

Mammalian Fat and Dachsous cadherins regulate apical membrane organization in the embryonic cerebral cortex

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Compartmentalization of the plasma membrane in a cell is fundamental for its proper functions. In this study, we present evidence that mammalian Fat4 and Dachsous1 cadherins regulate the apical plasma membrane organization in the embryonic cerebral cortex. In neural progenitor cells of the cortex, Fat4 and Dachsous1 were concentrated together in a cell–cell contact area positioned more apically than the adherens junction (AJ). These molecules interacted in a heterophilic fashion, affecting their respective protein levels. We

further found that Fat4 associated and colocalized with the Pals1 complex. Ultrastructurally, the apical junctions of the progenitor cells comprised the AJ and a stretch of plasma membrane apposition extending apically from the AJ, which positionally corresponded to the Fat4–Dachsous1-positive zone. Depletion of Fat4 or Pals1 abolished this membrane apposition. These results highlight the importance of the Fat4–Dachsous1–Pals1 complex in organizing the apical membrane architecture of neural progenitor cells.

Introduction

In the developing cerebral cortex, radial progenitor cells have an apico-basal polarity; their apical surfaces face the lateral ventricle, and their basal end feet are located on the roof of the cortex. Although several molecules have been identified as regulators of epithelial apico-basal polarity (Shin et al., 2006), precise mechanisms to regulate the apical domain organization in the cortical progenitor cells remain unknown.

Fat cadherins are featured by their huge extracellular portions having 34 cadherin repeats. In *Drosophila melanogaster*, there are two Fats, Fat and Fat-like (Tanoue and Takeichi, 2005), which are different in their functions. Fat is well known as a regulator of cell proliferation and planar cell polarity (PCP; Bryant et al., 1988; Mahoney et al., 1991; Yang et al., 2002; Ma et al., 2003; Casal et al., 2006). In the PCP regulation, Fat interacts with Dachsous, another huge cadherin, through the extracellular region of each. *dachsous* mutants show several morphogenetic defects (Clark et al., 1995), and both *fat* and *dachsous* mutants commonly exhibit defects in PCP (Yang et al., 2002; Ma et al., 2003).

In mammals, four types of Fats have been identified: Fat1, Fat2, Fat3, and Fat4 (Rock et al., 2005; Tanoue and Takeichi, 2005). Among them, Fat4 is thought to be the vertebrate counterpart of *Drosophila* Fat based on their sequence similarity. Recently, phenotypes of *Fat4*-null mice were reported; they exhibit complicated morphological abnormalities, including abnormal orientation of inner ear hair cells, cystic dilation of kidney tubules, and defects in organ shapes (Saburi et al., 2008). However, the molecular and cellular functions of Fat4 still remain to be analyzed. Regarding Dachsous, there are two types in mammals, Dachsous1 and 2. At the mRNA level, the former is broadly expressed in mouse embryos, whereas the latter exhibits a very restricted expression (Rock et al., 2005). Their functions also remain to be explored.

In this study, we analyzed the role of the mammalian Fat–Dachsous system in the embryonic cerebral cortex. Cell biological analysis showed that Fat4 and Dachsous1 heterophilically interacted with one another, as reported for their *Drosophila* counterparts. In the embryonic cortex, Fat4 and Dachsous1 proteins

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Abbreviations used in this paper: AJ, adherens junction; PCP, planar cell polarity; ZO-1, zonula occludens 1.

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accumulated at the cell–cell boundaries located apical to the adherens junction (AJ), which we defined as subapical membrane apposition. Fat4 knockdown resulted in a gross reduction in the area of this membrane domain. Furthermore, we found that the Pals1 complex was associated with the Fat4 cytoplasmic domain. Pals1 knockdown also caused the loss of the subapical membrane apposition. From these results, we propose that Fat4 and Dachsous1 regulate the subapical membrane organization in concert with the Pals1 complex in the developing cerebral cortex.

Results and discussion

Fat4 colocalizes with Dachsous1 at the apical portion of neural progenitor cells

We first generated antibodies against Fat4 and Dachsous1 and mapped their protein distributions in mouse embryos. Although there are two Dachsous in vertebrates, we examined only Dachsous1 because the expression level of Dachsous2 mRNA was in general low in our samples. Fat4 was distributed in several populations of mesenchymal and epithelial cells. In the epithelia, Fat4 was always detected in the apical portion of the cells. Dachsous1 protein expression was more restricted than that of Fat4 but tended to overlap with the Fat4 distribution (unpublished data). As both Fat4 and Dachsous1 were highly expressed in embryonic cerebral cortices, we decided to investigate the function of them in this tissue.

In the developing cortices, radial progenitor cells have an apico-basal polarity, forming the AJs at the ventricular side, which is a specific feature of neuroepithelial cells. We found that in embryonic day (E) 14.5 embryos, Fat4 and Dachsous1 were concentrated together in the apical portion of the cortex, although these molecules were distributed also in other areas of the cortex (Fig. 1, A and B). The apical signals of Fat4 and Dachsous1 overlapped with those of β -catenin, a component of the AJs, when these molecules had been double immunostained. However, high magnification views of these samples revealed that Fat4 and Dachsous1 were located more apically in the progenitor cells than β -catenin (Fig. 1 C). Horizontal views of the ventricular surface of the cortex showed that Fat4 was abundant along cell–cell boundaries (Fig. 1 D). However, contrasted with the linear and continuous distribution of β -catenin or zonula occludens 1 (ZO-1), another junctional marker, along the cell–cell boundaries, Fat4 immunostaining signals were discontinuous (Fig. 1 D). The distribution profile of Dachsous1 was similar to that of Fat4, and these two molecules colocalized well together (Fig. 1 E). The discontinuous distribution of Fat and Dachsous was also observed for the *Drosophila* homologues (Ma et al., 2003), suggesting that this unique localization pattern reflects certain conserved nature of these molecules.

Fat4 interacts with Dachsous1

To examine whether Fat4 interacts with Dachsous1 as in the case of *Drosophila*, we prepared L cells, classical cadherin-null fibroblasts, stably transfected with either one of these molecules (Fig. 2, A and B). Immunostaining observations showed that these molecules were diffusely distributed in each transfector. However, once these transfectants were mixed, both molecules

became concentrated together at their heterotypic boundaries along fine filopodial processes attaching onto the apical surface of neighboring cells (Fig. 2 A). These observations suggest that Fat4 interacts with Dachsous1 heterophilically. To further confirm this notion, we conducted cell aggregation assays. We dissociated Fat4 or Dachsous1 transfectants into single cells and allowed them to reaggregate in suspension cultures. However, these cells scarcely aggregated. When these transfectants had been mixed, they aggregated efficiently (Fig. 2 C), although their aggregates were less compacted as compared with those formed by E-cadherin transfectants. In those aggregates, Fat4 and Dachsous1 were sharply accumulated only at heterotypic cell–cell boundaries (Fig. 2 D), supporting the idea of their heterophilic interactions. We also examined whether they could interact in a cis-fashion by preparing cells expressing the two molecules together, but we obtained no evidence for supporting this possibility (Fig. S1).

Fat4 and Dachsous1 reciprocally regulate their protein level

In *Drosophila*, in the absence of *fat*, the level of Dachsous protein is down-regulated; conversely, without *dachsous*, the Fat protein level is up-regulated (Ma et al., 2003). To test whether such a relationship also exists for the mammalian homologues, we depleted these molecules in the embryonic cortices by electroporating siRNAs targeting their transcripts. Throughout this study, we routinely examined two different control siRNAs and two different siRNAs for a given gene to verify specific results obtained by using the siRNAs, and the results were confirmed by at least two independent experiments. We conducted the electroporation at E13.5 and examined the embryos at E15.5 or later and found specific knockdown of the expression of the targeted proteins at the electroporated regions of the cortex, as monitored by coelectroporation of GFP (Fig. 3 A and Fig. S2). When Fat4 was depleted, the staining signal of Dachsous1 was significantly attenuated in the apical portion of the cortex (Fig. 3 B and Fig. S2), suggesting that Fat4 has the ability to stabilize Dachsous1. Conversely, when Dachsous1 was knocked down, the staining signal of Fat4 was slightly up-regulated (Fig. 3 C and Fig. S2), suggesting that Dachsous1 negatively regulates Fat4.

Fat4 interacts with the Pals complex

To determine whether Fat and Dachsous are associated with any signaling systems, we sought molecules that interact with Fat4. By GST pull-down assays using a portion of the cytoplasmic region of Fat4 combined with liquid chromatography mass spectrometry/mass spectrometry analysis of the pulled-down materials, we identified MUPP1 as a protein that specifically coprecipitated with Fat4. MUPP1 is a parologue of Patj, which regulates apico-basal polarity and apical membrane organization in cultured cells as well as in *Drosophila* (Bachmann et al., 2001; Shin et al., 2005; Richard et al., 2006; Sugihara-Mizuno et al., 2007). The interaction between Fat4 and MUPP1 was confirmed by detecting MUPP1 from the pulled-down materials with anti-MUPP1 antibodies (Fig. 4, A and B). Using several deletion forms of the Fat4 cytoplasmic domain, we found the region of aa 4,708–4,797 was necessary and sufficient for its efficient binding to MUPP1.

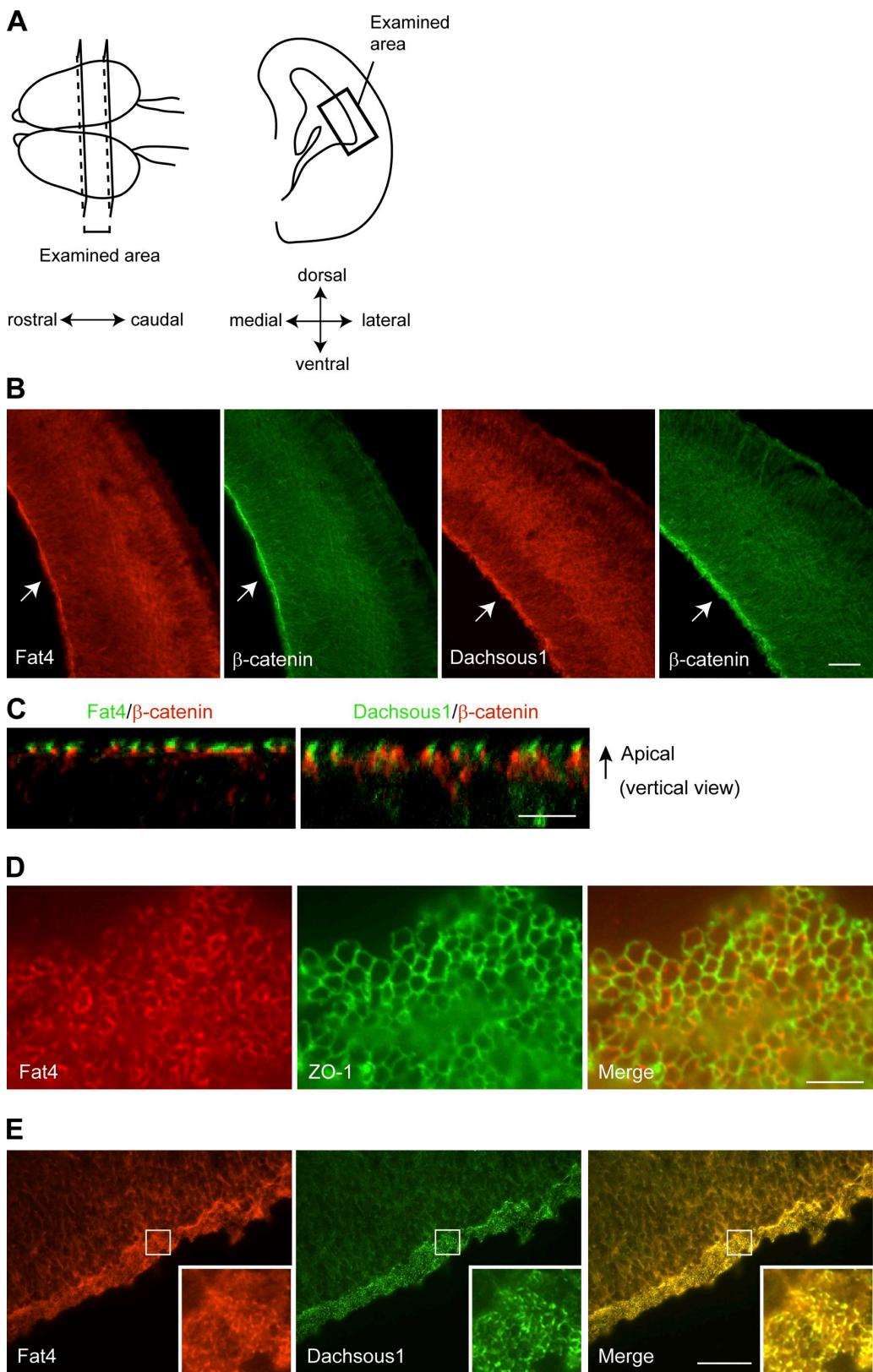


Figure 1. Apical localization of Fat4 and Dachsous1 in embryonic cerebral cortices. (A) The regions of the embryonic cortex examined are shown. (B) Coronal sections of an E14.5 cerebral cortex doubly immunostained for Fat4 or Dachsous1 and β -catenin. Arrows indicate the ventricular (apical) portion of the cortex, which is rich in these molecules. (C) Vertical images of the apical portion at a high magnification. Fat4 and Dachsous1 are localized more apically than β -catenin, an AJ marker. (D) Horizontal images of Fat4 distribution at the apical portion of an E14.5 cerebral cortex. The ventricular surface of a whole-mount cortex double immunostained for Fat4 and ZO-1 was photographed. Fat4 shows a discontinuous localization contrasted with the continuous signals of ZO-1. (E) A coronal section of an E14.5 cerebral cortex double immunostained for Fat4 and Dachsous1. An obliquely sectioned portion of the ventricular zone is enlarged in the inserts. Fat4 is colocalized with Dachsous1. Insets show a higher magnification view of the indicated regions. Bars: (B) 100 μ m; (C and D) 5 μ m; (E) 50 μ m.

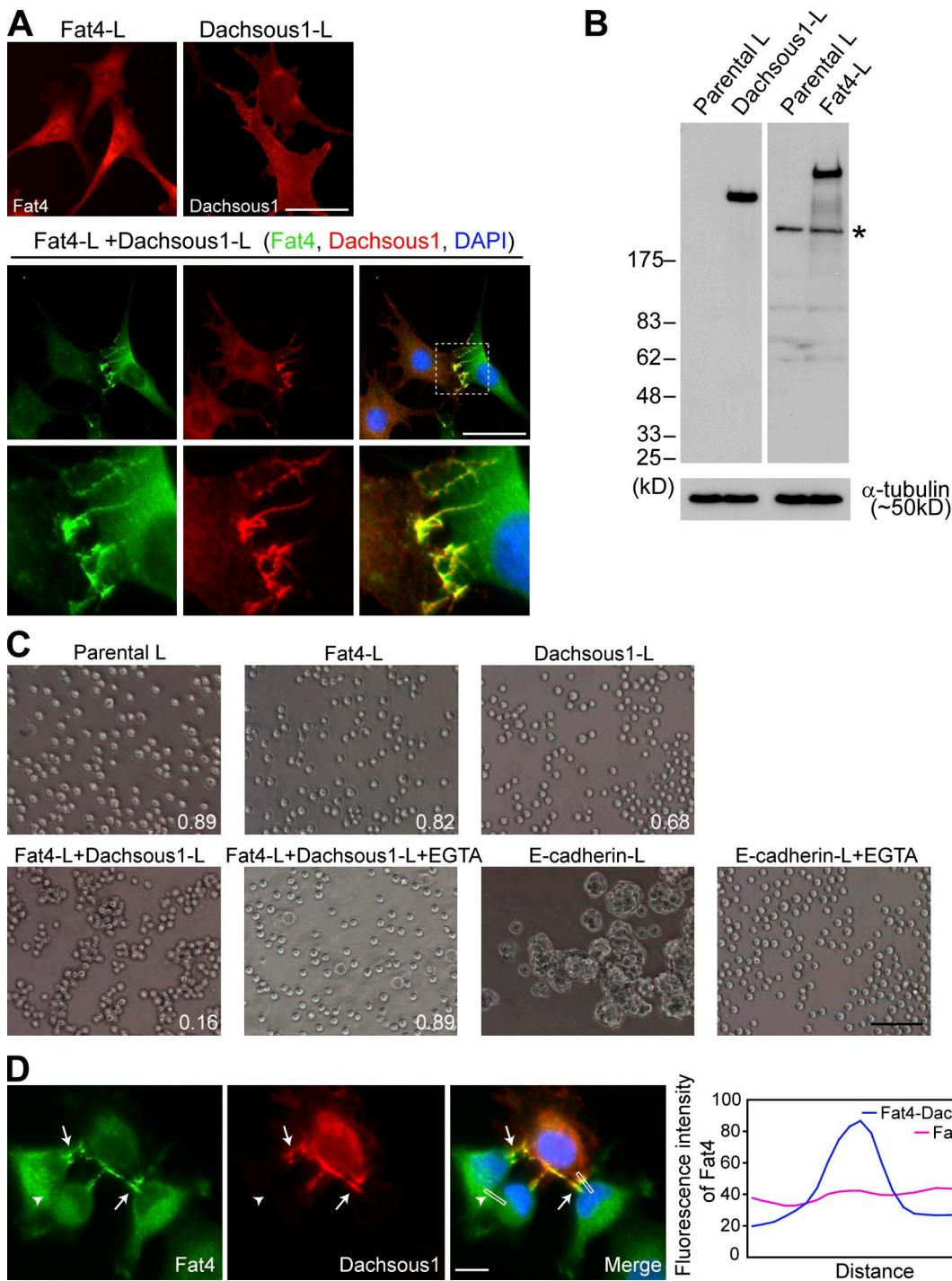


Figure 2. Interactions between Fat4 and Dachsous1. (A) L cells transfected with Fat4 (Fat4-L) or Dachsous1 (Dachsous1-L) cDNA and their mixed cultures (green, Fat4; red, Dachsous1) are shown. The boxed area is enlarged in the lower photos. Fat4 and Dachsous1 are concentrated and colocalized together along filopodial processes only at heterotypic cell-cell interfaces. (B) Western blots to detect Dachsous1 (left) or Fat4 (right) in the L cell transfectants. The asterisk indicates a nonspecific band. (C) Cell aggregation assays using Fat4-L and Dachsous1-L cells. Cells were dissociated with 4 mM EDTA and allowed to reaggregate for 3 h. The extent of aggregation was represented by an index of N/N_0 , where N is the number of cell clusters at 3 h, and N_0 is the number of total cells. Most vigorous aggregation was observed in the mixture of the two transfectants, and this aggregation was inhibited with 4 mM EGTA. As a positive control, the aggregation of E-cadherin-transfected L cells in the presence or absence of EGTA is also shown. (D) Cell aggregates formed in the aforementioned experiments were allowed to adhere to the dish and were double immunostained for Fat4 and Dachsous1. These molecules accumulate only at their heterotypic interfaces (arrows). The graph shows the fluorescence intensity of Fat4 signals scanned along the white rectangles at a homotypic (arrowheads) or heterotypic interface indicated in the merged image. Bars: (A) 50 μ m; (C) 200 μ m; (D) 20 μ m.

Intriguingly, the aa sequence of this region was considerably conserved in *Drosophila* Fat (Fig. 4 C). We also confirmed that endogenous MUPP1 could be coimmunoprecipitated with endogenous

Fat4 from lysates of E14.5 cerebral cortices and vice versa (Fig. 4 D). As previous studies showed that MUPP1 forms a complex with Pals1, a vertebrate counterpart of *Drosophila* Stardust

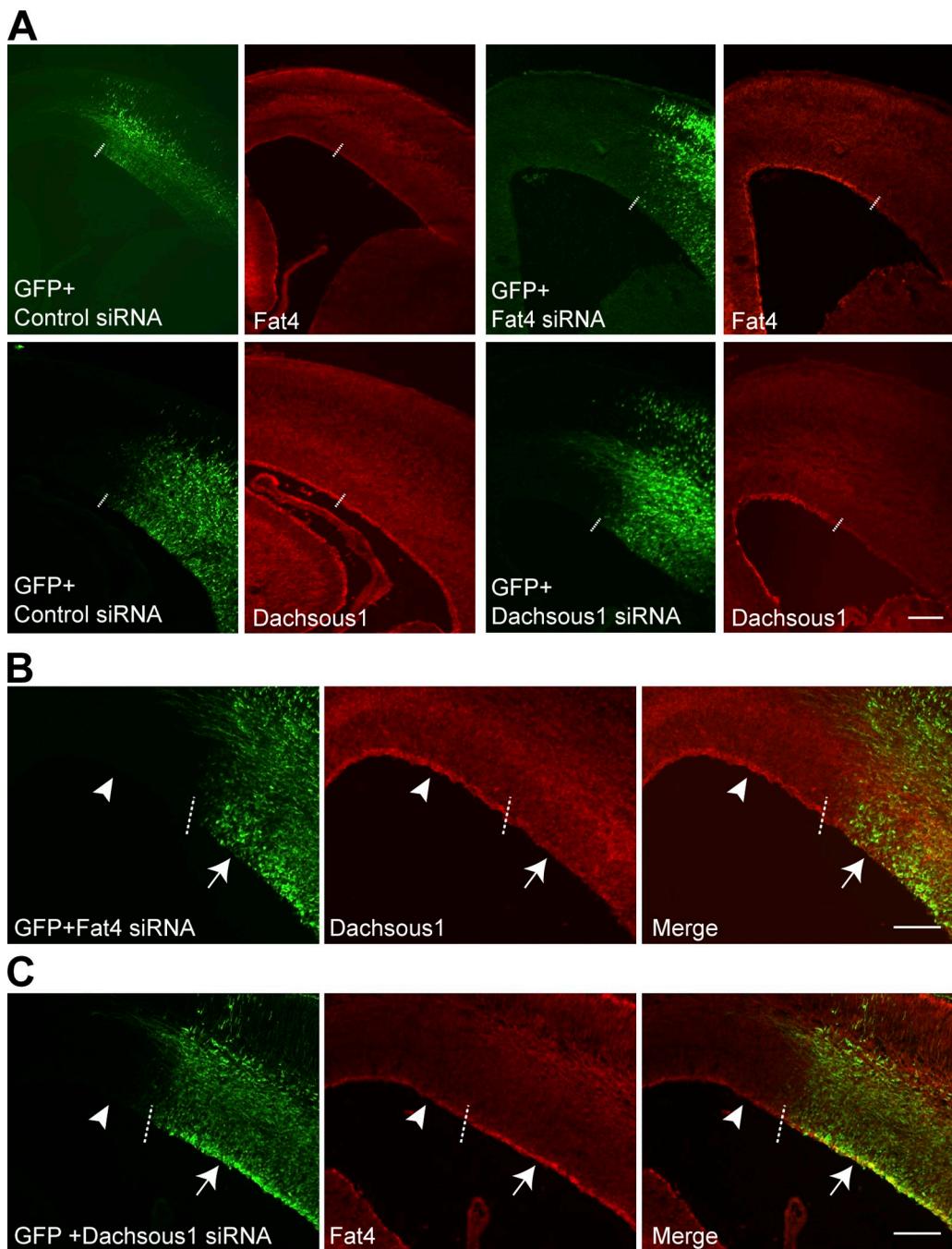


Figure 3. Fat4 and Dachsous1 reciprocally regulate protein levels. (A) *In vivo* knockdown of Fat4 and Dachsous1. GFP expression vector and siRNA were coelectroporated into E13.5 cerebral cortices *in utero* and immunostained at E15.5. Delivering Fat4 or Dachsous1 siRNAs reduced the apical expression of the respective molecules at the GFP-positive area, whereas control siRNAs did not. (B) Fat4 knockdown down-regulates the level of Dachsous1 protein. Apical expression of Dachsous1 is reduced in the GFP-positive area where Fat4 is knocked down (arrows) compared with that in the GFP-negative area (arrowheads). (C) Dachsous1 knockdown up-regulates Fat4 expression. Apical expression of Fat4 is up-regulated in the GFP-positive area where Dachsous1 is knocked down (arrows) compared with that in the GFP-negative area (arrowheads). See Fig. S2 for quantification of the data in B and C. Dashed lines indicate the boundaries between the GFP-positive and -negative areas. Bars, 200 μ m.

(van de Pavert et al., 2004; Sugihara-Mizuno et al., 2007), we examined whether this interaction also occurred in the cortex, and as a result, we indeed detected Pals1 in the MUPP1 immunoprecipitates prepared from cortical lysates (Fig. 4 D, right). Likewise, we found that the Fat4 immunoprecipitates contained Pals1 (Fig. 4 D, left). These results suggest that Fat4, MUPP1, and Pals1 form a multimolecular complex.

Next, we examined the localization of MUPP1 and Pals1 in the embryonic cerebral cortex. MUPP1 was exclusively localized at the apical portion of the cortex throughout development, well colocalizing with the apical Fat4 signals (Fig. 4, E and F). Pals1 exhibited a similar localization pattern as MUPP1, colocalizing together (Fig. 4 G). These results agree with the idea that Fat4, MUPP1, and Pals1 formed a complex at

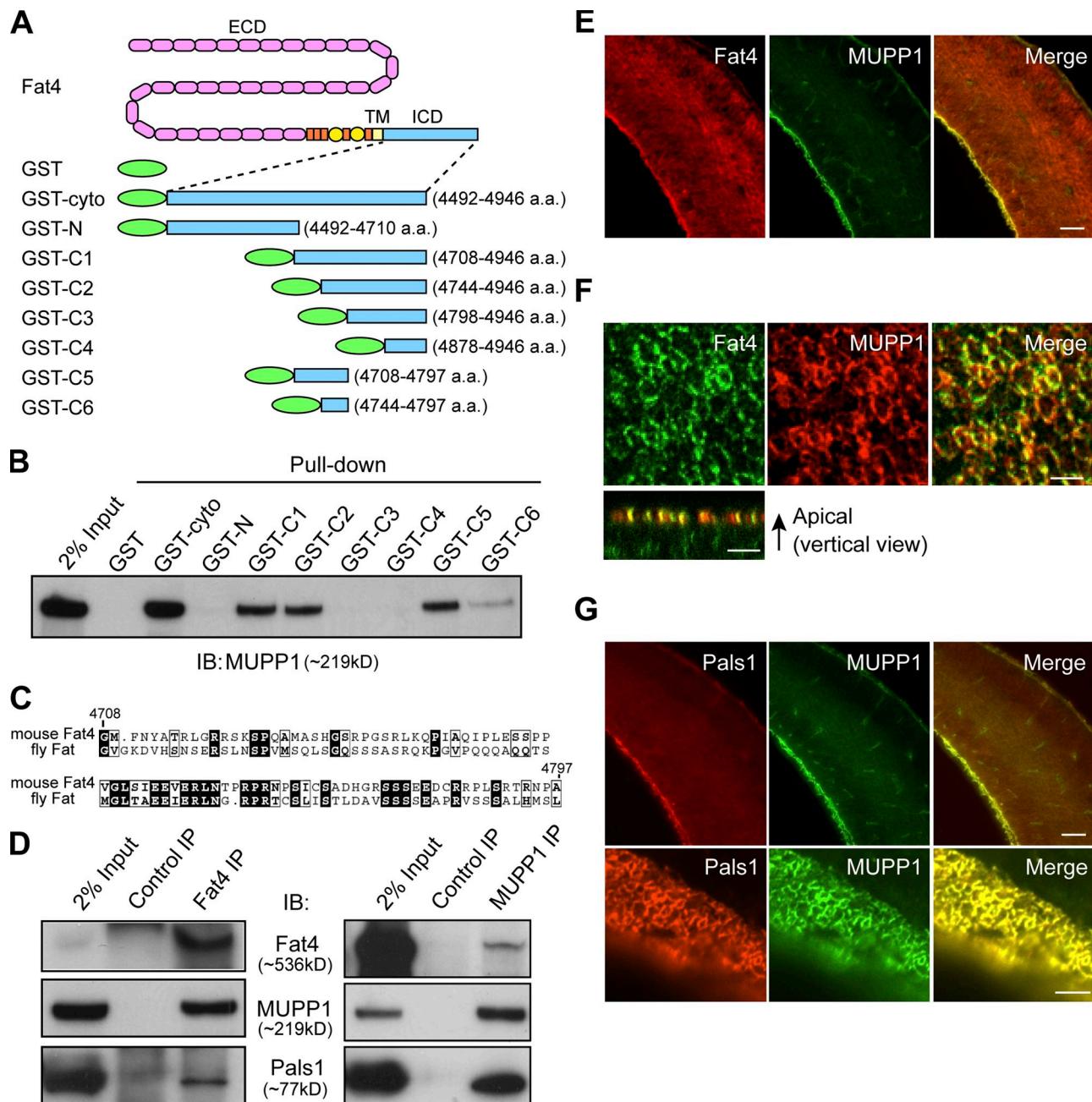


Figure 4. Fat4 interacts with MUPP1 and Pals1. (A) Fat4 deletion mutants. GST-cyto encodes the full-length cytoplasmic domain. The numbers represent aa sequences of mouse Fat4. ECD, extracellular cadherin domain; TM, transmembrane; ICD, intracellular domain. (B) Interaction of Fat4 mutants with MUPP1. GST pull-down assays were performed with GST or GST-Fat4 mutants. Endogenous MUPP1 was precipitated with GST-cyto or smaller fragments containing the C5-C6 portions. (C) aa sequences at 4,708–4,797 of mouse Fat4 and *Drosophila* Fat. Black or white boxed aa indicate identical or homologous ones, respectively. (D) Interaction between Fat4, MUPP1, and Pals1 detected by coimmunoprecipitation assays. Fat4 (left) or MUPP1 (right) were immunoprecipitated from a lysate of E14.5 brain. Immunoblotting (IB) was performed with the indicated antibodies. Rabbit or mouse IgG was used as a control. (E) E14.5 cerebral cortex doubly immunostained for Fat4 and MUPP1. MUPP1 are abundant in the apical portion of the cortex, colocalizing with Fat4. (F) Horizontal images of Fat4 and MUPP1 localization, as prepared for Fig. 1 C. A vertical view is also shown. Fat4 is well colocalized with MUPP1. (G) E14.5 cerebral cortex doubly immunostained for MUPP1 and Pals1. The bottom panels were prepared as explained in Fig. 1 E. These two proteins fully colocalize with each other. IP, immunoprecipitation. Bars: (E and G, top) 100 μm; (F) 5 μm; (G, bottom) 10 μm.

the apical portion of the cerebral cortex. However, depletion of Fat4 and Dachsous1 did not affect the MUPP1 and Pals1 expression and conversely, that of MUPP1 and Pals1 had no effects on the Fat4 and Dachsous1 distributions (unpublished data), suggesting that their apical recruitment depended on separate mechanisms or different mediators.

Fat4 and Pals1 regulate apical membrane organization in the embryonic cerebral cortex

Because the Stardust complex has been reported to regulate the apical membrane organization in *Drosophila* (Pellikka et al., 2002; Hong et al., 2003; Richard et al., 2006), we asked whether

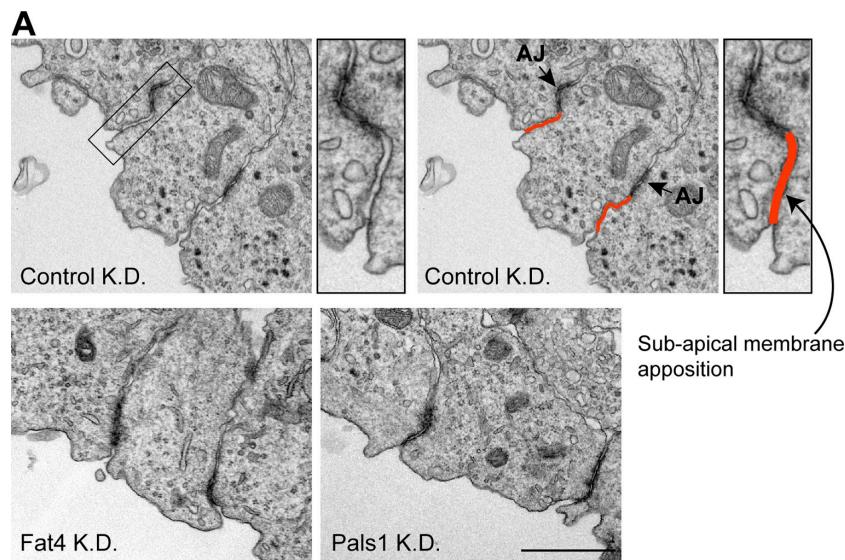
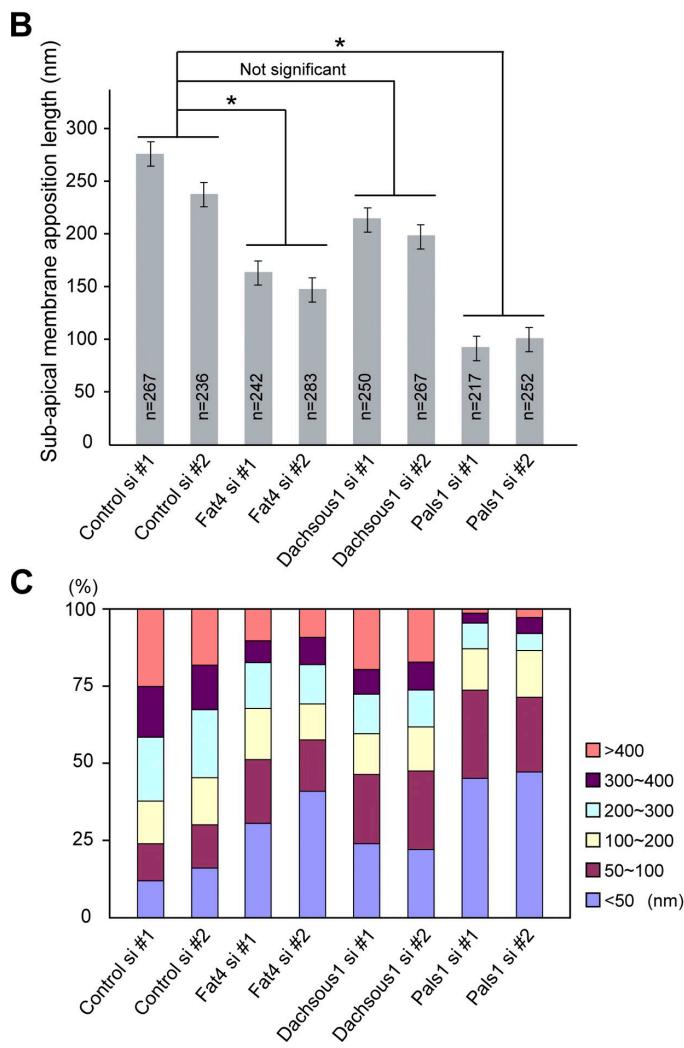


Figure 5. Fat4 and Pals1 regulate the subapical membrane apposition. (A) Representative transmission electron micrographs of the apical portion of the control, Fat4, or Pals1-depleted (KD) cortex. The magnified image of the boxed region is shown in the right (control KD). The subapical membrane apposition is marked in red at the right (identical to the left panels) of the control sample. The AJ can be recognized as electron-dense junctions. Note that the subapical membrane apposition is reduced or absent in the Pals1-depleted samples. Bar, 1 μ m. (B and C) Quantification of the length of the subapical membrane apposition. The results obtained with two siRNA constructs from two independent experiments are shown for each molecule. The stacked bar chart shows the relative frequencies of the lengths indicated. The reduction of the length of the subapical membrane apposition by Fat4 or Pals1 loss is statistically significant. Mean \pm SEM; *, $P < 0.001$ by Student's *t* test.



Fat4 and Dachsous1 had similar functions. We prepared ultra-thin sections for electron microscopy of E15.5 cortices, which had been electroporated with siRNAs targeting Fat4, Dachsous1, or Pals1 (Fig. S3) 2 d before the examination. Analysis of these sections showed that depletion of Fat4 and Pals1 affected

the apical membrane architecture. In the apical portions of untreated cortices, we could identify the AJs by their characteristic electron-dense appearance. Above the AJs, the plasma membranes were apposed to each other, showing a gap of 20–50-nm distances (Fig. 5 A, red), as observed by others (Ho et al., 2000).

This gap appeared to contain amorphous electron-dense materials, but the cytoplasmic sides exhibited no specific structures. We designated this specific membrane region the subapical membrane apposition. We first confirmed that control RNAi treatments had no particular effects on these membrane organizations. However, when Fat4 had been depleted, the area of the subapical membrane apposition became drastically reduced (Fig. 5, A–C). Removal of Pals1 gave similar effects. These results indicate that the apical membranes of neural progenitor cells require the Fat4–Pals1 complex to maintain the normal architecture at the subapical zone. However, depletion of Dachsous1 had no effects on the apical membrane morphology. Presumably, the action of Fat4 on the apical membrane organization was saturated, and therefore, removal of Dachsous1, which functions as a negative regulator of Fat4, could not further modify the apical structures. We did not examine the effect of MUPP1 removal because of the presence of its parologue, Patj, in the cortex (Srinivasan et al., 2008).

The findings presented in this study disclosed an unexpected role of the mammalian Fat–Dachsous system in the plasma membrane organization in the embryonic cerebral cortex. The AJ is known as the major cell junctional structure observed in the apical portions of neural progenitor cells (Ho et al., 2000; Lien et al., 2006; Kadokawa et al., 2007). We found that Fat4 and Dachsous1 were located more apical to the AJ and that the plasma membranes at the corresponding region showed a simple apposition, which we defined as the subapical membrane apposition. Such apically extended membrane appositions have not been described for general epithelial cells, and thus, these junctions might have uniquely developed for specific cell types, including neural progenitor cells. Depletion of Fat4 disrupted the subapical membrane apposition, indicating that it plays a key role in the maintenance of this specific structure.

We found that Fat4 and Dachsous1 could interact in a heterophilic fashion and regulated the protein level of the partner in a way similar to that found in *Drosophila* (Ma et al., 2003). Then, the question arises as to how the Fat4–Dachsous1 system regulates the apical membrane architecture. A simple answer would be that they function as heterophilic adhesion molecules to generate and maintain the subapical membrane apposition and that the loss of Fat4 caused disruption of this structure. However, the actual mechanism would not be so simple. First of all, Dachsous1 depletion did not affect the subapical membrane apposition. Furthermore, although depletion of Pals1 disrupted the subapical membrane apposition, it did not alter the apical localization of Fat4 and Dachsous1. These findings indicate that the adhesive interactions between Fat4 and Dachsous1 alone are not sufficient for them to maintain the subapical membrane apposition.

Stardust, the *Drosophila* counterpart of Pals1, and Crumbs are well known to regulate the apical membrane organization in *Drosophila* retinal cells (Pellikka et al., 2002). It is attractive to hypothesize that Fat may cooperate with these apical regulators and in turn takes part in the apical membrane organization. In such a case, Fat4 may function as a signaling component for apical membrane regulation rather than as a mechanical adhesion molecule. Because Crumbs also forms a complex with MUPP1/PatJ and Pals1 (van de Pavert et al., 2004), it is important to

determine how the Fat and Crumbs systems share these binding partners for their potential cooperation in future studies.

Notably, despite the broad distributions of Fat4 and Dachsous1 in the cortex, Pals1 and MUPP1 appeared to be restricted to the apical zone. We therefore can speculate that Fat4 and Dachsous1 may play pleiotropic morphogenetic roles, which could vary with the signaling systems present in the cells, and our study likely has deciphered one of these complex functions.

Materials and methods

Cell culture and immunostaining

Cells were cultured as described previously (Tanoue and Takeichi, 2004). For isolating stable cDNA transfectants, 400 µg/ml G418 (Invitrogen) or 250 µg/ml hygromycin B (Invitrogen) were used. Immunostaining was performed as described previously (Tanoue and Takeichi, 2004). Cell aggregation assays were performed as described previously (Nagafuchi et al., 1987).

Antibodies

Rabbit polyclonal antibodies against Fat4 and Dachsous1 and rat polyclonal antibody specific for Fat4 were raised against the cytoplasmic region of mouse Fat4 or Dachsous1. The specificities of these antibodies were confirmed by the observation that their immunostaining signals disappeared after RNAi-mediated depletion of each molecule as well as by transfection experiments (Fig. 2 B). The following antibodies were also used: mouse monoclonal antibodies against β-catenin (5H10; provided by M.J. Wheelock, University of Nebraska Medical Center, Omaha, NE), MUPP1 (Transduction), ZO-1 (Invitrogen), Flag (Sigma-Aldrich), rat monoclonal antibody against GFP (Nacalai), rabbit polyclonal antibodies against Pals1 (Millipore; provided by M. Adachi, Kyoto University, Kyoto, Japan), and GFP (MBL International). Primary antibodies were visualized with goat fluorescein-conjugated secondary antibodies. The fluorescences used were Alexa Fluor 488, 555, 568 (Invitrogen), and Cy3 (Millipore).

Plasmid construction and protein expression

Full-length rat Fat4 and Dachsous1 cDNAs were obtained by PCR and cloned into pCA-IRES-Neo or pCA-IRES-Hygro vector. N terminus-truncated forms of Fat4 and Dachsous1 were constructed by using pCA-Sig-IRES vector (Tanoue and Takeichi, 2004). For production of a GST-fused cytoplasmic region and deletion mutants of Fat4 in *Escherichia coli*, the pGEX-2T vector (GE Healthcare) was used.

In utero electroporation

Plasmids were prepared by using an EndoFree plasmid kit (QIAGEN). For the expression of EGFP, the pCA-EGFP vector was used. For DNA electroporation, DNA vectors were diluted in PBS to the final concentration of 0.5 mg/ml, whereas Stealth RNA (Invitrogen) was diluted in saline to the final concentration of 50 µM. DNA and/or Stealth siRNAs were microinjected into the lateral ventricle of E13.5 mice. The electroporation was performed by using a square wave electroporator (CUY21Edit; Nepagene). Five electrical pulses (40 V; 50-ms duration at 950-ms intervals) were delivered. At 2 d after the electroporation, the embryos were examined. We routinely examined the lateral portions of the cortex at its middle region (Fig. 1 A). Detailed procedures were described previously (Saito, 2006). The following Stealth siRNAs were used for the RNAi experiments: mouse Fat4-1, 5'-CAUGAUUCUUUCACGUGUCAGAAA-3'; mouse Fat4-2, 5'-CCG-AUGCAGAUGACGAAGAUAAUUA-3'; mouse Dachsous1-1, 5'-CGAUA-GCCAGUAGCUCUUUACAGAA-3'; mouse Dachsous1-2, 5'-GCGGAUG-GAUUGAGUGACAGCUCU-3'; mouse Pals1-1, 5'-CGUUGAAGACU-UGUUCUCUUCACUUU-3'; mouse Pals1-2, 5'-GGACAAGAACUAACUG-CCUUGUUGA-3'; control-1, 5'-UUUCUCGAGUGCACAAGUAAGA-AUG-3'; and control-2, 5'-UAAUAUAUCUUUGCCUUAUCCGCGG-3'.

Immunohistochemistry

Brains were fixed in 1% PFA in PBS for 1 or 2 h at 4°C. Tissue sectioning and immunohistochemistry were performed by standard procedures. For whole-mount preparations, brains of E14.5 mice were fixed in 1% PFA in PBS for 1 h at 4°C, made permeable in 0.2% Triton X-100 in PBS for 10 min at RT, and blocked with 3% BSA in PBS overnight at 4°C. The samples were incubated with primary antibodies in 3% BSA in PBS for 1 h at RT followed by extensive washing with PBS. After incubation with secondary

antibodies in 3% BSA in PBS for 1 h at RT, tissues were mounted in glycerol gelatin (Sigma-Aldrich).

GST pull-down, immunoprecipitation, and Western blotting

E14.5 mouse brains were homogenized in 20 mM Tris-HCl, pH 7.4, containing 1 mM MgCl₂, 2 mM EGTA, 150 mM NaCl, and protease inhibitor cocktail (Roche). Detailed procedures were described previously (Tanoue and Takeichi, 2004).

Photographic imaging

Microscopic images were obtained at RT by using a fluorescence microscope (Axioplan2; Carl Zeiss, Inc.) connected with a charge-coupled device camera (AxioCamHRc; Carl Zeiss, Inc.) through Plan Apochromat 63 \times 1.40 NA objectives using AxioVision software or a laser-scanning confocal microscope (LSM510; Carl Zeiss, Inc.) mounted on an inverted microscope (Axiovert100M; Carl Zeiss, Inc.) using Plan Apochromat 63 \times 1.40 NA or Plan Neofluar 40 \times 1.30 NA objectives using LSM510 software. Images were analyzed with the same software and with Photoshop software (Adobe).

Electron microscopy

The cerebral cortex was fixed overnight in 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at RT. After the fixation, the cortex was coronally sectioned at 50 μ m with a vibratome (Ted Pella, Inc.). The efficiency of the electroporation was checked by the intensity of the fluorescence of EGFP. The samples were next washed with 0.1 M cacodylate buffer and postfixed in 1% OsO₄ in 0.1 M cacodylate buffer on ice for 2 h. Thereafter, the samples were washed with distilled water, stained overnight with 0.5% aqueous uranyl acetate at RT, dehydrated with ethanol, and embedded in Polybed 812 (Polyscience). Ultrathin sections were cut with a diamond knife, double stained with uranyl acetate and lead citrate, and observed at 80 kV accelerating voltage using a transmission electron microscope (JEM-1010; JEOL) equipped with a charge-coupled device camera (2k; Hamamatsu Photonics). Images were processed with Photoshop software.

Online supplemental material

Fig. S1 shows a test for cis-interactions of Fat4 and Dachsous1. Fig. S2 shows the effects of different siRNAs on the level of Fat4 and Dachsous1. Fig. S3 shows the effects of Pals1 siRNAs on its expression. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200811030/DC1>.

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