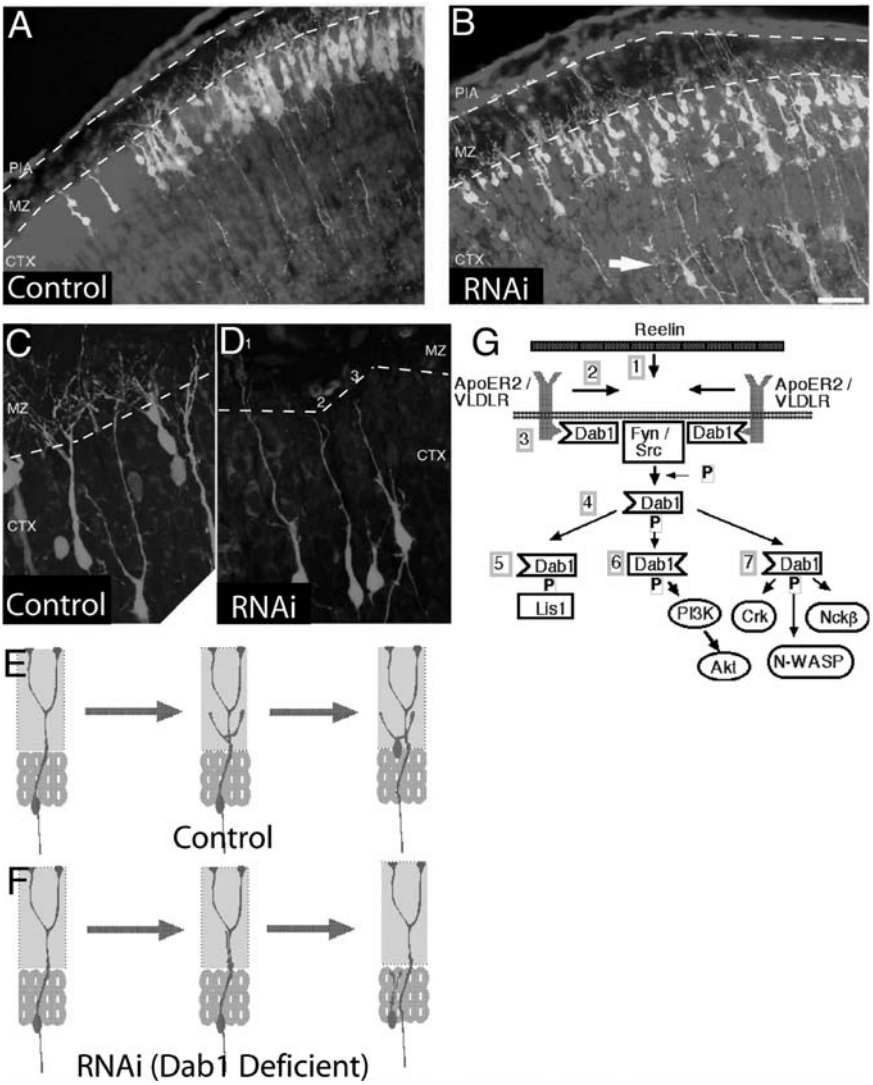

Fig. 7.1 Reelin Dab1 signaling in upper layer cortical neurons. (**A**, **B**) Low-magnification images of layer 2/3 cortical neurons on postnatal day 2 (P2), 7 days after *in utero* electroporation on E16 with either RNAi that suppresses Dab1 (RNAi) or control RNAi vector (Control). (**A**) Control electroporated neurons show precise lamination and exuberant dendritic growth in the MZ (dashed lines) on P2, whereas (**B**) Dab1-suppressed cells (RNAi) show disrupted lamination with occasional ectopic deep cells (arrow) and sparse dendrites in the MZ. (**C**, **D**) Higher-magnification images revealing extensive dendrites in (**C**) control cells and stunted dendrites in (**D**) RNAi-treated cells that either do not penetrate the MZ (cells 2 and 3) or stunted dendrites that do not show extensive secondary and tertiary branching in the MZ (cell 1). Scale bars: 50 μm (**A**, **B**); 20 μm (**D**). (**E**, **F**) Model of cell positioning and dendritogenesis in the developing cortex. (**E**) A control neuron (dark green) migrating on a radial glial process (red) extends a branched leading process into the MZ and then translocates through the upper ~50 μm of the CP, arresting migration at the first branch point of the leading process. (**F**) Dab1-deficient cells extend a leading process into the MZ but it remains simplified and the neuron does not translocate efficiently. (**G**) Dab1 interactions (after D'Arcangelo, 2006). Reelin secreted by CR cells (1) binds Reelin receptors (ApoER2 and VLDLR) in the migrating neuron causing (2) the clustering of Reelin receptors and Dab1.

deficient for either receptor show much more subtle phenotypes and are overtly normal (not ataxic), whereas mice deficient for both receptors phenocopy the *reeler* mouse (Trommsdorff *et al*., 1999). Both of these receptors are members of the LDLR (low-density-lipoprotein receptor) family and contain a single Dab1 binding site (NPXY) on their cytoplasmic tails. Upon binding of Reelin to VLDLR and ApoER2 (Hiesberger *et al*., 1999; D'Arcangelo *et al*., 1999), a rapid phosphorylation of Dab1 on tyrosine residues is induced. Neither ApoER2 nor VLDLR possesses kinase domains or displays kinase activity, however, leading to the suggestion that the Reelin-induced phosphorylation of Dab1 is achieved through Src family kinases (SFKs). Two SFKs, Fyn and Src, bind Dab1 (Howell *et al*., 1997a), phosphorylate Dab1, and are activated by Reelin binding to its receptors (Arnaud *et al*., 2003b; Bock and Herz, 2003; Jossin *et al*., 2003). Conclusive evidence for the involvement of SFKs in Reelin signaling is found in mice deficient in both Fyn and Src. These "double knockout" animals recapitulate major aspects of the *reeler* phenotype including cortical layer inversion and cerebellar abnormalities (Kuo *et al*., 2005). These genetic and biochemical findings establish Dab1 as a central link in an entirely novel receptor complex that includes Reelin, VLDLR or ApoER2, and the tyrosine kinases Fyn or Src (Fig. 7.1G).

4 Dab1 Isoforms and the Importance of p80

In both mouse and human, the genomic organization of *dab1* is complex with multiple isoforms generated from alternative poly-adenylation and splicing events (Bar *et al*., 2003). In the developing mouse nervous system, Dab1 appears to exist in a number of distinct isoforms represented in cDNAs encoding 555-, 271-, and 217-amino-acid proteins that all share the N-terminal PTB/PID domain (Howell *et al*., 1997a). Immunoblotting of whole brain lysates during the neurodevelopmental period reveals proteins with weights of p120, p80, and p60 (Howell *et al*., 1997a,b); however, the correspondence between the cDNAs and these different protein forms of Dab1 is only known for the p80 form, which is encoded by the Dab1 555 cDNA. The p80 form appears to be the only isoform expressed in brain that is required for normal histological development. The essential nature of p80 was confirmed in a "knockin" experiment; the Dab1 555 cDNA was inserted into the mouse Dab1 locus, knocking out the endogenous splice forms of Dab1 and creating an obligate p80-expressing mouse (Howell *et al*., 2000). This obligate p80-expressing mouse was histologically indistinguishable from normal mice that express multiple splice

Fig. 7.1 (continued) (3) The cytoplasmic clustering of Dab1 activates two SFKs (Fyn and Src) leading to (4) tyrosine phosphorylation of Dab1. (5) Phospho-Dab1 binds Lis1, a cytoplasmic dynein interacting protein encoded by *Lis1*, the gene underlying Miller-Dieker lissencephaly. (6) Phospho-Dab1 also activates PI3 kinase and Akt kinase and (7) binds adapter proteins Crk, Nckβ as well as N-WASP. Reelin signaling may regulate multiple cellular events including glial adhesion, somal positioning, and dendritogenesis. Panels **A–F** modified from Olson *et al*. (2006), copyright 2006 by the Society for Neuroscience (*See Color Plates*)

forms of the Dab1 message. Given the evidence pointing to the importance of the p80 isoform of Dab1 in development, it was surprising to find that a similarly constructed "knockin" mouse that was an obligate expresser of a p45 allele of Dab1, that lacks 284 amino acids at the C-terminus, showed normal cortical development (Herrick and Cooper, 2002). Although animals with a single copy of the p45 allele over a null allele ($Dab1^{p45/-}$) showed disruptions in the upper cortical layers, the observation that $Dab1^{p45/p45}$ homozygotes appear normal focuses efforts at understanding Dab1's role in cortical development at the N-terminal 271 amino acids.

5 PTB/PI Domains

The Dab1 PTB/PID binds F/YXNPXY domains in the cytoplasmic tails of the Reelin receptors ApoER2 and VLDLR. The PTB/PID is the major recognized feature within the N-terminal 271 amino acids and extends from amino acids 23 to 174 (Howell *et al*., 1997a). A new appreciation of the PTB/PID domain has emerged since the prototype PTB was identified in 1994 in the adapter Shc (Src homology 2 domain-containing protein; Blaikie *et al*., 1994; Kavanaugh and Williams, 1994). Originally, the PTB/PID was so named because Shc preferentially bound NPXY motifs when the tyrosine residue on the ligand was phosphorylated (NPXpY) (Kavanaugh *et al*., 1995; Songyang *et al*., 1995). For example, Shc binds the epidermal growth factor receptor (EGFR) when the NPXY sequence in the receptor's cytoplasmic tail is tyrosine phosphorylated (O'Bryan *et al*., 1998). One might assume that this phosphorylation-dependent binding of the PTB/PID would be a fundamental property of the domain, but subsequently identified PTB/PID proteins, including Dab1, appear to preferentially bind the dephosphorylated amino acid sequence NPXY sequences (Howell *et al*., 1999b). In fact, the dephosphorylated tyrosine-preferring PTB/PIDs appear to be the most common, comprising approximately 75% of the characterized PTB/PIDs (Uhlik *et al*., 2005). This emerging picture of the PTB/PID has complicated the terminology (Margolis, 1999) and has led to further studies of PTB/PID–ligand interactions.

6 Structure of the Dab1 PTB/PI Domain

The Dab1 PTB/PID has been crystallized in bound states with peptides corresponding to the cytoplasmic domains of ApoER2 (Stolt *et al*., 2003) and another Dab1 binding partner, the amyloid precursor protein (APP) (Yun *et al*., 2003). The Dab1 PTB/PID, like each of the 10 different solved PTB domains, adopts a structure similar to that of the pleckstrin homology (PH) superfold. Whereas the Shc PTB/PID has three critical basic amino acids in the binding pocket that interact with the phosphorylated tyrosine residue in NPXpY, Dab1 lacks two of these critical basic amino acids. Instead, binding of nonphosphorylated tyrosine is favored by van der Waals attraction between the PTB/PID and the NPXY target. The distinct characteristics of the Dab1

PTB/PID constitute a third PTB/PID family separate from the Shc-like and IRS-like PTB/PID domains (Uhlik *et al*., 2005) and have led to the term Dab homology (DH) domain to describe this particular class of PTB/PID (Yun *et al*., 2003).

6.1 Phospholipid Binding by the Dab1 PTB/PID

Another shared feature among many PTB/PID domain family members is the phospholipid binding subdomain within the PTB/PID. This phospholipid binding domain is a highly basic sequence that in Dab1 "crowns" the PTB/PID and binds the phosphatidylinositol (PtdIns) phosphate head groups of plasma membrane lipids. This binding localizes Dab1 to the plasma membrane and is apparently independent of NPXY peptide binding, as mutations have been identified that can separately and selectively abolish phospholipid or NPXY binding (Xu *et al*., 2005; Huang *et al*., 2005; Stolt *et al*., 2004). The crystal structure of Dab1 PTB/PID–ApoER2–PtdIns-4,5-P2 suggests that binding of PtdIns-4,5-P2 confines the peptide binding pocket to a vertical orientation with respect to the plasma membrane, possibly leading to more favorable interactions between the peptide binding pocket and the NPXY motifs in the cytoplasmic tails of the receptors (Stolt *et al*., 2003; Uhlik *et al*., 2005).

7 Dab1 Phosphorylation by Src Family Kinases

As mentioned above, a defining feature of the Reelin–Dab1 interaction is the rapid, Reelin-dependent tyrosine phosphorylation of Dab1. Although it was apparent early on that Dab1 is hypophosphorylated in the *reeler* mouse (Rice *et al*., 1998), it was not initially clear if Dab1 phosphorylation was directly dependent on Reelin. The development of an *in vitro* assay using cultured embryonic neurons enabled the demonstration that Dab1 is rapidly phosphorylated in response to recombinant Reelin application (Howell *et al*., 1999a). The essential nature of tyrosine phosphorylation of Dab1 was later demonstrated in an elegant "knockin" experiment, where a p80 allele of Dab1 was constructed that substituted phenylalanine (F) for tyrosine (Y) at five candidate phosphorylation sites on Dab1 (Howell *et al*., 2000). This so-called "5F" allele was then "knocked-in" to the Dab1 locus, thereby rendering a Dab1 allele that could not be phosphorylated at these positions. Homozygotes of the Dab1 5F displayed a *reeler*-like phenotype, demonstrating that these five tyrosines are essential for Dab1 function, likely because of their role as phosphorylation substrates.

7.1 Genetics of Fyn and Src Deficiency in Cortical Development

From the time of its discovery, Dab1 was known to bind nonreceptor tyrosine kinases including Src, Fyn, and Abl (Howell *et al*., 1997a), and thus these kinases became likely candidates for mediating Reelin-dependent Dab1 phosphorylation.

Consistent with this notion, broad inhibition of Src family kinases, using the pharmacological agent PP2, both blocked Reelin-dependent Dab1 phosphorylation (Arnaud *et al*., 2003b; Bock and Herz, 2003; Jossin *et al*., 2003) and caused *reeler*-like malformations in cerebral cortical slices (Jossin *et al*., 2003). Pharmacological blockade of SFKs specifically reduced Dab1 phosphorylation at tyrosines 198 and 220, the major sites of Reelin-dependent tyrosine phosphorylation (Keshvara *et al*., 2001). Inhibition of Abl, however, does not suppress Dab1 phosphorylation (Arnaud *et al*., 2003b). Mice singly deficient for Src (Soriano *et al*., 1991), Fyn (Stein *et al*., 1992), and Yes (Stein *et al*., 1994), however, do not reveal a *reeler* mouse phenotype, implying that multiple members of the SFK family contribute to normal Reelin signaling. This hypothesis was dramatically confirmed when a double knockout of Src and Fyn was created that recapitulated major histological features of the *reeler* phenotype, including deficiency in preplate splitting, an inversion of cortical layers, and ectopic clusters of Purkinje cells in the developing cerebellum (Kuo *et al*., 2005).

7.2 Dab1 Clustering Activates SFKs

One of the most important insights emerging from the studies of Dab1–SFK interaction is that simple clustering of Dab1 may be sufficient to stimulate its phosphorylation by SFKs (Fig. 7.1). Reelin is secreted as a multimer (Kubo *et al*., 2002) and likely acts as a multivalent ligand for the Reelin receptors (Strasser *et al*., 2004). Therefore, Reelin binding to the Reelin receptors likely causes the local clustering of both Reelin receptors and, by extension, Dab1 bound to the cytoplasmic tail of the receptors. In fact, Dab1 dimerization, independent of the Reelin receptors, is sufficient to induce Dab1 phosphorylation on Y198 and Y220 by SFKs (Strasser *et al*., 2004). Similarly, simple coexpression of Dab1 and SFKs (either Src or Fyn) in 293 kidney cells leads to a dramatic increase in SFK activity (Bock and Herz, 2003). This amplification of SFK activity is apparently attenuated by Dab1 protein degradation mediated by the polyubiquination and proteasomal degradation (Arnaud *et al*., 2003a; Bock *et al*., 2004). The observed overexpression of the Dab1 5F allele suggests that proteolysis of Dab1 is dependent on Dab1 phosphorylation (Howell *et al*., 2000; Arnaud *et al*., 2003a). Thus, Dab1 phosphorylation initially amplifies Fyn and Src activity minutes after Reelin exposure, followed by a presumed slower time-scale dampening of Reelin signaling ($t_{1/2}$=160 minutes), subsequent to Dab1 degradation (Arnaud *et al*., 2003a; Bock *et al*., 2004).

8 Protein Interactions Subsequent to Dab1 Phosphorylation

Given the essential nature of Dab1 tyrosine phosphorylation, the effort to decode Reelin signaling has focused primarily on protein interactions that occur subsequent to Dab1 phosphorylation (Fig. 7.1G). One branch of the Reelin signaling pathway

has the potential to regulate the actin cytoskeleton through N-WASP (Suetsugu *et al*., 2004), as well as through Nckβ (Pramatarova *et al*., 2003) and the Crk family of adapters (Ballif *et al*., 2004; Chen *et al*., 2004). Nckβ and Crk family adapters contain both SH2 domains that preferentially bind phosphorylated tyrosine motifs and SH3 domains that bind specific proline-containing sequences, such as XPXXP (Pawson and Scott, 1997). Nckβ appears to relocalize with phospho-Dab1 in neuronal growth cones in response to Reelin application, and this localization is correlated with regions of remodeled actin cytoskeleton (Pramatarova *et al*., 2003). Similarly, the Crk family of adapter molecules also associate with phosphorylated Dab1. Crk family adapters have been implicated in a wide range of cellular processes, including migration, endocytosis, and morphological reorganization (Chen *et al*., 2004, and references therein). A functional linkage between phospho-Dab1 and cell migration is suggested by the observation that phosphorylated Dab1 reduces migration rates in heterologous NBT-II (tumor cells), possibly by depletion of Crk protein from the focal adhesion contacts (Chen *et al*., 2004).

Other effectors of Reelin signaling have the potential to modify the microtubule cytoskeleton. Phospho-Dab1 binds p85, the regulatory subunit of PI3 kinase (Beffert *et al*., 2002; Ballif *et al*., 2003; Bock *et al*., 2003). The association of p85 with Dab1 liberates and activates the p110 catalytic subunit of PI3 kinase, leading to the subsequent activation of the serine threonine kinase Akt (also known as Protein Kinase B) and the subsequent inhibition of glycogen synthetase kinase 3β (GSK3β) (Beffert *et al*., 2002). The microtubule-associated protein (MAP) tau is a GSK3β target and is hyperphosphorylated in some strains of *reeler* mice (Hiesberger *et al*., 1999), possibly altering microtubule stability in these animals. The interaction of phospho-Dab1 with the lissencephaly gene Lis1 provides another route toward modification of microtubule dynamics (Assadi *et al*., 2003). Lis1 is encoded by the gene underlying autosomal dominant Miller-Dieker lissencephaly, a severe form of lissencephaly that corresponds to nearly complete absence of sulci and gyri in the human cerebral cortex (Reiner *et al*., 1993). Miller-Dieker lissencephaly is more severe than the lissencephaly with cerebellar hypoplasia (LCH) associated with Reelin deficiency (Hong *et al*., 2000). Compound mutants of Lis1- and Dab1-deficient mice show enhanced histological disorganization in the hippocampus, suggesting a genetic interaction between these loci. Lis1 is known also to bind the microtubule motor cytoplasmic dynein, and via this interaction regulates cytoskeletal dynamics, including the positioning of the centrosome (Wynshaw-Boris and Gambello, 2001; Tanaka *et al*., 2004). The interaction between phospho-Dab1 and Lis1 therefore has the potential to mediate complex regulation of microtubule dynamics and cell migration (Wynshaw-Boris and Gambello, 2001).

9 Dab1 Independent Reelin Signaling

Although interactions with phosphorylated Dab1 are clearly essential for normal Reelin signaling, Dab1 phosphorylation may not be sufficient for triggering all downstream aspects of Reelin signaling. As mentioned above, the secreted form

of Reelin is now known to be multivalent and to crosslink the Reelin receptors (Kubo *et al*., 2002). This crosslinking leads to the phosphorylation of Dab1 by SFKs. Dab1 phosphorylation can also be stimulated by the crosslinking of Reelin receptors using antibodies that recognize the extracellular domains of VLDLR and ApoER2. However, this antibody-dependent crosslinking apparently does not rescue the *reeler* phenotype in cortical slice cultures (Jossin *et al*., 2004), whereas exogenous Reelin application will provide rescue. This finding implies the existence of a Reelin signaling pathway that works in parallel with phospho-Dab1.

10 Promotion of Receptor Expression by Dab1

Additionally, Dab1 may have an upstream role in promoting Reelin receptor presence at the cell surface (Utsunomiya-Tate *et al*., 2000). Cultured neurons from *yotari* embryos that lack Dab1, bind approximately half the exogenously applied Reelin compared to wild-type. This deficiency in Reelin binding appears to be due to a reduction of the amount of Reelin receptors present on the cell surface and implies that experiments that rely on the analysis of Dab1-deficient or Dab1-suppressed cells may identify both the effect of deficient receptor expression and deficient downstream signaling.

11 Dab1 Expression During Development

In all areas of the CNS affected by the Reelin mutation, cells expressing Dab1 are found within a few cell diameters of cells expressing Reelin (Sheldon *et al*., 1997; Rice *et al*., 1998). In the mouse cerebral cortex, Dab1 expression is observed as a series of bands across the cerebral wall, including a band within the proliferative ventricular zone (Bar *et al*., 2003). The apparent highest levels of Dab1 expression are associated with cells settling beneath the marginal zone (MZ), the location of Reelin-secreting Cajal-Retzius (CR) cells. Similar spatial relationships are observed elsewhere in the developing brain. In the hippocampus, for example, Dab1-expressing pyramidal neurons settle underneath Reelin-expressing CR cells, and in the cerebellum Dab1 is expressed by migrating Purkinje cells, as they settle underneath the Reelin-expressing cells of the external granule layer (EGL) (Rice *et al*., 1998). In humans, Dab1 is first strongly detected at the seventh gestational week (GW) during the initial phases of cortical plate development (Meyer *et al*., 2003). As cortical development proceeds, Dab1 expression is greatest at the upper tier of the cortical plate underneath the MZ, similar to the pattern of Dab1 expression observed in rodents. A close spatial relationship between Dab1- and Reelin-expressing cells continues through the neuraxis into the spinal cord

(Yip *et al*., 2004) and underscores the tight functional relationship between Reelin and Dab1.

12 Cellular Response to Reelin/Dab1 Signaling

There is evidence that Reelin–Dab1 signaling modifies three possibly interrelated cellular activities during cortical development: cell adhesion (Pinto Lord *et al*., 1982; Hoffarth *et al*., 1995; Dulabon *et al*., 2000; Hack *et al*., 2002; Sanada *et al*., 2004), somal movement and positioning (Caviness and Sidman, 1973; Super *et al*., 2000; Magdaleno *et al*., 2002; Bock *et al*., 2003; Jossin *et al*., 2003; Olson *et al*., 2006), and dendritic or glial process growth (Pinto Lord *et al*., 1982; Forster *et al*., 2002; Hartfuss *et al*., 2003; Niu *et al*., 2004; Olson *et al*., 2006). In principle, modification of any one of these cellular activities could affect neuronal migration, and it is not clear if any single cellular activity represents the "primary response" to Reelin, while the other activities are derived or secondary. Defining the possible function(s) of Reelin signaling is facilitated by examination of cell autonomous Dab1 deficiency. In these experiments a subset of cells that are "blinded to Reelin" due to Dab1 deficiency are allowed to develop within an otherwise normal cortex. In this way, the cellular response to Reelin can be separated from cellular responses to the gross disorganization of the cortex caused by Reelin signaling deficiency.

12.1 Dab1-Dependent Cellular Positioning

Suppression of Dab1 in a subset of developing cortical cells by RNAi (RNA interference) causes migrating neurons to arrest a few cell diameters (~40–50 μm) short of their destination at the boundary of the cortical plate (CP) and marginal zone (MZ) (Fig. 7.1A,B) (Olson *et al*., 2006). An arrest of Dab1-deficient cells below Dab1-expressing cells is also observed in the cortices of chimeric mice composed of Dab1 −/− and Dab +/+ cells. In these animals, Dab1-deficient cells are found underneath a "supercortex" of wild-type cells (Hammond *et al*., 2001), suggesting that Dab1 has an important, cell autonomous role in promoting somal movement and migration through the cortex. Other studies have also argued for a role of Reelin signaling in the promotion of migration (Super *et al*., 2000; Magdaleno *et al*., 2002; Bock *et al*., 2003; Jossin *et al*., 2003). In a mutant *Drosophila* eye model, induced expression of mammalian Dab1 can rescue cellular positioning defects of photoreceptors (Pramatarova *et al*., 2006), suggesting a relatively direct interaction between Dab1 and the cellular positioning machinery. It is important to note, however, that Dab1-suppressed cells in mice can migrate appropriately for hundreds of micrometers through the intermediate zone (IZ) and lower CP (Olson *et al*., 2006), indicating that deficits in neuronal migration associated with Reelin signaling deficiency might be relatively specific to the final ~40–50 μm of migration.

12.2 Dab1-Dependent Cellular Adhesion

Reelin–Dab1 signaling is also associated with adherent interactions between developing neurons and glia. Whereas wild-type neurons appear to detach from the glial process at the end of their migration (Pinto Lord *et al*., 1982; Nadarajah *et al*., 2001; Sanada *et al*., 2004), neurons deficient in Reelin–Dab1 signaling maintain inappropriate glial adhesion (Pinto Lord *et al*., 1982; Sanada *et al*., 2004). This detachment at the end of migration is, however, a complex event at the molecular level, possibly involving modulation of $\alpha 3\beta 1$ integrin-mediated adhesion (Dulabon *et al*., 2000; Sanada *et al*., 2004; Schmid *et al*., 2005). However, mice deficient in $\beta 1$ integrin are unable to form functional $\alpha 3\beta 1$ heterodimeric receptors, yet show histological malformations that are more consistent with a basal lamina defect rather than cortical migration abnormalities (Graus-Porta *et al*., 2001). Nevertheless, a number of empirical findings indicate possible cell adhesion abnormalities in the *reeler* phenotype, particularly at the end of migration, and in this regard integrin interactions remain an intriguing aspect of Dab1 function.

12.3 Dab1-Dependent Process Outgrowth

Finally, Reelin–Dab1 signaling performs a major role in stimulating process outgrowth and branching in radial glia (Hartfuss *et al*., 2003), migrating neurons (Olson *et al*., 2006), and postmigratory neurons (Tabata and Nakajima, 2002; Niu *et al*., 2004; Olson *et al*., 2006). Dab1 suppression causes a cell autonomous reduction of the size and complexity of cerebral pyramidal neuron dendrites (Fig. 7.1A–D) (Olson *et al*., 2006). Similar dendritic reductions are also observed in cerebellar Purkinje cells (Trommsdorff *et al*., 1999) and hippocampal CA field pyramidal neurons (Niu *et al*., 2004) in *reeler* mice. Importantly, Reelin application appears to directly stimulate the dendritic growth of cultured hippocampal neurons (Niu *et al*., 2004) and the phosphorylation of the dendritic protein MAP1b (Gonzalez-Billault *et al*., 2005). In contrast, axonal growth is not affected by deficiency in Reelin signaling (Jossin and Goffinet, 2001), indicating that Reelin signaling selectively enhances dendritogenesis.

13 Models of Reelin/Dab1 Function

Reelin signaling deficiency may cause persistent and inappropriate neuron–glial adhesion at the end of migration, and this, in turn, may lead to altered cellular positioning. We propose a modified model that focuses on Reelin's demonstrated ability to stimulate dendritic growth (Niu *et al*., 2004) and leading process transformation at the MZ (Olson *et al*., 2006). In this new model, the leading process of the migrating neuron has a central role in directing cellular positioning (Fig. 7.1E,F). Reelin signaling

modifies the leading process in such a way to permit glial-independent leading process growth. Our data indicate that Reelin signaling stimulates the formation of a branched leading process in the MZ. Since the presence of this branched leading process correlates with movement of the cell soma through the last 50 μm, this leading process may well be essential for cellular movement and positioning. The first stable branch point formed by the leading process anticipates the arrest position of the moving nucleus, and thereby defines the arrest point of the cell body. In this way, cellular positioning (i.e., lamination) in the developing cortex is the indirect consequence of the Reelin-dependent transformation of the leading process into a maturing, branched dendrite. Emerging studies suggest that inhibition of PI3 kinase affects both Reelin-dependent cellular positioning and dendritogenesis, while inhibition of Akt affects dendritogenesis specifically (Jossin and Goffinet, 2006). This study raises hope that the multiple effects of Reelin signaling on developing neurons can be dissected at the molecular level through investigations of Dab1-dependent interactions. Further studies of this important signaling pathway are likely to provide new insights and additional surprises regarding essential aspects of cortical development.

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Chapter 8
Ultrastructural Localization of Reelin

Rosalinda C. Roberts and Emma Perez-Costas

Contents

Different chapters of the present book provide background about the molecular, genetic, and biochemical features of the reelin gene, its transcript, and final protein forms. It is not, therefore, our intention to provide an extensive background about the features of the reelin gene and protein, but to emphasize some relevant features that will be helpful for a better understanding of the data reviewed in the present chapter.

1 Reelin Expression and Distribution in Vertebrates

As far as we know, reelin is expressed throughout the vertebrate scale from the most ancient living vertebrate group to humans (for reviews see Lambert de Rouvroit and Goffinet, 1998; Tissir and Goffinet, 2003; Herz and Chen, 2006). The reelin gene has an 87.2% sequence homology between mice and humans, and the protein encoded by this gene presents an even higher homology (94.2%) between these two

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species (DeSilva *et al*., 1997). In addition, reelin is also present in other vertebrates, for which partial sequences of the gene have been obtained (Bernier *et al*., 1999, 2000; Goffinet *et al*., 1999; Costagli *et al*., 2002), or for which reelin mRNA and/or protein have been detected by different techniques (Pesold *et al*., 1998, 1999; Bernier *et al*., 1999, 2000; Goffinet *et al*., 1999; Rodriguez *et al*., 2000; Perez-Garcia *et al*., 2001; Costagli *et al*., 2002; Martinez-Cerdeno and Clasca, 2002; Martinez-Cerdeno *et al*., 2002, 2003; Perez-Costas *et al*., 2002, 2004; Tissir *et al*., 2003; Candal *et al*., 2005; Ramos-Moreno *et al*., 2006).

During embryonic development in mice, reelin is expressed by different neuronal groups in the brain and spinal cord (D'Arcangelo *et al*., 1995; Ikeda and Terashima, 1997; Schiffman *et al*., 1997; Alcantara *et al*., 1998; Kubasak *et al*., 2004). Reelin is also widely expressed in different neuronal groups during brain development in other vertebrates, including humans (Bernier *et al*., 1999, 2000; Goffinet *et al*., 1999; Costagli *et al*., 2002; Meyer and Goffinet, 1998; Meyer *et al*., 2002; Perez-Costas *et al*., 2002; Tissir *et al*., 2003; Abraham *et al*., 2004; Candal *et al*., 2005; Roberts *et al*., 2005). During embryonic development of mammals, reelin is synthesized and secreted in the cortex by Cajal-Retzius cells and in the cerebellum by cells in the external granular layer. In both cases, reelin secreted into the extracellular matrix is involved in the signaling for the correct positioning of postmitotic neurons that migrate radially from proliferative zones (for reviews see D'Arcangelo and Curran, 1998; Lambert de Rouvroit and Goffinet, 1998; Rice and Curran, 2001; Tissir and Goffinet, 2003). In addition, a possible role for reelin in neuronal positioning has also been reported in non-cortical areas of the mammalian brain, such as in the rhombencephalic motor nuclei, sympathetic preganglionic neurons, and even in autonomic neurons in the spinal cord (Yip *et al*., 2000; Ohshima *et al*., 2002; Phelps *et al*., 2002). Finally, several research groups have provided cumulative evidence indicating that reelin may be involved directly or indirectly in other important functions in the developing brain of vertebrates such as synaptogenesis and proper dendritic development (Del Rio *et al*., 1997; Miyata *et al*., 1997; Forster *et al*., 1998; Borrell *et al*., 1999; Ohshima *et al*., 2001; Rice *et al*., 2001; Niu *et al*., 2004; Yabut *et al*., 2007).

Even though early studies associated reelin expression almost exclusively with developmental stages of the brain and it was initially considered as a "developmental molecule," an increasing number of studies have shown that reelin is widely expressed in the adult vertebrate brain as well (Perez-Garcia *et al*., 2001; Costagli *et al*., 2002; Martinez-Cerdeno and Clasca, 2002; Martinez-Cerdeno *et al*., 2002, 2003; Perez-Costas *et al*., 2004; Roberts *et al*., 2005; Ramos-Moreno *et al*., 2006). This robust presence of reelin in adult brain contrasts with early works in which the presence of reelin in the adult brain was considered an almost residual expression that remained after development (e.g., see, the early descriptions of reelin expression in the mouse by Ikeda and Terashima, 1997, and Alcantara *et al*., 1998). It has been hypothesized that reelin in the adult brain is involved in synaptic function, in regulating neuronal plasticity, and could have a role in the modulation of learning and memory processes (Impagnatiello *et al*., 1998; Pesold *et al*., 1998, 1999;

Guidotti *et al*., 2000; Rodriguez *et al*., 2000, 2002; Weeber *et al*., 2002; Perez-Costas *et al*., 2004; Roberts *et al*., 2005). This hypothesis is supported by evidence showing that reelin plays a role in controlling synaptic plasticity in the adult brain (Weeber *et al*., 2002; Beffert *et al*., 2005; Chen *et al*., 2005; Qiu *et al*., 2006).

Some of the findings that have brought more attention to the study of reelin are the downregulation of reelin protein levels in several neurological and psychiatric diseases, including schizophrenia, bipolar disorder, major depression, autism, and Alzheimer's disease (Impagnatiello *et al*., 1998; Fatemi *et al*., 2000, 2001, 2002, 2005; Guidotti *et al*., 2000). However, none of these disorders are linked with direct genetic defects of the reelin gene. In fact, the only human disease that has been linked with defects in the reelin gene is a severe autosomal recessive form of lissencephaly with cerebellar hypoplasia, which is accompanied by severe delays in cognitive and motor development (Hong *et al*., 2000). Thus, the continued study of reelin is important not only to learn more about its normal function, but also to determine how abnormalities in reelin may contribute to the neuropathology of the aforementioned diseases.

In summary, reelin is a highly ubiquitous molecule in the vertebrate brain and is widely present in both the developing and adult brain. Currently, the mechanism of action of this protein is not completely understood in the developing brain and is even less clear in the adult. The high conservation of the reelin protein sequence throughout the vertebrate scale, as well as its high expression during development and in the adult brain, indicate that this protein may be involved in many roles, ranging from the most studied ones (such as its involvement in neuronal migration and proper neuronal positioning) to other possible functions, such as a role in adult neural plasticity related to learning and memory processes.

An important contribution toward a better knowledge of reelin function is provided by detailed studies of the cellular and subcellular localization of this protein. Detailed light microscopy can identify cellular types that express reelin and also perform a gross examination of its intracellular localization, but electron microscopy studies are necessary to discern where the protein is localized at the subcellular level. Detailed knowledge of the ultrastructural localization of this protein will help to shed light on some of the questions that are still open about reelin storage, transport, and secretion, as well as to identify cellular types that express reelin in the vertebrate brain.

2 Types of Cells that Express Reelin

The expression of reelin is not only widespread in different brain regions and virtually present in all vertebrates (Fig. 8.1 shows examples of reelin labeling in lamprey, rat, and human brain), but also is seen in a variety of cell types as detected by *in situ* hybridization, and/or antibodies against different epitopes of the reelin protein. As with other aspects of the study of reelin, early studies using mRNA *in situ* hybridization techniques reported a quite limited expression of reelin, largely confined to

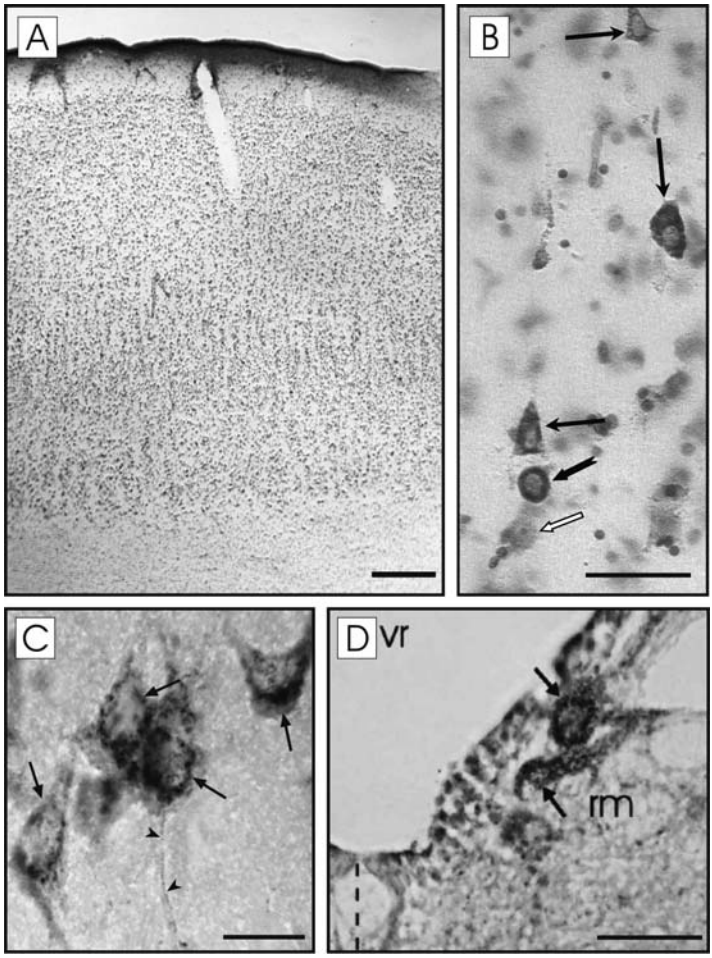

Fig. 8.1 Reelin-labeled neurons in the vertebrate brain. (**A**) Low magnification image of reelin labeling in the adult human cortex (BA39) demonstrating the abundant presence of reelin-labeled cells in all layers of the cortex (brown-stained cells). The section is counterstained with cresyl violet. (**B**) High magnification of the same cortical area as in **A** showing reelin-labeled pyramidal (plain black arrows) and nonpyramidal (notched arrow) cells. An unlabeled pyramidal cell is indicated with a white arrow. (**C**) Reelin-labeled cells of the adult rat entorhinal cortex. Arrows indicate the particle reelin labeling present in the cytoplasm, while arrowheads indicate reelin-labeled processes. (**D**) Reelin-labeled cells of the reticular rhombencephalic nucleus of the lamprey. Note the high similarity of the intracytoplasmic staining of these cells with the staining shown in **C**. vr, rhombencephalic ventricle; rm, nucleus reticularis medius. Scale bars: 500 µm (**A**); 50 µm (**B**); 15 µm (**C**); 150 µm (**D**). [**A**, **B** extracted from Roberts *et al*. (2005) *J. Comp. Neurol*. 482:294–308; **C** extracted from Perez-Costas (2002) Doctoral Thesis, p. 143; **D** extracted from Perez-Costas *et al*. (2004) *J. Chem. Neuroanat*. 27:7–21] (*See Color Plates*)

the cortex, cerebellum, and other laminar structures of the brain (Ikeda and Terashima, 1997; Alcantara *et al*., 1998). Initially, the expression of reelin in the adult cortex was attributed almost exclusively to GABAergic interneurons, and the presence of reelin-expressing neurons in other areas of the brain was timidly reported (Ikeda and Terashima, 1997; Alcantara *et al*., 1998). Subsequently, the improvement in reelin antibodies allowed more detailed studies that revealed that reelin is also widely expressed in noncortical structures of the vertebrate brain (Costagli *et al*., 2002; Martinez-Cerdeno *et al*., 2002, 2003; Perez-Costas *et al*., 2002, 2004; Ramos-Moreno *et al*., 2006). Moreover, the idea that reelin is almost exclusively present in a subset of GABAergic interneurons in the adult vertebrate cortex (Pesold *et al*., 1998, 1999) has been strongly challenged by several studies pointing out a much higher variety of cell types that express reelin in the cortex of adult vertebrates (Fig. 8.1; Martinez-Cerdeno and Clasca, 2002; Martinez-Cerdeno *et al*., 2002, 2003; Roberts *et al*., 2005, Ramos-Moreno *et al*., 2006). In fact, the high amount and variety of reelin-labeled cells observed in the adult human cortex (Fig. 8.1; Roberts *et al*., 2005) is comparable with what has been reported in the cortex of other adult mammals (Martinez-Cerdeno and Clasca, 2002; Martinez-Cerdeno *et al*., 2002, 2003; Deguchi *et al*., 2003; Ramos-Moreno *et al*., 2006).

In addition to the variety of neuronal types that express reelin, some studies indicate that reelin can also be present in glial cells (Perez-Garcia *et al*., 2001; Roberts *et al*., 2005) but at a much lower rate than in neurons, being only clearly distinguishable at the electron microscopic level (Roberts *et al*., 2005).

3 Subcellular and Ultrastructural Localization of Reelin

Currently, a considerable number of research works have provided data about reelin distribution in different species, and some of them include detailed studies using light microscopy. On the contrary, a surprisingly small number of studies have been done at the electron microscopic level. High magnification lenses in light microscopy allow an overall study of the presence or absence of reelin in a specific part of a cell (for example, in the soma or in the processes of the cell), making it possible to discern the "gross" subcellular localization of reelin. However, only the electron microscope provides the appropriate resolution to analyze which organelles or ultrastructural components of the cell may be taking part in the synthesis, storage, or transport of reelin.

3.1 Where Is Reelin Located at the Subcellular Level?

The subcellular localization of reelin is almost as ubiquitous as its regional distribution in the brain. Antibodies against reelin have allowed a thorough analysis of reelin localization at the subcellular level. In all vertebrates studied, reelin consistently appears located in the soma, axonal processes, and dendrites of reelin-containing

cells. Another consistent pattern is the abundance of extracellular matrix reelin labeling in developmental stages and a dramatic decrease of this kind of labeling in the adult brain (Perez-Garcia *et al*., 2001; Martinez-Cerdeno and Clasca, 2002; Martinez-Cerdeno *et al*., 2002, 2003; Perez-Costas *et al*., 2002, 2004; Tissir and Goffinet, 2003; Candal *et al*., 2005; Ramos-Moreno *et al*., 2006). One of the best-known examples is the presence of intense extracellular matrix labeling for reelin in cortical areas of mammals during development that is replaced by a predominantly intracellular staining in the adult brain (Tissir and Goffinet, 2003; Caruncho *et al*., 2004; Roberts *et al*., 2005). Another less-known phenomenon, but also consistent throughout the vertebrate scale, is the presence of a transient expression of reelin in some specific major fiber tracts, coinciding in time with the moment of their development and/or maturation. Examples of this are the transient labeling for reelin in the afferent tract of the habenula, or the transient expression of reelin in the optic tract (Fig. 8 2; Perez-Costas *et al*., 2002).

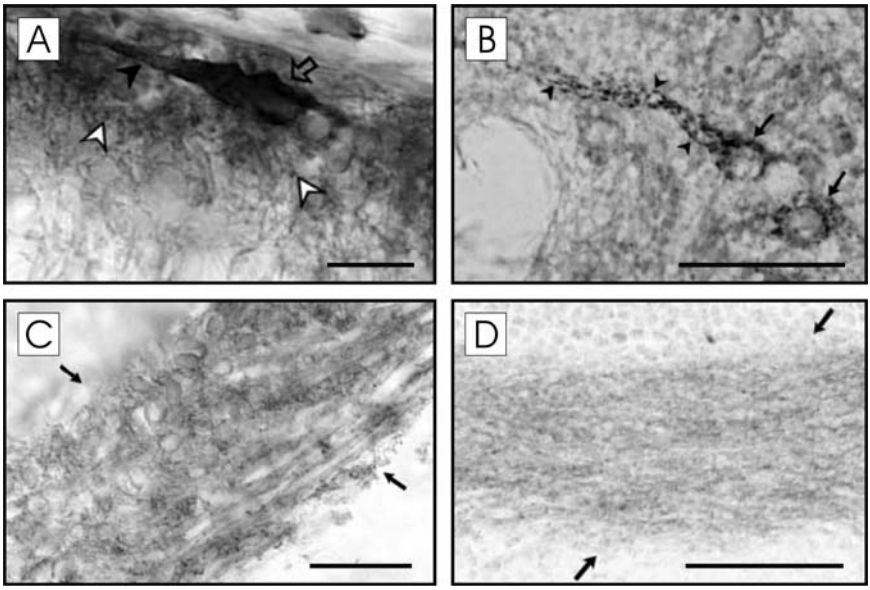

Fig. 8.2 Subcellular localization of reelin in vertebrates at the light microscopic level. (**A**) Reelin-labeled Cajal-Retzius cell in the cortex of a 17-day-old rat embryo. The labeled cell is surrounded by strong extracellular reelin labeling (white arrowheads). Note that the cell soma presents a solid reelin labeling, and the dendrite of the Cajal-Retzius cell is also labeled (black arrowhead). (**B**) Reelin-labeled cells of the retinopetal mesencephalic nucleus in the adult lamprey brain. Neuronal somata (arrows) present conspicuous particulate labeling that is also present in the initial segment of a process (arrowheads). (**C**) Transient reelin labeling in the afferent habenular tract (stria medullaris) of a 17-day-old rat embryo. (**D**) Transient reelin labeling in the optic tract of the larval lamprey brain. Scale bars: 10 µm (**A**); 150 µm (**B**); 25 µm (**C**); 50 µm (**D**). [**A**, **C**, and **D** extracted and modified from Perez-Costas *et al*. (2002) *J. Chem. Neuroanat*. 23:211–221; **B** extracted and modified from Perez-Costas *et al*. (2004) *J. Chem. Neuroanat*. 27:7–21]

An excellent paper by Martinez-Cerdeno *et al*. (2002) has shown the presence of two distinct patterns of staining for neuronal somata at the light microscopic level in different neuronal populations of the adult primate brain. One type of staining consisted in labeled particles located in the cell soma, and a second type consisted in a solid staining throughout the soma, as well as primary and secondary dendrites, the cell nuclei being devoid of reelin labeling in both cases. A review of the literature demonstrates that both types of staining are also present in other vertebrates (Fig. 8.2; see also Perez-Garcia *et al*., 2001; Martinez-Cerdeno *et al*., 2003; Perez-Costas *et al*., 2004; Ramos-Moreno *et al*., 2006). The possible biological meaning of the presence of these two different patterns of subcellular reelin labeling in the neuronal somata can be answered, in part, by electron microscopy. Ultrastructural analysis of the human cortex suggests that the different patterns of staining result from the labeling of specific organelles or other intracellular components, such as ribosomes (Fig. 8.3; Roberts *et al*., 2005). Within the somata of neurons, round membrane-bound profiles (probably endosomes) (Fig. 8.3A), and labeling deposited in outpockets of the nuclear membrane (Fig. 8.3B) may account for the particulate staining observed at the light microscopic level. The more diffuse staining observed at the light microscopic level in somata and proximal dendrites is probably due to the labeling of reelin on ribosomes and rough endoplasmic reticulum (Fig. 8.3A, 8.4A).

3.2 Ultrastructural Localization of Reelin

Studies of reelin at the electron microscopic level have been performed only in mammalian species, with detailed studies available in the mouse (Pappas *et al*., 2001) and human brain (Roberts *et al*., 2005). Reelin labeling at the electron microscopic level is consistent with what has been described using light microscopy, identifying reelin-labeled structures in neuronal somata, axons, and dendrites, as well as extracellular matrix labeling (Pesold *et al*., 1998; Rodriguez *et al*., 2000; Derer *et al*., 2001; Pappas *et al*., 2001, 2003; Martinez-Cerdeno *et al*., 2002; Roberts *et al*., 2005). In addition, the high resolution of the electron microscope has demonstrated that reelin can also be present in glial cells but in very low levels, compared with its presence in neurons (Roberts *et al*., 2005). Although most of the studies agree on the consistency of this pattern, some discrepancies are present.

3.2.1 Reelin in Neuronal Somata

Within the somata of human cortical neurons, reelin labeling is present in the euchromatin of the nucleus, ribosomes on the outer nuclear membrane, rough endoplasmic reticulum, and polyribosome rosettes (Fig. 8.3, 8.4). In these structures, the labeling is very discretely deposited (Fig. 8.4, 8.5). For example, spherical membrane-bound cytoplasmic organelles are labeled fairly regularly (Fig. 8.3A). In many instances, an outpocketing of the nuclear outer membrane appears filled

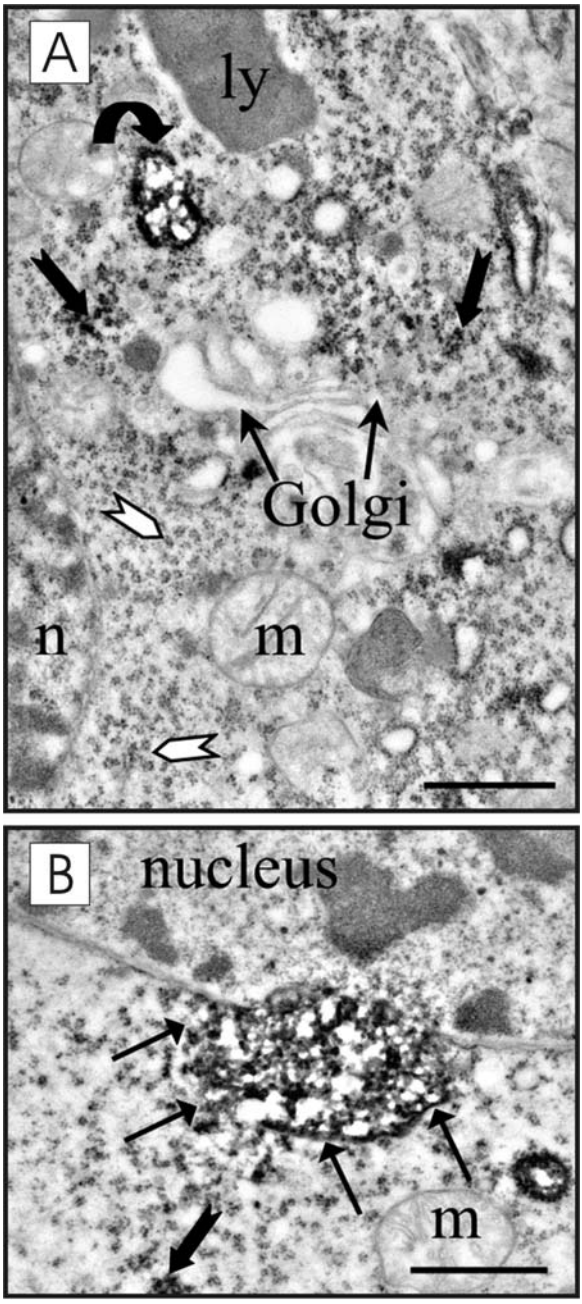

Fig. 8.3 Ultrastructural localization of reelin labeling in neuronal somata. (**A)** Many ribosomes are labeled (black arrows), while some are unlabeled (white arrows). Labeled oval organelles (curved arrow), which may be endoplasmic reticulum or endosomes, are present in some cell bodies. The Golgi apparatus is unlabeled as are mitochondria (m) and lysosomes (ly). n, nucleus. (**B**) An example of labeling in the space between the inner and outer nuclear membrane (plain black arrows). Labeled ribosomes (arrow) and unlabeled mitochondrion are shown. Scale bars: 1 µm. [Figures extracted and modified from Roberts *et al*. (2005) *J. Comp. Neurol*. 482:294–308]

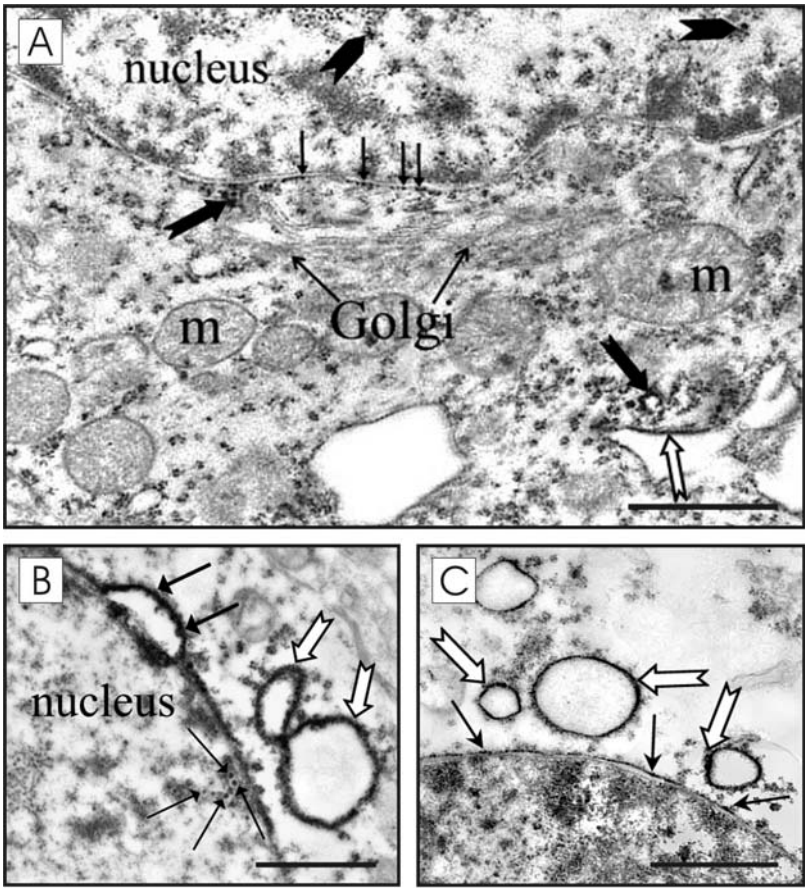

Fig. 8.4 Ultrastructural localization of reelin labeling in neuronal somata. (**A**, **B**) Reelin labeling in adult human cortex. (**A**) Reelin appears in ribosomes (thick arrows), rough endoplasmic reticulum and ribosomes of the outer nuclear membrane (thin arrows). Note also some particulate reelin labeling in the nucleus (arrowheads). (**B**) More detailed image from a different neuron showing reelin labeling in ribosomes on the outer nuclear membrane (arrows), as well as in the rough endoplasmic reticulum (white arrows). Note also the presence of reelin-labeled vesicles in the nucleus (arrows). (**C**) Reelin labeling in fetal human cortex. Reelin labeling appears in the rough endoplasmic reticulum (white arrows) as well as in ribosomes of the outer nuclear membrane (thin arrows). Note the similarity of this staining with that of the adult brain shown in **A** and **B**. Scale bars: 1 μm in A-C. [Figures extracted and modified from Roberts *et al*. (2005) *J. Comp. Neurol*. 482:294–308]

with reelin-labeled material (Fig. 8.3B). Ribosomes on the outer nuclear membrane and rough endoplasmic reticulum are robustly labeled (Fig. 8.4A,B), as well as vesicles within the nucleus at the nuclear membrane (Fig. 8.4B). In developing human brain (Fig. 8.4C), reelin is located on the euchromatin within the nucleus, in the innermost part of the nuclear membrane, on rough endoplasmic reticulum, and on ribosomes along the outer nuclear membrane (Roberts *et al*., 2005). Presence of reelin on ribosomes, polyribosome rosettes, rough endoplasmic reticulum, and

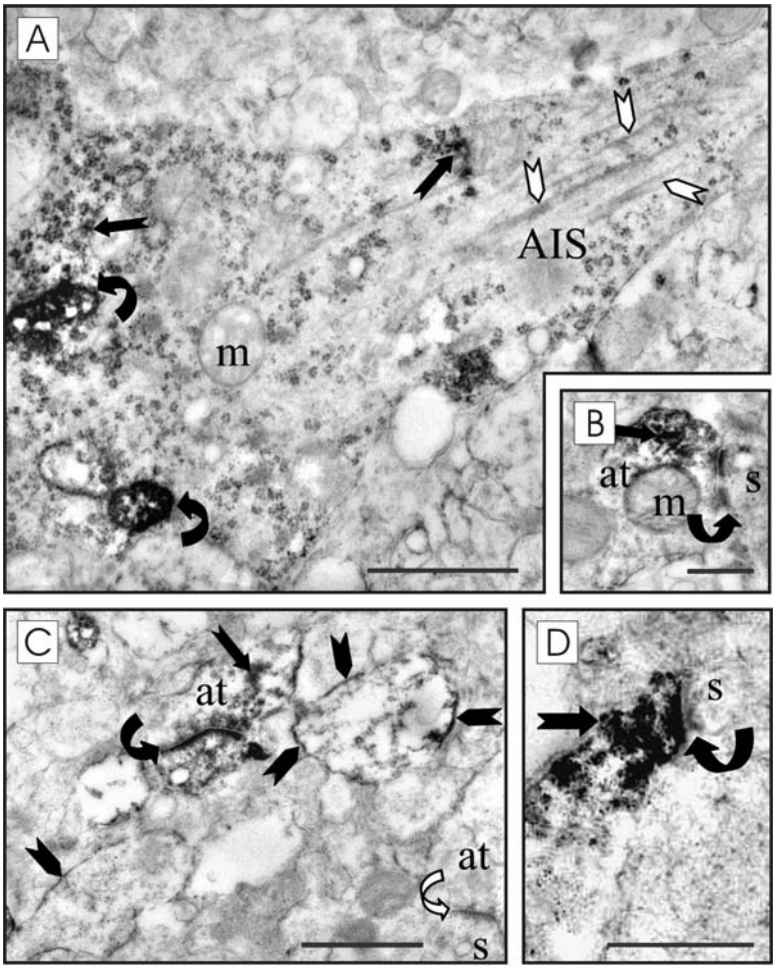

Fig. 8.5 Ultrastructural localization of reelin labeling in neuronal processes and synapses. (**A–C**) Reelin labeling in the adult human cortex. (**A**) Reelin-labeled axon initial segment (AIS) in which the axon hillock contains numerous reelin-labeled ribosomes (straight black arrows), as well as labeled oval organelles (curved arrows). (**B**) Labeled axon terminal (straight arrow) forming a synapse (curved arrow) with a spine (s). (**C**) Neuropil showing a labeled axon terminal (straight black arrow) forming a synapse (curved black arrow) with a labeled spine (s). (**D**) Reelin labeling in the human fetal cortex. A labeled axon terminal (straight arrow) forms a synapse (curved arrow) with a spine (s). Scale bars: 1 µm (**A**, **C**); 0.5 µm (**B**, **D**). [Figures extracted and modified from Roberts *et al*. (2005) *J. Comp. Neurol*. 482:294–308]

outer nuclear membrane of cells in the human cortex (Roberts *et al*., 2005) is in general agreement with what has been observed in rodent and nonhuman primates (Pappas *et al.,* 2001; Martinez-Cerdeno *et al*., 2002).

Reelin labeling in adult human cortex is conspicuously absent from heterochromatin, most mitochondria, and lysosomes (Fig. 8.3A, 8.4A; Roberts *et al*., 2005).

The Golgi apparatus appears almost devoid of reelin (Fig. 8.3A, 8.4A), while the extracellular matrix (Fig. 8.5C) and multivesicular bodies (Fig. 8.6A) appear occasionally labeled (Roberts *et al*., 2005). A major difference in the results found in human and those in other species for which electron microscopy was utilized is the paucity of reelin in the adult human extracellular space and the subcellular machinery in which secreted proteins are made and taken back up into the cell. In contrast, in the adult mouse, reelin has been found in the Golgi apparatus and extracellular space (Pappas *et al*., 2001) and is secreted in cell cultures of murine neurons (Lacor *et al*., 2000). In macaques, Martinez-Cerdeno *et al*. (2002) reported the presence of reelin in secretory organelles, but the most robust staining was present in the rough endoplasmic reticulum, rather than the smooth endoplasmic reticulum or the Golgi

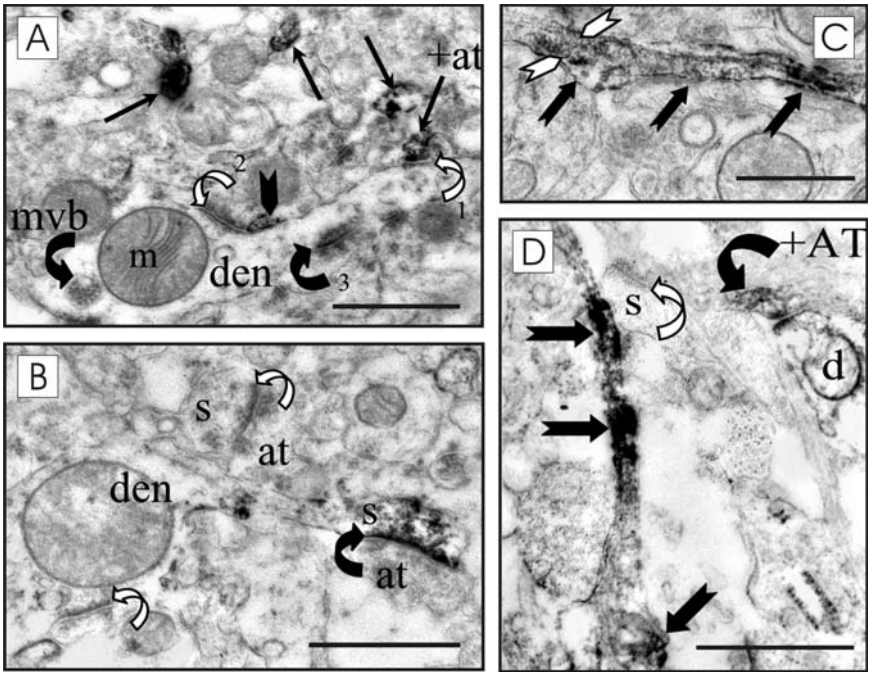

Fig. 8.6 Ultrastructural localization of reelin labeling in neuronal processes and synapses. (**A–C**) Reelin labeling in the neuropil of the adult human cortex. (**A**) Diffuse reelin labeling in profiles (straight arrows) that could correspond with small dendrites or spine necks. A symmetric synapse (curved black arrow) is formed by a labeled terminal (+at) but the postsynaptic density is not labeled (white arrow 1). Another labeled synapse (curved black arrow 3) is formed by an unlabeled terminal and a reelin-labeled postsynaptic density. Note also the presence of a labeled glial process (black arrowhead). (**B**) A long labeled spine (s) receives an asymmetric synapse (black arrow) from an unlabeled terminal (at). (**C**) Diffusely labeled unmyelinated axon (straight arrows). (**D**) In this image of the fetal human cortex, reelin labeling appears in long thin neuronal processes (black arrows) and in an axon terminal (+AT) forming a synapse with a dendrite (d). Scale bars: 1 µm (**A–C**); 0.5 µm (**D**). [Figures extracted and modified from Roberts *et al*. (2005) *J. Comp. Neurol*. 482:294–308]

apparatus. This group reported also that all reelin labeling was intracellular in the adult nonhuman primate brain (Martinez-Cerdeno *et al*., 2002).

3.2.2 Reelin in Neuronal Processes and Synapses (Neuropil)

The presence of abundant neuropil staining has been described at the light microscopic level in different vertebrates (Martinez-Cerdeno *et al*., 2002, 2003; Perez-Costas *et al*., 2002; Ramos-Moreno *et al*., 2006). Neuropil includes both dendritic and axonal processes and electron microscopy is necessary to identify the labeled profiles. Reelin labeling in the human cortical neuropil is richly distributed among axons, terminals, dendritic shafts, and spines (Roberts *et al*., 2005).

Reelin labeling in the adult human appears throughout the axon, including the axon hillock and initial segment, and axon terminals (Fig. 8.5B,C, 8.6A). In the axon hillock and axon initial segment, reelin labeling is deposited on polyribosome rosettes, and present diffusely in spherical membrane-bound cytoplasmic organelles (Fig. 8.5A). In addition, in small unmyelinated axons reelin labeling is diffusely deposited inside the axon (Fig. 8.6C). Reelin axonal labeling is also present in fetal human brain (Fig. 8.5D, 8.6D; Roberts *et al*., 2005). The ultrastructural localization of reelin-labeled axonal processes has also been described in rodents and nonhuman primates (Derer *et al*., 2001; Pappas *et al*., 2001; Martinez-Cerdeno *et al*., 2002). The presence of reelin labeling throughout the extent of axonal processes strongly suggests that reelin can be transported long distances through axonal tracts (Derer *et al*., 2001; Perez-Costas *et al*., 2002; Martinez-Cerdeno *et al*., 2003; Roberts *et al*., 2005).

In some axon terminals, it is possible to discern that reelin labeling is located around and between synaptic vesicles (Roberts *et al*., 2005). The labeled axon terminals formed both asymmetric (Fig. 8.5B,C) and symmetric synapses (Fig. 8.6A), indicating that reelin can be present in inhibitory and excitatory terminals. Labeling in axon terminals forming symmetric synapses is consistent with the localization of reelin in cortical interneurons, which form this type of synapse (DeFelipe *et al*., 2002). The location of reelin in pyramidal cells and in axon terminals that form asymmetric synapses suggests that reelin is involved in corticocortical signaling (DeFelipe *et al*., 2002). Thalamocortical projections represent another potential source of reelin-labeled terminals forming asymmetric synapses in the cortex (Peters, 2002).

Throughout the neuropil, reelin labeling in the human cortex also appears in dendrites and spines (Roberts *et al*., 2005). In dendritic shafts, reelin labeling appears in the rough endoplasmic reticulum and polyribosome rosettes, as well as in some of the postsynaptic densities (Fig. 8.6A). Dendritic spines contain diffuse labeling in the head and neck (Fig. 8.6B) and/or prominent labeling in the postsynaptic density (Fig. 8.5C, 8.6A). The postsynaptic densities that reveal reelin labeling in spines and dendritic shafts are associated with both asymmetric (Fig. 8.5C) and symmetric synapses (Fig. 8.6A). These results in human are consistent with the localization of reelin labeling in spines and/or the postsynaptic densities in adult mice (Pappas *et al*., 2001) and nonhuman primates (Rodriguez *et al*., 2000; Martinez-Cerdeno *et al*., 2002). Moreover, recent studies have shown that reelin

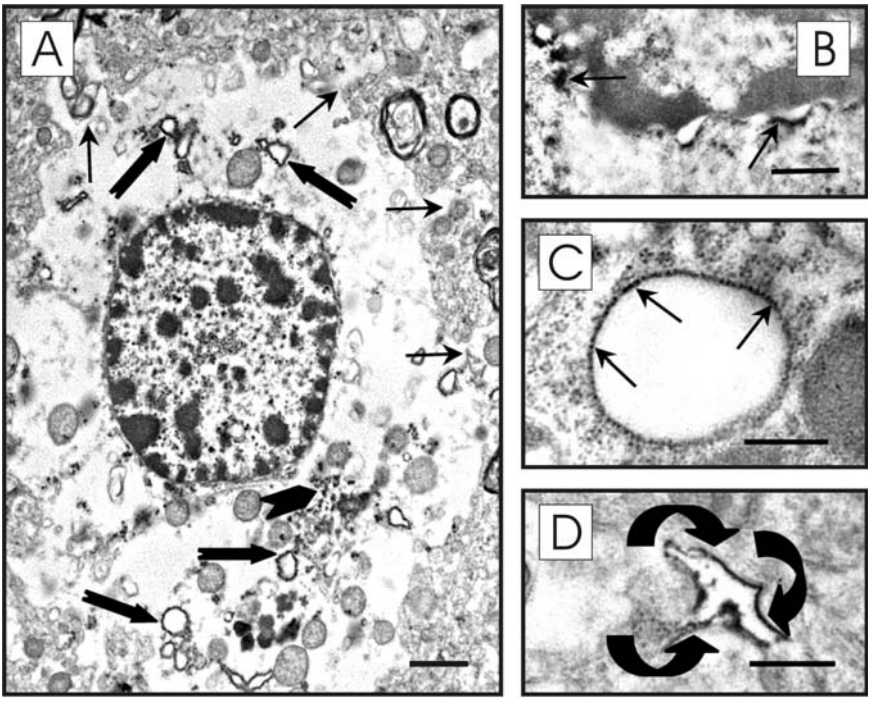

Fig. 8.7 Localization of reelin labeling in glial cells in the human cortex. (**A**) Astrocyte showing reelin labeling in the rough endoplasmic reticulum (thick arrows) as well as in free ribosomes (arrowheads). (**B**) Reelin labeling in the outer nuclear membrane of a glial cell (arrows). (**C**) Detail of the rough endoplasmic reticulum of a glial cell showing labeled ribosomes (arrows). (**D**) A small irregularly shaped labeled profile (curved arrows) that probably corresponds to an astrocytic process. Scale bars: 1 µm (**A**); 0.5 µm (**B–D**). [Figures extracted and modified from Roberts *et al*. (2005) *J. Comp. Neurol*. 482:294–308]

and its receptors can be pivotal regulators of *N*-methyl-D-aspartate receptor function and could also regulate GABA circuits (Herz and Chen, 2006).

3.2.3 Reelin Labeling in Glial Cells

At the electron microscopic level, reelin labeling is also found in glial cells of the human cortex (Fig. 8.7A) but in lower levels than in neurons. In cell bodies, the labeling appears in ribosomes attached to the outer nuclear membrane (Fig. 8.7B), and as discrete labeling on the rough endoplasmic reticulum and in polyribosome rosettes (Fig. 8.7A,C). Small labeled astrocytic processes are observed throughout the neuropil often associated with synapses (Fig. 8.7D; Roberts *et al*., 2005). The presence and extent of reelin labeling in glial cells in nonhuman primates and other species is somewhat unclear, and, if present, is probably less abundant than in humans. Ultrastructural studies in nonhuman primates (Martinez-Cerdeno *et al*.,

2002) and rodents (Pappas *et al*., 2001) did not report the presence of reelin labeling in glial cells. However, a study by Rodriguez *et al*. (2000) presenting electron microscopy data on reelin labeling in a nonhuman primate shows an image of a small irregularly shaped reelin-labeled process remarkably similar in morphology to that of an astroglial process, though the profile was interpreted by the authors to be a dendritic spine.

The lack of other studies describing reelin labeling in glial cells could reflect several technical factors, including that the low level of reelin labeling in glial cells could surpass the detection limits of light microscopy, as well as possible differences in tissue processing and, perhaps, misinterpretation of electron microscope images. On the other hand, it is quite possible that there are real differences in reelin content in glial cells among different vertebrate species, as occurs for other structures in the brain (Martinez-Cerdeno and Clasca, 2002; Deguchi *et al*., 2003; Martinez-Cerdeno *et al*., 2003). Thus, reelin labeling in human glial cells may represent a pattern reflecting differences in the phylogenetic scale.

4 Reelin Function and Malfunction

During neocortical development, reelin is present in the extracellular matrix and Cajal-Retzius cells and functions to guide developing neurons (Meyer and Goffinet, 1998; Meyer *et al*., 2002). However, once neocortical neuronal migration ceases during the first half of gestation (Rakic and Sidman, 1968; Rakic, 1978) and the degeneration of Cajal-Retzius cells occurs by the end of the second trimester (Meyer and Goffinet, 1998), the major function of reelin in human must change. At midgestation in human, the localization of reelin has both fetal and adult patterns (Meyer *et al*., 2002; Roberts *et al*., 2005). Thus, the second trimester of human development appears to represent the transition in location and function of reelin. This pattern of change in location across development is consistent with the pattern of other proteins and molecules whose function is critical during development but that continue to be expressed in adulthood.

At the synapse, reelin labeling has been found associated with presynaptic and postsynaptic elements (Rodriguez *et al*., 2000; Pappas *et al*., 2001; Roberts *et al*., 2005). The location of reelin in axon terminals, dendritic spines, the postsynaptic density, and astroglial processes in adult brain is consistent with the apparent role of reelin in synaptic plasticity (Rice *et al*., 2001; Weeber *et al*., 2002), which is a function of the tripartite synapse (spine, terminal, and astroglial process). In adult human cortex, reelin is present in 53% of synaptic complexes, where labeling is present in the axon terminal, the postsynaptic density, or both, suggesting an important role for reelin in synaptic processes (Roberts *et al*., 2005). The abundance of reelin associated with cortical synapses suggests its involvement in the function of many cortical connections.

In summary, the ultrastructural localization of reelin indicates that this protein can be synthesized by different types of neurons in the vertebrate brain, transported

even long distances through neuronal processes, and is abundantly present in synapses, both in the developing and adult brain. The prevalence of this protein in the adult brain also indicates that reelin's functions go far beyond its role in neuronal migration and correct positioning of neurons in the cortex. In fact, the remarkable abundance of reelin labeling in axon terminals, as well as dendrites and dendritic spines in adult brain is consistent with a role for reelin in normal mature synaptic function, including synaptic plasticity and appropriate remodeling of synaptic contacts. Altogether, these findings suggest that proper levels of reelin and its receptors could be crucial for the proper functioning of synaptic transmission in the adult brain.

An abundance of evidence suggests that the malfunction of reelin may have a role in several diseases of the brain. Reelin abnormalities are found in several brain regions in schizophrenia, bipolar disease, depression (Impagnatiello *et al*., 1998; Fatemi *et al*., 2000, 2001; Guidotti *et al*., 2000; Eastwood and Harrison, 2002; Caruncho *et al*., 2004), autism (Persico *et al*., 2001; Reichelt *et al*., 2001; Fatemi *et al*., 2002, 2005), and lissencephaly (Hong *et al*., 2000). The decrease in reelin mRNA in schizophrenia (Impagnatiello *et al*., 1998; Guidotti *et al*., 2000) is particularly interesting, considering the evidence from postmortem studies that schizophrenia is associated with impaired cell migration in the neocortex (Akbarian *et al*., 1996; Kirkpatrick *et al*., 1999, 2003), and the observation that reelin is present in dendritic spines and terminals, which are affected in this disease (Roberts *et al*., 1996; Glantz and Lewis, 2000). Moreover, it has been reported that heterozygous reeler mice (that have reduced levels of reelin in the brain) present anatomical and behavioral deficits that resemble some of the behavioral and anatomical deficits present in schizophrenia (Impagnatiello *et al*., 1998; Tueting *et al*., 1999; Guidotti *et al*., 2000; Ballmaier *et al*., 2002). Whether reelin is affected during development, adulthood, or in both of these stages in brain disease remains to be determined. In any case, reelin and/or molecules in its signaling pathway may be potential therapeutic targets for several brain disorders.

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