

# REVIEW

## Cell and molecular mechanisms that control cortical layer formation in the brain

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**Abstract.** Various classes of neurons in the mammalian central nervous system (CNS) migrate from their sites of origin to their final positions, where they are arranged in elaborate laminar structures. These precise patterns of neuronal alignment are disrupted in several human diseases and mouse mutants. Among them, *reeler*, an autosomal recessive mouse mutant discovered half a century ago, has been studied as a valuable material for investigations of neuronal layer formations. Recent identification of a gene mutated in *reeler* (*reelin*), and subsequent characterizations of other genes underlying mouse and human brain malformations have rapidly expanded our knowledge of the molecular programs underlying the normal brain layer formation. In this review, we summarize the cellular and molecular mechanisms that establish highly ordered structures in the brain, in particular in the cerebral cortex. (Keio J Med 52 (1): 8–20, March 2003)

**Key words:** Reelin, Dab1, VLDLR/ApoER2, Cdk5/p35, lissencephaly

### Introduction

The mammalian cerebral cortex is a highly ordered structure. The different classes of neurons reside in an organized radial array of six cellular layers ranging from the pial surface to the white matter. During development of laminated structures, neurons should migrate in a coordinated series of patterns from their sites of origin to their final positions, as neurons are usually generated in sites distant from their final locations.

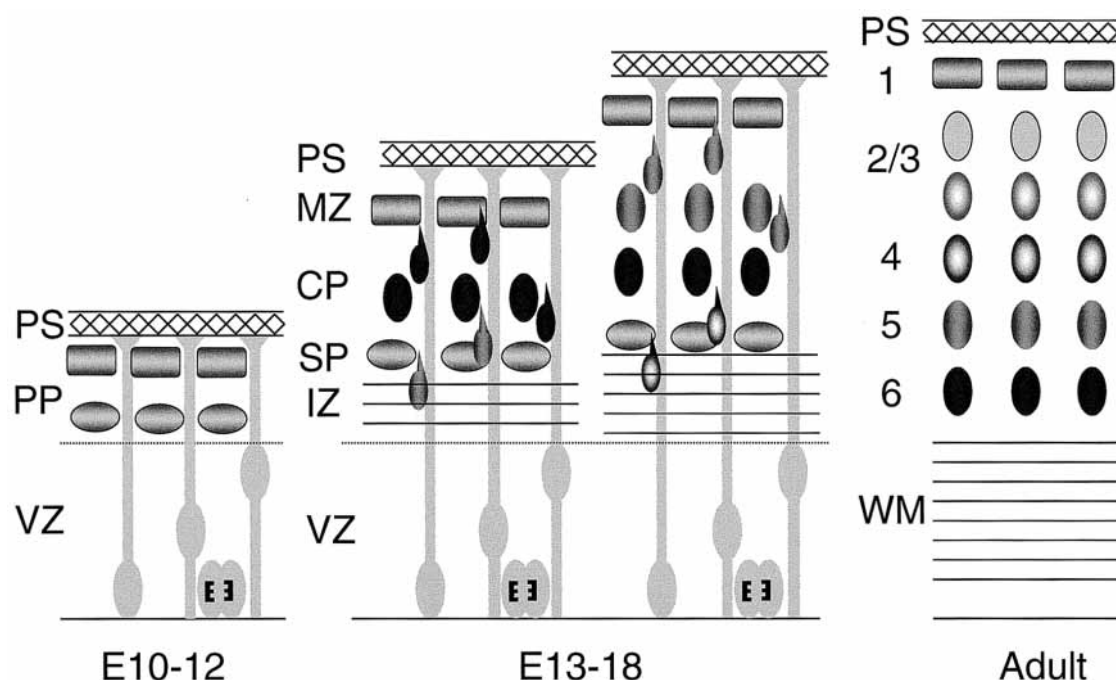
In the developing cerebral cortex, neurons are mainly generated in the cerebral ventricular zone and then move to the developing cortical plate via “radial migration”.<sup>1–4</sup> In contrast, most GABAergic interneurons originate in the ganglionic eminences and enter the developing cortical plate via “tangential migration” in mice.<sup>5–8</sup>

The failure of migration leads to defects of cortical-layer formation in several human diseases and mouse mutants.<sup>9–11</sup> However, the mutations of genes that are the base cause of severe cortical dysplasias in humans and mice have provided an entry point into the molecular pathway that controls neuronal migration and cor-

tical layer formation. In this review, we focus on radial migration, because most of the known mutants with cortical layering defects are due to the disruption of radial migration.

### Development of the cerebral cortex

The mammalian cerebral cortex is constructed in two distinct steps<sup>3,4,12</sup> (Fig. 1). The first step of neocortical development is the formation of the preplate or primordial plexiform layer above the ventricular zone. The preplate is composed of afferent and efferent fibers and the earliest generated neurons, including the Cajal-Retzius cells and the subplate neurons.<sup>12</sup> In the second step, cortical plate neurons are generated in the ventricular zone along the ventricle, and these earlier-born neurons invade the preplate. Migrating neurons move past the subplate, splitting this layer away from the Cajal-Retzius cells. Therefore, the preplate is split into a superficial marginal zone, in which the Cajal-Retzius cells remain adjacent to the pial surface, and a deep subplate. The late-born neurons are generated in the ventricular zone and migrate radially, passing through



**Fig. 1** Development of the neocortex. The first step of neocortical development is the formation of the preplate (PP) above the ventricular zone (VZ). In the second step, cortical plate neurons are generated in the ventricular zone, and these earlier-born neurons invade the preplate. Continued migration splits the preplate into a superficial marginal zone (MZ), in which the Cajal-Retzius cells remain adjacent to the pial surface (PS), and a deep subplate (SP). The late-born neurons are generated in the ventricular zone and migrate radially, passing through the intermediate zone (IZ) which consists of pioneer axons, then move past the subplate and earlier generated cortical plate neurons (CP). The systematic migration of late-generated neurons past predecessors results in the “inside-out” fashion of cortical layering, in which the later generated cortical plate neurons are located at more superficial positions than the earlier-born neurons. In adulthood, a six-layered neocortex is formed above the axonal tracts termed white matter (WM).

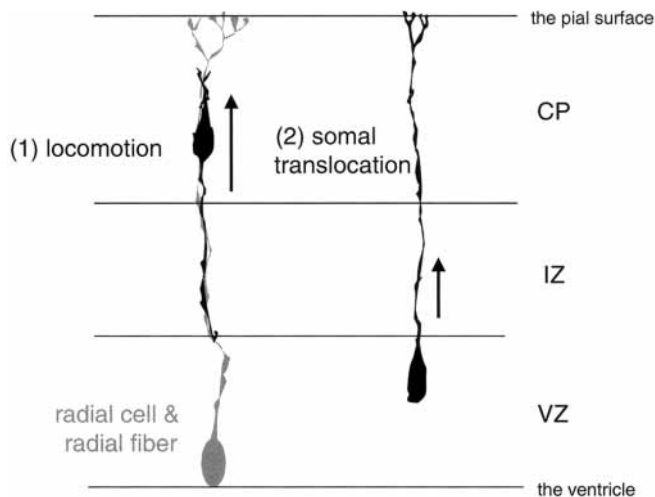
the intermediate zone, then move past the subplate and earlier generated cortical plate neurons. The systematic migration of late-generated neurons past predecessors results in the “inside-out” fashion of cortical layering, in which the later generated cortical plate neurons are located at more superficial positions than the earlier-born neurons.<sup>1,13</sup>

#### *Modes of radial migration*

Two distinct modes of radial migration, locomotion and somal translocation, have been reported (Fig. 2). The locomotion cell migrates along a radial fiber of a radial glial cell;<sup>2,14</sup> which traverse the entire thickness of the developing cerebral wall. Neurons migrating in this mode have a characteristic bipolar cell-morphology, with a thick leading process and a thin trailing process, and the entire cell moves along the radial fibers. In somal translocation, the cell has a long radially directed leading process that attaches to the pial surface. The cell soma moves toward the leading edge, which results in the shortening of the leading process, while its terminal remains attached to the pial surface.<sup>15–19</sup> A recent time-lapse study of radially mi-

grating neocortical neurons revealed that each mode of migration had distinct dynamics and occurred preferentially at different developmental stages.<sup>18</sup> At embryonic day E12–13 in mice, neurons use somal translocation to split the preplate, while at E15–16, locomotion predominates to transverse the expanding cortical plate. Although somal translocation occurs as a continuous movement and does not need the radial glial fibers, locomotion is interspersed by pauses and is dependent on the radial glial fibers.

In contrast to the bipolar or monopolar morphology of locomotion and somal translocation cells, histological analyses of fixed sections of developing neocortex using electron microscopy have demonstrated the existence of multipolar cells in the intermediate zone,<sup>20</sup> which could not be explained simply by the locomotion or somal translocation. Recently, Tabata and Nakajima established an *in utero* gene transfer system that allows specific labeling of migrating cells in the developing cortex with GFP or red fluorescent protein (DsRed).<sup>21,22</sup> Using this system, we performed time-lapse observations, and found that neurons dynamically change their morphology and migration modes during their journeys toward the pial surface (Tabata *et al.*,



**Fig. 2** Modes of radial migration. (1) The locomotion cell migrates along a radial fiber of a radial glial cell; which traverse the entire thickness of the developing cerebral wall. Neurons migrating in this mode have a characteristic bipolar cell-morphology, with a thick leading process and a thin trailing process. (2) In somal translocation, the cell has a long radially directed leading process that attaches to the pial surface. The cell soma moves toward the leading edge, while its terminal remains attached to the pial surface. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone.

in submission). These results imply that each neuron migrates via a combination of several modes of migration, depending on the migration phase and the developmental stage. The existence of various migration modes is important, as the responsible genes in defects of neocortical-layer formation might be involved in distinct modes of migration.

### Defects of Cortical Layer Formation in Mice

The analysis of malformations in mouse brains has led to dramatic progress in the identifications of genes important for cortical layering. Among them, the most studied and characterized is the *reeler* malformation in mice.

#### The *reeler* malformation

Over 50 years, *reeler* (*Reln<sup>rl</sup>*), the spontaneous mouse mutant, has been studied as an important experimental model. The *reeler* mouse exhibits an autosomal recessive mutation located on chromosome 5.<sup>23</sup> The *reeler* mutation causes ataxic and reeling gait in affected mice. Analysis of the central nervous system of mutant mice revealed severe architectonic abnormalities in the cortical structures of the cerebrum, hippocampus, and cerebellum.<sup>24–26</sup> In addition to these anomalies in the cortical structures, more subtle abnormalities in

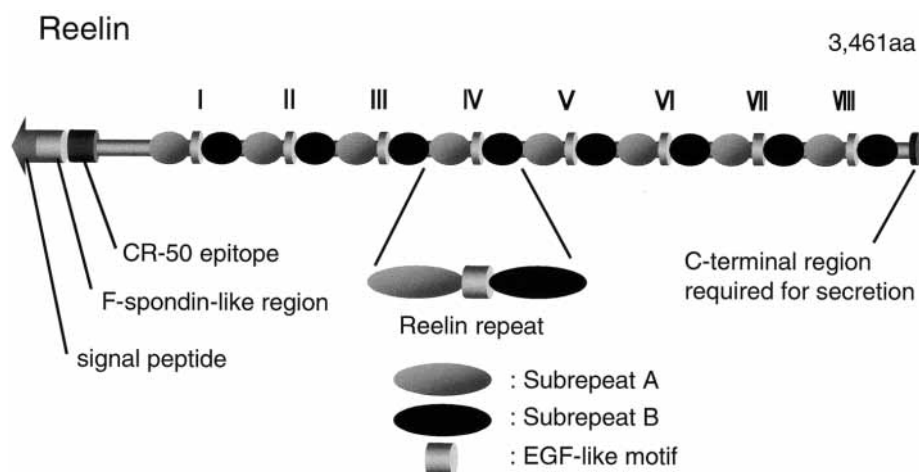
cell clustering or in laminar organizations have been reported in several subcortical structures, including the amygdala, olfactory tubercle, olfactory bulb, inferior olivary complex, dorsal cochlear nucleus, and facial nucleus.<sup>27–31</sup> Yip *et al.* showed that migration of sympathetic preganglionic neurons is affected in the spinal cord as well, although it had been generally thought that neuronal migration was not disrupted in the spinal cord of the *reeler* mutant.<sup>32</sup> The *reeler* mutation has its effect without impairing the survival or vitality of neocortical neurons,<sup>33,34</sup> and therefore has provided a valuable model for investigations of neuronal layer formations.

In the neocortex of the *reeler* mutant, the first cell position defects arise at the splitting of the preplate. The initial formation of the preplate proceeds normally.<sup>35,36</sup> However, the early-born cortical neurons fail to invade the preplate, and the subplate remains adjacent to the marginal zone in a structure known as the “superplate”.<sup>37</sup> Subsequent cohorts of late-generated neurons are unable to pass their predecessors, resulting in an “outside-in” pattern of development.<sup>24,37</sup> Consequently, a disorganized cortical plate is formed underneath the superplate, with an indistinct and almost inverted layering.

#### CR-50 and the *reeler* gene

The first material used to investigate the molecular deficit in the *reeler* mouse was obtained in a unique strategy. On the hypothesis that particular molecules encoded by the *reeler* gene may mediate the presumptive interactions between the preplate and the forming cortical plate neurons in normal mice, Ogawa *et al.* generated a monoclonal antibody (CR-50) by immunizing *reeler* mice with brain extracts from normal mice.<sup>38</sup> CR-50 recognized a distinct allelic antigen present in normal but not in *reeler* mice, expressed in the regions of normal mice where morphological abnormalities occur in *reeler* mice. In the neocortex, the CR-50 antigen was associated superficially with the Cajal-Retzius cells. Importantly, several experimental approaches revealed that CR-50 functions as a blocking antibody in culture studies.<sup>38–40</sup> In addition, Nakajima *et al.* reported that intraventricular injection of CR50 at the embryonic stage disrupted the normal development of the hippocampus and converted it to a *reeler* pattern *in vivo*.<sup>41</sup> These lines of studies strongly suggested that the mutation of the CR-50 antigen causes the *reeler* phenotype.

On the other hand, a transgene insertion produced another *reeler* allele (*Reln<sup>rl-tg</sup>*), providing a probe for the *reeler* locus,<sup>42</sup> which led to the isolation of the candidate *reeler* gene.<sup>43</sup> Positional cloning identified the same candidate *reeler* gene at the same period.<sup>44,45</sup>



**Fig. 3** Schematic representation of the Reelin structure. Reelin is a secreted extracellular matrix protein composed of 3,461 aa with a relative molecular mass of 388 kDa. The N-terminus of Reelin has 25% identity with that of F-spondin. These first 500 aa of Reelin are succeeded by eight Reelin repeats. Each Reelin repeat is composed of 350–390 aa and contains two related subrepeats A and B, separated by an EGF-like motif of 30 aa. CR-50 antibody is recognized upstream of the Reelin repeats, although this antibody clearly blocks several Reelin-mediated phenomena both *in vitro* and *in vivo*.

This gene was named *reelin*. No *reelin* mRNA was detected in the two *reeler* alleles (*Reln<sup>rl</sup>* and *Reln<sup>rl-tg</sup>*).<sup>43</sup> The *reelin* open-reading frame is 10,383 bases long, encoding a protein composed of 3461 amino acids (aa) which has a relative molecular mass of 388 K (Fig. 3). An epitope of Reelin, defined near the N-terminus, was shown to be recognized by CR-50.<sup>46</sup> Considering the functions of CR-50 as a blocking antibody, the deficiency of Reelin was in fact indicated to be responsible for the *reeler* phenotype.

The region more N-terminal to the CR-50 epitope contains a cleavable signal peptide, followed by a region which has 25% identity with that of F-spondin.<sup>47</sup> These first 500 aa of Reelin are succeeded by eight “Reelin repeats”. Each Reelin repeat is composed of 350–390 aa and contains two related subrepeats A and B, separated by an EGF-like motif of 30 aa.<sup>43</sup> In these sequences, Reelin resembles extracellular matrix proteins.

Reelin was secreted when constructed full-length *reelin* cDNA was transfected to COS cells, and deletion analysis demonstrated that a short region of positively charged amino acids near the C-terminus is required for secretion of Reelin.<sup>46</sup> In the *reeler Orleans* mutant (*Reln<sup>rl-Orl</sup>*), Reelin is made but not secreted due to the insertion of an L1 transposonable element that alters the amino acid sequence of the C-terminus.<sup>48,49</sup> Recently, it was shown that secreted Reelin molecules tend to form a homophilic dimer.<sup>50</sup> As Reelin-Reelin interaction was inhibited by CR-50, CR-50 might neutralize the Reelin function at least partly by inhibiting the homophilic assembly of Reelin proteins.<sup>51</sup>

In the developing brain of normal mice, Reelin is

secreted from a subset of neurons, for example, by the Cajal-Retzius cells in the cerebral cortex<sup>38</sup> and hippocampus,<sup>41</sup> and by granule cells in the cerebellum.<sup>39,52</sup> Importantly, the Reelin-producing neurons are associated with regions where first phenotypic defects of neuronal migration are detected in *reeler* mice. The spatial and temporal expression pattern suggested that Reelin is important as the extracellular signal for migrating neurons. This suggestion was confirmed by subsequent reports of mutations in genes encoding molecules that transmit Reelin signal.

#### *Yotari, scrambler, and Dab1 (disabled 1)*

A number of mutations that result in a brain phenotype similar to that of *reeler* have expanded our knowledge of the Reelin signal cascade (Fig. 4). They include *yotari*, an autosomal recessive mutant mouse which appeared during the generation of null mice for the gene encoding the receptor for inositol-1,4,5-triphosphate.<sup>53</sup> Despite the *reeler*-like phenotypes in *yotari*, alteration in Reelin expression was not observed. Detailed investigations revealed that a long interspersed nuclear element (L1) insertion caused the mutation in the *disabled 1* (*Dab1*) gene.<sup>54</sup> This allele is referred as *Dab1<sup>yot</sup>*, and another spontaneous mutant mice *scrambler* (*Dab1<sup>scm</sup>*)<sup>55</sup> with a mutation in the *Dab1* gene,<sup>56,57</sup> or mice with a target disruption of *Dab1* (*Dab1<sup>tm1Cpr</sup>*) were also found to exhibit phenotypes indistinguishable from those of *reeler*.<sup>58</sup> *Dab1* protein is drastically reduced in *Dab1<sup>scm</sup>* (~5% of the wild type level of *Dab1* protein), and not detected in *Dab1<sup>yot</sup>* and *Dab1<sup>tm1Cpr</sup>*.<sup>56,58</sup>

*Dab1* encodes an intracellular adaptor or docking protein originally identified as a Src-binding protein.<sup>59</sup> The Dab1 p80 is predominantly expressed in neurons in the developing CNS from one of several alternatively spliced mRNAs, and it is tyrosine phosphorylated during brain development. Near the N-terminus, Dab1 contains a region of approximately 150 aa known as a protein interaction/phosphotyrosine binding (PI/PTB) domain. The Dab1 PI/PTB domain binds to phospholipid bilayers containing phosphatidylinositol 4P (PtdIns4P) or PtdIns4,5P<sub>2</sub> and to the unphosphorylated sequence F/YXNPXY (in the single-letter amino-acid code, where X is any amino acid), found in the cytoplasmic tails of five members of the low density lipoprotein receptor (LDLR) family and of three members of the amyloid precursor protein (APP) family, and the cytoplasmic signaling protein Ship.<sup>60–62</sup> Unlike other PTB domains, the Dab1 PTB does not bind to tyrosine-phosphorylated peptide ligands (NPXpY), and binds phospholipids in a manner that does not interfere with protein binding.<sup>60</sup> A cluster of tyrosine residues downstream of the Dab1 PI/PTB domain serves as docking sites for SH2 domain-containing proteins such as the non-receptor type protein tyrosine kinases (PTKs), Src, Fyn, and Abl. Src can phosphorylate Dab1 *in vitro*, and tyrosine phosphorylation of Dab1 promotes the interaction with these PTKs.<sup>59</sup> These structures of Dab1 imply that Dab1 mediates protein-protein or protein-membrane docking functions in kinase signaling cascades during brain development.

The identical mutant phenotype of Reelin and Dab1 suggests that they are in a common signaling pathway that regulates layer formation. In *reeler*, the major populations of displaced neurons contain elevated levels of Dab1 protein, although they express normal levels of *Dab1* mRNA.<sup>63</sup> This suggests that Dab1 accumulates in the absence of a Reelin-evoked signal and functions downstream of Reelin. In addition, Dab1 is tyrosine-phosphorylated in a Reelin-dependent manner in primary cultured neurons,<sup>64</sup> indicating that Reelin regulates neuronal positions by stimulating Dab1 tyrosine phosphorylation. Genetically, the double-null mice for Reelin and Dab1 show no additional defect, supporting the concept that Reelin and Dab1 are acting on the same signaling cascade.<sup>64</sup>

Certain tyrosines on Dab1 are responsible for the Reelin signaling *in vivo*, as indicated by knock-in mutation of all these tyrosines in mice, which caused a brain phenotype that is identical to that of the *reeler* mice.<sup>65</sup> The major sites of Src-catalyzed phosphorylation *in vitro* are also preferentially phosphorylated by Reelin in primary cultures.<sup>65,66</sup> These findings suggest that Src family PTKs play important roles in the Reelin-induced tyrosine phosphorylation of Dab1.

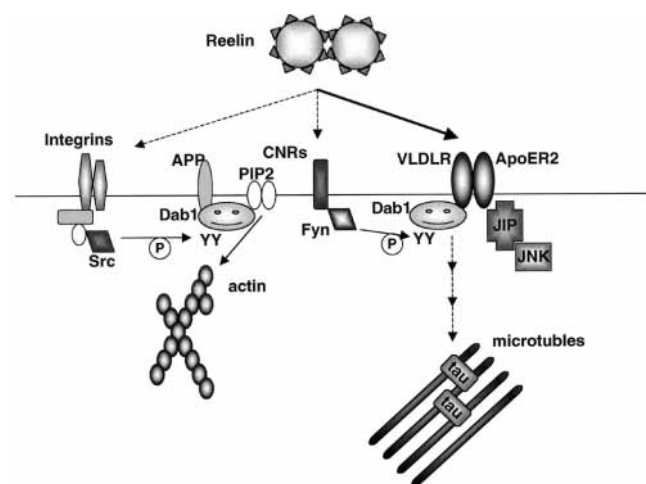
However, the mechanism by which Reelin induces Dab1 phosphorylation is not yet known.<sup>67,68</sup>

### *Reelin receptors*

After the discovery of Dab1 as a downstream molecule of Reelin, the identification of receptors that transmit the Reelin signal to intracellular Dab1 became the major interest of the field. Genetics contributed serendipitously again. The double-knockout mice lacking both the very low density lipoprotein receptor (VLDLR) gene<sup>69,70</sup> and the apolipoprotein E receptor 2 (ApoER2) gene<sup>71–73</sup> exhibit behavioral and neuro-anatomical defects that are indistinguishable from those of *reeler* mice.<sup>74</sup> Moreover, Dab1 protein is increased in the double knockout mouse, indicating an occurrence of a failure in transmitting the Reelin signal. Biochemically, Reelin was demonstrated to bind to the ligand binding domains of VLDLR and ApoER2.<sup>75,76</sup> The estimated K<sub>d</sub> for the interaction was 0.5 nM,<sup>75</sup> or 0.7 nM,<sup>76</sup> suggesting a high-affinity interaction. The intracellular domains of VLDLR and ApoER2 bind to Dab1 at least *in vitro*, implying the pivotal role of VLDLR and ApoER2 in transmitting the Reelin signal to intracellular Dab1.

VLDLR and ApoER2 are likely to function in concert, but the presence of distinct functions is also plausible. Mice lacking only *VLDLR* or *ApoER2* exhibit subtle but different defects, i.e., the deficiency in VLDLR predominantly affects the cerebellum, whereas a phenotype of the ApoER2 defect is obvious in the cerebrum and hippocampus.<sup>74</sup> Biochemically, the cytoplasmic domain of LDLR family members interacts with various proteins implicated in the regulation of mitogen-activating protein kinases, cell adhesion, vesicle trafficking, or neurotransmission.<sup>77</sup> ApoER2, but not VLDLR, contains a unique insertion sequence of 59 amino acids in its cytoplasmic domain<sup>71</sup> and binds to the family of JNK-interacting protein (JIPs), which act as molecular scaffolds for the JNK-signaling pathway, and to rhoGEF, an exchange factor for the small GTPase rhoA.<sup>78</sup> This suggests that ApoER2 is able to assemble to a multi-protein complex, which might participate in Reelin signaling. Very recently, it has been reported that JNK is important for neuronal migration in the neocortex.<sup>79</sup> These additional interactions might explain the distinct phenotypes of mice lacking either the VLDLR or ApoER2 alone.

Although the characterization of VLDLR and ApoER2 as Reelin receptors has shed light on the Reelin-Dab1 signal, the mechanism of Reelin-induced Dab1 phosphorylation remains unclear. VLDLR and ApoER2 do not possess intrinsic tyrosine kinase activity. Neither have they been shown to associate with tyrosine kinase or phosphatases. One hypothesis is that



**Fig. 4** The Reelin signaling pathway. Binding of Reelin to cell surface receptors results in tyrosine phosphorylation of Dab1. Src family protein kinases (Src, Fyn, Abl) are potential candidates that phosphorylate Dab1. The Reelin signal is transmitted to Dab1 through a very low density lipoprotein receptor (VLDLR) or ApoE receptor 2 (ApoER2). Cadherin-related neuronal receptors (CNRs) and  $\alpha 3\beta 1$  integrin are the candidates for co-receptors. Dab1 binds to the cytoplasmic tails of VLDLR and ApoER2 and to those of members of the amyloid precursor protein (APP) family. Dab1 can bind to phospholipids at the same time, including  $\text{PtdIns}(4,5)\text{P}_2$  which is a cofactor in signaling to the actin cytoskeleton.

Dab1 is tyrosine-phosphorylated by a coreceptor. It was reported that Reelin binds to cadherin-related neuronal receptors (CNRs),<sup>80</sup> and  $\alpha 3\beta 1$  integrin<sup>81</sup> in biochemical studies. As CNRs bind Fyn in their cytoplasmic tails<sup>82</sup> and Src associates with integrin-dependent cytoskeletal complexes, both receptors could contribute to Reelin-induced Dab1 tyrosine phosphorylation. Further studies are required to elucidate the complex interactions between Reelin and its various receptors. In particular, the functional importance should be shown genetically *in vivo*.

#### *How does Reelin regulate neocortical layering?*

Despite the identification of molecules in the Reelin signaling pathway, the response of cortical neurons to the Reelin signal remains unclear.

Several lines of studies indicate that Reelin influences neuronal migration by altering the adhesive properties of neurons. In *reeler* mutants, early-born neurons maintain abnormally extensive contacts with the radial fibers and obstruct the movement of late-born neurons.<sup>83</sup> Neurons from *reeler* mice exhibit increased adhesive properties in cell aggregation assays.<sup>84</sup> Earlier-born neurons from normal and *reeler* brains form aggregates with different properties, probably reflecting altered adhesion in *reeler* mice.<sup>38,85</sup> In addition, a recent study demonstrated that Reelin acts as a detach-

ment signal in chain-migrating interneuron precursors in the olfactory bulb.<sup>86</sup> Reelin might alter the adhesive properties of neurons to release neurons from radial fibers and/or to place them in distinct layers. The future identification of molecular cues, for instance, the adhesion molecules that interact with neurons within each layer and their altered properties in *reeler* mice, will be important to test this model.

Reelin was also suggested to detach neurons from radial fibers by binding to  $\alpha 3\beta 1$  integrin, which regulates neuron-glia interactions.<sup>81</sup> The Reelin-binding to these integrins has been thought to switch migrating neurons from a gliophilic adhesive preference to a neurophilic adhesive preference. However, the phenotypes of mice lacking the  $\beta 1$  gene are quite different from those of *reeler*, and a major role of  $\beta 1$  integrin in the brain seems to be in establishing the integrity of the pial basement membrane.<sup>87</sup> To what extent the inhibitory effects of Reelin binding to  $\alpha 3\beta 1$  contribute to neuronal migration remains unknown.

The phenotypes of mutant mice that lack the C-terminal region of Dab1 are interpreted in terms of the “stop signal” model,<sup>88</sup> but also consistent with the “detachment model”. Unlike full length *Dab1*, a single copy of the truncated gene does not support normal migration of the CA1 pyramidal cell in the hippocampus and the late-born cortical plate neurons. In the neocortex, the late-born neurons mistakenly enter layer 1, probably because they fail to receive the Reelin signal and over-migrate. It will be extremely interesting to test the conditional ablation of Reelin or Dab1 during late cortical development to verify the function of Reelin on late-born cortical neurons.

With the aim of elucidating the Reelin function a transgenic mouse was produced, in which an ectopic expression system was used to target Reelin to the ventricular zone under the control of the nestin promoter/enhancer.<sup>89</sup> In the presence of Reelin protein, ectopic Reelin did not alter cell migration. However, in the *reeler* background mice that express ectopic Reelin, some, but not all, cortical neurons migrate past the subplate in the presence of ectopic Reelin in the ventricular zone. Consequently, subplate neurons were split away from the Cajal-Retzius cells by the insertion of a subpopulation of cortical neurons. From these results, Reelin does not appear to function simply as a stop signal or an attractant. Rather, ectopic Reelin was thought to promote cell-cell interactions that are sufficient for the preplate splitting. However, studies are needed to elucidate where ectopic Reelin is secreted, as a small amount of Reelin might be transported to the marginal zone though the fibers and might influence the splitting of the preplate.

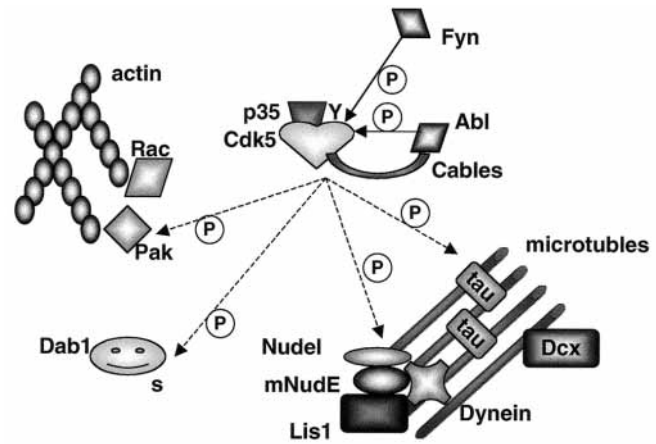
The role of Reelin in promoting splitting of the preplate may be distinct from its effect on lamination, as

laminar formation was not rescued despite the successful prelate splitting in *reeler* mice that express ectopic Reelin.<sup>89</sup> Similarly, in the brains of chimeras consisting of Dab1 mutant (Dab1<sup>-/-</sup>) and wild type (Dab1<sup>+/+</sup>) cells, the preplate was split by Dab1<sup>+/+</sup> neurons, but the Dab1<sup>-/-</sup> neurons could not pass the formed subplate, indicating failure in splitting the preplate is not the sole defect in Dab1<sup>-/-</sup> mutant (Dab1<sup>scm</sup>).<sup>90</sup> Thus Reelin might exert different influences on early- and late-born neurons. Moreover, Reelin does not function exclusively in layer formation. Reelin is thought to act as a branching signal for the entorhinohippocampal projections<sup>40</sup> and for the glial processes in the hippocampus<sup>91</sup>. In addition, abnormalities in retinal synaptic layering are also observed in *reeler*.<sup>92</sup> Different subsequent intracellular signaling events evoked by Reelin-Dab1 signal might underlie the specific responses. Identifications of effector molecules are hoped to elucidate the roles of Reelin-Dab1 signal in each phase.

#### Cdk5/p35 and cross talk

Genetics has provided another group of mutant mice that have defects of neocortical layer formation. They include mice with a targeted disruption of cyclin-dependent kinase 5 (Cdk5)<sup>93</sup> or its regulatory subunit p35.<sup>94</sup> Cdk5 is a serine/threonine kinase, and unlike other members of the Cdk family, its activity is detected mainly in postmitotic neurons.<sup>95</sup> Association of Cdk5 with a neuron-specific regulatory subunit, either p35 or its isoform p39, is critical for its kinase activity.<sup>96–98</sup> Null mice for Cdk5 exhibit perinatal death associated with cortical laminar defects,<sup>93</sup> whereas mice lacking p35 display a milder phenotype because of the redundancy of Cdk5 regulatory subunits.<sup>94,99,100</sup> Moreover, p35 and p39 double mutant mice show a phenotype identical to that of Cdk5 null mice, confirming that p35 and p39 are essential for Cdk5 function during brain development.<sup>101</sup>

In mice that are null for either Cdk5 or p35, the first cohort of migrating cortical neurons successfully invades and splits the preplate.<sup>93,94,99,102</sup> However, the late-generated neurons are unable to migrate past their predecessors and pile up in an inverted layer under the subplate. Consequently, neurons are packed in an “outside-in” pattern, in an indistinct laminated structure. Despite similarities between the phenotypes of the *reeler* mice and those of Cdk5 and p35 mutants, differences are also apparent. For instance, the marginal zone (layer 1) is not present in the *reeler* mice and present in Cdk5 and p35 mutants, albeit very thin in Cdk5 mutants. The corpus callosum is largely intact in *reeler* but diminished in Cdk5 and p35 mutants. Unlike *reeler*, Cdk5 mutants die during the perinatal period, while p35 mutants suffer from seizure activity. The



**Fig. 5** Cyclin dependent protein kinase 5 (Cdk5)/p35 signaling. Cdk5/p35 may regulate the dynamics of microtubules through phosphorylating microtubule-associated proteins Tau, MAP2, and Nudel. Associated with Nudel, mNudE, and dynein, lissencephaly 1 (Lis1) interacts with the microtubules, probably in concert with Doublecortin (Dcx). Cdk5/p35 also causes Pak1 hyperphosphorylation in a RacGTP-dependent manner and modulates dynamics of the actin cytoskeleton. Dab1 is serine-phosphorylated by Cdk5/p35. Active Fyn and Abl can tyrosine-phosphorylate Cdk5, which is enhanced by Cables.

large neurons of Cdk5 mutants in the brainstem and in the spinal cord show degenerative chromatolytic changes, partly explaining the perinatal mortality.<sup>93</sup>

These differences imply that Reelin-Dab1 and Cdk5/p35 have different roles in neuronal migration. Furthermore, the double-null mice for p35 and Reelin/Dab1 demonstrate more severe migration defects than the single mutants, indicating that Cdk5/p35 and Reelin/Dab1 act on the different signaling pathways.<sup>100</sup> This is in contrast with the phenotypes in mutant mice lacking both Reelin and Dab1 that resemble those of the individual mutants of these genes.<sup>64</sup>

However, it is also obvious that Cdk5/p35 and Reelin/Dab1 contribute synergistically to neuronal migration, and biochemical studies suggest cross talk between these pathways (Fig. 5). For instance, as above-mentioned, Dab1 is phosphorylated by Src family PTKs *in vitro*, and active c-Abl, a member of Src family PTKs, leads to Cdk5 phosphorylation on tyrosine 15 (Y15), which stimulates p35/Cdk5 kinase activity. This phosphorylation is enhanced by Cables, a protein which exhibits limited homology to cyclin A, suggesting that Cables may mediate the interaction between Cdk5 and Dab1 by binding to both Cdk5 and c-Abl.<sup>103</sup> Fyn, another member of Src family PTKs, also associates with Cdk5 and facilitates the activation of Cdk5 through Y15 phosphorylation, coordinately mediating neuronal guidance regulated by Semaphorin-3A.<sup>104</sup> Hyperphosphorylation of Tau, a microtubule-

stabilizing protein and a substrate of p35/Cdk5, is reported in mice that lack either Reelin or both VLDLR and ApoER2, supporting the links between Reelin signaling and p35/Cdk5.<sup>75</sup> However, it is not yet known whether Reelin signaling actually stimulates p35/Cdk5 kinase activity *in vitro* or *in vivo*.

On the other hand, Dab1 itself is a substrate for Cdk5, and is phosphorylated by Cdk5 on serine 491 *in vitro* and *in vivo*.<sup>105</sup> As already mentioned above, mutant mice that lack the C-terminal region containing serine 491 show normal development, implying that the C-terminal region might not be essential for Reelin signaling.<sup>88</sup> However, unlike full length *Dab1*, a single copy of the truncated gene does not support normal migration of the CA1 pyramidal cell in the hippocampus and late-born CP neurons. These results might imply that the Dab1 C-terminus affects the strength of Reelin signaling. Although *Dab1* knock-in mice should be made to assess the precise functional significance of Dab1 serine phosphorylation, Cdk5 might serve to modulate Reelin signaling.

How do p35/Cdk5 contribute to neuronal migration and neocortical layering? Several studies indicate that p35/Cdk5 may regulate actin/microtubule dynamics (Fig. 5). The interactions between p35/Cdk5 and Rac/Pak1 suggest that p35/Cdk5 may modulate dynamics of the actin cytoskeleton in neurons.<sup>106,107</sup> Microtubule associating proteins Tau, MAP2, and Nudel are substrates of p35/Cdk5.<sup>108–111</sup> Additionally, p35/Cdk5 may regulate cell adhesion by N-cadherin during cortical development by binding to  $\beta$ -catenin.<sup>112</sup>

### Defects of Cortical Layer Formation in Humans

In humans, the malformation due to abnormal neuronal migration often appears as lissencephaly<sup>9</sup> (“smooth brain”, from “lissos”, meaning smooth, and “encephalos”, meaning brain). Concerning lissencephaly, analogous mutations in mice have been produced by gene targeting in detailed investigations.

#### Type 1 (classical) lissencephaly

In patients with type 1, or classical lissencephaly (LIS), both agyric (convolution is absent) and pachygyric (convolution is broad) regions have a characteristic “four-layered” cortex, composed of (1) a molecular layer, (2) an outer cellular layer (true cortex), (3) a cell-sparse layer, and (4) a deep cellular layer composed of heterotopic incompletely migrated neurons.<sup>113,114</sup> Clinical manifestations are mainly epilepsy and mental retardation. Two genes associated with LIS have been cloned: lissencephaly 1 (*Lis1*, also known as Miller-Dieker syndrome chromosome region, *MDCR*, or more properly, the  $\beta$  subunit of platelet activating factor

acetylhydrolase, *PAFAH1B1*)<sup>115,116</sup> and doublecortin (*Dcx*, also known as *XLIS*).<sup>117,118</sup> In more than 90% of patients with Miller-Dieker syndrome (MDS) and ~40% of those with isolated lissencephaly sequence (ILS), heterozygous mutations in the *Lis1* gene are observed.<sup>115,119</sup> *Dcx* is responsible for X-linked ILS; only hemizygous males develop X-linked ILS, and heterozygous females have a subcortical band of heterotopia (SBH).<sup>117</sup> Whereas the brain malformation due to *Lis1* mutations was more severe over the parietal and occipital regions, *Dcx* mutations produced the reverse gradient, which was more severe over the frontal cortex.<sup>119</sup> The distinct LIS patterns suggest that *Lis1* and *Dcx* may work in a partly overlapping, but distinct manner, in human neuronal migration.

To further address the function of *Lis1*, mutant alleles in mouse *Lis1* were produced.<sup>120</sup> *Lis1* null mice die soon after implantation, indicating that *Lis1* is an essential gene. *Lis1* heterozygotes show cell-autonomous delayed neuronal migration. Cortical development progressed in a normal inside-out fashion, but formed cortical layers are indistinct. Further reduction of *Lis1* activity displays more severe brain disorganization, suggesting a dosage-sensitive role in neuronal migration. *Lis1* contains a WD (tryptophan-aspartic acid) repeat, and interacts with multiple proteins, including an ATP-driven microtubule motor dynein<sup>121–124</sup> and Nudel/mNudeE.<sup>110,111,125</sup> These interactions have profound effects on the microtubule structure of a cell, and may play central roles in microtubule dynamics for nuclear movement, cellular transport, and migration. Furthermore, Nudel is a substrate for Cdk5 and is regulated by Cdk5/p35 to modify neuronal morphology.<sup>110,111</sup> However, a precise model for how these interactions are regulated during brain development is still unclear and further investigations are required.

A mouse carrying a targeted mutation in the *Dcx* gene has also been created.<sup>126</sup> Hemizygous male *Dcx* mice, however, show neocortical lamination largely indistinguishable from the wild type and show normal patterns of neocortical neurogenesis and neuronal migration. Migrating neurons in the human brain may have a greater dependence on *Dcx* function than mice. Proteins homologous to *Dcx*, such as DCAMKL1,<sup>127</sup> may suffice to support migration in the *Dcx* mutant mouse. As *Dcx* binds to microtubules and stimulates the polymerization of purified tubulin,<sup>128,129</sup> *Dcx* seems to regulate the organization of microtubules in cooperation with *Lis1*.

Whereas hemizygous *Dcx* mutations and heterozygous *Lis1* mutations produce lissencephaly and profound neocortical disorganization in humans, analogous mutations in mice cause more subtle (in the case of *Lis1*) or negligible (in the case of *Dcx*) neocortical



defects. Perhaps in humans, in whom cortical neurons transverse a much longer absolute distance than in mice during brain development, migrations are more subject to various impairments, resulting in more severe disorganizations.

### *Type 2 (cobblestone) lissencephaly*

Type 2 or cobblestone lissencephaly is the common brain abnormality seen in some types of congenital muscular dystrophies (CMDs), in which abnormal neuronal migration results in a brain with a bumpy, cobblestone appearance and loss of the normal folding pattern.<sup>130</sup> In cobblestone lissencephaly, the pia is disrupted and discontinuous, and through the gaps cortical neurons migrate over the outer surface of the pia, forming piles of neurons.

Two of these for which human gene mutations have been found are Fukuyama congenital muscular dystrophy (FCMD) and muscle-eye-brain disease (MEB). The FCMD gene *fukutin*,<sup>131</sup> shares homology with *fringe*-like glycosyltransferases, and the MEB gene, *POMGnT1*, seems to be a new glycosyltransferase.<sup>132</sup> Very recently, it was reported that reduced (=hypo) glycosylation of  $\alpha$ -dystroglycan is involved in these two diseases, as well as in a spontaneous mouse mutant, the myodystrophy (*myd*) mouse.<sup>133</sup> In the *myd* mouse, a mutation in the *LARGE* gene, which is thought to encode another glycosyltransferase, has been linked to a muscular dystrophy phenotype. The hypoglycosylation of  $\alpha$ -dystroglycan results in impairment in the binding components of the basement membrane and disruption of the pia. Moreover, brain-selective deletion of dystroglycan in mice is sufficient to cause CMD-like brain malformation, convincing proof of the role of dystroglycan in stabilizing the pial basement membrane.<sup>134</sup>

Similar cortical dysplasias are also found in mice with targeted disruption of the basement membrane protein heparan sulfate proteoglycan Perlecan,<sup>135</sup> of the nidogen-binding site of Laminin  $\gamma 1$ ,<sup>136</sup> of  $\alpha 6$  integrin,<sup>137</sup> and of a brain-specific  $\beta 1$  integrin.<sup>87</sup> In all mutants, including human CMDs, the continuity of the pial basement membrane is disrupted, accompanied by cortical dysplasia. Perlecan, Nidogen1, and Laminin1 are distributed in the pial basement membrane, while Laminin receptors  $\alpha 6$  integrin and  $\beta 1$  integrin are expressed in the developing neocortex, implying that the interaction between the pia and neocortex is indispensable for normal neuronal migration, as well as the continuity of the pial basement membrane.

### *Lissencephaly caused by mutation in the reelin gene*

The nomenclature for lissencephaly with cerebellar hypoplasia (LCH) is only now evolving, and comprises

six broad classes, LCHa-f, that are grouped according to their distinguishing features.<sup>10,138</sup> Among them, it has recently been reported that an autosomal recessive form of human lissencephaly (LCHb) was mapped to chromosome 7q22 and was associated with two independent mutations in the human gene encoding *reelin* (*RELN*).<sup>139</sup> This distinctive pattern possesses a moderately thickened cortex and pachygyria, markedly abnormal hippocampal formation, and severe cerebellar hypoplasia with absent folia.<sup>139,140</sup> Some humans with *reelin* mutations show a severe delay in neurological and cognitive development, accompanied with epilepsy.<sup>139</sup>

Additionally, some patients show abnormal neuromuscular connectivity and congenital lymphedema, and one showed accumulation of fatty ascites fluid that required peritoneal shunting.<sup>139</sup> These unsuspected symptoms might reflect Reelin functions via LDL receptor families outside the brain.

## Concluding Remarks

Recent genetic studies have provided detailed information about molecules important for neuronal migrations over a relatively short period of time. On the other hand, direct observation of migrating neurons is expanding our knowledge of the different behavior of migrating neurons in the formation of the cortex. In the coming years, it is hoped that these molecular and cellular approaches will work together to rapidly elucidate the mechanisms that regulate cortical layer formations, the basis of the elaborate networks in the brain.

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