FAK-mediated extracellular signals are essential for interkinetic nuclear migration and planar divisions in the neuroepithelium

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Summary

During the development of the vertebrate nervous system, mitosis of neural progenitor cells takes place near the lumen, the apical side of the neural tube, through a characteristic movement of nuclei known as interkinetic nuclear migration (INM). Furthermore, during the proliferative period, neural progenitor cells exhibit planar cell divisions to produce equivalent daughter cells. Here, we examine the potential role of extracellular signals in INM and planar divisions using the medaka mutant *tacobo* (*tab*). This *tab* mutant shows pleiotropic phenotypes, including neurogenesis, and positional cloning identified *tab* as *laminin γ1* (*lamc1*), providing a unique framework to study the role of extracelluar signals in neurogenesis. In *tab* mutant neural tubes, a number of nuclei exhibit abnormal patterns of migration leading to basally mislocalized mitosis. Furthermore, the orientation of cell division near the apical surface is randomized. Probably because of these defects, neurogenesis is accelerated in the *tab* neural tube. Detailed analyses demonstrate that extracellular signals mediated by the FAK pathway regulate INM and planar divisions in the neuroepithelium, possibly through interaction with the intracellular dynein-motor system.

Key words: Interkinetic nuclear migration, Laminin, Medaka, Neuroepithelial cells, Planar divisions

Introduction

During the morphogenesis of the neural tube, neural progenitors continuously divide, and post mitotic neurons then migrate to form complex neural networks. It is widely conserved among vertebrates that this mitotic process takes place near the lumen, the apical side of the neural tube (Gotz and Huttner, 2005). When viewed at the cellular level, this apically localized mitosis is the region of output for the cell-cycle-dependent oscillation of the nuclei within the neuroepithelium. Mitotic (M phase) nuclei are located in close proximity to the apical surfaces, whereas nuclei undergoing DNA synthesis (S phase) are displaced more basally. This characteristic movement of nuclei is known as interkinetic nuclear migration (INM) (Fujita, 1964; Sauer, 1935) and is thought to be important for maintaining a neural progenitor pool during neurogenesis (Baye and Link, 2007; Del Bene et al., 2008; Murciano et al., 2002; Xie et al., 2007). Furthermore, planar cell divisions near the apical surface are thought to be important to maintain the progenitor pool (Gotz and Huttner, 2005). Indeed, recent works suggest that most divisions during both proliferative and neurogenic stages are planar (that is, parallel to the apical surface), whereas vertical or oblique divisions are rarely seen, and daughter cells that inherit both the apical and basal components are suggested to remain in the progenitor pool (Konno et al., 2008). Although INM and planar cell divisions have been regarded as hallmarks of vertebrate neural progenitors, their cellular and molecular mechanisms are just beginning to be elucidated. Previous studies have suggested the involvement of several intracellular components such as the actin and microtubule cytoskeletons, molecules involved in adherens junctions, polarity complex and the centrosome (Cappello et al., 2006; Imai et al., 2006; Lien et al., 2006; Murciano et al., 2002; Siller and Doe, 2009; Xie et al., 2007). However, it remains unclear whether extracellular molecules, such as components of the extracellular matrix (ECM), have any role in INM and mitotic orientation of neural progenitors, and if so, how they interact with the intracellular machinery that drives this process. Indeed, the ECM has long been shown to participate in neurogenesis (see below), but there are no reports of vertebrate ECM mutants with defective INM and mitotic orientation. In this context, the medaka mutant *tacobo* (*tab*) used in the present study provides a unique framework. The medaka fish, *Oryzias latipes*, is an emerging vertebrate model and now has a high quality draft genome and a

number of unique mutants (Furutani-Seiki et al., 2004; Kasahara et al., 2007; Wittbrodt et al., 2002). Our recent mutagenesis screen in medaka identified *tab* as a mutant showing pleiotropic phenotypes including impaired INM in the neuroepithelium. Here, we report that *tab* encodes laminin γ1 (Lamc1) and that a mutation in this gene causes the impaired INM and mitotic orientation.

Laminins are heterotrimeric glycoprotein complexes consisting of individual α-, β- and γ-chain subunits, and are major structural components of basement membranes (BMs). To date, 15 mammalian laminin complexes have been described comprising five α-chains, three β-chains and three γ-chains. Among these different chain types, laminin γ1 is the most commonly found, contributing to ten laminins including laminin 1, which is essential for early embryogenesis (Miner and Yurchenco, 2004). In addition to their structural roles, the laminins act as signaling molecules through their cell surface receptors, which include integrins (Yurchenco et al., 2004). A variety of laminin functions have been elucidated through genetic studies in humans, mice and zebrafish, and include cell migration, proliferation, differentiation and cell polarity (Miner and Yurchenco, 2004; O'Brien et al., 2001). As an example, recent work in both mouse and human has revealed a role of laminin 5 (α3β3γ2) in the epidermis; mutations in one of the chains cause defective skin morphogenesis in mouse or Herlitz junctional epidermolysis bullosa in human (Aberdam et al., 1994; Ryan et al., 1999). Furthermore, studies of zebrafish laminin mutants have highlighted the broader role of these molecules in organogenesis of such structures as the notochord and blood vessels (Parsons et al., 2002; Pollard et al., 2006).

In the case of neurogenesis, the proper functioning of the laminins was found to be a prerequisite for the formation of the cortical BMs, defects in which cause impaired cortical histogenesis whereby cortical neurons protrude into the subarachnoid (Halfter et al., 2002). Genetic analyses of laminins is sometimes hampered by their requirement in early developmental processes and their functional redundancy; for example, a lack of laminin α1, β1 and γ1 in mice leads to early post implantation lethality (Alpy et al., 2005; Miner et al., 2004; Smyth et al., 1999). Moreover, compensation of one chain in the absence of another has been frequently reported (Miner et al., 2004; Yang et al., 2005). As a likely result of these difficulties, the role of laminins in early neurogenesis, particularly in INM and mitotic orientation, has not yet been fully addressed (Halfter et al., 2002; Haubst et al., 2006).

In our current study, we use medaka wild-type and *tab* mutant embryos for analysis of neurogenesis. We focus on the early stage neural tube in which mitotically active neuroepithelial cells exhibit typical INM: they maintain cytoplasmic connections to both the apical (ventricular) and basal surfaces, and their nuclei migrate over the entire thickness of the neuroepithelium. Taking advantage of the transparency of the medaka fish embryos and simple structure of their early neural tubes, a live-imaging technique was successfully applied to record the behavior of individual nuclei in both the wild-type and *tab* neuroepithelium. Our genetic and imaging analyses clearly show that INM and planar cell divisions are dependent upon FAK-mediated signals downstream of laminins. Furthermore, we reveal a genetic interaction between FAK-mediated signals and the dynein system for the regulation of INM and mitotic orientation. To our knowledge, this is the first report to genetically demonstrate the important role of ECM molecules in these processes, linking extracellular signals with the intracellular machinery.

Results

The *tacobo* locus in medaka encodes laminin γ1

The *tacobo* (*tab*) medaka mutant was recessive and isolated in our screening of ENU-induced mutant fish with disrupted axis elongation and neuronal development (see below) (Ishikawa, 1996). We undertook a positional cloning approach to identify the *tab* gene. Mapping analysis with EST markers indicated that *tab* was located close to the Ola2909h marker (4/1504 recombinants/meiosis) on LG17. Chromosome walking using BAC clones was then carried out to narrow down the responsible region. We found that the gene encoding Lamc1 mapped close to the responsible region and we therefore tested whether *tab* encodes Lamc1. A comparison of genomic *laminin γ1* (*lamc1*) sequences between the wild-type (wt) and *tab* mutant revealed a T to A substitution in the presumptive splice-donor site (the 20th intron) in the mutant (Fig. 1A). This mutation produces three kinds of abnormal splicing products which were all detected in *tab* embryos by RT-PCR, in addition to two other unexpected fragments (Fig. 1B). The aberrant transcript gave rise to a presumptive truncated form of Lamc1 that lacks most of the coiled-coil domain within the C-terminus (Fig. 1B). Because the coiled-coil domain in the laminin chains is required for the formation of heterotrimers, the deduced truncated protein was expected to be nonfunctional (Beck et al., 1993). In addition, *lamc1* is expressed almost ubiquitously during neurulation and neurogenesis with stronger expression in the midbrain-hindbrain boundary region, which is largely reduced in the *tab* mutant (data not shown). Furthermore, injection of two individual MOs specific for *lamc1* (designed to target the first methionine and the splicing donor site that is affected in *tab*) phenocopied the *tab* phenotype (Fig. 1C). We thus concluded that the *tab* locus encodes the medaka laminin γ1 chain.

The *tab* mutation causes pleiotropic phenotypes

Tab is a recessive lethal mutant showing pleiotropic morphological defects such as small eye, a shortened body axis, an enlarged midbrain-hindbrain boundary, defective notochord vacuolation, loss of jaw and malformed pectoral fins (Fig. 1C,D). Blood vessel formation is also severely affected in the *tab* mutant as seen in the zebrafish *lamc1* mutant *sleepy* (*slp*) (Fig. 1C). Furthermore, histological analysis revealed that *tab* develops defects in retinal lamination (Fig. 1D). These phenotypes are again reminiscent of the zebrafish laminin mutants, *bashful* (*bal*) (*lama1*), *grumpy* (*gup*) (*lamb1*) and *slp* (*lamc1*) (Parsons et al., 2002; Pollard et al., 2006), but the overall phenotype of *tab* appears to be more severe than any of the zebrafish single laminin mutants.

tab shows an abnormal mitotic pattern in the neural tube

Since early neural tube patterning appears normal in the *tab* mutant as indicated by the normal expression of regional markers (*wnt1*, *shh* and *krox20*; supplementary material Fig. S1), the aberrant morphogenesis that manifests in the neural tube could therefore be the result of abnormal cell proliferation at earlier stages. We thus examined the frequency and distribution pattern of neuroepithelial cells in late G2-M phase at the 10-somite stage using phosphorylated histone H3 (PH3) as a marker. At this stage, the gross morphology of the neural tube still appears normal in the *tab* mutant. We first calculated the mitotic index (the ratio of PH3-positive nuclei to total nuclei in a defined area) and found no significant difference between the wild-type and *tab* neural tubes (Fig. 2B). Also, the cell-cycle length was found to be unaffected in the *tab* neural tube by the BrdU pulse-labeling analysis (Fig. 2B, supplementary material Fig. S2). Interestingly, a significant difference was observed

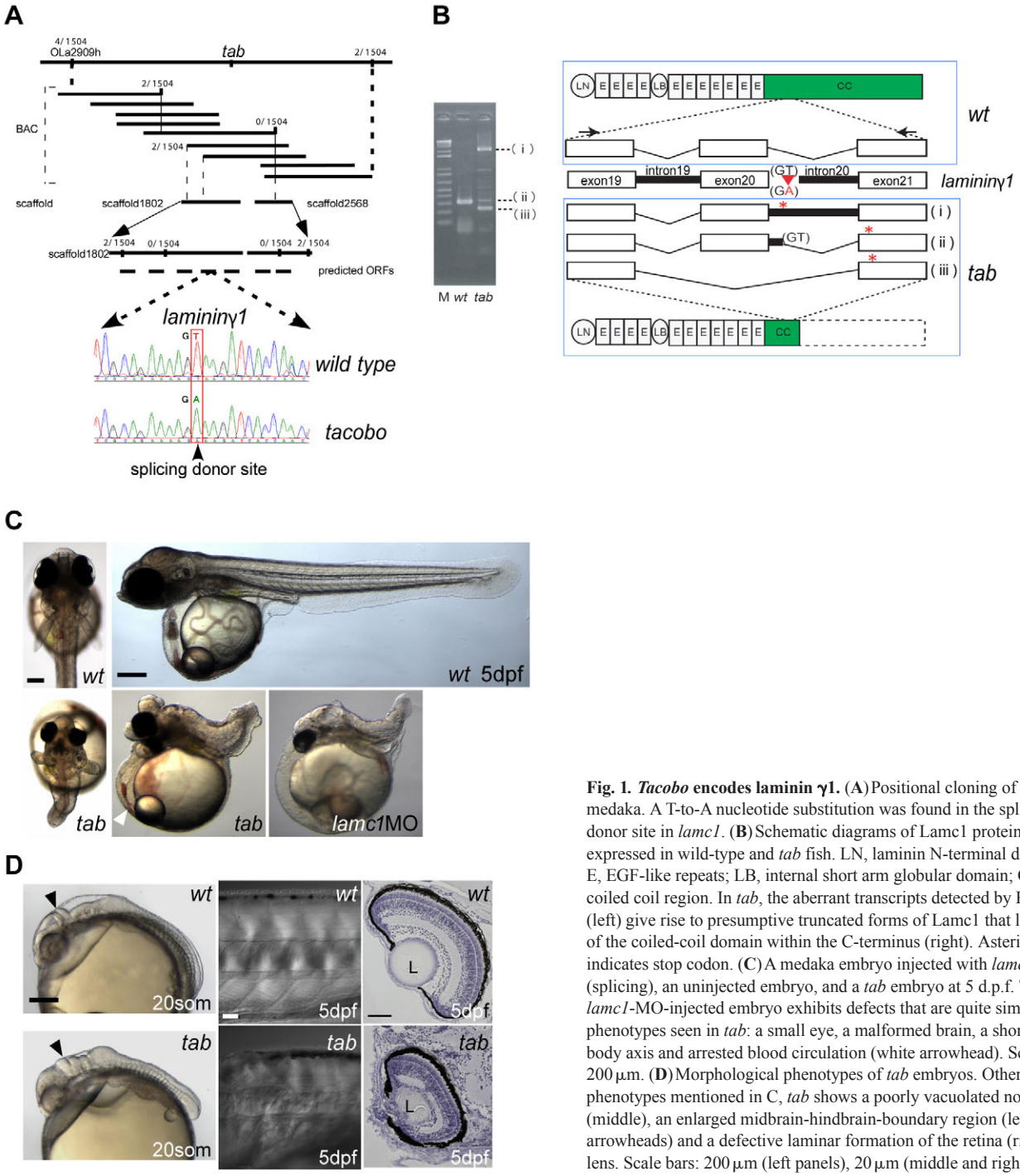

Fig. 1. *Tacobo* encodes laminin γ1. (**A**) Positional cloning of *tab* in the medaka. A T-to-A nucleotide substitution was found in the splicing donor site in *lamc1*. (**B**) Schematic diagrams of Lamc1 protein expressed in wild-type and *tab* fish. LN, laminin N-terminal domain; E, EGF-like repeats; LB, internal short arm globular domain; CC, coiled coil region. In *tab*, the aberrant transcripts detected by RT-PCR (left) give rise to presumptive truncated forms of Lamc1 that lack most of the coiled-coil domain within the C-terminus (right). Asterisk indicates stop codon. (**C**) A medaka embryo injected with *lamc1* MO (splicing), an uninjected embryo, and a *tab* embryo at 5 d.p.f. The *lamc1*-MO-injected embryo exhibits defects that are quite similar to the phenotypes seen in *tab*: a small eye, a malformed brain, a shortened body axis and arrested blood circulation (white arrowhead). Scale bars: 200 μm. (**D**) Morphological phenotypes of *tab* embryos. Other than the phenotypes mentioned in C, *tab* shows a poorly vacuolated notochord (middle), an enlarged midbrain-hindbrain-boundary region (left, black arrowheads) and a defective laminar formation of the retina (right). L, lens. Scale bars: 200 μm (left panels), 20 μm (middle and right panels).

in the positioning of the PH3-positive nuclei between wild type and *tab*. In wild-type medaka embryos, the labeled cells were mostly localized near the apical side of the neural tube (Fig. 2A). In the *tab* mutant, however, these cells were broadly dispersed along the apico-basal axis (Fig. 2A, arrowheads). The basal displacement of mitotic nuclei was observed along the entirety of the *tab* neural tube. This tendency was confirmed further by quantitative analysis (Fig. 2C): the relative distance of the labeled nuclei from the apical surface was found to have significantly increased in *tab*.

In the analysis below, we focused on this basal displacement of mitosis in the hindbrain region, because this phenotype has not been reported in laminin-related mutants in other vertebrate models, and because in the hindbrain region of *tab*, the phenotype is at its most pronounced and the imaging of nuclear migration can be readily performed.

Laminin deposition and the basement membrane are affected in *tab* mutants

To explore the role of *lamc1* in the positioning of mitotic nuclei in the neuroepithelium, we first examined the laminin deposition around the neural tube using an antibody raised against laminin 1. Laminin 1 is a heterotrimer of α1, β1, γ1 laminin chains, and

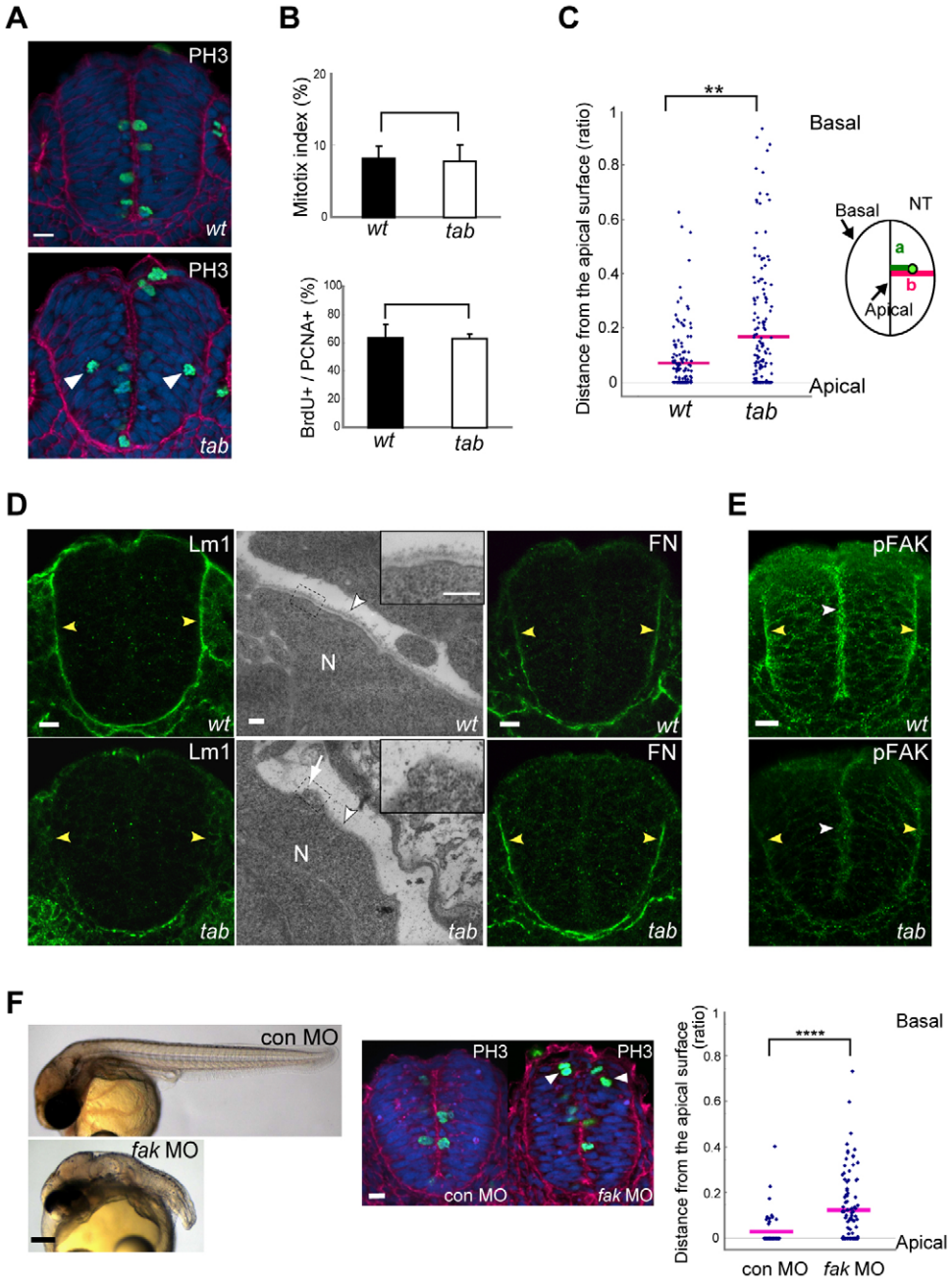

Fig. 2. Laminin regulates the mitotic nuclei positioning in neuroepithelial cells. (**A-C**) *tab* shows a defect in the positioning of its mitotic nuclei. (A) Transverse sections of the neural tube of wild-type and *tab* medaka embryos stained with anti-phospho-histone H3 (PH3) (green) antibodies and phalloidin (magenta) at the 10-somite stage. PH3-positive (mitotic) nuclei are localized apically in the wild-type neural tube, but are dispersed in the *tab* mutant (arrowheads). (B) Quantification of the mitotic index, i.e. the percentage of nuclei in the neural tube that are PH3-positive (upper panel). P=0.74, Student's t-test; wild type, n=5, 481 cells; *tab*, n=5, 522 cells. The cell-cycle length is not affected in *tab* neural tubes. The ratio of BrdU-positive nuclei to PCNA-positive (proliferative) nuclei is compared (lower panel). P=0.81, Student's t-test; wt, n=5, 1936 cells; *tab*, n=5, 1853 cells. Error bars indicate s.d. (C) To describe the position of the nuclei, following parameters shown in the right panel were used: (a) the distance of PH3-positive nuclei from the apical surface of the neural tube, and (b) the width of the neural tube. As revealed by the a/b ratio, *tab* mitotic nuclei are displaced basally, compared with that in wild-type nuclei. **P<0.005, Student's t-test, n=5 (wild type, 177 cells; *tab*, 183 cells). (**D**) Laminin deposition and the basement membrane are affected in *tab*. Representative images of the neural tube stained with anti-laminin-1 (Lm1; left) and anti-fibronectin (FN; right) antibodies at the 10-somite stage. Yellow arrowheads indicate the basal surface. Scale bars: 10 μm. Electron micrographs of the BMs of the neural tube at the 10-somite stage are shown in the middle panel. *tab* BM is thinner and disorganized with some gaps (arrow). N, neuroepithelium; white arrowheads, BMs. Scale bars: 200 nm. (**E**,**F**) FAK-mediated signaling is essential for localized mitosis in the neuroepithelium. (E) Representative images of the neural tube stained with an anti-FAK phospho-specific antibody at the 10-somite stage. White and yellow arrowheads indicate the apical and basal surface, respectively. Scale bar: 10 μm. (**F**) Mitotic nuclei are misplaced in the absence of FAK-mediated signaling. Dorsal view of *fak*-MO-injected embryos (5 d.p.f.) (left). Representative images of the neural tube stained with anti-PH3 antibody (10-somite stage) are shown in the middle panel. Arrowheads indicate the mislocalized mitosis. graph shows a quantitative analysis of the position of the mitotic nuclei in *fak* morphants. ****P<0.00005, Student's t-test; n=5 (control MO, 154 cells; *fak* MO, 137 cells).

is the major heterotrimer of the laminin γ1 subunit. As reported previously in zebrafish embryos using the same antibody we used in this study (Parsons et al., 2002), strong laminin 1 immunoreactivity was observed around the basal surface of medaka wild-type neural tubes throughout the segmentation stages, whereas in *tab*, this immunoreactivity almost disappeared (Fig. 2D, left panels). This suggests that the loss of the laminin γ1 chain causes a severe reduction in laminin 1 accumulation on the basal side of the neural epithelium. This reduction in immunoreactivity occurs in various organs, including blood vessels (data not shown). As laminins are essential for basement membrane (BM) assembly (Miner and Yurchenco, 2004), we examined the ultrastructure of the BM beneath the neural tube. In wild-type embryos, the basal surface of the neural tube was covered by a continuous BM sheet. By contrast, in *tab*, thinner and disorganized BMs were widely observed (Fig. 2D, middle panels). We also found that the basal surface of the *tab* neural tube was not as smooth as that in the wild type. Disorganized BMs were also found in the notochord sheath of the zebrafish *sly* mutant and retina of *Lamc1*-knockout mice (Halfter et al., 2002; Parsons et al., 2002). By contrast, fibronectin, which was reported to be localized to the basal surface of the neural tube (Mayer et al., 1981), appeared to be normally distributed around the *tab* neural tube (Fig. 2D, right panels).

FAK-mediated signaling is essential for localized mitosis in the neuroepithelium

In our present study, we focused on integrins because laminin signals are, to a large extent, mediated by these molecules and laminin-integrin interactions have been implicated in neuronal cell migration and cell shape change (Colognato et al., 2005; Georges-Labouesse et al., 1998). Among the downstream targets of laminin-integrin signaling, the protein tyrosine kinase FAK is known to have a prominent role in neurogenesis (Beggs et al., 2003; Parsons, 2003; Xie et al., 2003). Since the phosphorylation of tyrosine on position 397 (Y397) is a hallmark of FAK activation, we investigated both its phosphorylation state and cellular distribution in the neural tube using an anti-phosphorylated FAK (pFAK) antibody (see supplementary material Fig. S3 for antibody specificity).

In wild-type embryos, phosphorylated FAK was accumulated at both the basal and apical side of the neuroepithelium. In *tab* embryos, however, the accumulation of pFAK is greatly downregulated on both sides of the neuroepithelium (Fig. 2E), suggesting that the activation of FAK mostly depends on laminin signals in the neural tube. The downregulation of FAK was also founded in the retina of zebrafish *lama1* mutant *bal* (Semina et al., 2006). It is also worth noting that FAK was found to be normally activated at the somite boundary in *tab*, and consistently, no somite segmentation phenotype could be observed in *tab* embryos. This suggests that FAK is activated by

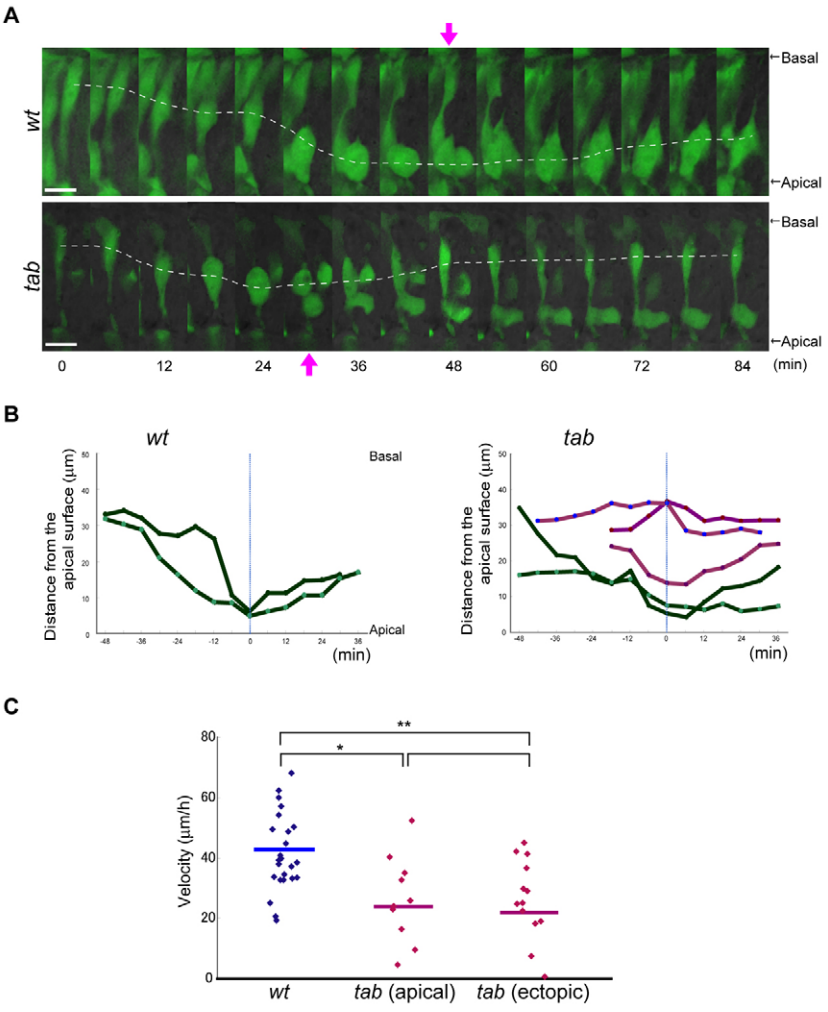

Fig. 3. Laminin regulates nuclear migration. (**A**) Kymographs of nuclear migration in the neuroepithelial cells at the 9- to 10-somite stage. The white dashed lines indicate the center of nuclei. In wild-type embryos, the nuclei migrate apically and divide at the apical surface of the neural tube. In the *tab* mutant embryos, the nuclei divide before reaching the apical surface. Arrows indicate the time point of nuclear division. Scale bars: 10 μm. (**B**) The distance from the center of the nuclei to the apical surface of the neural tube around the nuclear division plotted against time. Of daughter cells the one that migrates faster from its birth place is plotted for simplicity. Dark green and red lines indicate nuclei that divide apically and ectopically, respectively. Blue lines indicate the time point of nuclear division. (**C**) Velocities of nuclear migration before mitosis. *tab* nuclei showing apical and ectopic mitosis both have significantly decreased migration velocities. **P*<0.05, ***P*<0.005, Student's *t*-test; wild type, *n*=5, 26 cells; *tab*, *n*=5, 23 cells.

various signaling pathways, such as fibronectin, depending on contexts and tissues (Koshida et al., 2005).

We next examined by morpholino (MO) knockdown to what degree FAK-mediated signaling accounts for neural tube phenotypes in *tab*. *Fak*-MO-injected embryos exhibited mislocalized mitosis along the entire neural tube as well as a shortened body axis and dysplastic eyes, a phenotype identical to that of *tab* (Fig. 2F). These results indicate that FAK-mediated signals indeed play a major role in *lamc1*-dependent morphogenesis, including the positioning of mitotic nuclei in the neuroepithelium.

INM is perturbed in the *tab* neuroepithelium

To address the cellular mechanism underlying mislocalized mitosis in the *tab* mutant medaka, we tested whether the interkinetic nuclear migration (INM), a characteristic feature of neuroepithelial cells, was affected in *tab* neural tubes. For this analysis, we used confocal time-lapse imaging of neuroepithelial cells labeled with EGFP at around the 9- to 10-somite stages. In wild-type neural tubes, the typical nuclear migration associated with INM was seen, i.e. most nuclei had migrated from the basal side and divided when they reach the apical region (Fig. 3A, and supplementary material Movie 1). However, in *tab* mutant neural tubes, many nuclei exhibited abnormal and variable patterns of migration. A typical abnormal behavior in *tab* nuclei was slower apical migration and entry into mitosis prior to reaching the apical region (Fig. 3A, and supplementary material Movie 2). Superimposed traces demonstrate impaired nuclear migration around the time of mitosis in *tab*. Some nuclei showed normal migration, but in the most extreme cases, they migrated in an opposite direction (i.e. basally), followed by mitosis in the basal region (Fig. 3B). Most nuclei showed intermediate phenotypes, leading to mislocalized mitosis. Furthermore, we retrospectively measured the basal-to-apical migration velocity of the two populations of *tab* nuclei, one showing normal apical mitosis and the other showing basally displaced mitosis. In both cases, the migration velocity of *tab* nuclei was found to significantly decrease (Fig. 3C). These results indicate that abnormal INM in *tab* leads to the increased number of mislocalized mitoses.

The polarity and shape of neuroepithelial cells are not affected by the absence of laminin γ1

We speculated that the reduced nuclear migration activity in the *tab* mutant could be the result of disrupted cell polarity and/or cell-shape change. Previous reports have demonstrated that the apico-basal polarity is a prerequisite for correct mitotic nuclei positioning, which possibly affects nuclear migration and spindle orientation (Horne-Badovinac et al., 2001; Imai et al., 2006), and that laminins have an essential role in the establishment of this polarity (Li et al., 2002; O'Brien et al., 2001). However, our analysis of a series of apical markers (aPKCζ, ZO-1, γ-tubulin) showed that they were correctly localized in the mutant neuroepithelium (Fig. 4A).

We next assessed the morphology of neuroepithelial cells, which have apical and basal cellular processes extending to the basal and apical surfaces (ventricular and pial surfaces), a typical characteristic of neuroepithelial cells. For this analysis, embryos were injected at the 8- to 16-cell stage with *egfp* mRNA to label cells in a mosaic manner. In both wild-type and *tab* neural tubes, almost all cells displayed cellular processes extending to the apical and basal surfaces of the neural tube (wt, 73/74, *n*=5; *tab*, 72/73, *n*=5) (Fig. 4B, left panel). The detached processes observed in the wild type and *tab* are both basal processes of newly born daughter cells

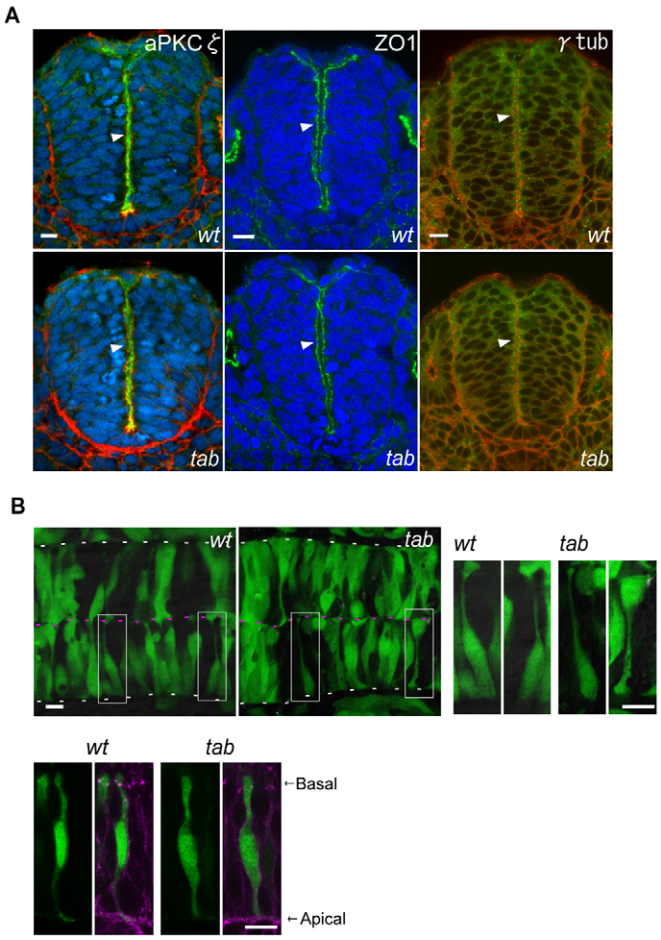

Fig. 4. The polarity and shape of neuroepithelial cells are not affected by the absence of laminin γ1. (**A**) Apical polarity is maintained in the *tab* neural tube. Transverse sections of the neural tube stained with aPKCζ (green, left panels), ZO-1 (green, middle panels), γ-tubulin (green, right panels). Arrowheads indicate apical surfaces. Red, Phalloidin; blue, DAPI. Scale bars: 10 μm. (**B**) Apical and basal processes of neuroepithelial cells are maintained in the *tab* mutant. Dorsal views of the neural tube taken by the time-lapse imaging analysis (left). Magenta and white dots indicate the apical and basal surface, respectively. A single whole cell including these cellular processes is indicated by white rectangles and higher magnification views are shown to the right. Lower panels show that apical and basal processes are also present in fixed embryos. Red, Phalloidin. Scale bar: 10 μm.

extending their processes toward basal surface of the neural tube. The attachment was also confirmed with the fixed embryos injected with *egfp* mRNA and stained with phalloidin (Fig. 4B, right panels). The attachment of cells to apical and basal surface of the neural tube was also maintained in *fak*-MO-injected embryos (supplementary material Fig. S4; 70/70, *n*=5).

Taken together, both cell polarity and morphology of neuroepithelial cells are not affected in *tab*. Recent works suggested that several molecules such as integrin-linked kinase (ILK) are also important downstream molecules of integrin signaling and are essential for cell polarization and cell morphology (Legate et al., 2006; Sakai et al., 2003). We speculate that ILK could compensate for the disrupted BMs. The results thus do not support the idea that cell polarity and/or cell shape defects are the primary cause of the basally displaced mitotic events in *tab* neural tubes, suggesting that

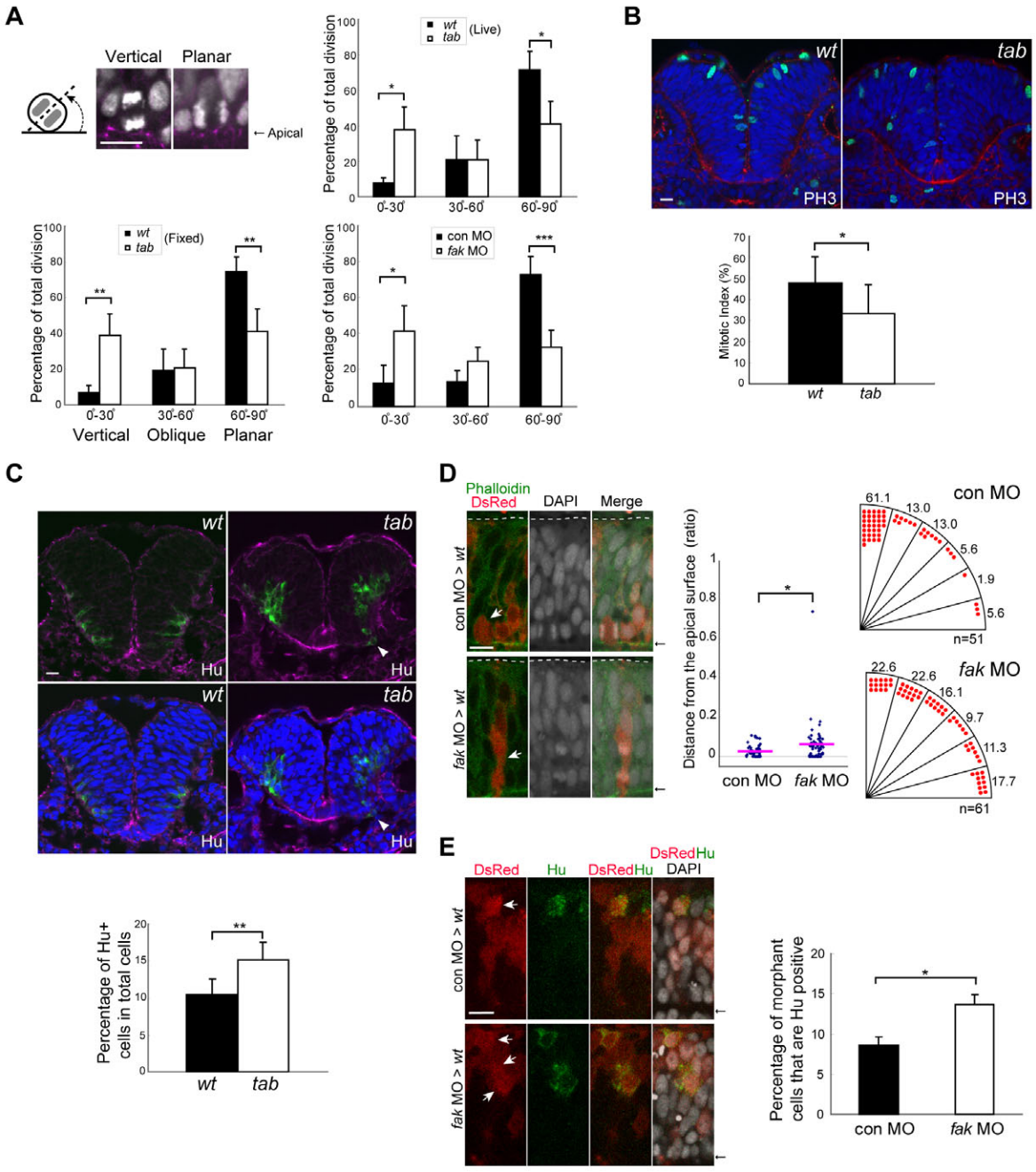

Fig. 5. *tab* shows affected planar divisions and accelerated neurogenesis. (**A**) *tab* shows randomized mitotic orientation in the apical region. Quantification of cleavage plane orientation with time-lapse imaging data [top right, **P*<0.05, Student's *t*-test; *n*=5 (wt, 72 cells; *tab*, 75 cells)] and fixed embryos stained with DAPI and phalloidin [lower left, ***P*<0.01, Student's *t*-test; *n*=5 (wt, 65 cells; *tab*, 89cells)]. Cleavage plane orientation with respect to the apical surface of the neural tube was scored and is presented in three categories: 0-30° (vertical division), 30-60° (oblique), 60-90° (planar division). Representative images are shown above. Graph in lower right panel shows quantification of cleavage plane orientation in *fak*-MO-injected embryos. **P*<0.05, ****P*<0.005, Student's *t*-test; control MO, *n*=5, 70 cells; *fak* MO, *n*=5, 69 cells. Error bars indicate s.d. (**B**,**C**) *tab* shows accelerated neurogenesis. (B) Transverse sections of neural tube stained with PH3 antibody at the 15-somite stage. Graph shows mitotic index is significantly decreased in *tab* neural tube. *P*<0.05, Student's *t*-test, *n*=5. (C) Transverse sections of the neural tube stained with a HuC/D antibody, phalloidin and DAPI at the 15-somite stage. Arrowhead indicates the ectopia of the neurons outside the neural tube. Graph shows the percentage of Hu-positive cells to the total cells in the neural tube was significantly increased in *tab*. *P*<0.05, Student's *t*-test, *n*=6 (wt, 4241 cells; *tab*, 4051 cells). Three sections from each embryo were measured. Scale bars: 10 μm. Error bars indicate s.d. (**D**) FAK autonomously acts on localized mitosis, spindle orientation. (left) Representative images showing transplanted MO cells in the neural tube stained with DAPI and phalloidin at the 10-somite stage. White arrows indicate the transplanted cells that undergo cell division. Black arrows and white dashed line indicate the apical and basal surface of the neural tube, respectively. Scale bar: 10 μm. Graph shows quantification of the distribution of mitotic nuclei of transplanted cells. **P*<0.05, Student's *t*-test, control MO, *n*=9, 51 cells; *fak* MO, *n*=14, 61 cells. Quantification of cleavage plane orientation in the transplanted cells is shown in charts on the right. (**E**) Fak autonomously acts on neurogenesis. Representative images showing MO-injected cells in the neural tube stained with a Hu antibody at the 15-somite stage. White arrows indicate the transplanted cells labeled by Hu. Graph shows quantification of the percentage of MO-injected cells that are Hu positive. **P*<0.05, Student's *t*-test, *n*=5 (control MO, 2019 cells; *fak* MO, 1511 cells). Black arrows indicate the apical surface of the neural tube. Scale bar: 10 μm. Error bars indicate s.d.

the intracellular machinery directing nuclear migration is impaired in these mutant cells.

Planar divisions are affected in the *tab* neuroepthelium

Recent works show that integrin signals regulate spindle orientation in both polarized and non-polarized cells (Lechler and Fuchs, 2005; Toyoshima and Nishida, 2007). We thus examined whether cleavage-plane orientation is affected in the *tab* neural tube using time-lapse imaging (Fig. 5A). In wild-type neural tubes, most mitotic cells in the apical surface show vertical cleavage (planar division), i.e. the cleavage plane is vertical to the apical surface (79%, 57/72). By contrast, in *tab*, the cleavage plane becomes nearly randomized: 45% (34/75) for vertical, 19% (14/75) for oblique and 36% (27/75) for horizontal. In mislocalized mitosis, the cleavage plane was horizontal, irrespective of the genotype (wt, 2/2; *tab*, 31/31). The cleavage plane also appeared to be randomized when mitotic orientation was examined in fixed sections (Fig. 5A). The same tendency was obtained in *fak*-MO-injected embryos (Fig. 5A). These data indicate that FAK-mediated signaling is essential for planar division in the apical surface of the neuroepithelium.

Accelerated neurogenesis in the *tab* neuroepithelium

To assess the impact of the above mentioned defects on neurogenesis, we first measured the mitotic index (percentage of phosphorylated histone-3-positive cells) in the neural tube at the 15-somite stage, the stage corresponding to the neurogenic phase. This index was significantly decreased in *tab* neural tubes compared with that in the wild type (Fig. 5B), suggesting that there is a reduced proportion of M-phase progenitors at this stage in *tab*. To determine whether this possible reduction in M-phase progenitors is accompanied by the increased number of postmitotic neurons, we stained differentiated neurons with Hu C/D, a pan neuronal maker at the 10-somite (proliferative phase) and 15-somite stages. Although no significant difference was observed at the 10-somite stage, a significant increase in Hu-positive cells was observed at the 15-somite stage (Fig. 5C and supplementary material Fig. S5), suggesting that accelerated neurogenesis is occurring in the *tab* neural tube during the neurogenic phase.

We also found some Hu-positive cells located outside the neural tubes of *tab* and *fak* morphants (i.e. ectopia), which was never observed in the wild type (Fig. 5C and supplementary material Fig. S6, arrowheads). Similar ectopias were also reported in the cortices of *Lamc1*-knockout mice, in which neurons were suggested to migrate outward from the neural tube through a gap in BMs of the neural tube (Halfter et al., 2002).

FAK autonomously acts on localized mitosis, spindle orientation and neurogenesis

To test cell autonomy of the FAK function, we performed mosaic analysis using *fak* morphants. We used *fak* morphants instead of *tab* mutants, because the *tab* mutation was expected to function non-cell-autonomously. Compared with the control-MO-injected cells and host wild-type cells, *fak*-MO-cells, when transplanted, specifically exhibited a significant increase in mislocalized mitotic nuclei (Fig. 5D, middle panels, and supplementary material Fig. S7), planar cell division (Fig. 5D, right panels, and supplementary material Fig. S7) and accelerated neurogenesis (Fig. 5E, supplementary material Fig. S7). These results indicate the cell-autonomous function of FAK, and also suggest the correlation between defects in mitosis at the earlier stage and accelerated neurogenesis at the later stage.

The dynein-dynactin complex and FAK-mediated signaling coordinately regulate INM and planar divisions

Recent studies have implicated dynein-dynactin as a mediator for nuclear migration in retinal neuroepithelium, nuclear positioning in photoreceptor cells (Del Bene et al., 2008; Tsujikawa et al., 2007) and spindle orientation in various cell types (Siller and Doe, 2009; Toyoshima et al., 2007), highlighting the importance of the dynein system in these processes. Dynactin is a large multi-subunit activator of dynein, which mediates the interaction of its cargo and enhances dynein processivity (Gross, 2003; King and Schroer, 2000). These led us to hypothesize that the activity of dynein could be impaired in the neural tube of the *tab* mutant, leading to defects in INM and planar divisions. To test this hypothesis, we attempted to decrease the activity of the dynactin complex in medaka embryos. In several experimental systems, the overexpression of dynamitin (p50, Dynactin2), a dynactin component (King and Schroer, 2000), was found to cause a dissociation of the dynactin complex and consequently to block its function (Burkhardt et al., 1997). Taking advantage of this, we overexpressed dynamitin in medaka embryos by injecting medaka *dynamitin* mRNA at the one-cell stage. Overexpression of dynamitin produced randomized orientation of apical cell divisions (Fig. 6A) and basally localized mitosis at the 10-somite stage (Fig. 6A). The mislocalized mitosis was reminiscent of the phenotype of *mikre oko* (*mok*), a zebrafish dynactin mutant in which INM in the retina is defective (Del Bene et al., 2008). Similar to *mok*, *dynamitin*-injected embryos displayed a severe defect in eye morphogenesis at 4 d.p.f., confirming the block in dynactin function by dynamitin overexpression in medaka embryos (Fig. 6A). These results indicate that the dynein-dynactin complex indeed regulates INM and mitotic orientation in the neural tube.

As the inhibition of laminin-FAK and dynein-dynactin activities produced similar phenotypes, we investigated the possible interplay between these two signals by performing a genetic interaction study. *dynamitin* mRNA and *fak* MO were titrated to suboptimal doses (*dynamitin* mRNA, 50 ng/μl; *fak* MO, 100 μM) and co-injected into medaka embryos to inhibit both pathways. As shown in Fig. 6B, single injections of a suboptimal dose of either mRNA or MO caused no significant defects. However, co-injection caused significantly greater effects on the positioning of mitotic nuclei and mitotic orientation in the neural tube (Fig. 6B). Together, these results suggest that the dynein-microtubule system and FAK-mediated signaling cooperate for INM and planar divisions in the neural tube.

Finally, we examined the distribution of dynactin p150 by staining wild-type neural tubes with the anti-dynactin p150 antibody and found that dynactin is enriched in both the apical and basal regions of the neural tube, in addition to the low levels of uniform distribution in the cytoplasm. In the neural tubes of *fak* morphants, the dynactin signal appeared to be evenly reduced compared with the control-MO-injected embryos (supplementary material Figs S3, S8A). However, the distribution of phosphorylated FAK was not affected by dynamitin overexpression (supplementary material Fig. S8C). Together, these results strengthen the notion that the dynein-microtubule system works in the FAK pathway in the neural tube.

Discussion

INM has been the focus of numerous studies of neurogenesis since it was first reported in 1930s (Del Bene et al., 2008; Murciano et al., 2002; Sauer, 1935; Xie et al., 2007). The importance of cleavage orientation of neural progenitors in maintaining self-renewability has also been recognized for many years (Gotz and

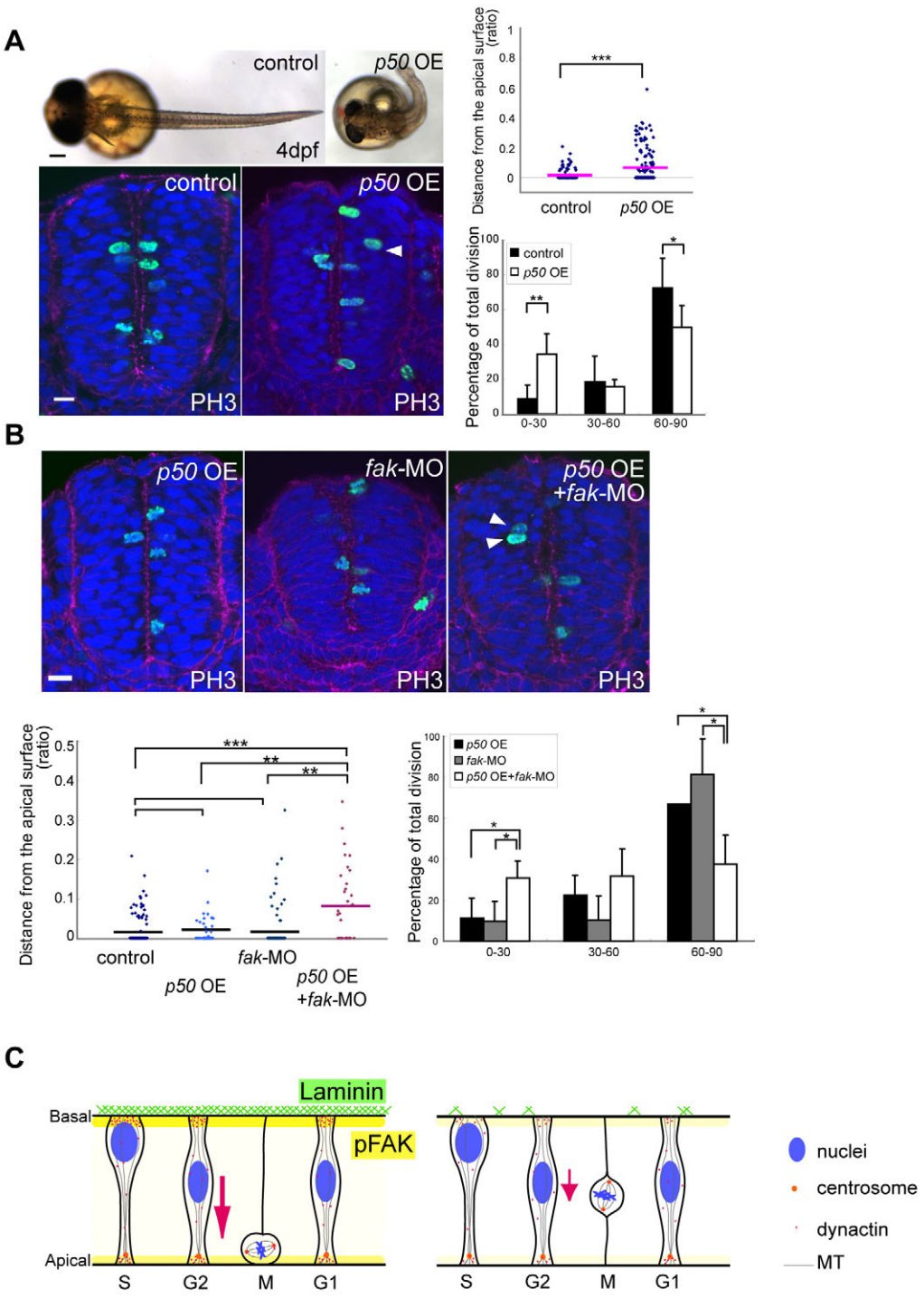

Fig. 6. The dynein-dynactin complex and FAK-mediated signaling coordinately regulate INM and planar divisions. (**A**) Dynactin functions in the positioning of mitotic nuclei and planar divisions. Upper left panels are dorsal views of dynamitin-overexpressing embryos at 4 d.p.f. Scale bar: 200 μm. Lower left panels show transverse sections of the injected embryos stained with PH3 at the 10-somite stage. Scale bar: 10 μm. Top graph shows quantitative analysis of the positions of the mitotic nuclei in the injected embryos. ***P<0.005, Student's t-test, n=5 [control LacZ, 153 cells; dynamitin (p50) OE, 183 cells]. Bottom graph shows quantitative analysis of the cleavage plane orientation in dynamitin-overexpressing embryos. *P<0.05, **P<0.01, Student's t-test; n=6 (control LacZ, 82 cells; *p50* OE, 109 cells). Error bars indicate s.d. (**B**) FAK regulates INM coordinately with the dynein-dynactin complex. Transverse sections of 10-somite stage embryos injected with a suboptimal dose of *dynamitin* mRNA, *fak* MO, or a mixture of both, and then stained with anti-PH3 antibody. Embryos coinjected with *dynamitin* mRNA and *fak* MO show significantly more severe defects in the positioning of mitotic nuclei than either of the single injected embryos. Graph on left shows quantitative analysis of the distribution of mitotic nuclei. **P<0.005, ***P<0.001, Student's t-test, n=5 [control, 153 cells; *fak* MO, 115 cells; dynamitin (p50) OE, 91 cells; *fak* MO + p50 OE, 104 cells]. Graph on right is a quantitative analysis of the cleavage plane orientation. *P<0.05, Student's t-test; [*fak* MO, n=5, 42 cells; dynamitin (p50) OE, n=5, 44 cells; *fak* MO + p50 OE, n=5, 62 cells]. Scale bar: 10 μm. Error bars indicate s.d. Arrowheads in A and B indicate the mislocalized mitosis. (**C**) Schematic representation of our proposed model for the mechanism underlying the regulation of INM by laminin and other ECM molecules.

Huttner, 2005; Konno et al., 2008). We have addressed the role of extracellular signals in these characteristic features of neurogenesis using the *lamc1* medaka mutant *tab*. We demonstrate for the first time that extracellular signals mediated by FAK regulate the activity of nuclear migration and the mitotic orientation in the vertebrate neuroepithelium. Our data also suggest that the dynein motor system functions in these two processes, possibly through interaction with FAK signaling. The *tab* mutant thus reveals the novel role of extracellular signals during vertebrate early neurogenesis (Fig. 6C).

FAK-mediated ECM signals are essential for INM and planar divisions in the neuroepithelium

The *tab* locus was found to encode the medaka laminin γ1 chain, and a mutated *tab* locus produces abnormally spliced transcripts that are translated into truncated forms of laminin γ1 chain which lack a coiled-coil domain. This domain is essential for heterotrimer formation (Beck et al., 1993). Although RT-PCR detected two other transcripts of unexpected size, *tab* is likely to be a null allele because of the severe loss of laminin 1 reactivity and the successful

phenocopy by MO injection. The phenotype of the *tab* mutants largely resembles that of the zebrafish laminin mutants, *bal*, *gup* and *sly* (Parsons et al., 2002; Pollard et al., 2006; Semina et al., 2006). However, defects in INM and planar divisions are found only in *tab* embryos. This might reflect a difference in the redundancy of the laminin isoforms and/or in the degree of the maternal contribution of laminins between the two fish systems. Indeed, *lamc1* is maternally provided as RNA and protein both in zebrafish and medaka (data not shown), and this contribution might alleviate the neural tube phenotype more in fast-developing zebrafish than in the medaka. In spite of this difference, however, *tab* and *sly* both exhibit an abnormal MHB morphology at relatively later stages. We consider this not to be a direct consequence of defective INM but rather a failure in laminin-dependent basal constriction, as previously reported (Gutzman et al., 2008).

As in zebrafish *sly* mutants, we observed a large reduction in laminin 1 immunoreactivity in the *tab* medaka, most notably around the mutant neural tubes, which exhibit basally mislocalized mitosis and a randomized cleavage plane in the apical region. The simplest interpretation of this finding is that the reduction in laminin accumulation directly induces the observed INM and mitotic defects in the neuroepithelium. However, the *tab* situation might not be so simple. The BM is a complex structure consisting of a variety of ECM proteins and proteoglycans, including laminins, and is enriched with a variety of growth factors (Yurchenco et al., 2004). Since laminin families are thought to have an important role in the maintenance of BM integrity (Yurchenco et al., 2004), other components of the BM might not be properly organized or lost in the absence of laminin 1. Indeed, other previous and our present studies demonstrate that the loss of laminin chains causes thinner and disintegrated BMs, suggesting that the phenotypes in *tab* are caused by defects in overall BM signals mediated by laminin proteins and other ECM molecules. Regardless of this complexity, we found in the present study that the FAK-mediated pathway accounts for the phenotypes observed in the *tab* mutant, because the *tab* mutation abrogates the activation of FAK and an MO knockdown of medaka *fak* phenocopies neural tube defects in *tab*. The mosaic experiment confirmed the cell-autonomous function of FAK in neuroepithelial cells (Fig. 5D). ECM proteins exert their effects through a variety of cell-surface molecules, and integrins are most commonly used as signaling receptors (DeMali et al., 2003). Integrins are heterodimeric transmembrane receptors composed of α- and β-chains and more than 20 have so far been identified, of which α1β1, α3β1, α6β1, α7β1 and α6β4 are major laminin receptors (Desban et al., 2006; Nishiuchi et al., 2006). Although integrins activate a variety of tyrosine kinases and GTPases to induce multiple downstream signaling pathways, FAK is known to have a prominent role in integrin signaling (DeMali et al., 2003; Parsons, 2003).

We observed that activated FAK is localized on both the apical and basal sides of the neuroepithelial cells in wild-type embryos. The positive apical staining was unexpected, but a similar pattern for phosphorylated FAK was also observed in the neural tube of chick embryos (supplementary material Fig. S9), suggesting a conserved pattern of FAK activation in the vertebrate neural tube. Since both basally and apically localized pFAK becomes undetectable in *tab* mutants, their activation must depend on γ1-associated laminins. Although laminin 1 immunoreactivity was confined to the BM of the neural tube under our experimental conditions, we cannot rule out the possibility that other undetectable isoforms of laminins activate FAK on the apical side. Indeed, the apical region of the E10 murine cortex expresses *Lama2*, *Lama4* and *Lamb2* (Lathia et al., 2007), all of which are associated with *Lamc1* and could thus account for the *Lamc1*-dependent apical activation of FAK.

Abnormal behavior of mutant nuclei during INM

Time-lapse imaging of living medaka embryos enabled us to monitor abnormal nuclear behavior in the mutant neuroepithelium before and after their division. A number of cells exhibit a reduced rate of nuclear migration toward the apical side without affecting the length of cell cycle, and prematurely undergo mitosis before they reach the surface region. Some mutant nuclei exhibit reversed migration toward the basal side before entering mitosis. This series of observations clearly demonstrates that mitosis and nuclear migration are decoupled in the *tab* mutant, suggesting the presence of a coupling mechanism under normal conditions. A similar situation has been reported for INM in the *Pax6* mutant cortex in rat (Tamai et al., 2007). Furthermore, time-lapse imaging of wild-type and *tab* nuclei revealed an interesting feature of nuclear migration: wherever mitosis takes place (one exception in 25 *tab* mutants), the nuclei change their direction after having undergone mitosis. A mitotic event must therefore activate a specific signal that dictates the direction of the ensuing nuclear migration. These factors could regulate the direction of migration by switching motor molecules from dynein to kinesin (or vice versa), upon mitosis. Thus, using the medaka *tab* as a model system will provide a good starting point to further understand this putative switching mechanism.

The *tab* neural tube simultaneously exhibits abnormal INM and randomized mitotic orientation on the apical side, raising the question as to whether the misorientation of the cleavage plane affects INM. However, the two events are likely to be independent, because Konno and co-workers (Konno et al., 2008) have demonstrated that INM normally occurs in the *LGN*-mutant mice in which the orientation of normally planar divisions are randomized in the developing neocortex.

Accelerated neurogenesis in *tab* neural tubes

In *tab* neural tubes, the number of neurons is increased whereas the mitotic index is decreased at the neurogenic stage (15-somite stage) (Fig. 5B,C). Although we failed to examine the cell cycle exit rate because of technical difficulties, the result strongly suggests that neurogenesis in *tab* is accelerated at the expense of progenitor cells. This notion is consistent with previous reports in which impaired INM caused accelerated neurogenesis in the mouse cerebral cortex and zebrafish retina (Del Bene et al., 2008; Xie et al., 2007). Basally located mitosis might reduce the chance of daughter cells to be exposed to proliferative signals such as the Notch signal, which was reported to be high in the apical region of the zebrafish retina (Del Bene et al., 2008). Furthermore, Konno and colleagues recently demonstrated in the mammalian cortex that only planar division gives rise to apical progenitors which inherit both the apical and basal component of the mother cell (Konno et al., 2008). Our mosaic experiments provide a possible link between early defects in mitosis and accelerated neurogenesis at the later stage. Taken together, it is reasonable to conclude that both defective INM and mitotic orientation caused the observed accelerated neurogenesis in *tab*.

Cooperation of FAK-mediated signaling and the dynein-microtubule system in neurogenesis

Previous studies implicated actin and the microtubule cytoskeleton in INM (Messier and Auclair, 1973; Messier and Auclair, 1974)

and cleavage plane orientation (Siller and Doe, 2009; Toyoshima et al., 2007). Recently, Norden and co-workers (Norden et al., 2009) showed that INM in the zebrafish retina largely depends on the activity of actomyosin. However, in our present study, we put a special emphasis on studying the role of microtubules in medaka neurogenesis, partly because treatment with the actin-depolymerizing agent, cytochalasin B, was less effective in this system. Under our experimental conditions, treatments with high doses of cytochalasin B, which completely disrupted the actin network, induced mislocalized mitosis and misorientation of the mitotic cleavage plane in the medaka neural tube. However, this does not seem to reflect the *tab* mutant situation, because the actin network appeared normal in mutant neuroepithelial cells as indicated by staining of actin filaments in *tab* mutant neural tubes (supplementary material Fig. S10).

Dynein and microtubules are known to play evolutionarily conserved roles in spindle orientation and nuclear positioning (Siller and Doe, 2009). Furthermore, a recent zebrafish study revealed a crucial role for dynein and microtubules in INM during retinal neurogenesis (Del Bene et al., 2008). The zebrafish *mok* locus, the associated mutation of which causes severe INM defects in the retina, was found to encode the dynactin 1 subunit of the dynactin complex, a key regulatory complex during dynein motor activity. This led us to analyze the role of dynein and microtubules in INM and mitotic orientation in medaka and we found that the basal displacement of mitotic nuclei and randomized mitotic plane was induced when the dynactin complex was disrupted.

At present, little is known about a direct link between FAK-mediated signaling and the dynein-dynactin complex, although they both are involved in many common biological phenomena such as cell migration, proliferation and the establishment of cell morphology (Gomes et al., 2005). However, a recent report demonstrated that phosphorylated FAK is crucial for both neuronal migration and nuclear movement through the regulation of a small network of microtubules that encompass the nucleus (Xie et al., 2003). In more recent reports, phosphatidylinositol 3-kinase (PI3K), which is one of the downstream molecules of integrin signaling, was found to be essential for spindle orientation control by regulating accumulation of dynactin to the midcortex of a cell which astral microtubules contact (Toyoshima et al., 2007; Toyoshima and Nishida, 2007). Because FAK is also known to activate PI3K (Chen et al., 1996), FAK is likely to regulate mitotic machinery through the PI3K-mediated dynactin function, whereas in the case of INM, the activity of perinuclear dynein-dynactin can be a target of FAK signaling. Consistently, the velocity of nuclear migration decreases whereas the distribution pattern of stabilized and dynamic microtubules appears intact in *tab* neural tubes (supplementary material Fig. S11).

In support of the possible functional link between FAK-mediated signaling and the dynein-dynactin complex, co-injection experiments using suboptimal doses of *dynamitin* mRNA and *fak* MO demonstrated that they genetically interact for the regulation of INM and planar cell divisions. Furthermore, neural tubes of the *fak* morphants exhibit reduced dynactin 1 signal, which otherwise largely co-localizes with phosphorylated FAK in the apical and basal regions (supplementary material Fig. S8A,B). In *tab* neuroepithelial cells, the basal accumulation of dynactin appeared to slightly decrease (data not shown). However, overexpressed dynamitin did not alter the distribution pattern of phosphorylated FAK in the neural tube (supplementary material Fig. S8C), suggesting that the function of dynein-dynactin is downstream of FAK. It is still unknown how FAK regulates dynactin.

Since the overall phenotype following the co-injection resembles that obtained from a single injection of either *dynamitin* mRNA or *fak* MOs, the interaction between dynein and FAK appears to occur not only in the neural tube, but also in other developing organs. Further analysis, especially by biochemical approaches, will be needed to address how FAK-mediated signaling interacts with dynein-dynactin complexes to regulate a variety of biological processes, including INM and planar divisions in the developing neuroepithelium.

Materials and Methods

Fish strains and mutant screening

All studies of wild-type medaka were carried out using the d-rR strain of a closed colony. ENU-based mutagenesis was performed as described previously (Ishikawa, 1996). For mosaic analysis, medaka DsRed transgenic line was used.

Genetic mapping

Tab heterozygous fish were mated with wild-type HNI fish to generate F1 families. Homozygous mutant embryos were obtained from F1 crosses. Mapping was performed by using EST markers, BAC chromosome walking and using information in the medaka genome database.

Injection of morpholino oligonucleotides and RNAs

Morpholinos (GeneTools) and capped RNAs synthesized using mMESSAGE mMACHINE (Ambion) were injected into 1-cell-stage drR embryos at a concentration of 150 μM for *lamc1* MO1, 5′-TGTGTTGGTGATCTTACTTTTCTGC-3′; *lamc1* MO2, 5′-CACAGTCCGCACAAAACTCTGCATC-3′, 100 or 300 μM for *fak* MO1, 5′-CCAGGTAGGAAGACGATGCCATACC-3′; *fak* MO2, 5′-GCTAACTGGCC-ATCATATTCTGAAA-3′; and *fak* 5mis MO, 5′-CCAcGTAgcAAGACcATG-CgATCC, 0.05 or 0.4 mg/ml for *dynamitin* mRNA, and *LacZ* mRNA. For live imaging of nuclear migration and cell shapes, 0.3 mg/ml EGFP mRNA was injected at the 8- to 16-cell stage to label cells in a mosaic fashion.

Histology

For histological analysis, fixed embryos were embedded in Technovit 8100 (Heraeus Kulzer). For ultrastructural studies, embryos were fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed in 1% OsO_4, stained with uranyl acetate and embedded in Epon (NEM). Sections were stained according to standard procedures and visualized with a JEM-1011 microscope (JEOL).

Immunohistochemistry

Whole-mount immunostaining of medaka embryos was performed as described previously (Koshida et al., 2005). The antibodies used were as follows; phospho-Histone H3 (Upstate, 1:200), laminin 1 (Sigma, 1:200), FAK [pY397] (BioSource International, 1:200), fibronectin (Sigma, 1:200), aPKCξ (Santa Cruz, 1:200), ZO-1 (Sigma, 1:200), γ-tubulin (Sigma, 1:1000) and dynactin p150 Glued (BD, 1:200). Alexa Fluor 488/555 goat anti-rabbit/mouse IgG (Molecular Probes) were used as secondary antibodies with DAPI and/or Rhodamine or Alexa Fluor 488-Phalloidin (Molecular Probes). For analysis of laminin 1 and γ-tubulin, embryos were permeabilized with acetone at –20°C for 5 minutes. For analysis of Hu C/D (BD, 1:500), BrdU (Serotec, 1:500) and PCNA (Sigma, 1:500), embryos were processed for cryosectioning (12 μm). To detect BrdU, cryosections were treated with 2N HCl for 1 hour. These specimens were observed by Zeiss LSM710 confocal microscope.

BrdU labeling

10 mM BrdU (Sigma) was injected into the yolk at the 10-somite stage. After incubation at 28°C for 2 hours, injected embryos were fixed.

Confocal time-lapse imaging of nuclear migration and cell shape

Labeled medaka embryos were dechorionated with medaka hatching enzyme, immobilized with 3.5 mM heptanol and embedded in low melting point agarose (Sigma) filled with the heptanol solution. Optical *z* sections were collected at 2 μm steps at 3 minute intervals, starting at the 9-somite stage until the 12-somite stage with an Olympus FV500 confocal microscope at an ambient room temperature of 28°C. For measurement of the velocity of nuclear migration, the distance traveled by individual nuclei from when they started migration was measured with Imaris and ImageJ programs. For observation of cell shape, *z* sections were collected at 1 μm steps, and also using fixed embryos labeled with EGFP and phalloidin.

Immunoblotting

Protein extracts were prepared from medaka embryos at 5 d.p.f. as described previously (Henry et al., 2001). Anti-pFAK (1:2000) or anti-dynactin p150 (1:250) antibodies, and anti-rabbit/mouse HRP secondary antibodies (1:5000; Amersham) were used.

Mosaic analysis

For mosaic analysis, cell transplantation experiments were performed as reported previously (Shimada et al., 2008) with the following modifications. A medaka transgenic line with ubiquitous expression of DsRed was used as a donor, and the donor embryos were injected with *fak* MO or 5mis MO. The transplanted embryos were fixed at either the 10- or 15-somite stage.

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Supplementary material available online at
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References

Aberdam, D., Galliano, M. F., Vailly, J., Pulkkinen, L., Bonifas, J., Christiano, A. M., Tryggvason, K., Uitto, J., Epstein, E. H., Jr, Ortonne, J. P. et al. (1994). Herlitz's junctional epidermolysis bullosa is linked to mutations in the gene (LAMC2) for the gamma 2 subunit of nicein/kalinin (LAMININ-5). *Nat. Genet.* **6**, 299-304.

Alpy, F., Jivkov, I., Sorokin, L., Klein, A., Arnold, C., Huss, Y., Kedinger, M., Simon-Assmann, P. and Lefebvre, O. (2005). Generation of a conditionally null allele of the laminin alpha1 gene. *Genesis* **43**, 59-70.

Baye, L. M. and Link, B. A. (2007). Interkinetic nuclear migration and the selection of neurogenic cell divisions during vertebrate retinogenesis. *J. Neurosci.* **27**, 10143-10152.

Beck, K., Dixon, T. W., Engel, J. and Parry, D. A. (1993). Ionic interactions in the coiled-coil domain of laminin determine the specificity of chain assembly. *J. Mol. Biol.* **231**, 311-323.

Beggs, H. E., Schahin-Reed, D., Zang, K., Goebbels, S., Nave, K. A., Gorski, J., Jones, K. R., Sretavan, D. and Reichardt, L. F. (2003). FAK deficiency in cells contributing to the basal lamina results in cortical abnormalities resembling congenital muscular dystrophies. *Neuron* **40**, 501-514.

Burkhardt, J. K., Echeverri, C. J., Nilsson, T. and Vallee, R. B. (1997). Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. *J. Cell Biol.* **139**, 469-484.

Cappello, S., Attardo, A., Wu, X., Iwasato, T., Itohara, S., Wilsch-Brauninger, M., Eilken, H. M., Rieger, M. A., Schroeder, T. T., Huttner, W. B. et al. (2006). The Rho-GTPase cdc42 regulates neural progenitor fate at the apical surface. *Nat. Neurosci.* **9**, 1099-1107.

Chen, H. C., Appeddu, P. A., Isoda, H. and Guan, J. L. (1996). Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. *J. Biol. Chem.* **271**, 26329-26334.

Colognato, H., ffrench-Constant, C. and Feltri, M. L. (2005). Human diseases reveal novel roles for neural laminins. *Trends Neurosci.* **28**, 480-486.

Del Bene, F., Wehman, A. M., Link, B. A. and Baier, H. (2008). Regulation of neurogenesis by interkinetic nuclear migration through an apical-basal notch gradient. *Cell* **134**, 1055-1065.

DeMali, K. A., Wennerberg, K. and Burridge, K. (2003). Integrin signaling to the actin cytoskeleton. *Curr. Opin. Cell Biol.* **15**, 572-582.

Desban, N., Lissitzky, J. C., Rousselle, P. and Duband, J. L. (2006). alpha1beta1-integrin engagement to distinct laminin-1 domains orchestrates spreading, migration and survival of neural crest cells through independent signaling pathways. *J. Cell Sci.* **119**, 3206-3218.

Fujita, S. (1964). Analysis of neuron differentiation in the central nervous system by tritiated thymidine autoradiography. *J. Comp. Neurol.* **122**, 311-327.

Furutani-Seiki, M., Sasado, T., Morinaga, C., Suwa, H., Niwa, K., Yoda, H., Deguchi, T., Hirose, Y., Yasuoka, A., Henrich, T. et al. (2004). A systematic genome-wide screen for mutations affecting organogenesis in Medaka, Oryzias latipes. *Mech. Dev.* **121**, 647-658.

Georges-Labouesse, E., Mark, M., Messaddeq, N. and Gansmuller, A. (1998). Essential role of alpha 6 integrins in cortical and retinal lamination. *Curr. Biol.* **8**, 983-986.

Gomes, E. R., Jani, S. and Gundersen, G. G. (2005). Nuclear movement regulated by Cdc42, MRCK, myosin, and actin flow establishes MTOC polarization in migrating cells. *Cell* **121**, 451-463.

Gotz, M. and Huttner, W. B. (2005). The cell biology of neurogenesis. *Nat. Rev. Mol. Cell. Biol.* **6**, 777-788.

Gross, S. P. (2003). Dynactin: coordinating motors with opposite inclinations. *Curr. Biol.* **13**, R320-R322.

Gutzman, J. H., Graeden, E. G., Lowery, L. A., Holley, H. S. and Sive, H. (2008). Formation of the zebrafish midbrain-hindbrain boundary constriction requires laminin-dependent basal constriction. *Mech. Dev.* **125**, 974-983.

Halfter, W., Dong, S., Yip, Y. P., Willem, M. and Mayer, U. (2002). A critical function of the pial basement membrane in cortical histogenesis. *J. Neurosci.* **22**, 6029-6040.

Haubst, N., Georges-Labouesse, E., De Arcangelis, A., Mayer, U. and Gotz, M. (2006). Basement membrane attachment is dispensable for radial glial cell fate and for proliferation, but affects positioning of neuronal subtypes. *Development* **133**, 3245-3254.

Henry, C. A., Crawford, B. D., Yan, Y. L., Postlethwait, J., Cooper, M. S. and Hille, M. B. (2001). Roles for zebrafish focal adhesion kinase in notochord and somite morphogenesis. *Dev. Biol.* **240**, 474-487.

Horne-Badovinac, S., Lin, D., Waldron, S., Schwarz, M., Mbamalu, G., Pawson, T., Jan, Y., Stainier, D. Y. and Abdelilah-Seyfried, S. (2001). Positional cloning of heart and soul reveals multiple roles for PKC lambda in zebrafish organogenesis. *Curr. Biol.* **11**, 1492-1502.

Imai, F., Hirai, S., Akimoto, K., Koyama, H., Miyata, T., Ogawa, M., Noguchi, S., Sasaoka, T., Noda, T. and Ohno, S. (2006). Inactivation of aPKClambda results in the loss of adherens junctions in neuroepithelial cells without affecting neurogenesis in mouse neocortex. *Development* **133**, 1735-1744.

Ishikawa, Y. (1996). A recessive lethal mutation, tb, that bends the midbrain region of the neural tube in the early embryo of the medaka. *Neurosci. Res.* **24**, 313-317.

Kasahara, M., Naruse, K., Sasaki, S., Nakatani, Y., Qu, W., Ahsan, B., Yamada, T., Nagayasu, Y., Doi, K., Kasai, Y. et al. (2007). The medaka draft genome and insights into vertebrate genome evolution. *Nature* **447**, 714-719.

King, S. J. and Schroer, T. A. (2000). Dynactin increases the processivity of the cytoplasmic dynein motor. *Nat. Cell Biol.* **2**, 20-24.

Konno, D., Shioi, G., Shitamukai, A., Mori, A., Kiyonari, H., Miyata, T. and Matsuzaki, F. (2008). Neuroepithelial progenitors undergo LGN-dependent planar divisions to maintain self-renewability during mammalian neurogenesis. *Nat. Cell Biol.* **10**, 93-101.

Koshida, S., Kishimoto, Y., Ustumi, H., Shimizu, T., Furutani-Seiki, M., Kondoh, H. and Takada, S. (2005). Integrinalpha5-dependent fibronectin accumulation for maintenance of somite boundaries in zebrafish embryos. *Dev. Cell* **8**, 587-598.

Lathia, J. D., Patton, B., Eckley, D. M., Magnus, T., Mughal, M. R., Sasaki, T., Caldwell, M. A., Rao, M. S., Mattson, M. P. and ffrench-Constant, C. (2007). Patterns of laminins and integrins in the embryonic ventricular zone of the CNS. *J. Comp. Neurol.* **505**, 630-643.

Lechler, T. and Fuchs, E. (2005). Asymmetric cell divisions promote stratification and differentiation of mammalian skin. *Nature* **437**, 275-280.

Legate, K. R., Montanez, E., Kudlacek, O. and Fassler, R. (2006). ILK, PINCH and parvin: the tIPP of integrin signalling. *Nat. Rev. Mol. Cell. Biol.* **7**, 20-31.

Li, S., Harrison, D., Carbonetto, S., Fassler, R., Smyth, N., Edgar, D. and Yurchenco, P. D. (2002). Matrix assembly, regulation, and survival functions of laminin and its receptors in embryonic stem cell differentiation. *J. Cell Biol.* **157**, 1279-1290.

Lien, W. H., Klezovitch, O., Fernandez, T. E., Delrow, J. and Vasioukhin, V. (2006). alphaE-catenin controls cerebral cortical size by regulating the hedgehog signaling pathway. *Science* **311**, 1609-1612.

Mayer, B. W., Jr, Hay, E. D. and Hynes, R. O. (1981). Immunocytochemical localization of fibronectin in embryonic chick trunk and area vasculosa. *Dev. Biol.* **82**, 267-286.

Messier, P. E. and Auclair, C. (1973). Inhibition of nuclear migration in the absence of microtubules in the chick embryo. *J. Embryol. Exp. Morphol.* **30**, 661-671.

Messier, P. E. and Auclair, C. (1974). Effect of cytochalasin B on interkinetic nuclear migration in the chick embryo. *Dev. Biol.* **36**, 218-223.

Miner, J. H. and Yurchenco, P. D. (2004). Laminin functions in tissue morphogenesis. *Annu. Rev. Cell Dev. Biol.* **20**, 255-284.

Murciano, A., Zamora, J., Lopez-Sanchez, J. and Frade, J. M. (2002). Interkinetic nuclear movement may provide spatial clues to the regulation of neurogenesis. *Mol. Cell Neurosci.* **21**, 285-300.

Nishiuchi, R., Takagi, J., Hayashi, M., Ido, H., Yagi, Y., Sanzen, N., Tsuji, T., Yamada, M. and Sekiguchi, K. (2006). Ligand-binding specificities of laminin-binding integrins: a comprehensive survey of laminin-integrin interactions using recombinant alpha3beta1, alpha6beta1, alpha7beta1 and alpha6beta4 integrins. *Matrix Biol.* **25**, 189-197.

Norden, C., Young, S., Link, B. A. and Harris, W. A. (2009). Actomyosin is the main driver of interkinetic nuclear migration in the retina. *Cell* **138**, 1195-1208.

O'Brien, L. E., Jou, T. S., Pollack, A. L., Zhang, Q., Hansen, S. H., Yurchenco, P. and Mostov, K. E. (2001). Rac1 orientates epithelial apical polarity through effects on basolateral laminin assembly. *Nat. Cell Biol.* **3**, 831-838.

Parsons, J. T. (2003). Focal adhesion kinase: the first ten years. *J. Cell Sci.* **116**, 1409-1416.

Parsons, M. J., Pollard, S. M., Saude, L., Feldman, B., Coutinho, P., Hirst, E. M. and Stemple, D. L. (2002). Zebrafish mutants identify an essential role for laminins in notochord formation. *Development* **129**, 3137-3146.

Pollard, S. M., Parsons, M. J., Kamei, M., Kettleborough, R. N., Thomas, K. A., Pham, V. N., Bae, M. K., Scott, A., Weinstein, B. M. and Stemple, D. L. (2006). Essential and overlapping roles for laminin alpha chains in notochord and blood vessel formation. *Dev. Biol.* **289**, 64-76.

Ryan, M. C., Lee, K., Miyashita, Y. and Carter, W. G. (1999). Targeted disruption of the LAMA3 gene in mice reveals abnormalities in survival and late stage differentiation of epithelial cells. *J. Cell Biol.* **145**, 1309-1323.

Sakai, T., Larsen, M. and Yamada, K. M. (2003). Fibronectin requirement in branching morphogenesis. *Nature* **423**, 876-881.

Sauer, F. C. (1935). Mitosis in the neural tube. *J Comp. Neurol.* **62**, 377-405.

Semina, E. V., Bosenko, D. V., Zinkevich, N. C., Soules, K. A., Hyde, D. R., Vihtelic, T. S., Willer, G. B., Gregg, R. G. and Link, B. A. (2006). Mutations in laminin alpha 1 result in complex, lens-independent ocular phenotypes in zebrafish. *Dev. Biol.* **299**, 63-77.

Shimada, A., Yabusaki, M., Niwa, H., Yokoi, H., Hatta, K., Kobayashi, D. and Takeda, H. (2008). Maternal-zygotic medaka mutants for fgfr1 reveal its essential role in the migration of the axial mesoderm but not the lateral mesoderm. *Development* **135**, 281-290.

Siller, K. H. and Doe, C. Q. (2009). Spindle orientation during asymmetric cell division. *Nat. Cell Biol.* **11**, 365-374.

Smyth, N., Vatansever, H. S., Murray, P., Meyer, M., Frie, C., Paulsson, M. and Edgar, D. (1999). Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. *J. Cell Biol.* **144**, 151-160.

Tamai, H., Shinohara, H., Miyata, T., Saito, K., Nishizawa, Y., Nomura, T. and Osumi, N. (2007). Pax6 transcription factor is required for the interkinetic nuclear movement of neuroepithelial cells. *Genes Cells* **12**, 983-996.

Toyoshima, F. and Nishida, E. (2007). Integrin-mediated adhesion orients the spindle parallel to the substratum in an EB1- and myosin X-dependent manner. *EMBO J.* **26**, 1487-1498.

Toyoshima, F., Matsumura, S., Morimoto, H., Mitsushima, M. and Nishida, E. (2007). PtdIns(3,4,5)P3 regulates spindle orientation in adherent cells. *Dev. Cell* **13**, 796-811.

Tsujikawa, M., Omori, Y., Biyanwila, J. and Malicki, J. (2007). Mechanism of positioning the cell nucleus in vertebrate photoreceptors. *Proc. Natl. Acad. Sci. USA* **104**, 14819-14824.

Wittbrodt, J., Shima, A. and Schartl, M. (2002). Medaka-a model organism from the far East. *Nat. Rev. Genet.* **3**, 53-64.

Xie, Z., Sanada, K., Samuels, B. A., Shih, H. and Tsai, L. H. (2003). Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. *Cell* **114**, 469-482.

Xie, Z., Moy, L. Y., Sanada, K., Zhou, Y., Buchman, J. J. and Tsai, L. H. (2007). Cep120 and TACCs control interkinetic nuclear migration and the neural progenitor pool. *Neuron* **56**, 79-93.

Yang, D., Bierman, J., Tarumi, Y. S., Zhong, Y. P., Rangwala, R., Proctor, T. M., Miyagoe-Suzuki, Y., Takeda, S., Miner, J. H., Sherman, L. S. et al. (2005). Coordinate control of axon defasciculation and myelination by laminin-2 and -8. *J. Cell Biol.* **168**, 655-666.

Yurchenco, P. D., Amenta, P. S. and Patton, B. L. (2004). Basement membrane assembly, stability and activities observed through a developmental lens. *Matrix Biol.* **22**, 521-538.