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Cajal-Retzius Cells Instruct Neuronal Migration by Coincidence Signaling between Secreted and Contact-Dependent Guidance Cues

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SUMMARY

Cajal-Retzius (CR) cells are a transient cell population of the CNS that is critical for brain development. In the neocortex, CR cells secrete reelin to instruct the radial migration of projection neurons. It has remained unexplored, however, whether CR cells provide additional molecular cues important for brain development. Here, we show that CR cells express the immunoglobulin-like adhesion molecule nectin1, whereas neocortical projection neurons express its preferred binding partner, nectin3. We demonstrate that nectin1- and nectin3-mediated interactions between CR cells and migrating neurons are critical for radial migration. Furthermore, reelin signaling to Rap1 promotes neuronal Cdh2 function via nectin3 and afadin, thus directing the broadly expressed homophilic cell adhesion molecule Cdh2 toward mediating heterotypic cell-cell interactions between neurons and CR cells. Our findings identify nectins and afadin as components of the reelin signaling pathway and demonstrate that coincidence signaling between CR cell-derived secreted and short-range guidance cues direct neuronal migration.

INTRODUCTION

Developmental processes frequently depend on transient cell populations to guide migrating cells. One such population in the CNS is that of the Cajal-Retzius (CR) cells, which have crucial functions in the developing neocortex and hippocampus (Soriano and Del Río, 2005). In the neocortex, CR cells reside in the marginal zone (MZ) and secrete reelin, which signals to projection neurons to control their radial migration (Franco et al., 2011; Gupta et al., 2003; Jossin and Cooper, 2011; Olson et al., 2006; Sekine et al., 2011). At early stages of neocortical development, radially migrating neurons enter the cortical plate (CP) using a migration mode called glia-independent somal translocation, which is characterized by the movement of neuronal cell bodies along their leading processes that are located in the marginal zone (MZ) (Nadarajah et al., 2001; Tabata and Nakajima, 2003). Later-born neurons must migrate further, and thus use several modes of migration (Noctor et al., 2004; Tabata and Nakajima, 2003), but ultimately complete their migration by switching to glia-independent somal translocation once their leading processes enter the MZ (Nadarajah et al., 2001). Reelin specifically regulates glia-independent somal translocation in early- and late-born neurons (Franco et al., 2011) but is dispensable for other modes of motility (Franco et al., 2011; Jossin and Cooper, 2011). During glia-independent somal translocation, reelin regulates the activity of cadherin 2 (Cdh2) to maintain neuronal leading processes in the MZ (Franco et al., 2011), possibly through their interaction with CR cells.

Cdh2 is widely expressed in radial glial cells (RGCs) and neurons of the developing neocortex and is critical for a variety of cellular processes. In migrating neurons, Cdh2 is required not only for forming stable attachments to cells in the MZ (Franco et al., 2011), but also for establishing dynamic adhesions with RGCs during glia-dependent migration (Kawauchi et al., 2010). In contrast, Cdh2 forms stable adherens junctions between RGCs at the ventricular surface (Kadowaki et al., 2007; Rasin et al., 2007). We therefore hypothesized that migrating neurons and other neocortical cell types, such as RGCs and CR cells, might express additional cell-surface receptors that direct the specificity of the homophilic cell adhesion molecule Cdh2 toward the establishment of heterotypic cell-cell contacts with distinct functional properties. Candidate molecules for such interactions are the nectins, a branch of the immunoglobulin superfamily that consists of four members (Takai et al., 2008). Outside the nervous system, nectins cooperate with cadherins in the assembly of adherens junctions (Takahashi et al., 1999; Takai et al., 2008). Within the nervous system, nectins have important functions at synaptic sites (Rikitake et al., 2012). Importantly, some nectins, such as nectin1 and nectin3, preferentially engage in heterophilic interactions that play critical roles during development (Honda et al., 2006; Inagaki et al., 2005; Okabe et al., 2004; Rikitake et al., 2012; Togashi et al., 2011; 2006). However, the functions of nectins in the developing neocortex are not known.

Here, we show that nectin1 and nectin3 are expressed in complementary patterns in the neocortex, in which radially migrating neurons express nectin3 and CR cells express nectin1. We demonstrate that nectin1 in CR cells mediates heterotypic interactions with nectin3 in the leading processes of migrating projection neurons. These nectin-based adhesions control radial

migration by acting in concert with reelin and Cdh2 to promote interactions between migrating neurons and CR cells. Overall, our findings reveal that CR cells instruct the directional migration of neocortical projection neurons by coincident presentation of secreted molecules, such as reelin, and cell-surface-bound guidance cues, such as cadherins and nectins. Our results also clarify how the homophilic cell adhesion molecule Cdh2, which is expressed in many neocortical cell types, mediates specific interactions between two defined cell types by combinatorial signaling with other cell adhesion molecules.

RESULTS

Nectin Expression in the Developing Neocortex

Previous studies have shown that nectins cooperate with cadherins in adherens junction assembly (Takahashi et al., 1999; Takai et al., 2008). Since Cdh2 regulates radial neuronal migration (Franco et al., 2011; Jossin and Cooper, 2011; Kawauchi et al., 2010), we hypothesized that nectins might regulate Cdh2 function during migration. We therefore analyzed the expression patterns of all four nectin family members in the developing neocortex by in situ hybridization. At embryonic day 13.5 (E13.5), nectin2 and nectin4 showed weak, if any, expression in the neocortex (data not shown). In contrast, nectin1 was prominently expressed in the cortical hem and MZ (Figure 1A; Figures S1A and S1B available online), whereas nectin3 was expressed in the neocortical ventricular zone, subventricular zone (SVZ), and intermediate zone (IZ) (Figure 1H). The adaptor protein afadin, which binds to the cytoplasmic domains of all nectins (Miyahara et al., 2000; Takahashi et al., 1999), was expressed throughout the neocortical wall (Figure 1K).

We next used immunohistochemistry to determine the cell types that express nectins and afadin. At E14.5, Nectin1 was confined to the cortical hem and MZ (Figure 1B; Figure S1B), the major source and destination of CR cells, respectively (Meyer et al., 2002; Yoshida et al., 2006; Zhao et al., 2006). Costaining with calretinin, a marker for CR cells (Weisenhorn et al., 1994) and interneurons (Gonchar and Burkhalter, 1997), revealed nectin1 expression in calretinin$^+$ cells (Figure 1C). Even though interneurons are rare in the MZ at E14.5 (Xu et al., 2004), we wanted to confirm that the nectin1$^+$ cells were CR cells. We therefore generated a *Wnt3a-Cre* mouse line (Figure S1C) that expresses Cre in CR cells (Louvi et al., 2007; Yoshida et al., 2006) and crossed them with *Ai9* mice (Figure 1D), which carry a Cre-inducible tdTomato allele (Madisen et al., 2010). tdTomato$^+$ cells in the MZ expressed reelin, confirming their identity as CR cells (Figure 1E). These cells also expressed nectin1 in vivo (Figure 1F) and in vitro (Figure 1G).

Next, we determined the expression pattern of nectin3, the preferred binding partner for nectin1 (Satoh-Horikawa et al., 2000; Togashi et al., 2006; 2011). In contrast to nectin1, nectin3 was present throughout the neocortical wall, including the subplate (SP), CP, and MZ (Figure 1I). In the CP and MZ, nectin3 was enriched in Tuj1$^+$ leading processes of radially migrating neurons (Figure 1J). Similarly, nectin3 was prominently localized to the processes of cultured neocortical neurons (Figure S1D). Costaining for nectin3 and nestin revealed additional staining in the endfeet of RGCs (Figure S1F). A similar expression pattern in neurons (Figures 1L and 1M; Figure S1E) and RGCs (Figure S1G) was observed for afadin.

Early-Born Projection Neurons Require Nectin3 and Afadin for Glia-Independent Somal Translocation

To define the functions of nectin1, nectin3, and their effector afadin during radial neuronal migration, we used small hairpin RNAs (shRNAs) to knock down their expression. We first focused on nectin3 and afadin, which are expressed in radially migrating neurons. shRNAs for nectin3, afadin, or a nonsilencing control were introduced by in utero electroporation into the neocortical primordium of E12.5 embryos (Figure 2A). We chose E12.5 because neurons generated at this time point largely migrate by glia-independent somal translocation (Nadarajah et al., 2001), thus allowing us to specifically study the roles of these molecules in this mode of migration. Our shRNA vectors coexpress EGFP for detection of electroporated cells. At 4 days after electroporation, control cells had migrated into the CP and populated emerging layers V and VI (Figure 2C). In contrast, knockdown of nectin3 or afadin with two different shRNA constructs each blocked migration and caused neurons to accumulate in the IZ (Figures 2B and 2C; Figures S2A and S2B). The knockdown efficiency of each shRNA was confirmed by western blotting (Figure S2C), and the migratory defects were rescued by coelectroporation with nectin3 or afadin complementary DNAs (cDNAs), which are not targeted by the shRNAs (Figures 2B and 2C), thus confirming the shRNA on-target specificity.

Nectin3 Acts in Migrating Neurons

The vector used in the knockdown experiments expresses shRNAs in RGCs and neurons. To define the cell type that is affected and to determine the mechanisms responsible for the migration defects, we first evaluated neuronal differentiation using molecular markers. Knockdown of nectin3 or afadin did not affect the normal differentiation process. Cells that failed to migrate were positive for the neuronal marker Tuj1 (Figures S2D and S2E), but not for the RGC marker Pax6 (Figures S2F and S2G), the intermediate progenitor marker Tbr2 (Figures S2H and S2I), or the proliferation marker Ki67 (Figures S2J and S2K).

Next, we analyzed the extent to which nectin3 and afadin act cell autonomously in migrating neurons using a doublecortin (Dcx) promoter construct that is expressed in migrating neurons, but not in RGCs (Figure 2D) (Franco et al., 2011; Wang et al., 2007). The Dcx vector contains an internal ribosome entry site (IRES) for simultaneous expression of cDNAs and EGFP. We cloned the following into the Dcx vector: (i) full-length control cDNAs for nectin3 and afadin (Dcx-nectin3-iGFP, Dcx-afadin-iGFP); (ii) truncated nectin3 (Dcx-DN-nectin3-iGFP) that lacks the afadin binding site and acts as a dominant negative (Brakeman et al., 2009; Takahashi et al., 1999); and (iii) truncated afadin (Dcx-DN-afadin-iGFP) lacking the F-actin binding site that is critical for stabilizing adherens junctions (Mandai et al., 1997; Ozaki-Kuroda et al., 2002). As an additional control, we used the Dcx-iGFP vector lacking a cDNA insert. When embryos were electroporated at E12.5 and analyzed at E16.5, the positions of neurons expressing full-length nectin3 or afadin were identical to those expressing the Dcx-iGFP control (Figures 2E

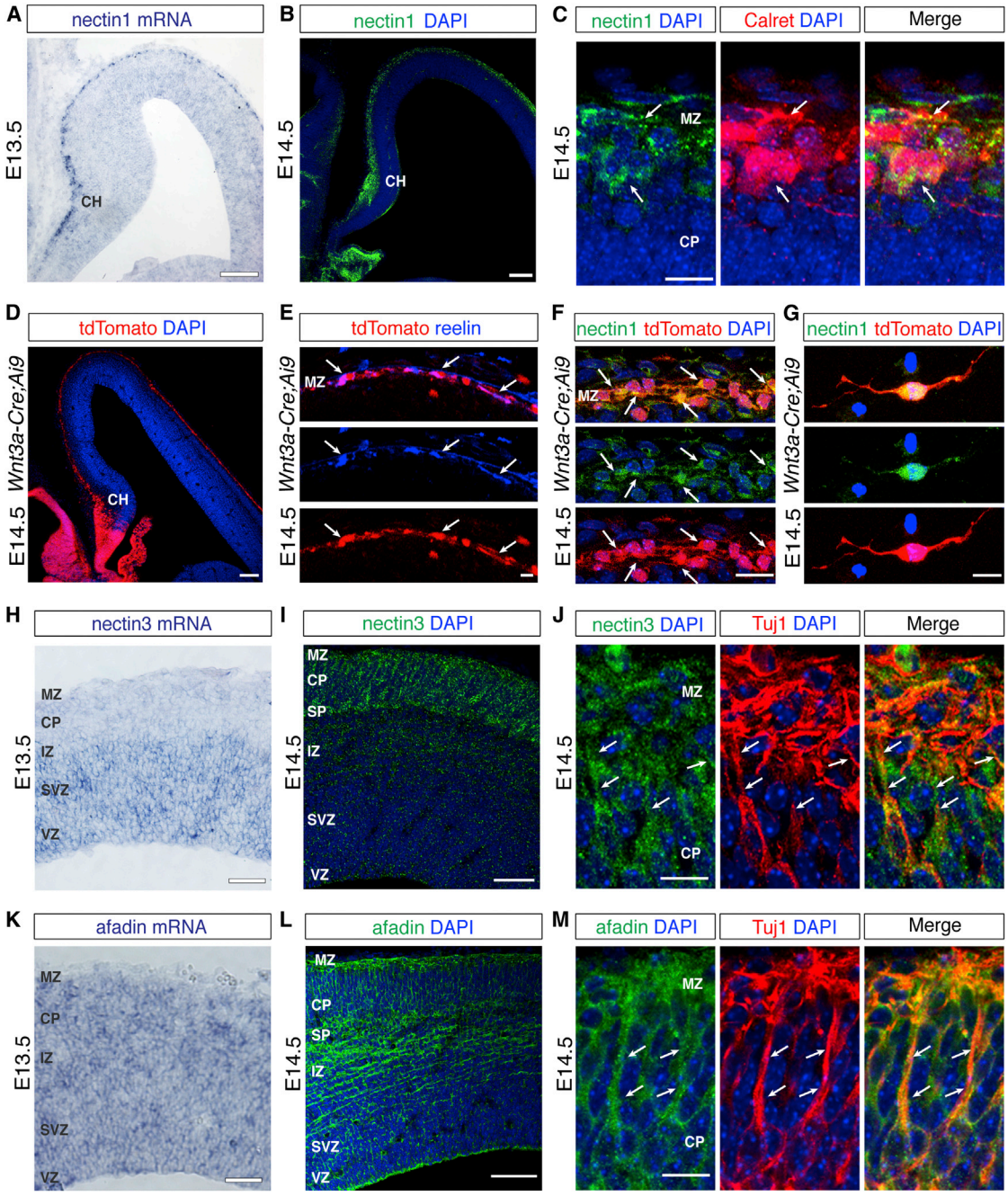

Figure 1. Expression Patterns of Nectins and Afadin in the Developing Neocortex

(A) Nectin1 mRNA is expressed in the cortical hem (CH) and marginal zone. Scale bars = 100 μm.

(B) Nectin1 protein is expressed in the cortical hem (CH) and marginal zone. Scale bars = 100 μm.

(C) High-magnification views show coexpression (arrows) of nectin1 with calretinin (Calret) in CR cells. Scale bars = 5 μm.

(D–G) Analysis of nectin1 expression in *Wnt3a-Cre;Ai9* mice. (D) Low-magnification view of tdTomato expression in the CH and marginal zone. Scale bars = 100 μm. (E) tdTomato$^+$ cells in the MZ (arrows) are reelin$^+$ CR cells. Scale bars = 10 μm. (F) tdTomato$^+$ CR cells in the MZ express nectin1. Scale bars = 10 μm. (G) Nectin1 expression is maintained in cultured tdTomato$^+$ CR cells. Scale bars = 10 μm. (H–M) Nectin3 and afadin are expressed in the leading processes of projection neurons. (H) Nectin3 mRNA expression is high in the ventricular zone (VZ), subventricular zone (SVZ), and intermediate zone (IZ). Scale bars = 50 μm. (I) Nectin3 protein is prominently expressed in the subplate (SP), cortical plate (CP), and MZ. Scale bars = 50 μm. (J) High-magnification views show that nectin3 is expressed in the leading processes (arrows) of Tuj1$^+$ neurons. Scale bars = 5 μm. (K) Afadin mRNA and (L) protein are widely expressed in the neocortical primordium. Scale bars = 50 μm. (M) Afadin is expressed in the leading processes (arrows) of Tuj1$^+$ neurons. Where indicated, nuclei were stained with DAPI (blue). Scale bars = 5 μm. See also Figure S1.

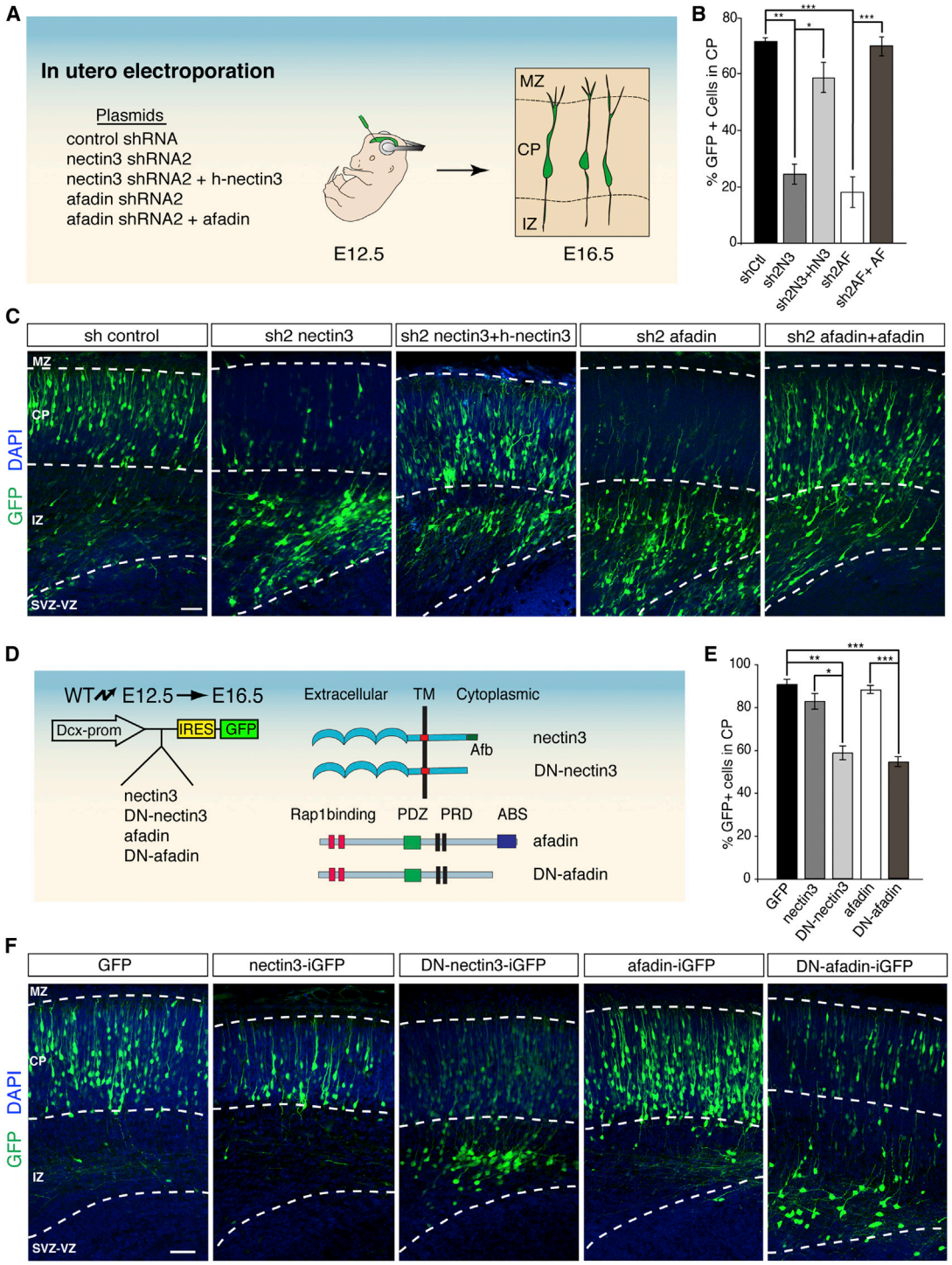

Figure 2. Nectin3 and Afadin Regulate Glia-Independent Somal Translocation of Early-Born Projection Neurons

(A) Strategy to perturb the functions of nectin3 and afadin. Embryos were electroporated in utero at E12.5 with control, nectin3, or afadin shRNAs to target early-born neurons that migrate by glia-independent somal translocation. Positions of the electroporated cells were analyzed at E16.5.

(B) Quantification (mean ± SEM) of the percentage of electroporated neurons from (C) that migrated into the CP. *p < 0.001, **p < 0.0001, ***p < 0.00001 by Student's t test.

(C) Representative images showing the positions of electroporated neurons (green). Nuclei were stained with DAPI (blue). Knockdown of nectin3 (sh2 nectin3) or afadin (sh2 afadin) blocks migration into the CP compared to a nonsilencing control (sh control). Migration defects are rescued by coexpression of shRNAs and their respective refractory cDNAs (sh2 nectin3 + h-nectin3; sh2 afadin + afadin).

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and 2F), indicating that overexpression of the wild-type proteins does not disrupt migration. In contrast, expression of DN-nectin3 or DN-afadin caused electroporated cells to accumulate near the IZ (Figures 2E and 2F), indicating that nectin3 and afadin act in neurons, at least in part, to regulate glia-independent somal translocation.

Nectin3 and Afadin Stabilize Leading Neuronal Processes in the Cortical MZ

We next determined the mechanism by which nectin3 and afadin regulate radial migration. We reasoned that the two proteins might help to anchor the leading processes of neurons in the MZ. We therefore evaluated neuronal morphology following perturbation of nectin3 or afadin function by knockdown and dominant-negative approaches, which gave similar results. Although neurons largely failed to migrate into the CP following perturbation of nectin3 or afadin, they still properly polarized the Golgi apparatus ahead of the nucleus (Figure 3A) and also developed stereotypical polarized morphologies characterized by leading processes (Figures 3B–3D). At 2–3 days after electroporation, leading processes that extended toward or even into the MZ were observed in both control neurons and neurons expressing shRNAs against nectin3 or afadin (Figures 3B and 3C). A small decrease in the number of branches was observed after 3 days in the case of afadin shRNA electroporation, suggesting onset of leading-process retraction. However, only control neurons had their cell bodies located close to the MZ, indicative of somal translocation. Cell bodies in the knockdown experiments failed to translocate toward the MZ (Figures 3B and 3C) and remained near the IZ and lower CP, as nonelectroporated cells bypassed them to expand the CP. This CP expansion initially caused the leading processes of affected neurons to appear longer than those of controls neurons 2–3 days after electroporation (Figures 3B and 3C), but many of these processes were subsequently retracted by 4 days after electroporation (Figures 2C and 2F). Additionally, whereas the leading processes of control neurons extensively branched in the MZ, no such branching was observed after nectin3 or afadin knockdown (Figure 3D). Together, these data indicate that nectin3 and afadin are not required for neuronal polarization or initial process extension, but are important for leading-process anchorage and arborization in the MZ and subsequent somal translocation.

To directly determine whether nectin3 and afadin are required for somal translocation, we carried out time-lapse imaging experiments. Neurons from E13.5 animals were electroporated with control, nectin3, or afadin shRNAs, and neocortical slice cultures were prepared at E15.5. As reported (Franco et al., 2011), control neurons translocated their cell bodies along their leading processes toward the MZ (Figure 3E). In contrast, neurons expressing shRNAs for afadin or nectin3 extended leading processes but failed to undergo somal translocation (Figure 3E).

Interactions between the Leading Processes of Migrating Neurons and CR Cells

Since nectin3 mediates cell-cell adhesion, we reasoned that it might mediate interactions of the leading neuronal processes with CR cells in the cortical MZ. To test this model, we again took advantage of *Wnt3a-Cre:Ai9* mice to study interactions between migrating neurons and tdTomato$^+$ CR cells in vitro and in vivo. We electroporated E13.5 embryos with Dcx-GFP to label migrating neurons and then isolated these primary neurons at E15.5. In parallel, we isolated primary tdTomato$^+$ CR cells from *Wnt3a-Cre:Ai9* embryos by magnetic-activated cell sorting (Figure 4A). Next, we combined the GFP$^+$ neurons with tdTomato$^+$ CR cells for in vitro cocultures. Using this paradigm, we consistently observed pairs of cells in which a GFP$^+$ neuron interacted with a tdTomato$^+$ CR cell. Some of the cells were in tight apposition and aligned their membranes (Figure 4B, arrowheads), while some neurons sent out processes to CR cells (Figure 4B, arrows). These data indicated that neurons and CR cells can engage in cell-cell interactions with one another, at least in vitro.

To extend these findings in vivo and to determine whether nectin3 and afadin are required in neurons to mediate interactions with CR cells, we electroporated *Wnt3a-Cre;Ai9* embryos with shRNA vectors at E12.5 to obtain differentially labeled neurons and CR cells within the same brain. Neuronal processes were visualized at E15.5 in single confocal sections in relation to tdTomato$^+$ CR cells. Similar to the in vitro experiments, the branched leading processes of control neurons overlapped with the cell bodies and projections of CR cells (Figure 4C). Moreover, these interactions and the branching of the leading processes were disrupted upon knockdown of nectin3 or afadin (Figure S3A), providing evidence that nectin3 expression in neurons is important for the formation of these contacts in vivo.

Nectin1 and Nectin3 Colocalize at Contact Sites between the Leading Processes of Migrating Neurons and CR Cells

To directly assess whether nectin1 and nectin3 are recruited in vivo to interaction sites between the leading processes of migrating neurons and CR cells, we established a protocol with sufficient resolution to visualize individual cell-cell contacts. For this purpose, we electroporated the cortical hem at E11.5, during peak times of CR cell generation (Yoshida et al., 2006), to express in CR cells full-length nectin1 and a blue fluorescence protein (BFP) (Figure 4D). The same embryos were re-electroporated at E13.5, but the neocortical VZ was targeted to introduce GFP-tagged nectin3 into migrating neurons. At 3 days after the second electroporation, BFP$^+$ CR cells overexpressing nectin1

(D) Strategy to perturb nectin3 and afadin specifically in neurons. Wild-type or dominant-negative versions of nectin3 or afadin (depicted at right) were expressed with GFP from the neuron-specific Dcx promoter. In utero electroporations were performed as in (A).
(E) Quantification (mean ± SEM) of the percentage of electroporated neurons from (F) that enter the CP. *$p < 0.005$, **$p < 0.0002$, ***$p < 0.00005$ by Student's t test.
(F) Representative examples of neuron-specific perturbations. Overexpression of wild-type nectin3 (nectin3-iGFP) or afadin (afadin-iGFP) does not affect neuronal migration compared to control (GFP). In contrast, expression of dominant-negative versions of nectin3 (DN-nectin3-iGFP) or afadin (DN-afadin-iGFP) impairs migration. Ctl, control; N3, nectin3; AF, afadin; TM, transmembrane domain; Afb, afadin binding site; PRD, proline-rich domain; ABS, actin binding site. Scale bar = 50 μm. See also Figure S2.

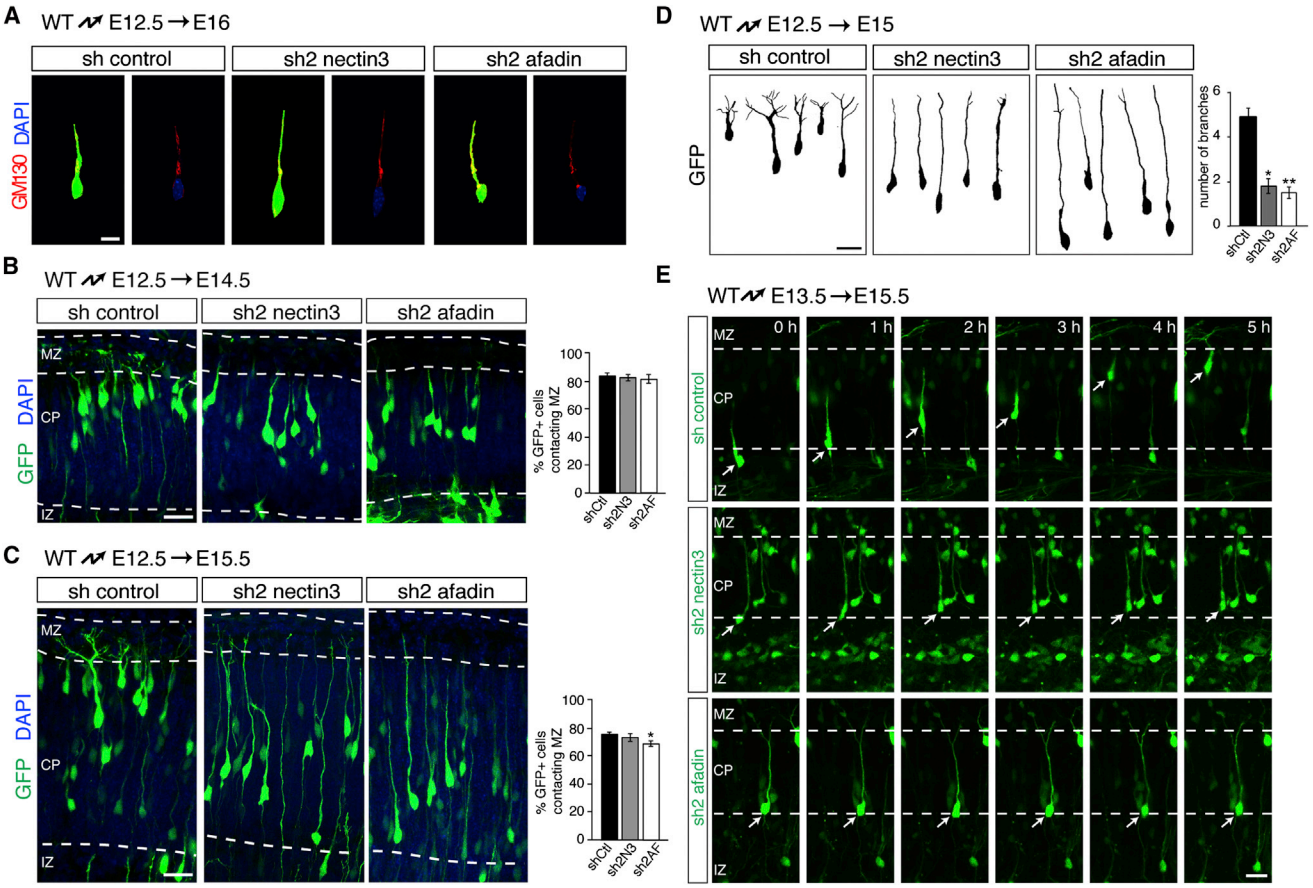


Figure 3. Nectin3 and Afadin Are Dispensable for Cell Polarization and Process Extension but Required for Leading-Process Branching and Somal Translocation

(A–D) Perturbation of nectin3 or afadin does not affect neuronal polarization or leading-process extension. E12.5 brains were electroporated in utero with nonsilencing control shRNA or shRNAs targeting nectin3 or afadin. (A) Staining of E16.5 brains with GM130 (red) and DAPI (blue) reveals normal polar localization of the Golgi apparatus ahead of the nucleus. Analysis at (B) E14.5 and (C) E15.5 demonstrates similar numbers of leading processes contacting the marginal zone in the three conditions at these time points. Quantification is mean ± SEM. A small reduction in the number of processes contacting MZ is observed 3 days after electroporation. *$p < 0.05$ by Student's t test. (D) Morphological analysis of migrating neurons. shRNAs were electroporated into E12.5 embryos, followed by three-dimensional (3D) reconstruction of GFP-positive neurons at E15.5. Leading processes of neurons electroporated with control shRNA profusely arborize in the MZ. Processes are poorly arborized in cells expressing shRNAs targeting nectin3 or afadin. Quantification is mean ± SEM of the number of branches in the MZ 3 days after electroporation. *$p < 5 \times 10^{-5}$, **$p < 5 \times 10^{-6}$ by Student's t test. Scale bars = 10 μm.

(E) Time-lapse imaging of neurons migrating at E15.5 after electroporation of shRNAs at E13.5. Neurons in the IZ were imaged for 5 hr while undergoing glia-independent somal translocation into the CP. Control cells migrate out of the IZ to the top of the CP, and cells expressing nectin3 or afadin shRNA fail to undergo somal translocation. Arrows point to neuronal cell bodies. Abbreviations are as in Figure 1. Scale bars = 20 μm.

had migrated tangentially from the hem into the cortical MZ, while nectin3-GFP$^+$ neurons had migrated radially to populate the emerging CP (Figure 4E). The BFP$^+$ cells expressed calretinin (Figure S3B) and reelin (data not shown), confirming their identity as CR cells. Staining of the electroporated brains with antibodies to nectin1 revealed a punctate staining in CR cells in the cortical MZ (Figure 4E). At a higher resolution, nectin1 could be seen concentrated around the tips of the leading processes of migrating neurons, which also contained high levels of nectin3-GFP (Figure 4F). Colocalization between nectin1 and nectin3 was observed at multiple locations within the cell bodies and distal processes of CR cells (Figure S3C). Together, these data demonstrate that nectin1 and nectin3 are appropriately localized to mediate interactions between CR cells and migrating neurons.

Nectin1 in CR Cells Nonautonomously Regulates Glia-Independent Somal Translocation of Neurons

Because nectin3 preferentially forms heterotypic adhesions with nectin1 (Satoh-Horikawa et al., 2000), we next determined whether nectin1 expression in CR cells is required for the radial migration of nectin3-expressing neurons. For this purpose, we took advantage of our double-electroporation strategy (Figure 5A). We first electroporated hem-derived CR cells at E11.5 with a DN-nectin1 construct that lacks the afadin binding site (Brakeman et al., 2009; Takahashi et al., 1999). The same embryos were re-electroporated at E13.5 with a Dcx-mCherry expression vector to label migrating neurons and then analyzed at E17.5.

CR cells expressing DN-nectin1 still migrated along their normal route within the cortical MZ (Figures S4A–S4D).

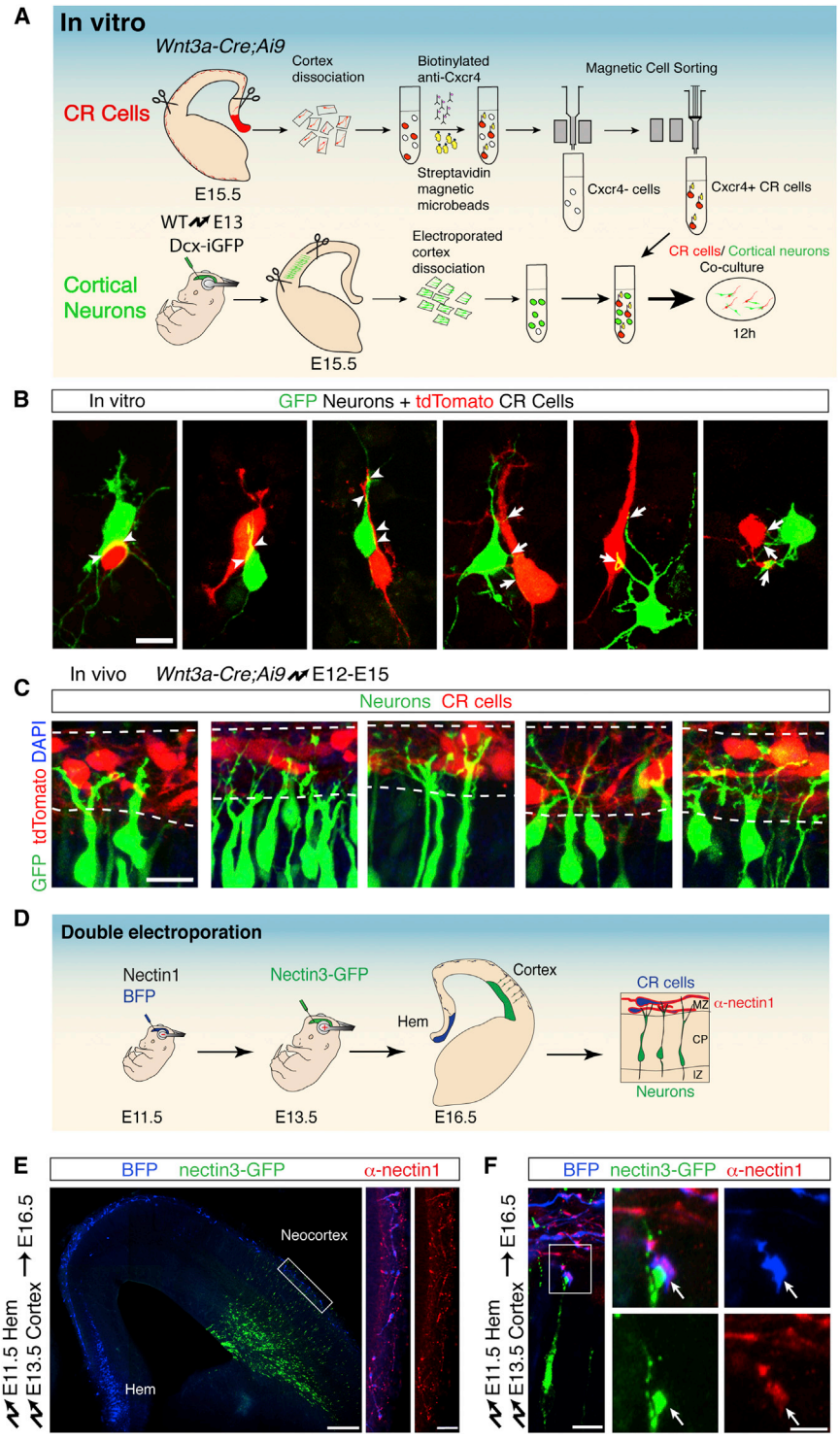


Figure 4. Interactions between Neurons and CR Cells

(A) Schematic of the coculture experiment to visualize in vitro interactions between migrating neurons and CR cells. tdTomato$^+$ CR cells (red) were isolated from E15.5 *Wnt3a-Cre;Ai9* brains using anti-Cxcr4 magnetic bead purification. Cortical neurons (green) were microdissected from E15.5 brains electroporated at E13.5 with Dcx-GFP. Equal numbers of each cell type were mixed and cocultured for 12 hr.

(B) Representative examples of cortical neurons (green) contacting CR cells (red). Arrowheads point to closely aligned membranes between neurons and CR cells. Arrows point to neuronal branches that contact CR cells. Scale bars = 10 μm.

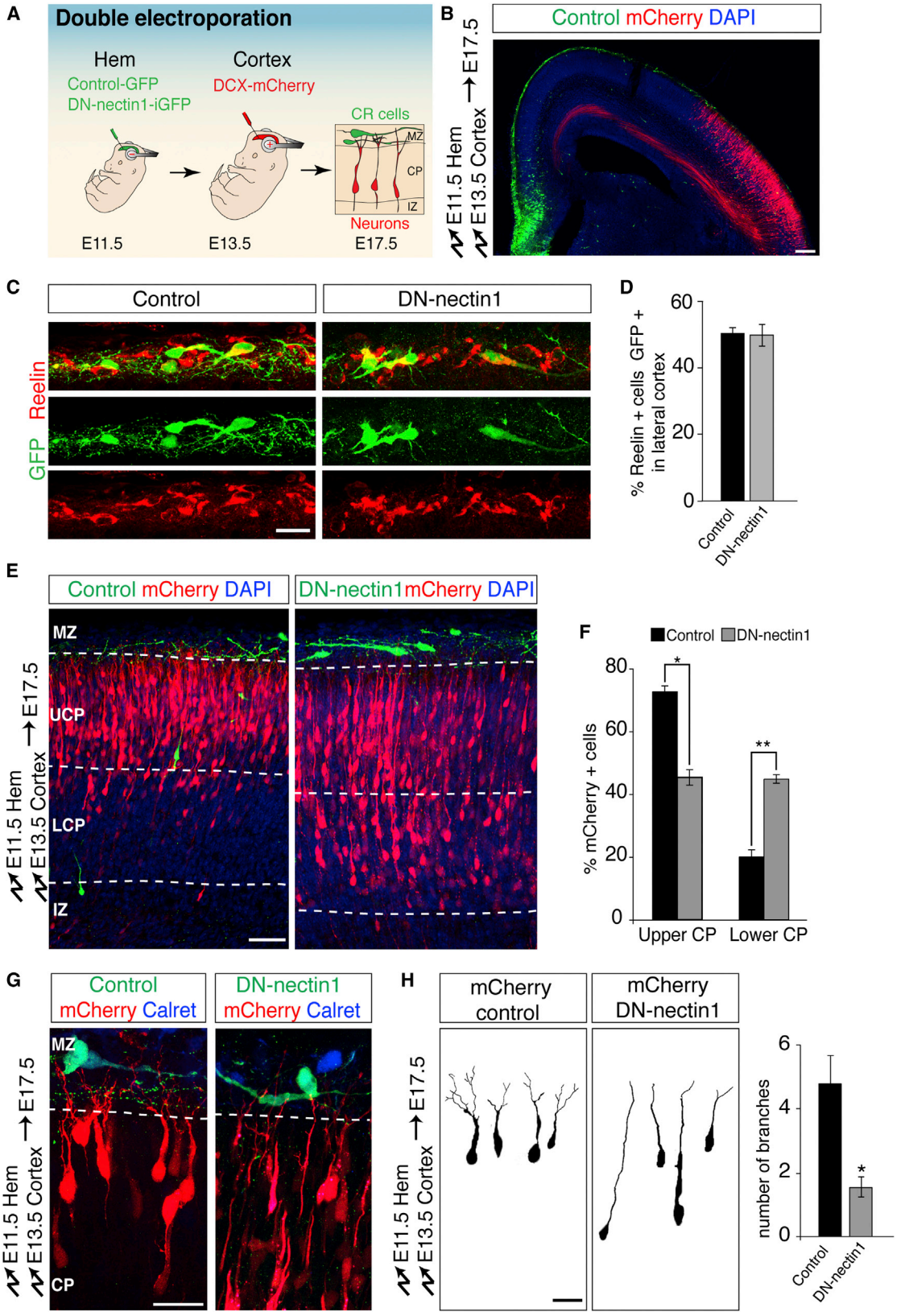
(C) In vivo interactions between the leading processes of migrating neurons and CR cells. *Wnt3a-Cre;Ai9* embryos were electroporated at E12.5 to express GFP in cortical neurons. Coronal sections showing the MZ at E15.5 demonstrate branched leading processes of projection neurons (green) in contact with CR cells (red). Scale bars = 10 μm.

(D) Double-electroporation protocol to visualize nectin1 and nectin3 at contact sites between CR cells and cortical neurons. CR cells in the cortical hem were electroporated at E11.5 with vectors expressing blue fluorescent protein (BFP) and nectin1. Migrating neurons in the same embryos were then electroporated at E13.5 with nectin3-GFP. Double-electroporated brains were analyzed at E16.5. To obtain cellular resolution in dense tissue, overexpressed nectin1 was detected using an antibody dilution 10-fold higher than that normally used to detect endogenous protein.

(E) Coronal section of an E16.5 double-electroporated brain to visualize interactions between CR cells and migrating neurons. Hem-electroporated cells express BFP (blue), and projection neurons express GFP (green). Scale bar = 200 μm. Magnification on the right shows the MZ after nectin1 immunohistochemistry (red). Scale bar = 50 μm.

(F) High magnification showing the leading process of an individual nectin3-GFP$^+$ projection neuron contacting a branch of a nectin1$^+$ CR cell. Panels on the right show the colocalization of nectin1 and nectin3 at the contact point (arrows) between cells. Scale bars = 2.5 μm. See also Figure S3.

Quantitative evaluation confirmed that ~50% of all reelin$^+$ CR cells expressed DN-nectin1, even in the lateral cortex at a substantial distance from the cortical hem (Figures 5C and 5D). These findings show that our electroporation method targets half of all CR cells and that DN-nectin1 does not significantly affect their tangential migration. However, the positions of radially migrating neurons were strikingly altered after nectin1 perturbation in CR cells. Neurons in controls had migrated into the upper part of the CP, whereas large numbers of neurons remained in the lower part of the CP following expression of DN-nectin1 in CR cells (Figures 5E and 5F). Neurons in controls had normal bipolar morphologies with leading processes that branched in the MZ, whereas branch density was drastically decreased following expression of DN-nectin1 in CR cells (Figures 5G and 5H). Similar defects in migration and leading-process arborization were found when nectin1 function



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in CR cells was perturbed using shRNAs (Figures S4E–S4I). Finally, nectin1 perturbation in CR cells did not produce obvious changes in the morphologies of RGC processes or the localization of RGC endfeet (Figure S4J). We conclude that perturbation of nectin1 function in CR cells affects interactions between neuronal leading processes and CR cells, thereby nonautonomously perturbing somal translocation of radially migrating neurons into the CP.

Cdh2 Acts in Neurons in Concert with Nectin3

We have previously shown that Cdh2 in neurons is required for glia-independent somal translocation (Franco et al., 2011); we now show that nectin3 and afadin in neurons are also required for this process. In epithelial cells, nectins form nascent cell-cell adhesion sites, to which afadin is recruited by binding to the cytoplasmic tails of nectins. Afadin then stabilizes cadherin molecules at the cell surface to promote the establishment of stable adherens junctions containing nectins and cadherins (Figure 6A) (Hoshino et al., 2005). We hypothesized that nectin3 and afadin, in migrating neurons, may also cooperate with Cdh2 to regulate the attachment of neuronal leading processes in the MZ.

We first determined the extent to which nectin3 and afadin act in a common pathway in migrating neurons. The similarity in the migration defects caused by knockdown of nectin3 or afadin suggested a functional link between the two. Further supporting this conclusion, nectin3 lacking the afadin binding site (Figure 2D) acts as a dominant negative (Brakeman et al., 2009; Takahashi et al., 1999) and affects radial migration (Figures 2E and 2F), likely by preventing nectin-mediated recruitment of afadin to the cell membrane. We therefore reasoned that overexpression of afadin might rescue the defects caused by nectin3 inactivation, presumably by targeting sufficient amounts of afadin to the cell surface to regulate Cdh2 function. We coexpressed nectin3 shRNA with a full-length afadin cDNA in neurons at E12.5 and analyzed their positions at E16.5. Overexpression of afadin partially rescued the migration defect caused by knockdown of nectin3 (Figures 6C and 6D). Similarly, expressing full-length Cdh2 also rescued the migration defect caused by expression of nectin3 shRNA (Figures 6C and 6D) or afadin shRNA (Figures S5A and S5B). Taken together, these findings suggest that Cdh2 acts in migrating neurons in concert with nectin3 and afadin to regulate glia-independent somal translocation. In support of this model, Cdh2 also colocalized with nectin1 and nectin3 at contact sites between the leading processes of migration neurons and CR cells in vivo (Figures S5C and S5D) and in vitro (Figure S5E). In addition, nectin1-coated latex beads attached to cultured cortical neurons and recruited nectin3, Cdh2, and the Cdh2-binding protein p120 catenin (p120ctn) to the bead/neuron interface (Figure S5F).

p120ctn Function in Glia-Independent Somal Translocation

Stabilization of cadherins at adherens junctions by the nectin/afadin complex depends on p120ctn, which binds to the cytoplasmic domain of Cdh2 and regulates its endocytosis (Figure 6A) (Davis et al., 2003; Hoshino et al., 2005; Sato et al., 2006). We therefore determined whether p120ctn is required in neurons for nectin3 and afadin function during migration. We first evaluated the extent to which a mutated form of Cdh2 (E780A) (Figure 6B) that does not bind p120ctn (Thoreson et al., 2000) can rescue the migratory defects caused by knockdown of nectin3 or afadin. In contrast to wild-type Cdh2 (Figures 6C and 6D), Cdh2 (E780A) was unable to rescue the migratory defect caused by nectin3 and afadin knockdown (Figures 6C and 6D; Figures S5A and S5B).

To independently confirm that binding of p120ctn to Cdh2 is important for Cdh2 function during migration, we took advantage of a dominant-negative cadherin construct (DN-Cdh) that consists of the cytoplasmic domain common to classical cadherins (Figure 6B). This construct blocks neuronal migration (Franco et al., 2011; Jossin and Cooper, 2011), most likely by sequestering cytoplasmic binding partners of endogenous cadherins. Deletion of the binding site for p120ctn within DN-Cdh (Figure 6B) released the dominant-negative effect (Figures 6E and 6F), likely because p120ctn was no longer sequestered, indicating that p120ctn binding to Cdh2 is important for glia-independent somal translocation.

Figure 5. Nectin1 in CR Cells Is Nonautonomously Required for Glia-Independent Somal Translocation of Radially Migrating Projection Neurons

(A) Double electroporation was used to target CR cells and then label radially migrating neurons in the same embryo. Control-iGFP or DN-nectin1-iGFP was electroporated into the cortical hem at E11.5, and then migrating neurons were electroporated with Dcx-mCherry at E13.5. Neuronal position was analyzed at E17.5.

(B) Example of a double-electroporated brain showing GFP-expressing cells (green) in the hem and MZ and mCherry-expressing projection neurons (red). DAPI-stained nuclei are shown in blue. Scale bars = 100 μm.

(C) High-magnification view of the lateral cortex showing that hem-electroporated cells in the MZ (green) have typical CR cell morphologies and express reelin (red). Note that half of all reelin+ CR cells in the lateral cortex are electroporated, as quantified in (D). Scale bars = 10 μm.

(D) Quantification (mean ± SEM) of the percentage of CR cells in the lateral cortex that express control GFP or DN-nectin1 after hem electroporation.

(E) Perturbation of nectin1 function in CR cells causes a non-cell-autonomous migration defect of early-born projection neurons. Neurons in controls reach the upper cortical plate (UCP) by E17.5, whereas expression of DN-nectin1 in CR cells (green) causes radially migrating neurons (red) to accumulate in the lower CP (LCP). Scale bars = 50 μm.

(F) Quantification (mean ± SEM) of the data in (D). *$p < 0.00005$, **$p < 0.000005$ by Student's t test.

(G) Leading processes of migrating neurons (red) show reduced arborization compared to controls when they encounter CR cells expressing DN-nectin1 (green). The CR cell marker caretinin is shown in blue. Scale bars = 10 μm.

(H) 3D reconstructions of mCherry$^+$ projection neurons in contact with the MZ after double electroporation. Leading processes that contact control CR cells arborize in the MZ, whereas neurons contacting CR cells expressing DN-nectin1 display minimal arborization. Quantification is mean ± SEM of the number of branches in the MZ 4 days after electroporation. *$p < 0.001$ by Student's t test. Scale bars = 10 μm. See also Figure S4.

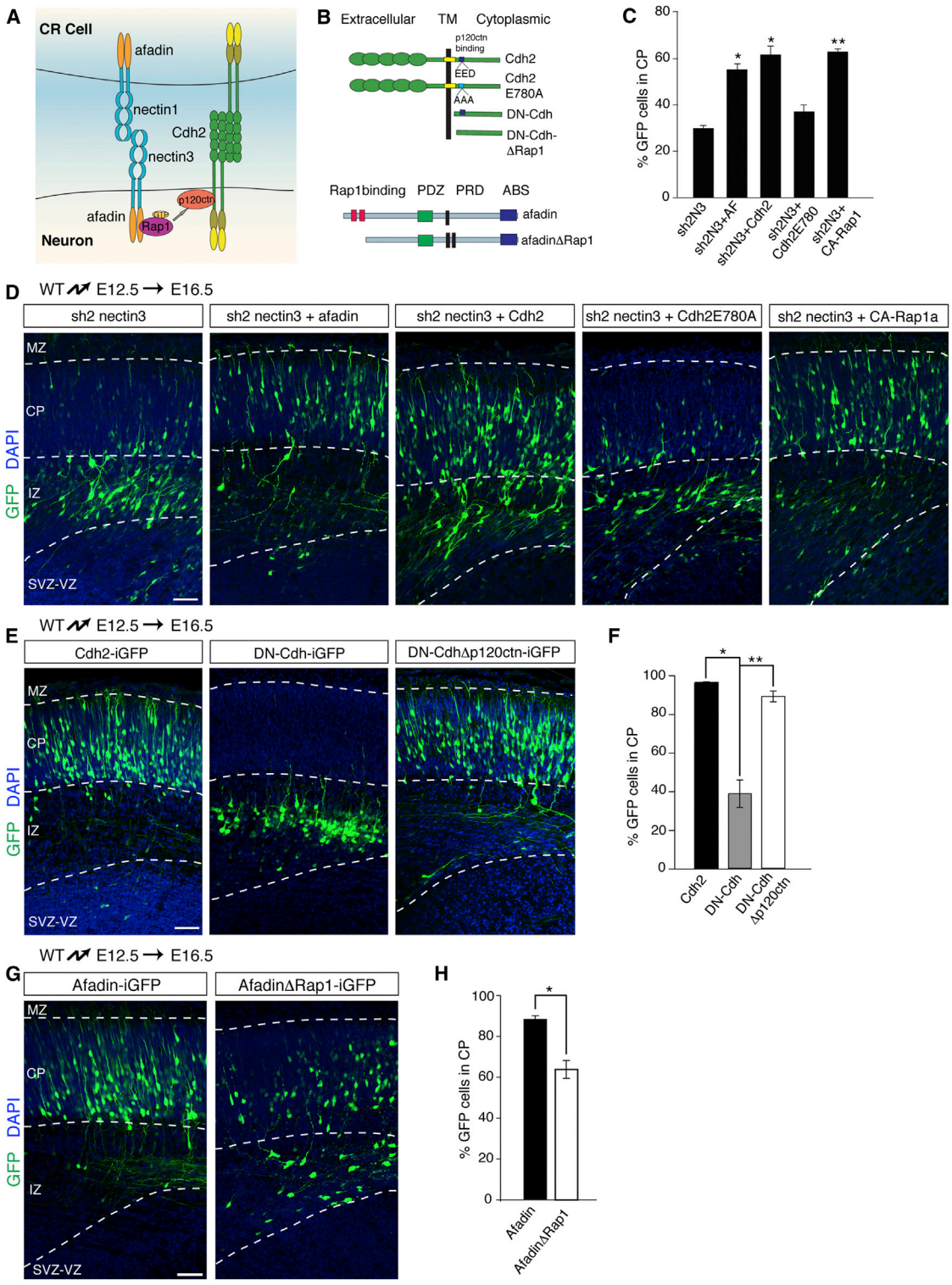

Figure 6. Nectins Act Upstream of Afadin, Rap1, p120ctn, and Cadherins during Glia-Independent Somal Translocation

(A) Illustration of the signaling pathway connecting nectins and cadherins.

(B) Schematic of constructs to study the involvement of p120ctn binding to Cdh2 (upper part) and afadin binding to Rap1 (lower part) in glia-independent somal translocation.

(C) Quantification (mean ± SEM) of the percentage of electroporated neurons from (D) that migrated into the CP. *$p < 0.001$, **$p < 0.00005$ by Student's t test (comparing cells expressing sh2N3, which targets nectin3, to those expressing sh2N3 and rescue constructs).

(legend continued on next page)

Rap1 Acts in Concert with Afadin and p120ctn to Link Nectin3 and Cdh2 Functions

The nectin/afadin complex does not bind p120ctn directly, but does so via the small guanosine triphosphatase (GTPase) Rap1, which binds to both afadin and p120ctn (Figure 6A) (Hoshino et al., 2005; Sato et al., 2006). We hypothesized that Rap1 might be the crucial link between nectin3 and afadin and Cdh2 and p120ctn pairs. Several lines of evidence support this model. First, Rap1 is required for glia-independent somal translocation, and overexpression of Cdh2 can rescue the migration defect caused by Rap1 loss of function, demonstrating that Cdh2 acts downstream of Rap1 in this process (Franco et al., 2011). In addition, we now show that a constitutively active form of Rap1 rescued the migration defect caused by nectin3 knockdown (Figures 6C and 6D). Finally, an afadin construct lacking the Rap1 binding site (Figure 6B) acted as a dominant negative and disrupted radial migration (Figures 6G and 6H). Taken together, our data suggest that nectin3 in migrating neurons recruits an afadin/Rap1 complex that regulates Cdh2 function via p120ctn, thereby promoting leading-process attachment in the MZ and glia-independent somal translocation.

Cdh2 in CR Cells Is Required for Glia-Independent Somal Translocation

At adherens junctions, cadherins are recruited between neighboring cells through nectin and afadin to form stable adhesions. We therefore reasoned that CR cells might also express Cdh2 that acts in concert with nectin1 to mediate interactions with neurons. Indeed, Cdh2 was expressed in CR cells (Figures 7A and 7B). For functional tests, we electroporated the cortical hem at E11.5 with Dcx-iGFP or Dcx-DN-Cdh-iGFP then electroporated the neocortical VZ of the same embryos at E13.5 with Dcx-mCherry to label migrating neurons. By E17.5, GFP^+ CR cells had migrated into the neocortical MZ (Figure 7C), while $mCherry^+$ radially migrating neurons populated the emerging CP (Figure 7D). Expression of DN-Cdh did not inhibit the migration of CR cells within the MZ (Figure 7C), but the positions of radially migrating neurons were significantly altered (Figures 7D and 7E). Neurons in controls migrated into the upper CP, whereas large numbers of neurons remained in the lower CP following expression of DN-Cdh in CR cells (Figures 7D and 7E). In addition, neurons in controls had leading processes that branched extensively in the MZ, but branch density was decreased following expression of DN-Cdh in CR cells (Figures 7F and 7G). The defects in cell morphology and migration resembled those observed after perturbation of nectin1 in CR cells (Figures 5E–5H; Figures S4E–S4G), indicating that nectin1 and Cdh2 in CR cells cooperate to establish stable interactions between neuronal leading processes and CR cells.

Reelin Regulates Recruitment of Cdh2 to the Cell Surface of Neurons Engaged in Nectin-Mediated Adhesive Interactions

Reelin regulates glia-independent somal translocation by activating Cdh2 function via the adaptor protein Dab1 and the small GTPase Rap1 (Franco et al., 2011). However, the mechanism that links Dab1 and Rap1 to Cdh2 function is unclear. Since Rap1 binds to afadin and p120ctn, we reasoned that afadin might provide the critical link between reelin signaling, nectins, and Cdh2 (Figure 8A). We hypothesized that nectins initially mediate heterophilic interactions between migrating neurons and CR cells, leading to the subsequent recruitment of Cdh2 in a reelin-dependent manner to stabilize these nascent adhesion sites (Figure 8A).

To test this hypothesis in vitro, we modeled in vivo interactions between $nectin3^+$ neurons and $nectin1^+$ CR cells by coating glass-bottom wells with recombinant nectin1 and plating dissociated neurons on the coated surface (Figure 8B). We then used total internal reflection fluorescence (TIRF) microscopy to study the recruitment of Cdh2 to the adhesive interface between neurons and the nectin1-coated surface. When neurons were cultured overnight, neuronal Cdh2 was recruited to the cell-substrate interface in nectin1-coated wells, but not on control poly-L-lysine-coated glass (Figure S6). As predicted by our model (Figure 8A), this recruitment of Cdh2 was inhibited upon afadin knockdown in neurons (Figure S6), demonstrating that Cdh2 recruitment was dependent on afadin.

Next, we modified our TIRF assay to allow us to quantitatively evaluate effects of reelin on Cdh2 recruitment. Using primary neurons from *reeler* embryos to maximize response to reelin, we allowed dissociated neurons to make initial contacts with different substrates by plating them for only 1–2 hr (Figure 8B). We then measured the effects of recombinant reelin on Cdh2 recruitment to the interface between neurons and the substrate. We also evaluated Cdh2 adhesive function. Recruitment of Cdh2 to nectin1 substrates was enhanced by treatment of neurons with recombinant reelin (Figure 8D), whereas reelin had no effect on Cdh2 recruitment to poly-L-lysine (Figure 8C). A similar increase in Cdh2 recruitment was observed by overexpression of constitutively active Rap1, but not by overexpression of afadin alone (Figure 8E), suggesting that reelin signaling via Rap1 does not simply act by increasing afadin levels within the cell. Furthermore, interactions of afadin with p120ctn were enhanced by reelin treatment (Figure 8F), suggesting that the reelin/Rap1

(D) Overexpression of afadin, Cdh2, or constitutively active Rap1 in neurons rescues the migration defects caused by nectin3 shRNA expression. Expression of a Cdh2 cDNA mutated in the p120ctn binding site (Cdh2-E780A) does not rescue the migratory phenotype caused by nectin3 knockdown. Constructs were electroporated at E12.5, and neuronal position was determined at E16.5. Electroporated neurons are in green and nuclei in blue.

(E) Deletion of part of the p120ctn binding site in DN-Cdh relieves its inhibitory effect on radial migration. Constructs were electroporated at E12.5, and neuronal position was determined at E16.5. Electroporated neurons are in green and nuclei in blue.

(F) Quantification (mean ± SEM) of the percentage of electroporated neurons from (E) that enter the CP. $^*p < 0.0005$, $^{**}p < 0.00005$ by Student's t test.

(G) Binding of Rap1 to afadin is required to regulate somal translocation. Electroporation of a truncated form of afadin lacking the Rap1-binding domain perturbs migration into the CP. Constructs were electroporated at E12.5, and neuronal position was determined at E16.5. Electroporated neurons are in green and nuclei in blue.

(H) Quantification (mean ± SEM) of the percentage of electoporated neurons from (G) that enter the CP. $^*p < 0.003$ by Student's t test. Abbreviations are as in Figures 1 and 6. Scale bars = 50 μm. See also Figure S5.

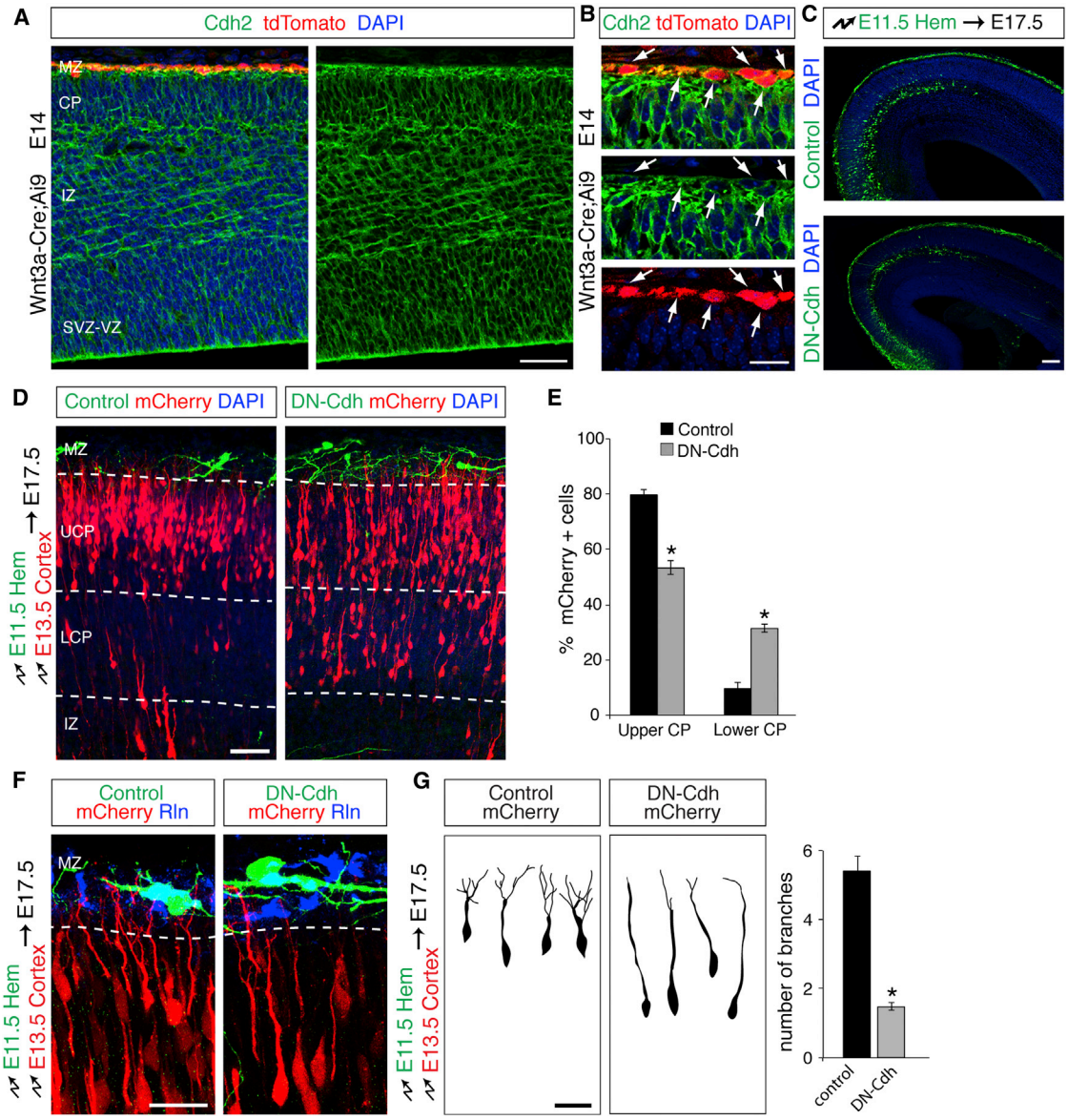

Figure 7. Cdh2 in CR Cells Is Required Nonautonomously for Glia-Independent Somal Translocation of Radially Migrating Projection Neurons

(A) Coronal section of an E14.5 brain from a *Wnt3a-Cre;Ai9* embryo showing expression of Cdh2 protein in the developing neocortex. tdTomato$^+$ CR cells in the MZ express Cdh2. Nuclei are shown in blue. Scale bar = 50 μm.

(B) Higher-magnification view of (A) showing the MZ. Arrows point to CR cells coexpressing Cdh2 and tdTomato. Scale bar = 10 μm.

(C) CR cells targeted with DN-Cdh migrate normally to populate the MZ. Coronal sections of brains electroporated with control or DN-Cdh at E11.5 in the cortical hem and analyzed at E17.5. Electroporated CR cells are shown in green, and DAPI stained nuclei are shown in blue. Scale bar = 100 μm.

(D–F) Double-electroporation experiments as described in Figure 5 were carried out to perturb Cdh2 function in CR cells and determine effects on radially migrating neurons. (D) Coronal sections of double-electroporated brains in which CR cells (green) express either GFP alone (control) or together with dominant-negative cadherin (DN-Cdh). Migrating projection neurons express mCherry (red). Nuclei are in blue. Radially migrating neurons accumulate in the lower CP (LCP) upon expression of DN-Cdh in CR cells, whereas neurons in controls populate the upper CP (UCP). Scale bar = 50 μm. (E) Quantification (mean ± SEM) of the data in (D). *$p < 0.0002$ by Student's t test. (F) Leading-process arborization of migrating neurons (red) is reduced when they contact CR cells (green) expressing DN-Cdh compared to when they contact control GFP-expressing CR cells. Reelin staining is shown in blue. Scale bar = 10 μm.

(G) 3D reconstructions of mCherry$^+$ projection neurons in contact with the MZ after double electroporation. Leading processes that contact control CR cells arborize in the MZ, whereas neurons contacting CR cells expressing DN-Cdh display reduced arborization. Quantification is mean ± SEM of the number of branches in the MZ 4 days after electroporation. *$p < 0.0001$ by Student's t test. Abbreviations are as in Figure 5. Scale bar = 10 μm.

pathway facilitates complex formation between the two proteins. Finally, adhesion of dissociated primary neurons to Cdh2-coated coverslips was substantially increased following reelin treatment (Figure 8G), confirming that cell-surface-expressed Cdh2 was functionally active in mediating homophilic interactions. In conclusion, since p120ctn binding to cadherins stabilizes their expression at the cell surface (Hoshino et al., 2005), our findings suggest that reelin stabilizes Cdh2 at the surface by facilitating afadin- and Rap1-mediated recruitment of p120ctn to nascent nectin-based cell-cell adhesions.

DISCUSSION

Here we provide insights into the mechanisms by which CR cells instruct neocortical development and identify nectins as components of the reelin signaling pathway. Previous studies have shown that CR cell-derived reelin regulates the Cdh2-dependent anchorage of the leading processes of radially migrating neurons with yet-to-be-defined cells in the cortical MZ (Franco et al., 2011). We now identify CR cells as the adhesion partners for migrating neurons and demonstrate that heterotypic binding specificity between the two cell types is achieved by a combinatorial adhesion code consisting of the homophilic cell adhesion molecule Cdh2 and the heterophilic cell adhesion molecules nectin1 and nectin3. Unlike ubiquitously expressed Cdh2, nectin1 and nectin3 are expressed specifically in CR cells and migrating neurons, respectively. Using functional perturbations, we show that nectin1 and nectin3 mediate heterotypic interactions between CR cells and the leading processes of migrating neurons. Cdh2 is then likely required to consolidate these initial interactions into stable contacts to facilitate translocation of the neuronal cell bodies along the leading processes.

Our findings also define components of the signaling pathway that couple reelin to nectins and cadherins. Reelin regulates Cdh2 function during glia-independent somal translocation via the adaptor protein Dab1 and the small GTPase Rap1 (Franco et al., 2011). We now show that nectin3 and afadin provide a critical link connecting reelin, Dab1, and Rap1 to Cdh2. Accordingly, perturbation of nectin3 or afadin disrupts glia-independent somal translocation, and overexpression of Cdh2 in neurons rescues these migratory defects. Reelin signaling facilitates Cdh2 recruitment to nectin1- and nectin3-based adhesions, indicating that reelin promotes the assembly of adhesion sites consisting of nectins and cadherins. Afadin apparently serves a critical function in connecting reelin signaling to adhesion by binding to nectins and Rap1. In addition, afadin binds p120ctn in a Rap1-dependent manner, reelin signaling enhances recruitment of p120ctn to afadin, and p120ctn binding to Cdh2 is critical for glia-independent somal translocation. These results reveal a resemblance to the mechanism of adherens junction assembly in epithelial cells in which nectins establish weak nascent adhesion sites that are then consolidated into stable adherens junctions by the nectin-dependent stabilization of cadherin function via afadin, Rap1, and p120ctn (Hoshino et al., 2005; Sato et al., 2006). Since p120ctn inhibits cadherin endocytosis (Davis et al., 2003; Hoshino et al., 2005), this model is consistent with the observation that reelin increases Cdh2 cell-surface levels (Jossin and Cooper, 2011).

Our findings also provide insights into the mechanisms by which heterotypic cell-cell interaction specificity is achieved by combinatorial interactions between homophilic cell adhesion molecules (Cdh2) and receptors that preferentially engage in heterophilic interactions, such as nectin1 and nectin3 (Satoh-Horikawa et al., 2000). This combinatorial signaling is likely critical in several developmental contexts. For example, previous studies have shown that nectin1 is expressed in hippocampal mossy fibers, whereas nectin3 is expressed in CA3 pyramidal neurons. These nectins are localized at synaptic contacts formed between the two cell types, and perturbation of their function leads to synaptic defects (Honda et al., 2006). Cdh2 is also recruited to the synaptic sites and is required for their function (Brigidi and Bamji, 2011), suggesting that cooperation between nectins and cadherins determines synaptic specificity. However, in some instances, nectins appear to function independently of cadherins. For example, nectin1 and nectin3 regulate pathfinding of commissural axons at the spinal cord midline independently of cadherins (Okabe et al., 2004). Instead, axonal pathfinding depends on the secreted signaling molecule netrin1 (Serafini et al., 1994). Nevertheless, there is a striking similarity between axonal pathfinding and radial neuronal migration in that directional motility in both cases is regulated by combinations of secreted signaling molecules, such as reelin and netrin1, together with cell adhesion molecules, such as nectins and cadherins. The combinatorial code of these molecular cues likely varies depending on the cell type and developmental context, resulting in different functional outputs. In this regard, it will be interesting to analyze nectin and cadherin functions during other stages of neocortical development, for example during the formation of axonal processes or dendrites within neocortical cell layers.

Mice with mutations in *nectin1* and *nectin3* show defects in hippocampal synapse formation, but no neocortical defects have been reported in these mice when analyzed by general histology (Honda et al., 2006). In light of the current findings, it will be important to analyze the formation of neocortical cell layers in these mice further, for example by using molecular markers that define the identity and position of subtypes of projection neurons. In addition, it is feasible that in these knockout mice, which lack nectin1 or nectin3 throughout development, other cell adhesion molecules might be upregulated to functionally compensate for the loss of nectin1 and nectin3. This compensation may not be triggered by acute perturbations. Similar observations have been made in other instances, for example when the function of doublecortin was disrupted genetically or by RNAi. Only in the latter case were functional defects observed (Bai et al., 2003), whereas defects following genetic perturbation were compensated for, at least in part, by expression of doublecortin kinase (Deuel et al., 2006; Koizumi et al., 2006).

Our findings extend the notion that the neocortical MZ is an important signaling center for brain development. The MZ contains extracellular matrix (ECM) molecules and various cell types, including interneurons, meningeal fibroblasts, and CR cells. Although CR cells are best known for controlling neocortical lamination via reelin secretion, they are thought to regulate several other important developmental events and thus might provide additional molecular cues besides reelin. For example, CR cell subpopulations that have distinct extracortical origins

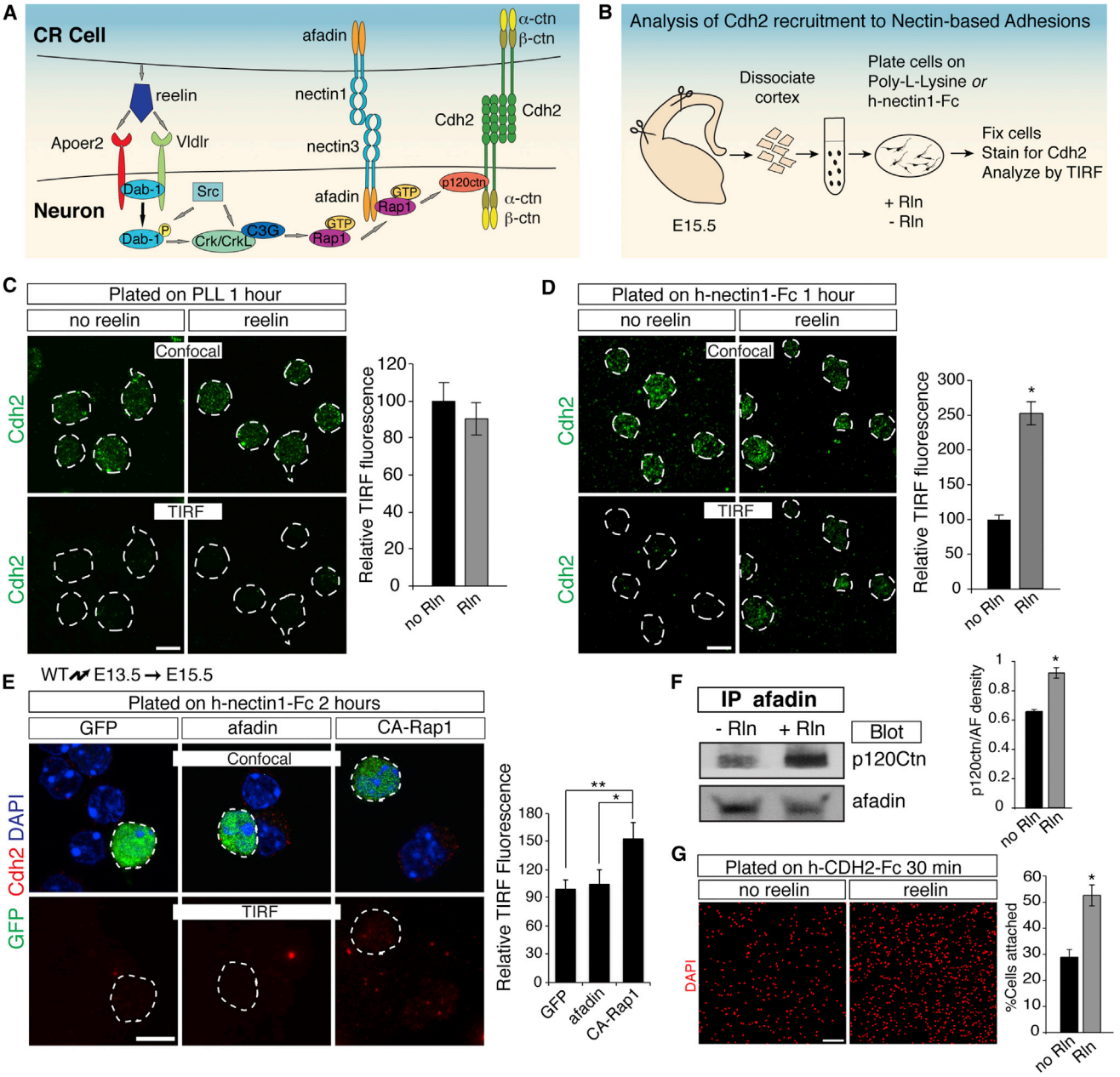

Figure 8. Reelin Signaling Promotes Recruitment of p120ctn and Cdh2 to Nectin and Afadin Complexes and Enhances Cdh2-Mediated Adhesion

(A) Signaling pathways proposed to act during neuronal migration.

(B) Experimental strategy to study the effects of reelin signaling on recruitment of Cdh2 to nectin-based adhesions. Dissociated neocortical neurons were plated on glass coated with poly-L-lysine or nectin1-Fc in the presence or absence of reelin. Recruitment of Cdh2 to the cell-substrate interface was analyzed by TIRF microscopy.

(C and D) Reelin promotes recruitment of Cdh2 to nectin-based adhesions. Neocortical cells from E15.5 *reeler* brains were plated onto (C) poly-L-lysine-coated or (D) nectin1-Fc-coated glass ± reelin and immunostained for Cdh2 (green). Cells (dotted outlines) were identified by confocal microscopy (upper panels) and Cdh2 at the cell-substrate interface by TIRF microscopy (lower panels). Quantification (mean ± SEM) is the Cdh2 mean pixel intensity per cell in TIRF images, normalized to control (no reelin). *$p < 5 \times 10^{-13}$ by Student's t test. Scale bars = 10 μm.

(E) Cortical neurons were electroporated in utero with GFP alone or together with afadin or constitutively active Rap1a (CA-Rap1) at E13.5, dissociated at E15.5, and plated on nectin1-Fc-coated glass for 2 hr before staining for Cdh2 (red). Electroporated cells (green) are outlined. Quantification (normalized mean ± SEM) of the Cdh2 TIRF signal (red) demonstrates that overexpression of afadin does not affect Cdh2 recruitment compared to control, whereas CA-Rap1 promotes Cdh2 recruitment to the nectin1 surface. *$p < 0.04$, **$p < 0.01$ by Student's t test. Scale bars = 10 μm.

(F) Reelin treatment promotes coimmunoprecipitation of p120ctn with afadin in neurons. Quantification (mean ± SEM) is the optical density of western blot bands for p120ctn, normalized to afadin levels. *$p < 0.005$ by Student's t test.

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populate different regions of the neocortical surface, suggesting that they might be involved in patterning the neocortex (Griveau et al., 2010). Projection neurons and CR cells also interact after projection neurons have settled into neocortical cell layers, raising the possibility that CR cells regulate the maturation of dendrites and synapses (Marín-Padilla, 1998; Radnikow et al., 2002). Finally, CR cells and GABAergic interneurons in the cortical MZ show synchronized neuronal activity (Aguiló et al., 1999; Radnikow et al., 2002; Schwartz et al., 1998; Soda et al., 2003), and CR cells receive synaptic inputs from the thalamus, entorhinal cortex, and brainstem (Janusonis et al., 2004; Supèr et al., 1998). The functions of these developmental circuits are not known. Intriguingly, recent molecular profiling studies have identified secreted molecules and transmembrane proteins that are expressed in CR cells (Yamazaki et al., 2004), some of which likely instruct the formation of neocortical circuits by mechanisms that have yet to be explored.

EXPERIMENTAL PROCEDURES

Procedures are described in detail in Supplemental Experimental Procedures.

Mice

Experiments using mice were carried out under the oversight of an institutional review board. *Wnt3a-Cre* mice were generated by targeting an IRES-Cre cassette into the 3′ UTR of the *Wnt3a* gene. *Ai9* mice have been described (Madisen et al., 2010). *Reeler* mice were purchased from Jackson Laboratory (Stock 000235). C57BL/6J mice were used for in utero electroporations.

Expression Constructs

shRNAs for nectin3 and afadin were expressed from the U6 promoter in vectors also containing a CMV-GFP cassette. cDNAs were expressed in RGCs and neurons using the CAG-iGFP vector containing the chicken β-actin promoter (CAG) and an IRES-EGFP (Hand et al., 2005). Neuron-specific expression was achieved using Dcx-iGFP, which contains the doublecortin promoter and an IRES-EGFP (Franco et al., 2011).

In Utero Electroporation and Time-Lapse Imaging

Electroporations and time-lapse imaging were carried out as described (Franco et al., 2012). Static images were taken using a Nikon C2 laser-scanning confocal microscope. For quantification, the mean percentage of GFP^+ or $mCherry^+$ cells located in the CP or MZ ± SEM was determined. At least four animals from three separate experiments were analyzed for each condition. Statistical significance was evaluated by Student's t test.

In Situ Hybridizations, Immunohistochemistry, Immunoprecipitations, and Western Blots

In situ hybridizations, immunostainings, immunoprecipitations, and western blots were carried out as described (Belvindrah et al., 2007; Franco et al., 2011, 2012; Tiveron et al., 1996). Probes and antibodies are summarized in the Supplemental Experimental Procedures. Images were captured using a Nikon C2 laser-scanning confocal microscope or an Olympus AX70 microscope for bright-field images.

Cdh2 Adhesion Assays

Coverglass was coated with 0.01% poly-L-lysine (Sigma) or recombinant human CDH2-Fc (0.5 μg/ml; R&D Systems) as detailed in the Supplemental Experimental Procedures. Cortical neurons were plated in the presence or absence of recombinant reelin (0.5 μg/ml; R&D). Cells were washed, fixed, and stained with DAPI (Molecular Probes). The number of attached cells was counted in 9 fields (10× magnification) for each coverslip using ImageJ software. Five independent experiments were performed. The number of cells attached was normalized as a percentage of cells attached to poly-L-lysine. Values are mean ± SEM. Statistical significance was evaluated by Student's t test.

Coculture of Cortical Neurons with CR Cells

Embryos were electroporated with Dcx-GFP at E13.5, brains were dissected at E15.5, and primary neocortical cells were prepared as described (Belvindrah et al., 2007). E15.5 *Wnt3a-Cre;Ai9* cortices were dissociated into single-cell suspensions and enriched for CR cells by magnetic cell sorting with biotinylated anti-CD184 (Cxcr4) (BD Biosciences) and Anti-Biotin MicroBeads (Miltenyi Biotec). Equal numbers of GFP^+ neurons and $tdTomato^+$ CR cells were mixed and plated on poly-L-lysine-coated coverslips (Sigma) for 12 hr at 37°C. Coverslips were processed for immunocytochemistry and imaged on a confocal microscope. Three independent experiments were performed.

TIRF Microscopy

Coverglass was coated with 0.01% poly-L-lysine (Sigma) or recombinant human nectin1-Fc (38 μg/ml; Sino Biological) as detailed in the Supplemental Experimental Procedures. cDNAs and shRNAs were introduced into neurons by in utero electroporation at E13.5. Neurons were dissociated at E15.5, plated, cultured on substrates with or without recombinant reelin (0.5 μg/ml; R&D), washed, fixed, and immunostained. Cdh2 was detected by immunocytochemistry on a Nikon Ti Eclipse TIRF microscope. Excitation was carried out with a 488 nm Coherent laser. Images were collected with an Andor iXon DU-897 EMCCD camera. Pixel intensity of the TIRF signal was quantified using NIS-Elements software (Nikon). Three independent experiments were performed. Values are mean ± SEM. Statistical significance was evaluated by Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2013.06.040.

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(G) Reelin enhances cadherin-mediated cell adhesion. E15.5 primary neocortical cells were plated on recombinant human h-CDH2-Fc-coated coverslips in the presence or absence of recombinant reelin and allowed to adhere for 30 min prior to washing and analysis. For quantification, the percentage of cells adhering to poly-L-lysine was considered maximum adhesion, and cell attachment to CDH2-Fc is shown as the percent of maximum adhesion (mean ± SEM). *$p < 0.002$ by Student's t test. Rln, reelin. Scale bars = 100 μm. See also Figure S6.

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