The Pafah1b Complex Interacts with the Reelin Receptor VLDLR

Guangcheng Zhang1,2,*, Amir H. Assadi1,2,*, Robert S. McNeil1,2, Uwe Beffert3, Anthony Wynshaw-Boris4, Joachim Herz5, Gary D. Clark1,2,3,4,*, Gabriella D’Arcangelo1,2,3,5,6,*

1 The Cain Foundation Laboratories, Texas Children’s Hospital, Houston, Texas, United States of America, 2 Department of Pediatrics, Baylor College of Medicine, Houston, Texas, United States of America, 3 Department of Neuroscience, Baylor College of Medicine, Houston, Texas, United States of America, 4 Department of Neurology, Baylor College of Medicine, Houston, Texas, United States of America, 5 Program in Developmental Biology, Baylor College of Medicine, Houston, Texas, United States of America, 6 Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, Texas, United States of America, 7 Department of Pediatrics and Medicine, University of California, San Diego School of Medicine, La Jolla, California, United States of America

Reelin is an extracellular protein that directs the organization of cortical structures of the brain through the activation of two receptors, the very low-density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor 2 (ApoER2), and the phosphorylation of Disabled-1 (Dab1). Lis1, the product of the Pafah1b1 gene, is a component of the brain platelet-activating factor acetylhydrolase 1b (Pafah1b) complex, and binds to phosphorylated Dab1 in response to Reelin. Here we investigated the involvement of the whole Pafah1b complex in Reelin signaling and cortical layer formation and found that catalytic subunits of the Pafah1b complex, Pafah1b2 and Pafah1b3, specifically bind to the NPxYL sequence of VLDLR, but not ApoER2. Compound Pafah1b1+/−;Apoer2−/− mutant mice exhibit a reeler-like phenotype in the forebrain consisting of the inversion of cortical layers and hippocampal disorganization, whereas double Pafah1b1+/−;Vldlr−/− mutants do not. These results suggest that a cross-talk between the Pafah1b complex and Reelin occurs downstream of the VLDLR receptor.

INTRODUCTION

Heterozygous mutations in the PAFAH1B1 (LIS1) gene in humans cause a reduction in the number of cortical gyri (lissencephaly) [1]. Homozygous mutations in the RELN (REELN) gene also result in lissencephaly, with additional cerebellar hypoplasia [2]. Reelin, a secreted glycoprotein controlling neuronal positioning, functions by clustering its receptors VLDLR and ApoER2, causing the activation of src-family kinases (SRKs) and the phosphorylation of the adapter molecule Dab1 [reviewed by [3–5]]. Disruption of Reelin (Reln) in homozygous reeler mice results in cortical layer disruption, cerebellar hypoplasia and ataxia. Mice deficient for Dab1 [6–8], both VLDLR and ApoER2 receptors [9] and SFKs Fyn and Src [10] exhibit a reeler-like phenotype.

Homologous deletions of the Pafah1b1 gene (encoding Lis1) in the mouse result in early embryonic lethality, whereas heterozygous mutations lead to hippocampal lamination defects [11]. Further reduction of Lis1 activity in compound hypomorphic mutants led to the disruption of cortical layers [12]. Lis1 was initially identified as the non-catalytic β subunit of the Pafah1b complex [1,13]. This complex also contains two catalytic α subunits encoded by the Pafah1b2 and Pafah1b3 genes that hydrolyze the platelet-activating factor [14]. The product of the Pafah1b2 gene (α2) is 30 kDa, whereas the product of the Pafah1b3 gene (α1) is a 29 kDa protein. The entire Pafah1b complex resembles a G-protein signaling complex [15,16]. In the mouse, mutations in the α subunit genes cause no overt neurological phenotype, but loss of Pafah1b2 disrupts spermatogenesis [16,17]. In humans, PAFAH1B3 hemizygosity is associated with mental retardation and ataxia [18], suggesting that the catalytic subunits of Pafah1b may be important for brain development or function. In addition to its involvement with the Pafah1b complex, Lis1 participates in cytoskeletal dynamics as a component of an evolutionary conserved pathway that mediates nucleokinesis [reviewed by [19]]. Lis1 regulates the function of cytoplasmic dynein/dynactin motor complex [20,21] through binding to several of its components. These interactions are thought to be important for several aspects of brain development, including neural stem cell proliferation and neuronal migration [22].

We previously showed that Lis1 binds Dab1 in a Reelin-dependent manner and that Lis1 and Reelin functionally interact [23]. Given the association of Lis1 with the Pafah1b α subunits, we investigated the interaction between this complex and the Reelin pathway. Here we demonstrated that both α subunits bind the Reelin receptor VLDLR but not ApoER2. Genetic experiments further demonstrated that loss of ApoER2 combined with Lis1 reduction results in a reeler-like phenotype, suggesting that the Pafah1b complex modulates the Reelin pathway.

* To whom correspondence should be addressed. E-mail: gangelo@bcm.tmc.edu

These authors contributed equally to this work. (GZ and AA; GC and GD)
RESULTS
Pafah1b3 and Pafah1b2 bind to VLDLR but not to ApoER2

Previous observations revealed the genetic interaction of Pafah1b1 with the genes encoding components of the Reelin signaling pathway, and the direct binding of Lis1 to phosphorylated Dab1 [23]. In this study we turned our attention to the catalytic subunits of the Pafah1b complex. To investigate potential biochemical interactions of Pafah1b catalytic subunits with the Reelin receptors VLDLR and ApoER2, co-immunoprecipitation assays were performed. Plasmids encoding Pafah1b3 (tagged with a MYC epitope) and VLDLR (tagged with either GFP or HA epitopes) were transfected in 293T cells. Results show that VLDLR was co-immunoprecipitated with antibodies directed against the Pafah1b3 tag and, conversely, that Pafah1b3 was co-immunoprecipitated with antibodies directed against the VLDLR tag (Fig. 1A). To determine whether this binding was direct, individual

Figure 1. Pafah1b2 and Pafah1b3 bind VLDLR but not ApoER2. (A) Pafah1b3 binds VLDLR in transfected cells. Pafah1b3-MYC was co-expressed in 293T cells with GFP- (lanes 1–3) or HA-tagged VLDLR (lanes 4–6). VLDLR was co-precipitated with MYC antibodies directed against Pafah1b3 (lanes 2 and 5), but not with control antibodies (lanes 3 and 6). Conversely, Pafah1b3 was co-precipitated with HA (lane 9) or GFP (lane 10) directed against VLDLR, but not with control antibodies (lanes 11–12). Lanes 1 and 4 show VLDLR, and lanes 7–8 show Pafah1b3 in the WCL. Blots were probed with the GFP (lanes 1–3), HA (lanes 4–6) or Myc antibodies (lanes 7–12). (B) Pafah1b2 and Pafah1b3 bind VLDLR in a cell-free-system (TNT). In vitro translated proteins were radiolabeled with 35S and analyzed by SDS-PAGE (lanes 1–4). Individual Pafah1b subunits were combined with equal amounts of VLDLR and immunoprecipitated with FLAG (lanes 5–7) or a negative control antibody (lanes 8). Proteins were detected by autoradiography. Note that VLDLR co-precipitated with Pafah1b α subunits, but not with Lis1. (C) Pafah1b2 and Pafah1b3 do not bind ApoER2 in transfected cells. ApoER2-GFP was co-expressed in 293T cells with the indicated proteins, and co-immunoprecipitated with HA antibodies directed against Dab1 (lane 1). FLAG antibodies directed against the Pafah1b subunits (lanes 2–4) or a control antibody (lane 5) did not co-precipitate the receptor. Blots were probed with antibodies against GFP to detect co-precipitated ApoER2 (upper panel) or total ApoER2 expression in the corresponding WCLs (middle panel). FLAG or HA antibodies were used to detect Pafah1b subunits (lower left panel) or Dab1 (lower right panel) in the corresponding WCLs. ApoER2-GFP was co-precipitated exclusively with Dab1. (D) Pafah1b2 and Pafah1b3 do not bind ApoER2 in a cell-free-system (TNT). In vitro translated proteins were radiolabeled with 35S and analyzed by SDS-PAGE (lanes 1–4). Equal amounts of ApoER2 were combined with the indicated proteins and immunoprecipitated with a control antibody (lane 5), or with antibodies against Dab1 (lane 6), Pafah1b2 (lane 7) or Pafah1b3 (lane 8). Proteins were detected by autoradiography. ApoER2 again co-precipitated only with Dab1. IP, immunoprecipitate; WCL, whole cell lysate. doi:10.1371/journal.pone.0000252.g001
FLAG-tagged Pafah1b subunits were expressed in a cell-free system and incubated in the presence of in vitro-translated VLDLR. The receptor was efficiently co-immunoprecipitated with either Pafah1b2 or Pafah1b3, but not with Lis1 (Fig. 1B), indicating that the z subunits are capable of binding VLDLR directly.

Similar experiments were performed to determine whether Pafah1b3 and Pafah1b2 interact with ApoER2. When this receptor was co-expressed with either Pafah1b2 or Pafah1b3 in 293T cells, immunoprecipitation experiments failed to reveal any interaction (Fig. 1C). As a positive control we used Dab1, which is known to bind lipoprotein receptors [9]. Immunoprecipitation assays using in vitro-translated proteins also revealed no interaction between z subunits and ApoER2, whereas Dab1 was able to bind the receptor, as expected (Fig. 1D). Together, these results demonstrate that both Pafah1b2 and Pafah1b3 z subunits interact specifically with the Reelin receptor VLDLR, but not with ApoER2.

The cytoplasmic NPxY domain of lipoprotein receptors enables binding of proteins containing PTB domains such as Dab1 [9]. To determine whether the NPxY motif is required for Pafah1b2 and Pafah1b3 binding to VLDLR, a series of C-terminal truncation mutants of the receptor tagged with GFP were produced (Fig. 2A). Co-immunoprecipitation experiments were conducted in COS7 cells using antibodies against the z subunit tag. The VLDLR ectodomain fragment (VLDLRΔ809) and the C-terminal truncation mutant VLDLRΔ825, both lacking the NPxY domain, showed no interaction with Pafah1b3 (Fig. 2B) or with Pafah1b2 (Fig. 2D). On the other hand, the truncation construct VLDLRΔ855 that retained the NPxY motif did exhibit binding to Pafah1b3 (Fig. 2B and C) and to Pafah1b2 (Fig. 2D). Finally, the specific NPxY mutant, VLDLRΔ(AAxA), showed loss of interaction with either Pafah1b3 (Fig. 2C) and with Pafah1b2 (Fig. 2D). These results demonstrated that z subunit binding to VLDLR requires the presence of an intact NPxY motif.

The cytoplasmic regions of VLDLR and ApoER2 that include the NPxY binding domain possess significant sequence homology, yet we found that Pafah1b2 and Pafah1b3 bind only to VLDLR. To understand this apparent discrepancy, we examined the protein sequence of the receptor intracellular domains. The NPxY motif sequence in both receptors is NPVY, but the first amino acid downstream of this motif differs. In VLDLR this residues corresponds to a leucine (Leu774), whereas in ApoER2 is an arginine (Arg774) (Fig. 3A). To determine whether a leucine at this position is important for Pafah1b2 and Pafah1b3 binding, an ApoER2 mutant receptor was generated in which Arg774 was substituted by a leucine (R774L). Co-immunoprecipitation experiments in transfected cells demonstrated that ApoER2 carrying the R774L mutation was able to bind Pafah1b2 and Pafah1b3, unlike the wild type receptor (Fig. 3B). These observations suggest that the NPVY sequences of VLDLR mediates its unique property of binding to the Pafah1b3 z subunits. In contrast, Dab1 does not appear to discriminate between lipoprotein receptors as it binds to both, the NPVYL sequence of VLDLR and the NPVYR sequence of ApoER2.

Since the Pafah1b z subunits and Dab1 bind a similar region of VLDLR, we reasoned that they might compete for receptor occupancy. Indeed, increasing concentrations of Pafah1b2 were found to displace Pafah1b3 from VLDLR in co-immunoprecipitation experiments in vitro (Fig. 4A). Furthermore, increasing concentrations of Pafah1b3 were able to partially displace Dab1 from VLDLR, consistently with our observation that they bind similar residues of the receptor intracellular domain (Fig. 4B).

Figure 2. Pafah1b2 and Pafah1b3 interact specifically with the NPxY motif of VLDLR. (A) Diagram of the VLDLR expression constructs used in this study. TM = transmembrane region. CT = cytoplasmic tail. (B) The NPxY motif is required for VLDLR binding to Pafah1b3. Pafah1b3-MYC was co-expressed in 293T cells with the indicated GFP-tagged VLDLR constructs. Proteins were immunoprecipitated with Myc antibodies and the blot was probed with GFP antibodies to detect VLDLR receptors (upper panel). Only full-length VLDLR and the NPxY-containing VLDLR855 co-precipitated with Pafah1b3. WCLs were probed with GFP (middle panels) or Myc (lower panels) antibodies to ensure that similar amounts of VLDLR or Pafah1b3 proteins were present in each sample. (C) The NPxY motif is required for VLDLR binding to Pafah1b2. FLAG-Pafah1b2 was co-expressed in 293T cells with GFP-tagged VLDLR constructs and immunoprecipitated with FLAG antibodies. Blots were probed with GFP antibodies to detect VLDLR proteins in the IP (upper panel) or WCLs (middle panel), or FLAG antibodies to detect Pafah1b2 in the WCLs (lower panel). Only full-length VLDLR and VLDLR855 co-precipitated with Pafah1b2. IP, immunoprecipitate; WCL, whole cell lysate.

doi:10.1371/journal.pone.0000252.g002
In vivo analysis of the Pafah1b complex function in cortical lamination

The biochemical data presented above suggests that the subunits may bring the whole Pafah1b complex in proximity of the VLDLR receptor. Since this receptor also binds Dab1, our findings raise the possibility that the Pafah1b complex may modulate Reelin signaling. To address this possibility in vivo, we set out to generate double mutant mice carrying mutations in genes encoding Pafah1b subunits as well as in genes encoding Reelin receptors. Given that both, Apoer2 and Pafah1b2 male knock out mice are sterile [16,24], we could not generate double mutants that lacked these proteins. We were however able to readily generate compound mice that were heterozygous for Pafah1b1 and homozygous for either Apoer2 or Vldlr. Cortical lamination in these mutants was analyzed as a read-out of Reelin activity in brain development and compared to single mutants or to double Apoer2/Vldlr mutants, which exhibit a reeler-like phenotype. Cortical sections were stained with two cellular layer-specific neuronal markers, Calbindin (layer II/III) and Foxp2 (layer VI) and the layer distribution of immunolabeled cells was analyzed quantitatively. This analysis revealed no obvious lamination defects in single Pafah1b1+/− or Vldlr−/− mice, whereas some layer abnormalities were observed in single Apoer2−/− mice, as previously reported [9,25] (Fig. 5). The cortex of Pafah1b1+/−;Vldlr−/− double mutants also appear fairly normal, however, that of Pafah1b1+/−;Apoer2−/− double mutants presented a severe abnormality similar to the cortical layer inversion typically seen in Reln−/−, Dab1−/− or Apoer2−/−;Vldlr−/− mice (Fig. 5). Calbindin-positive neurons destined for upper layers were ectopically located in the lower cortex, whereas Foxp2-positive neurons destined for a lower layer were ectopically located into the upper cortex. The cortex of Pafah1b1+/−;Apoer2−/− mutants also revealed hypercellularity of layer I, another typical feature of the reeler phenotype that is also seen in double Apoer2−/−;Vldlr−/− mutants (Fig. 5).

To gain further evidence of the occurrence of a reeler-like phenotype in Pafah1b1+/−;Apoer2−/− double mutants, we also examined the anatomy of hippocampal structures (Fig. 6). Cellular layers in the hippocampus proper and dentate gyrus were normal in heterozygous Apoer2+/− mice, whereas a modest split of the pyramidal layer in area CA1 and CA3 was observed in Pafah1b1+/− and homozygous Apoer2−/− mice. However, a reeler-like phenotype characterized by profound dyslamination of all cellular layers was observed in double Pafah1b1+/−;Apoer2−/− mice (Fig. 6). No gross abnormalities were observed in the cerebellum of Pafah1b1+/−;Apoer2−/− double mutants, unlike reeler mice which exhibit profound cerebellar hypoplasia (not shown). Together, these data demonstrate that Pafah1b1, like Vldlr mutations, synergize with Apoer2 disruption and contribute to the appearance of a reeler-like phenotype, at least in the forebrain.

Reelin signaling is largely intact in Pafah1b1+/−; Apoer2−/− cortical neurons

In normal neurons, Reelin treatment induces Dab1 phosphorylation on tyrosine residues [26–28] and the activation of PI3K, which leads to the phosphorylation of Akt on serine residue 473 [29–31]. In double Vldlr+/−;Apoer2−/− mutant mice the appearance of the anatomical phenotype correlates with loss of these Reelin-dependent signaling events [29]. Since double Pafah1b1+/−;
Apoer2<sup>−/−</sup> mutants exhibit a reeler-like phenotype in the forebrain, which we sought to determine whether Reelin signaling was also affected. Cortical neurons were obtained from mice carrying Pafah1b1, Apoer2 and Vldlr mutations, alone or in combination. As for normal mice, Reelin treatment was found to induce Dab1 and Akt phosphorylation in all mutants examined, including Pafah1b1<sup>−/−</sup>;Apoer2<sup>−/−</sup> mutants, even though these animals exhibit cortical layers defects (Fig. 7). These data indicate Dab1 and Akt phosphorylation is not sufficient to induce cortical layer formation.

**DISCUSSION**

We have previously demonstrated the existence of an interaction between Reelin and Lis1 signaling consisting of the direct binding of Lis1, the regulatory subunit of the Pafah1b complex, to the adapter Dab1 [23]. This interaction takes place when Dab1 is phosphorylated on tyrosine residues in response to Reelin. In the present study we have examined the interaction of individual Reelin receptors with the subunits of the Pafah1b complex. We showed that the catalytic z subunits of the Pafah1b complex, Pafah1b2 and Pafah1b3, bind specifically VLDLR and that a reduction in Lis1 activity mimics the loss of this receptor in the forebrain. The binding of Pafah1b3 and Pafah1b2 to VLDLR requires the NPxY domain and the presence of a leucine residue immediately following this sequence. The catalytic z subunits cannot bind the NPxYR sequence of ApoER2, but a point mutation that converts the arginine residue adjacent to the NPxY motif to a leucine rescued Pafah1b z subunit binding, demonstrating that this residue is critical for coupling the Pafah1b complex selectively with VLDLR. Given the low abundance of this receptor in neurons, we could not confirm that the interactions we observed in transfected cells and in vitro also take place in normal neurons. However, given the specificity of the Pafah1b z subunits for VLDLR and the strict requirement for the NPVYL sequence, it seems reasonable to conclude that the binding may indeed occur in vivo.

Through our genetic studies, we demonstrated that the biochemical interaction of the Pafah1b complex with VLDLR has physiological consequences for forebrain development. Consistent with our biochemical data, we observed that Pafah1b1<sup>−/−</sup> mutations had no effect on the appearance of brain structures in Vldlr<sup>−/−</sup> mutants, suggesting that the products of these genes may function in a linear pathway. On the other hand Pafah1b1<sup>−/−</sup> mutations exacerbated the phenotype of Apoer2<sup>−/−</sup> mutants to an extent that the appearance of cortical and hippocampal structures in double Pafah1b1<sup>−/−</sup>;Apoer2<sup>−/−</sup> mutants resembled that of reeler mice. Since a reeler-like phenotype is also observed in double Vldlr<sup>−/−</sup>;Apoer2<sup>−/−</sup> mutants [9], these data suggest that Lis1 is an important component of the Reelin signaling pathway downstream of VLDLR (Fig. 8). The simplest interpretation of our data is that the z subunits function as signaling adapter molecules by bringing Lis1 in proximity of the VLDLR receptor and Dab1, thus facilitating Reelin signaling through this receptor. An alternative interpretation of our genetic findings is that ApoER2 is the dominant Reelin receptor in forebrain development, and that the consequences of Pafah1b1<sup>−/−</sup> mutations on Reelin signaling can only be appreciated when this receptor is missing. This view is
supported by the observation that ApoER2<sup>−/−</sup> mutations in isolation already result in a noticeable cortical and hippocampal phenotype, unlike VLDLR<sup>−/−</sup> mutations [9,25]. Both interpretations of our data are consistent with a functional role for the Pafah1b complex in Reelin signaling during brain development.

Despite the disorganization of cortical layers, we observed that the induction of Dab1 and Akt phosphorylation by Reelin was fairly normal in neurons isolated from Pafah1b<sup>+/−</sup>;ApoER2<sup>−/−</sup> double mutant brains. This is in striking contrast to the results obtained using VLDLR<sup>−/−</sup>;ApoER2<sup>−/−</sup> double mutant neurons, in which Reelin does not appear capable to elicit any signaling events [25,29]. Similarly, Reelin treatment did not induce Akt phosphorylation in Dab1<sup>−/−</sup> neurons [29,31]. Our findings indicate that the Pafah1b complex and Lis1 are not required for many of the signaling events which are normally stimulated by Reelin mainly through clustering of the ApoER2 receptor. In the absence of ApoER2, Reelin signaling events such as Dab1 and Akt phosphorylation still occur, albeit to a lower level, mediated by the VLDLR receptor and irrespective of the presence of Pafah1b proteins. However, under these reduced signaling conditions, Lis1 deficiency prevents the formation of cortical layers. Together with our previous observation that Lis1 binds exclusively phospho-Dab1 [23], the present findings suggest that Lis1 functions downstream of SFK activity and it is not predicted to interfere with the interaction between Dab1 and other signaling molecules such as PI3K [29–31], Nckβ [32], Crk family proteins [28,33], or N-WASP [34].

We and others have previously shown that loss of Pafah1b or subunits in the mouse does not result in a neurological phenotype, but affects spermatogenesis [16,17]. These studies indicate that the catalytic subunits of the Pafah1b complex are not absolutely required for brain development. Based on the present...
data we propose that they may modulate Reelin signaling downstream of VLDLR, possibly by promoting Lis1 and Dab1 interaction.

VLDLR and ApoER2 are both individually capable of binding Reelin on the extracellular side and Dab1 on the intracellular side, and both contribute to cortical layer formation [9,25,35,36]. In addition, ApoER2 is known to bind JNK Interacting Protein (JIP) 1 and 2 and PSD-95 through a unique intracellular domain encoded by an alternatively spliced exon [37–39]. Recent studies demonstrated that ApoER2 interacts with the NMDA receptor, thereby mediating a Reelin-dependent function in learning and memory in the adult brain [40]. Here we have shown that VLDLR is also capable of unique interactions that may affect Reelin signaling and forebrain cellular layer formation. It has recently been reported that VLDLR deletions in humans result in a neurological disorder characterized by lissencephaly and cerebellar hypoplasia, malformations similar but less severe than those associated with RELN deletions [41]. Thus, in humans an overt neurological phenotype is seen even in the presence of the ApoER2, further underscoring the importance of VLDLR in brain development. It remains to be determined whether the unique ability of VLDLR to bind the Pafah1b complex affects postnatal brain function in addition to neuronal positioning during embryogenesis.

MATERIALS AND METHODS

Generation of plasmid constructs

Human VLDLR cDNA encoding the 873 amino acids isoform A (accession # NP_003374) was cloned into pEGFP-N1 (Clontech) to generate the VLDLR-GFP fusion protein. Alternatively, the same cDNA was tagged at the C terminus with the HA epitope by PCR. Truncation constructs VLDLR-F809-GFP (containing VLDLR amino acids 1-809), VLDLR-F825-GFP (containing VLDLR amino acids 1-825) and VLDLR-F855-GFP (containing VLDLR amino acids 1-855) were generated using the Expand High Fidelity PCR System. To produce VLDLR(AAxA)-GFP, site directed mutagenesis was performed on the VLDLR-GFP plasmid using the Stratagene QuickChange Mutagenesis Kit. Mouse Apoer2 cDNA (a gift from J. Nimpf, Medical University of Vienna, Austria) encoding the full-length receptor (accession # CAC38356) minus the alternatively spliced 59 amino acids exon 19 was cloned in frame with GFP or HA as described above for VLDLR. The FLAG-ApoER2(WT) construct was generated by subcloning ApoER2-HA into pCMV-Tag (Stratagene). This was further mutagenized to generate the FLAG-ApoER2(R774L) in which Arg 774 was replaced by a Leu. All constructs were sequenced to verify the intended mutations. Mouse Dab1 cDNA (accession # NP_796233) encoding the 555 amino acids isoform 2 was HA-tagged by PCR and subcloned into pcDNA3.1 vector (Invitrogen). Mouse Pafah1b3 cDNA (accession #Q61205) encoding the 29 kDa ± subunit, mouse Pafah1b2 cDNA (accession #Q61206) encoding the 30 kDa ± subunit, and mouse Pafah1b1 cDNA (accession #NP_038653) encoding the 45 kDa ± subunit of the Pafah1b complex, were cloned into the pEGFP-C1 (Clontech), pcDNA3.1(+)-myc/his (Invitrogen) or pCMV-Tag (Stratagene) to introduce the GFP, Myc or FLAG tag, respectively.

Cell culture and protein analysis

COS7 or 293T cells (ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum, and transfected with expression vectors using the Fugene 6 reagent (Roche). After 30–40 hours, the cells were harvested and the proteins extracted in lysis buffer (PBS, 5 mM EDTA, 1% Triton X-100, pH 7.4) in the presence of protease inhibitors (Mini Complete protease inhibitor cocktail tablets, Roche). For immunoprecipitation, the lysates were incubated with appropriate antibodies for 1–2 hours at 4°C, followed by protein A/G agarose beads (Pierce). Samples were analyzed by SDS-PAGE. To assay Reelin signaling, primary
cortical neurons were cultured from embryonic mice and treated with Reelin-containing conditioned medium for 20 min. Cells were lysed and proteins were subjected to Western blot analysis as previously described [29].

**In vitro binding assay**

Vldlr, Apoer2, Pafah1b, Pafah1b3, Pafah1b2, and Dab1 cDNAs were produced in vitro using the TNT Quick Coupled Transcription/Translation System (Promega), according to the manufacturer instructions using 35S-labeled methionine (Amersham Biosciences). Proteins were separated by SDS-PAGE and detected by autoradiography on dried gels. Quantitative analysis of autoradiography bands density was performed using ImageJ software (NIH image).

**Animals**

Reeler mutant mice were obtained from The Jackson Laboratories on a C57BL/6×C3H hybrid background. Vldlr, Apoer2 and Dab1 knock out mice were on a hybrid C57BL/6×129S6/SvEv. Pafah1b1 (Pafah1b1neo, a null, was utilized in these studies) [11] was on a 129S6/SvEv background. Mutants were genotyped by PCR as described previously for Pafah1b1 [11], Reelin [42], Apoer2 and Vldlr [9], and Dab1 [6].

**Quantification and statistical analysis of cortical sections**

Sagittal paraffin sections (5 μm) of the brain from siblings (when possible) of each genotype were stained with cresyl violet or processed for immunohistochemistry using antibodies against...
Callardin [layer II–III] or Foxp2 [layer VI]. Four paramedian sagittal images at a level caudal to the corpus callosum were utilized for quantitative analysis as previously described [23].

ACKNOWLEDGMENTS

We thank T. Curran and J. Nimipf for plasmid constructs, G. Eichele for Pafah1b3 mutant mice, B.A. Antalyy for help with histology.

REFERENCES

1. Reiner O, Carrozzi R, Shen Y, Wehner M, Faustinella F, et al. (1995) Isolation of a Miller-Dieker leukocyte gene containing G protein beta-subunit-like repeats. Nature 370: 736–738.
2. Lei X, Shugart YY, Huang DT, Al Shahwan S, Grant PE, et al. (2000) Interaction of reelin and L1 in brain development. Neuron 26: 95–106.
3. Rice DS, Curran T (2001) Role of the Reelin signaling pathway in central nervous system development. Ann Rev Neurosci 24: 1005–1039.
4. Tissir F, Goffinet AM (2003) Reelin and brain development. Nat Rev Neurosci 4: 496–505.
5. D’Arcangelo G (2005) The reeler mouse: anatomy of a mutant. In: D’Arcangelo G, ed. International Review of Neurobiology. San Diego, CA: Elsevier Inc. pp. 383–417.
6. Howell BW, Hawkes R, Soriano P, Cooper JA (1997) Neuronal position in the mammalian Lis1. Nat Cell Biol 2: 767–775.
7. Sheldon M, Rice DS, D’Arcangelo G, Yoneshima H, Nakajima K, et al. (1997) Scrumbled and yotari disrupt the disabled gene and produce a reeler-like phenotype in mice. Nature 389: 730–733.
8. Ware ML, Fox JW, Gonzales JL, Davis NM, Lambert de Rouvoir C, et al. (1997) Abrupt splicing of a mouse disabled homolog, mDabl, in the scrumbler mouse. Neuron 19: 239–249.
9. Trommsdorff M, Gotthardt M, Hiesberger T, Shelton J, Stockinger W, et al. (1999) Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDLR receptor and ApoE receptor 2. Cell 97: 689–701.
10. Kuo G, Arnaud L, Kronstad-O’Brien P, Cooper JA (2003) Absence of Fyn and Src causes a reeler-like phenotype. J Neurosci 23: 1719–1729.
11. Hattori M, Adachi H, Tsujimoto M, Arai N, Inoue K (1994) Miller-Dieker leukocyte gene encodes a subunit of brain platelet-activating factor acetylhydrolase. Nature 370: 216–218.
12. Albrecht U, Abu-Issa R, Ratz B, Hattori M, Aoki J, et al. (1996) Platelet-activating factor acetylhydrolase expression and activity suggest a link between neuronal migration and platelet-activating factor. Dev Biol 180: 579–593.
13. Ho YS, Svenson L, Derwenda U, Serre L, Wei Y, et al. (1999) Reduced expression of Pafah1b1 (Lis1) in neural precursors is associated with early embryonic lethality. Nat Genet 19: 333–339.
14. Gambello MJ, Darling DL, Yingling J, Tanaka T, Glosser JG, et al. (2003) Multiple dose-dependent effects of Lis1 on cortical development. J Neurosci 23: 1719–1729.
15. Reiner O, Carrozzi R, Shen Y, Wehner M, Faustinella F, et al. (1995) Isolation of a Miller-Dieker leukocyte gene containing G protein beta-subunit-like repeats. Nature 370: 736–738.
16. Sheldon M, Rice DS, D’Arcangelo G, Yoneshima H, Nakajima K, et al. (1997) Scrumbled and yotari disrupt the disabled gene and produce a reeler-like phenotype in mice. Nature 389: 730–733.
17. Ware ML, Fox JW, Gonzales JL, Davis NM, Lambert de Rouvoir C, et al. (1997) Abrupt splicing of a mouse disabled homolog, mDabl, in the scrumbler mouse. Neuron 19: 239–249.
18. Trommsdorff M, Gotthardt M, Hiesberger T, Shelton J, Stockinger W, et al. (1999) Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDLR receptor and ApoE receptor 2. Cell 97: 689–701.
19. Kuo G, Arnaud L, Kronstad-O’Brien P, Cooper JA (2003) Absence of Fyn and Src causes a reeler-like phenotype. J Neurosci 23: 1719–1729.
20. Faulkner NE, Dujardin DL, Tai CY, Vaughan KT, O’Connell CB, et al. (2000) Dysneuropathy due to a PAFAH1B3-CLK2 fusion gene in a female with mental retardation, ataxia and atrophy of the brain. Hum Mol Genet 9: 969–975.
21. Smith DS, Niethammer M, Ayala R, Zhou Y, Gambello MJ, et al. (2000) Functional hemizygosity of PAFAH1B3 due to a PAFAH1B3-CLK2 fusion gene in a female with mental retardation, ataxia and atrophy of the brain. Hum Mol Genet 9: 969–975.
22. Assadi AH, Zhang G, Beffert U, McNeil RS, Renfro AL, et al. (2003) Interaction of reelin signaling and L1 in brain development. Nat Genet 33: 270–276.
23. Andersen OM, Yan Y, Vorum H, Wellner M, Andreassen TK, et al. (2003) Essential role of the apolipoprotein E receptor-2 in synaptic plasticity and memory by Reelin. J Biol Chem 278: 23993–23999.
24. Keshvarya L, Benhayon D, Magdaleno S, Curran T (2001) Identification of reelin-induced sites of tyrosyl phosphorylation on disabled 1. J Biol Chem 276: 16006–16014.
25. Ballif BA, Arnaud L, Arthur WT, Gurs D, Imamoto A, et al. (2004) Activation of a Dabl/Cril/C3G/Rap1 pathway in Reelin-stimulated neurons. Curr Biol 14: 606–610.
26. Beffert U, Morfini G, Bock IH, Reyna H, Brady ST, et al. (2002) Reelin-mediated signaling locally regulates protein kinase B/Akt and glycogen synthase kinase beta. J Biol Chem 277: 49956–49964.
27. Bock HH, Josso Y, Luo P, Forster E, May P, et al. (2003) PI3-Kinase interacts with the adaptor protein Dab1 in response to Reelin signaling and is required for normal cortical lamination. J Biol Chem 278: 30772–30779.
28. Ballif BA, Arnaud L, Cooper JA (2003) Tyrosine phosphorylation of Disabled-1 is essential for Reelin-stimulated activation of Akt and Src family kinases. Brain Res Mol Brain Res 117: 152–159.
29. Pramataratana V, Ochalaki PG, Chen K, Gropman A, Myers S, et al. (2003) Nckbeta interacts with tyrosine-phosphorylated disabled 1 and redistributes in Reelin-stimulated neurons. Mol Cell Biol 23: 7210–7221.
30. Huang Y, Magdaleno S, Hopkins R, Slaugther C, Curran T, et al. (2004) Tyrosine phosphorylated Disabled-1 recruits Crk family adaptor proteins. Biocchim Biophys Acta 1698: 204–212.
31. Santusso S, Trznka T, Morimura T, Hattori M, Mikoshiba K, et al. (2004) Regulation of actin cytoskeleton by mDab1 through N-WASP and ubiquitination of mDab1. Biochim J 384: 1–8.
32. D’Arcangelo G, Homoyouni R, Keshvarya L, Rice DS, Sheldon M, et al. (1999) Reelin is a ligand for lipoprotein receptors. Neuron 24: 471–479.
33. Hiesberger T, Trommsdorff M, Howell BW, Goffinet AM, Murphy MG, et al. (1999) Direct binding of Reelin to VLDLR receptor and ApoE receptor 2 induces tyrosine phosphorysation of Disabled-1 and modulates Tau phosphorylation. Neuron 24: 481–489.
34. Stockinger W, Brandes C, Fasching D, Hermann M, Gotthardt M, et al. (2000) The reelin receptor ApoER2 recruits JNK-interacting proteins-1 and -2. J Biol Chem 275: 25625–25632.
35. Gotthardt M, Trommsdorff M, Nevitt MF, Shelton J, Richardson JA, et al. (2000) Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. J Biol Chem 275: 25616–25624.
36. Brandes C, Kahr L, Stockinger W, Hiesberger T, Schneider WG, et al. (2001) Alternative splicing in the ligand binding domain of mouse ApoE receptor-2 produces receptor variants binding reelin but not alpha 2-macroglobulin. J Biol Chem 276: 22160–22169.
37. Beffert U, Weber EJ, Duradas A, Qiu S, Masulini L, et al. (2005) Modulation of synaptic plasticity and memory by Reelin involves differential splicing of the lipoprotein receptor ApoER2. Neuron 47: 567–579.
38. Boycott KM, Favelle S, Bureau A, Glais HC, Fujiwara TM, et al. (2005) Homozygous deletion of the very low density lipoprotein receptor gene causes autosomal recessive cerebellar hypoplasia with cerebral gyral simplification. Am J Hum Genet 77: 477–483.
39. Niu S, Renfro A, Quattrocchi CC, Sheldon M, D’Arcangelo G (2004) Reelin promotes hippocampal dendrite development through the VLDLR/ApoER2-Dabl pathway. Neuron 41: 71–84.

Author Contributions

Conceived and designed the experiments: GC GD. Performed the experiments: GZ AA UB GZ. Analyzed the data: RM. Contributed reagents/materials/analysis tools: JH AW AW. Wrote the paper: GD.

Pafah1b Binds Vldlr