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PATJ regulates directional migration of mammalian epithelial cells

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Directional migration is important in wound healing by epithelial cells. Recent studies have shown that polarity proteins such as mammalian Partitioning-defective 6 (Par6), atypical protein kinase C (aPKC) and mammalian Discs large 1 (Dlg1) are crucial not only for epithelial apico-basal polarity, but also for directional movement. Here, we show that the protein associated with Lin seven 1 (PALS1)-associated tight junction protein (PATJ), another evolutionarily conserved polarity protein, is also required for directional migration by using a wound-induced migration assay. In addition, we found that aPKC and Par3 localize to the leading edge during migration of epithelia and that PATJ regulates their localization. Furthermore, our results show that microtubule-organizing centre orientation is disrupted in PATJ RNA interference (RNAi) MDCKII (Madin–Darby canine kidney II) cells during migration. Together, our data indicate that PATJ controls directional migration by regulating the localization of aPKC and Par3 to the leading edge. The migration defect in PATJ RNAi cells seems to be due to the disorganization of the microtubule network induced by mislocalization of polarity proteins.

Keywords: epithelial migration; PALS1-associated tight junction protein; Par3 protein

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INTRODUCTION

The establishment of polarity is crucial for the correct function of many cell types. Epithelial cells have distinct apico-basal polarity that is crucial for its barrier function (Tsukita *et al*, 2001; Matter & Balda, 2003). Neurons polarize to develop dendrites and one long axon required for neurotransmission (Craig & Banker, 1994; Shi *et al*, 2003). Cells such as fibroblasts and astrocytes polarize in the direction of cell movement (Nobes & Hall, 1999; Etienne-Manneville & Hall, 2001). In spite of the diverse nature of polarity in different organisms, recent studies suggest that certain proteins are conserved in this process.

The Par3–Par6–aPKC complex (Par for Partitioning-defective; aPKC for atypical protein kinase C), originally identified as polarity proteins required to establish anterior–posterior polarity of the *Caenorhabditis elegans* embryo, is crucial for apico-basal polarity of *Drosophila* and mammalian epithelial cells (Macara, 2004). In migrating astrocytes, the Par6–aPKC complex is required for directional migration (Etienne-Manneville & Hall, 2001). The correct localization of the Par6–Par3–aPKC complex at the tip of the growing axon in the developing hippocampal neuron is crucial for its polarized axonal growth (Shi *et al*, 2003). Furthermore, a recent study showed that Discs large 1 (Dlg1), which has a role in *Drosophila* epithelial apico-basal polarity, is required for the polarization of migrating astrocytes (Etienne-Manneville *et al*, 2005).

Although the function of polarity proteins, including the Par6–aPKC complex and Dlg1, in directional migration has been well studied, little is known about the involvement of other evolutionarily conserved polarity proteins, such as Crumbs (CRB), protein associated with Lin seven 1 (PALS1, also known as Stardust) and PALS1-associated tight junction protein (PATJ), in directional movement. CRB–PALS1–PATJ is an evolutionarily conserved multiprotein complex that is important in the determination of epithelial polarity (Shin *et al*, 2006). In our previous study, we generated Madin–Darby canine kidney II (MDCKII) cells with a reduction in PATJ expression by using RNA interference (RNAi) and showed that PATJ regulates tight junction formation and epithelial polarity (Shin *et al*, 2005). However, the function of PATJ in epithelial migration is still unknown. In this study, we undertook in-depth analysis of PATJ function in migration by using previously generated PATJ RNAi MDCKII cells.

RESULTS AND DISCUSSION

PATJ localizes to the leading edge

To study epithelial migration, we used an *in vitro* wound-healing model (Nobes & Hall, 1999; Fenteany *et al*, 2000). Migration was induced after disruption of the confluent monolayer of MDCKII cells by using a scratch. Before investigating the function of PATJ in epithelial migration, we studied the localization of PATJ in migrating epithelia. Migrating MDCKII cells were fixed and PATJ,

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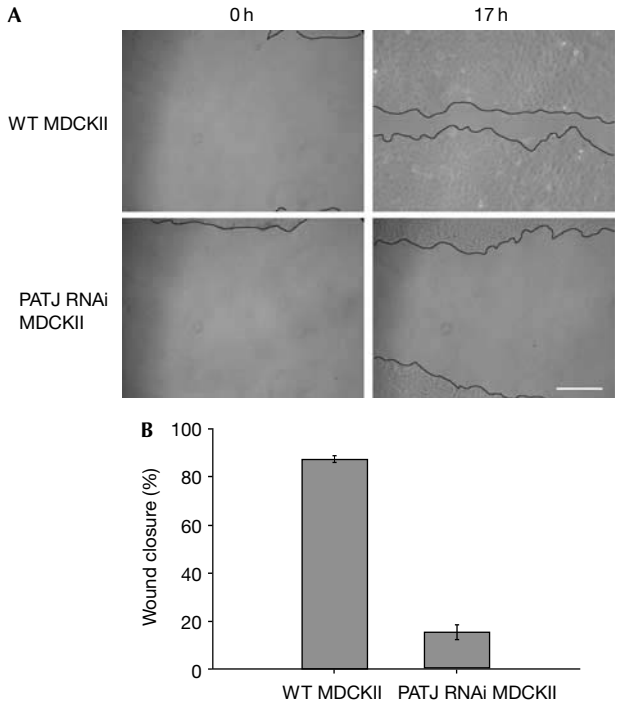

Fig 1 | PATJ is required for the migration of epithelial cells. (**A**) Wound-closure migration assays were performed with wild-type (WT) and PATJ RNAi, MDCKII cells. Wound areas are outlined. Scale bar, 100 μm. (**B**) Quantification of epithelial migration after wounding. Wild-type MDCKII cells show 87.4±1.4% wound closure, whereas PATJ RNAi MDCKII cells show 15.4±3.2% wound closure 17 h after wounding; $P<0.0001$, unpaired *t*-test. Relative areas of the wound were measured in three independent experiments. Standard deviations are shown as error bars ($n=3$). MDCKII, Madin–Darby canine kidney II; PALS1, protein associated with Lin seven 1; Par3, partitioning-defective 3; PATJ, PALS1-associated tight junction protein; RNAi, RNA interference.

as well as actin, localized 6 h after the scratch. At the front line of migrating epithelia, PATJ localized to the leading edge along the actin cortex (supplementary Fig 1A online); however, apical polarity proteins, including CRB3 and GP135, were not localized to the leading edge, suggesting specific localization of PATJ to the leading edge (supplementary Fig 1B online).

PATJ is required in wound healing

We found that PATJ localized to the leading edge of migrating epithelia; therefore, we investigated next the function of PATJ in directional migration. The wound-healing migration assay was performed by using previously developed PATJ RNAi MDCKII cells (Shin *et al*, 2005). As shown in Fig 1, 17 h after wounding wild-type MDCKII cells showed 87.4±1.4% wound closure; however, PATJ RNAi MDCKII cells showed only 15.4±3.2% wound closure (Fig 1). We were able to rescue this migration defect by re-expressing wild-type PATJ in the knockout cells (see below).

PATJ regulates the reorientation of MTOC

One of the characteristics of polarized migrating cells is the reorientation of the microtubule-organizing centre (MTOC) to point in the direction of migration (Palazzo *et al*, 2001). To study the reorientation of MTOC in migrating epithelia, we assessed the reorientation of MTOC by immunostaining with γ-tubulin to visualize the MTOC 6 h after wounding with wild-type, PATJ RNAi and enhanced green fluorescent protein (EGFP)–PATJ full-length rescue cells (supplementary Fig 2 online). In wild-type MDCKII cells, 65.7±1.3% of front-row cells had MTOC in the direction of migration 6 h after wounding (supplementary Fig 2 online); however, only 29.9±2.1% of PATJ RNAi cells showed the correct reorientation of MTOC (supplementary Fig 2B,D online). EGFP–PATJ full-length cells showed significant rescue in the MTOC reorientation, with 50.0±2.0% of cells having MTOC in the direction of migration (supplementary Fig 2C,D online). These data suggest that PATJ controls the reorientation of MTOC during epithelial migration.

PATJ controls aPKC and Par3 localization in migration

In our previous study, we found that the correct localization of Par3 to the tight junction is disrupted in the absence of PATJ expression in monolayers of MDCKII cells (Shin *et al*, 2005). Furthermore, in astrocytes, the Par6–aPKC complex is important in polarized migration (Etienne-Manneville & Hall, 2001; Etienne-Manneville *et al*, 2005). On the basis of these findings, we generated a hypothesis that the Par complex localizes to the leading edge of migrating MDCKII cells and that PATJ is responsible for the correct localization of this complex. To test this hypothesis, we performed a wound-healing migration assay followed by immunostaining for aPKC and Par3. As shown in Fig 2A, in wild-type MDCKII cells, aPKC and Par3 colocalized with PATJ to the leading edge of migrating cells. Both Par3 and aPKC failed to localize to the leading edge of migrating MDCKII cells when PATJ expression was knocked down by RNAi, suggesting that PATJ regulates aPKC and Par3 localization to the leading edge during migration (Fig 2B,D). To confirm these results, EGFP–PATJ rescue cells were immunostained for aPKC and Par3 6 h after wounding. As expected, the mislocalization of aPKC and Par3 was significantly reversed (Fig 2C,D).

Next, we investigated the cytoskeleton network in migrating epithelia, as actin and microtubule networks are crucial for directional migration. Fig 3 shows actin and microtubules in wild-type and PATJ RNAi MDCKII cells. The organization of actin does not seem to be significantly affected in PATJ RNAi cells (Fig 3A,B). Furthermore, the localization of cortactin at the leading edge is not affected by the reduction of PATJ expression, suggesting that the formation of lamellipodia is not significantly altered in PATJ RNAi MDCKII cells (supplementary Fig 3 online). By contrast, microtubule organization was disrupted in PATJ RNAi MDCKII cells compared with the wild-type MDCKII cells, which showed elongation of microtubules in the direction perpendicular to the wound (Fig 3A,B). Moreover, microtubule organization was assessed in PATJ rescue MDCKII cells to confirm that these defects are caused by the reduced expression of PATJ. As shown in Fig 3C, the elongation of microtubules towards the leading edge is partially restored. These results indicate that the defect in migration in PATJ RNAi MDCKII cells is, in part, due to the disorganization of microtubules, which could be related to the mislocalization of aPKC and Par3.

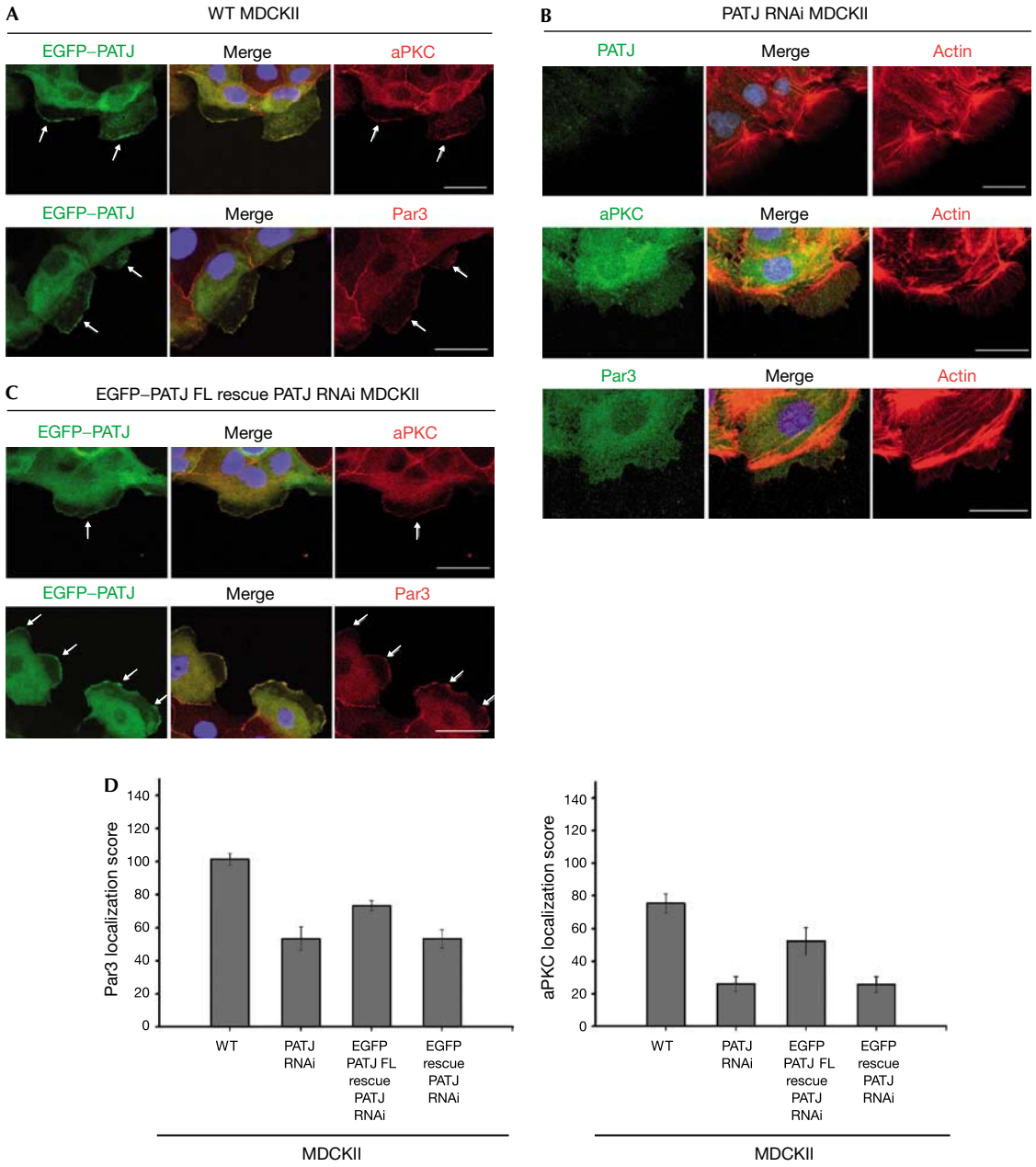

Fig 2 | PATJ is required for the localization of aPKC and Par3 to the leading edge of migrating epithelial cells. PATJ, aPKC and Par3 were immunostained 6 h after wounding in wild-type (WT; **A**), PATJ RNAi (**B**) and EGFP–PATJ full-length (FL) rescue PATJ RNAi MDCKII cells (**C**). Arrows show the localization of PATJ, aPKC and Par3 to the leading edge. Nuclei are shown in blue. Scale bar, 10 μm. (**D**) Quantification of aPKC and Par3 localization during migration. The score of Par3 localization decreases from 101.3±3.5 (WT MDCKII) to 53.5±6.7 (PATJ RNAi MDCKII cells); $P<0.0005$, unpaired *t*-test. The score of aPKC localization decreases from 75.3±5.7 (WT MDCKII) to 26.0±4.6 (PATJ RNAi MDCKII cells); $P<0.0005$, unpaired *t*-test. Standard deviations are shown as error bars ($n=3$). aPKC, atypical protein kinase C; EGFP, enhanced green fluorescent protein; MDCKII, Madin–Darby canine kidney II; PALS1, protein associated with Lin seven 1; Par3, Partitioning-defective 3; PATJ, PALS1-associated tight junction protein; RNAi, RNA interference.

The molecular mechanisms by which aPKC and Par3 regulate microtubule orientation are still unclear. Studies from other research groups indicate a role for the dynein motor complex, as this complex is known to control MTOC reorientation by regulating the plus ends of microtubules possibly through aPKC (Koonce *et al*, 1999; Etienne-Manneville & Hall, 2001; Palazzo *et al*, 2001). In addition, a recent study showed that the Par6–aPKC complex interacts directly with and phosphorylates glycogen synthase kinase-3β, and phosphorylated glycogen synthase kinase-3β induces the interaction of adenomatous

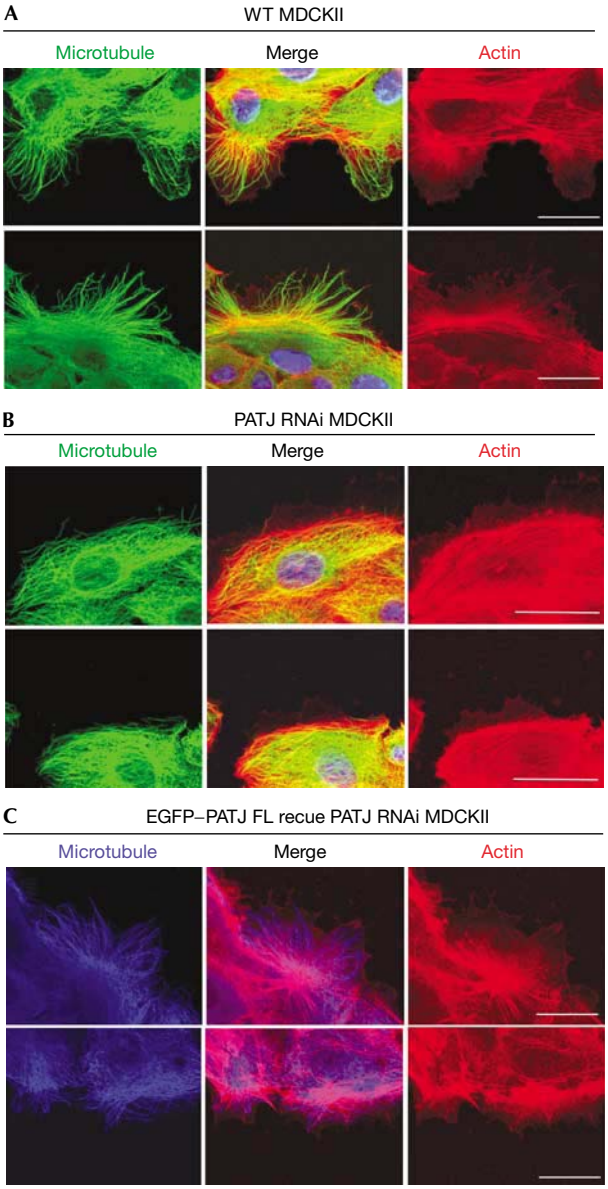

Fig 3 | Organization of microtubules is disrupted in PATJ RNAi MDCKII cells during wound healing. (**A**,**B**) Microtubules (green) and actin (red) were visualized in wild-type (WT; **A**) and PATJ RNAi (**B**) MDCKII cells 6 h after wounding. Merged images are shown in the middle column. Scale bars, 10 μm. (**C**) Microtubules (blue) and actin (red) were visualized in EGFP–PATJ full-length (FL) rescue PATJ RNAi MDCKII cells. Scale bars, 10 μm. EGFP, enhanced green fluorescent protein; MDCKII, Madin–Darby canine kidney II; PALS1, protein associated with Lin seven 1; PATJ, PALS1-associated tight junction protein; RNAi, RNA interference.

polyposis coli with the microtubule network (Etienne-Manneville & Hall, 2003). Adenomatous polyposis coli could regulate microtubule dynamics directly or through interactions with the dynein complex (Berrueta *et al*, 1999; Nakamura *et al*, 2001; Zumbrunn *et al*, 2001).

Structure–function analysis of PATJ

We were able to show that reintroducing wild-type PATJ into PATJ knockdown cells (Shin *et al*, 2005) rescued their migration defect (Fig 4A). This allowed us to perform a structure–function analysis using the mutant PATJ rescue system. Three mutants of EGFP–PATJ, ΔL27, ΔPDZ1–5 and ΔPDZ6–10 (PDZ for PSD95/DLG/ZO1), were introduced into PATJ RNAi MDCKII cells, followed by wound-healing assays. As shown in Fig 4A,B, the PATJ mutants were not able to rescue the migration defects as effectively as wild-type PATJ. In particular, deletions of the PDZ domains had a marked effect on PATJ function. To investigate how these domains are involved in migration, EGFP–PATJ mutants were immunoprecipitated from rescue MDCKII cells, followed by western blotting for Par3. As shown in Fig 4C, full-length EGFP–PATJ successfully interacted with Par3. Mutants without the L27, PDZ1–5 or PDZ6–10 domains were not able to interact with Par3, suggesting that these domains are involved in the interaction between PATJ and Par3. In addition, these mutants could not recruit Par3 or aPKC to the leading edge (supplementary Fig 4 online). However, the targeting of these PATJ mutants to the leading edge was also defective in migrating cells (Fig 4D,E). Thus, it is not clear if the inability of the PATJ mutants to rescue migration and protein targeting is due to their inability to interact with Par3 or secondary to defective transport of these mutants. It is possible that the targeting of PATJ and Par3 is mutually dependent, such that a PATJ protein that cannot bind to Par3 cannot target properly in cells. It is also possible that these PATJ mutants cannot bind to Par3 owing to their defective transport.

Our previous study showed that the L27 domain of PATJ interacts with the L27 domain of PALS1, and that PALS1 binds to the Par6–Par3–aPKC complex in MDCK cells (Hurd *et al*, 2003; Wang *et al*, 2004). Indeed, our results show that PALS1 is also required for the correct migration of epithelial cells (supplementary Fig 5 online). However, as shown in our previous studies, the loss of PALS1 expression also suppresses the expression of PATJ (Straight *et al*, 2004; Shin *et al*, 2005). This makes it difficult to separate the effect of PALS1 in epithelial migration from those of PATJ. However, further studies are necessary to explain the mechanism by which PDZ1–5 and PDZ6–10 are required for the transport of PATJ and rescue of cell migration. Recent studies have suggested other scaffold proteins that bind to the PDZ domains of PATJ (Wells *et al*, 2006).

Although previous studies with astrocytes suggested a role for Par6–aPKC and not Par3 in migration, more recent studies suggest the functional importance of Par3 in polarity, especially the activation of aPKC, in two different types of epithelial cells—keratinocytes and MDCKII cells (Chen & Macara, 2005; Mertens *et al*, 2005). Our study investigates the migration of epithelial cells and shows the strong localization of Par3 to the leading edge; therefore, we argue that Par3 and aPKC might be important in the migration of epithelial cells.

One of the interesting questions raised by this study is how PATJ initially localizes to the leading edge of migrating epithelia. A recent study indicates that engagement of integrins on wounding recruits and activates Cell division cycle 42 (Cdc42), and activated Cdc42 targets the Par6–aPKC complex to the leading edge (Etienne-Manneville & Hall, 2001). Interestingly, our study suggests that PATJ localization is required for the correct targeting of aPKC and Par3 to the leading edge. Previous results

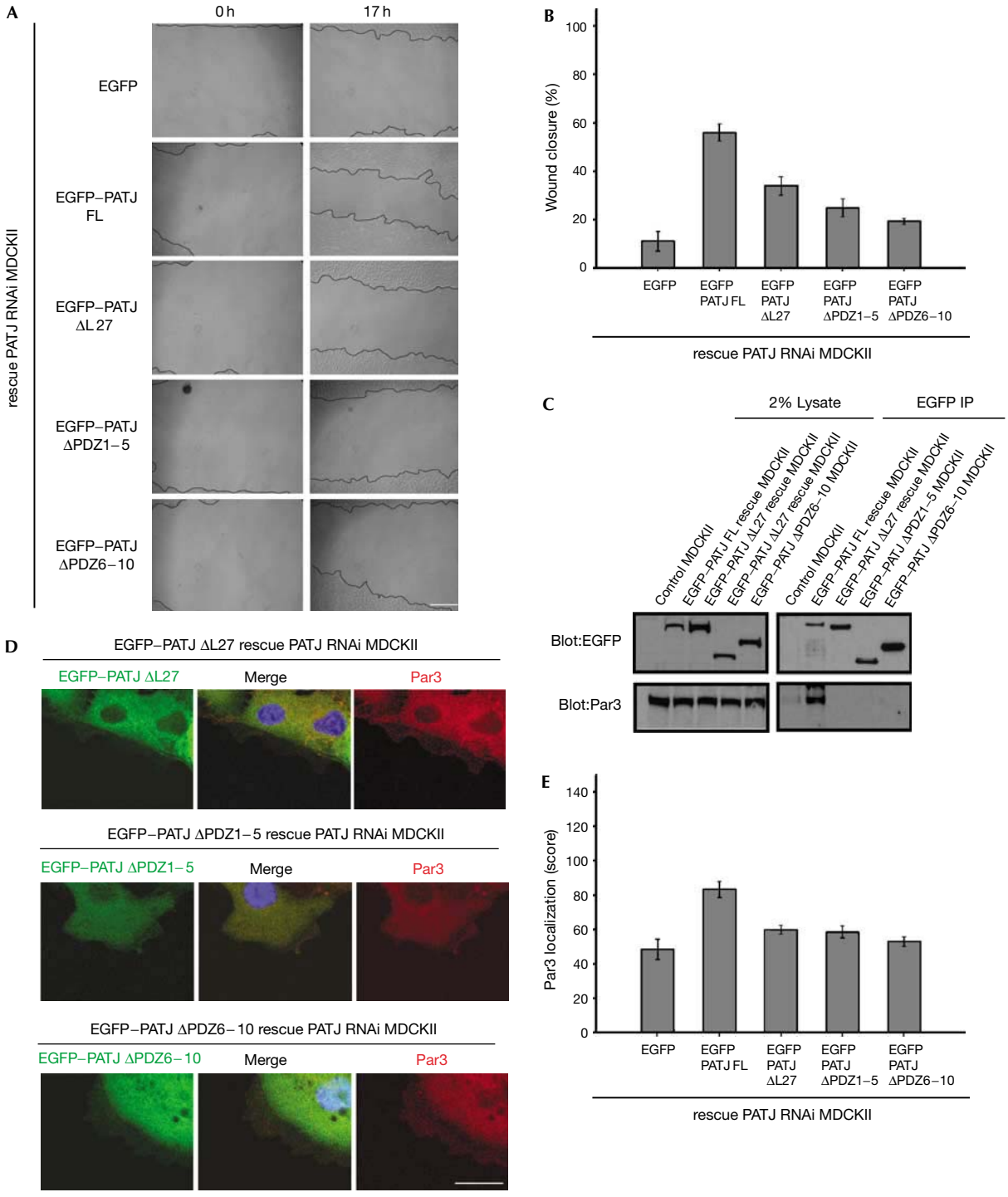

Fig 4 | Structure–function analysis of PATJ. (**A**) Wound closure migration assays were performed with EGFP, EGFP–PATJ full length (FL), EGFP-PATJ ΔL27, EGFP–PATJ ΔPDZ1–5 and EGFP–PATJ ΔPDZ6–10 rescue PATJ RNAi MDCKII cells. Wound areas are outlined. Scale bar, 100 μm. (**B**) Quantification of epithelial migration with mutant PATJ rescue cell lines after wounding. Relative areas of the wound were measured in three independent experiments. Standard deviations are shown as error bars ($n = 3$). Results as percentage wound closure at 17 h are as follows: EGFP rescue PATJ RNAi: 11.2 ± 4.1%; EGFP PATJ FL rescue PATJ RNAi: 56.1 ± 3.6%; EGFP PATJ ΔL27 rescue PATJ RNAi: 33.9 ± 3.8%; EGFP PATJ ΔPDZ1–5 rescue PATJ RNAi: 24.9 ± 3.8%; EGFP PATJ ΔPDZ6–10 rescue PATJ RNAi: 19.3 ± 1.2%. (**C**) Immunoprecipitation of EGFP–PATJ mutant proteins followed by western blot for Par3. (**D**) Par3 was immunostained 6 h after wounding with EGFP–PATJ mutant rescue PATJ RNAi cell lines. Scale bar, 10 μm. (**E**) Quantification of Par3 localization during migration of EGFP–PATJ mutant rescue PATJ RNAi MDCKII cells. EGFP, enhanced green fluorescent protein; MDCKII, Madin–Darby canine kidney II; PALS1, protein associated with Lin seven 1; Par3, Partitioning-defective 3; PATJ, PALS1-associated tight junction protein; RNAi, RNA interference.

have suggested that Cdc42 can enhance the interaction between the PATJ-binding protein, PALS1 and Par6 (Hurd *et al*, 2003; Wang *et al*, 2004). However, this does not explain what targets PATJ in the first place. CRB3, the principal apical binding partner for PALS1 and PATJ, does not seem to localize to the leading edge of migrating cells (supplementary Fig 1B online) and we found that CRB3 knockdown does not affect epithelial migration (supplementary Fig 6 online). One can hypothesize that other transmembrane proteins such as claudin, occludin and junctional adhesion molecule might have a role in the localization of PATJ to the leading edge, as PATJ interacts with many proteins directly or indirectly. For example, junctional adhesion molecule interacts with Zonula Occludens (ZO) 1 and occludin (Bazzoni *et al*, 2000; Ebnet *et al*, 2000). Occludin interacts with ZO3 by means of ZO1, and ZO3 and claudin directly bind to PATJ at the tight junction (Roh *et al*, 2002). Thus, further work is necessary to understand what is upstream of PATJ in migration and how PATJ localizes to the leading edge.

Speculation

On the basis of our findings, we propose a model to explain how PATJ regulates the migration of epithelial cells. PATJ localizes to the leading edge of migrating epithelia after wounding. PATJ then assists in recruiting aPKC and Par3 to the leading edge. This hypothesis is not surprising because recent studies have shown the direct interaction between CRB3–PALS1–PATJ and Par6–Par3–aPKC in mammals and *Drosophila* (Hurd *et al*, 2003; Nam & Choi, 2003; Lemmers *et al*, 2004). Once aPKC and Par3 localize to the leading edge, they might control the microtubule and MTOC reorientation, which is crucial for directional migration.

METHODS

Cell lines, antibodies and immunostaining. PATJ RNAi and EGFP–PATJ-expressing MDCKII cell lines were generated as described previously (Shin *et al*, 2005). EGFP–PATJ mutant rescue MDCKII cells were generated with the same vector system as that used in EGFP–PATJ full-length rescue MDCKII cells described in a previous study (Shin *et al*, 2005). CRB3 RNAi MDCKII cell lines were generated as previously described (Fan *et al*, 2004). PALS1 RNAi MDCKII cell lines were generated using pSilencer 2.1-U6 hygro (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The following sequence of PALS1 was used for the target of small interference RNA (siRNA) designed for knockdown of PALS1 expression: 5′-GGGGATATACTTCATATCA-3′. PATJ and Par3 antisera were generated as described previously (Makarova *et al*, 2003). Antibody to aPKC was obtained from Upstate (Lake Placid, NY, USA). Rhodamine–phalloidin was used for visualization of actin (Invitrogen, Carlsbad, CA, USA). Mouse γ- and α-tubulin antibodies were obtained from Sigma-Aldrich (St Louis, MO, USA). Immunostaining was performed as described previously (Shin *et al*, 2005). Anti-cortactin, clone 4F11, Alexa Fluor 488 conjugate was obtained from Upstate. All images were visualized using a Nikon Eclipse TE2000U (Melville, NY, USA) fluorescence microscope and an Olympus FV500 (Melville) confocal microscope, and analysed with MetaMorph software (Downingtown, PA, USA), Olympus Fluoview Ver.1.3b (Melville) Viewer and Adobe Photoshop software.

Wound-healing assay. Wound-healing assays for MDCK cells have been reported previously (Fenteany *et al*, 2000). For wound-closure experiments, relative wound areas were measured using ImageJ software (NIH, Bethesda, MD, USA) 17 h after wounding. For immunostaining of PATJ, Par3, aPKC and MTOC, cells were fixed 6 h after initial wounding for further analysis.

Statistical analysis. For wound-closure assays, wound areas were measured in triplicate. Means and standard deviation were calculated and plotted using Sigma Plot and Microsoft Excel software. For MTOC reorientation assays, a minimum of 100 cells from the front row of migrating epithelial sheets were counted, and an MTOC in the forward-facing quadrant was counted as positive (Etienne-Manneville & Hall, 2001). Experiments were repeated three times and statistical analyses were performed with the same software as described above. For localization of Par3 and aPKC, 100 ruffling cells were examined. Cells with complete, partial or no localization of Par3 and aPKC to the leading edge were scored as 2, 1 or 0, respectively. Each experiment was repeated in triplicate.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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