

# The LIM protein Ajuba influences p130Cas localization and Rac1 activity during cell migration

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Cell migration requires extension of lamellipodia that are stabilized by formation of adhesive complexes at the leading edge. Both processes are regulated by signaling proteins recruited to nascent adhesive sites that lead to activation of Rho GTPases. The Ajuba/Zyxin family of LIM proteins are components of cellular adhesive complexes. We show that cells from Ajuba null mice are inhibited in their migration, without associated abnormality in adhesion to extracellular matrix proteins, cell spreading, or integrin activation. Lamellipodia

production, or function, is defective and there is a selective reduction in the level and tyrosine phosphorylation of FAK, p130Cas, Crk, and Dock180 at nascent focal complexes. In response to migratory cues Rac activation is blunted in Ajuba null cells, as detected biochemically and by FRET analysis. Ajuba associates with the focal adhesion-targeting domain of p130Cas, and rescue experiments suggest that Ajuba acts upstream of p130Cas to localize p130Cas to nascent adhesive sites in migrating cells thereby leading to the activation of Rac.

## Introduction

Cell migration is important during early development, adult homeostasis, inflammatory responses to infection, wound healing, and pathologically during tumor invasion and metastasis. To migrate, a cell must coordinate a number of different inputs into appropriate cellular responses. First, a cell must sense the direction to move and orient itself (i.e., polarize) so as to effect directed migration. Lamellipodia and filopodia are then extended from the leading edge in the direction of migration. New adhesions to the ECM are initiated at the leading edge, and these serve to pull the cell forward (Beningo et al., 2001). The actomyosin contractile machinery, as well as microtubules, must then pull the trailing part of the cell whereas sites of adhesion at the rear of the cell are disassembled (Dunn, 1980; Nobes and Hall, 1999). Central to this process is regulated adhesion and de-adhesion of cells to surrounding ECM.

Adhesion of migrating cells to ECM is highly dynamic. Engagement of integrin receptors at the leading edge results in their clustering and formation of focal complexes (Eklblom et al., 1986). These small focal complexes subsequently mature into larger focal adhesions which serve to link the cellular actin cytoskeleton to sites of adhesion to the ECM. Many cytosolic proteins, such as talin, paxillin,  $\alpha$ -actinin, FAK, c-Src, and p130Cas are recruited to newly forming adhesive complexes

and play structural and/or signaling roles (Zamir et al., 1999). Precisely how and when these proteins are recruited is not completely understood and are an area of active investigation.

Many proteins at leading edge focal complexes or focal adhesions are tyrosine phosphorylated in response to migration cues. For example, after integrin activation, FAK autophosphorylates on Tyr-397 (Cary et al., 1996), which serves as a binding site for c-Src family members and p130Cas. c-Src, and possibly FAK, then phosphorylate p130Cas in response to integrin clustering (Petch et al., 1995; Vuori et al., 1996). Tyrosine phosphorylated p130Cas serves to recruit the SH2/SH3 adaptor protein Crk into the evolving adhesive complex (Sakai et al., 1994). The p130Cas–Crk complex leads to activation of the small Rho GTPase, Rac1, via an interaction with the DOCK180–ELMO guanine nucleotide exchange factor (GEF; Kiyokawa et al., 1998a,b; Brugnera et al., 2002). Rac1 is a prominent regulator of cell migration through activation of the Arp2/3 complex (Miki et al., 2000) and de novo branched actin filaments at the leading edge resulting in lamellipodia. Rac1 also influences the formation of nascent focal complexes at the leading edge (Nobes and Hall, 1999).

The p130Cas–Crk–DOCK180–ELMO-signaling complex is highly conserved across species. Highlighting their central role in the regulation of cell migration, mutations in DOCK180, ELMO, and Rac1 homologues in both *C. elegans* and *Drosophila* all result in phenotypes with defects in cell migration and phagocytosis (Nolan et al., 1998; Reddien and Horvitz, 2000).

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Abbreviations used in this paper: ES, embryonic stem; GEF, guanine nucleotide exchange factor; wt, wild-type.

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Cytosolic LIM domain-containing proteins are also components of cellular adhesive complexes. Examples of LIM proteins at these sites include paxillin, PINCH, and members of the Zyxin/Ajuba family (Turner et al., 1990; Crawford et al., 1992; Tu et al., 1999). The Zyxin/Ajuba family of LIM proteins is characterized by three tandem, homologous LIM domains at the COOH terminus (LIM region) and divergent proline rich NH<sub>2</sub> termini (PreLIM region). The members of this family include: Ajuba (Goyal et al., 1999), LIMD1 (Kiss et al., 1999), LPP (Petit et al., 1996), Trip6 (Wang and Gilmore, 2001), and Zyxin (Crawford et al., 1992). What role these LIM proteins play in cell adhesion function and migration is not completely understood. Zyxin, LPP, and Trip6, but not Ajuba and LIMD1, contain FPPPP binding motifs for EVH1 domains present in Ena/VASP proteins and may serve to recruit these proteins to the leading edge (Renfranz and Beckerle, 2002). Zyxin and LPP, but not Ajuba and LIMD1, also bind  $\alpha$ -actinin and may contribute to the bundling of actin fibers (Drees et al., 1999). In epithelial cells Ajuba is recruited to newly forming cadherin-based cell-cell contacts, through an interaction with  $\alpha$ -catenin (Marie et al., 2003). Ajuba also associates directly with filamentous actin, and thus, contributes to linking and/or strengthening of epithelial cell-cell adhesive receptors to the actin cytoskeleton (Marie et al., 2003). All Zyxin/Ajuba family members contain nuclear export signals in their respective PreLIM regions and shuttle in/out of the nucleus, suggesting that they may also function as transducers of signals from adhesive receptors (Nix and Beckerle, 1997; Kanungo et al., 2000; Wang and Gilmore, 2001; Petit et al., 2003).

Genetic manipulation of many focal adhesion proteins, such as FAK, p130Cas, Src kinases, and paxillin has been shown to result in defects in cell adhesion, cell spreading, and migration (Honda et al., 1998; Xu et al., 1998; Sieg et al., 1999; Monkley et al., 2000; Hagel et al., 2002). Zyxin/Ajuba LIM proteins have also been implicated in cell motility regulation. Blocking the interaction of Zyxin with  $\alpha$ -actinin displaces it from its normal subcellular location and inhibits random cell migration and spreading (Drees et al., 1999). Overexpression of Trip6 in fibroblasts inhibits cell motility (Yi et al., 2002).

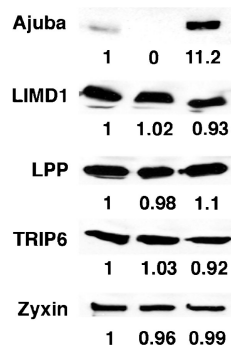


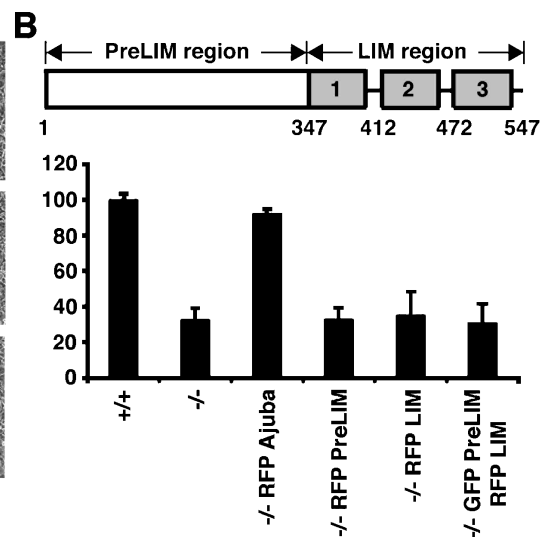
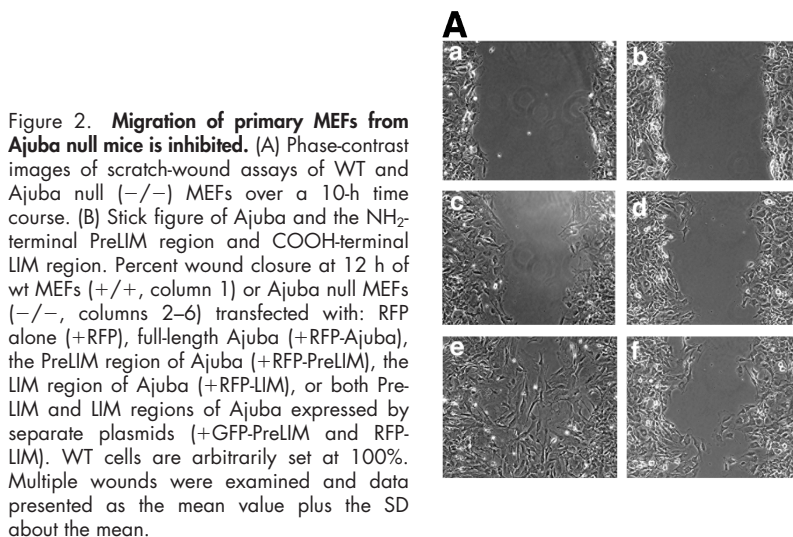
Figure 1. **Quantitative Western blot analyses of Zyxin/Ajuba family members.** WT (+/+, lane 1), Ajuba null (-/-, lane 2), and Ajuba rescued Ajuba null MEFs (-/- Rescue, lane 3). Equal amounts of protein were loaded in each lane. Densitometry was performed and the level of each protein is displayed below each lane. The wt sample is arbitrarily set at 1.

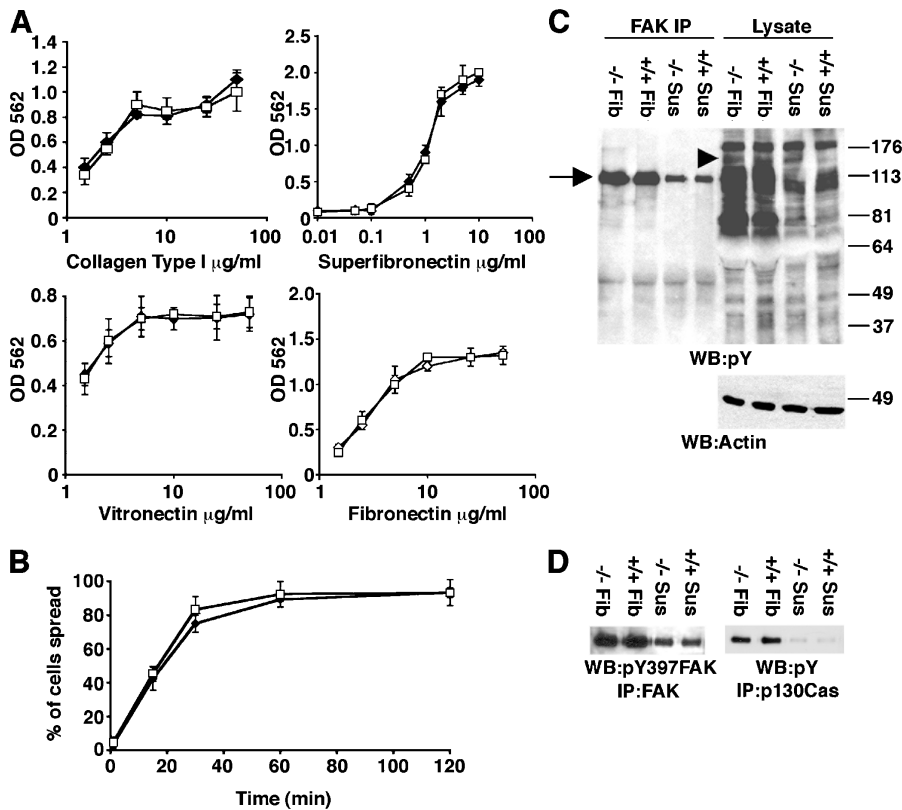
How these LIM proteins regulate cell motility is not understood, however. Here, we describe the generation and characterization of knockout mice lacking Ajuba. Ajuba was found to influence cell migration without affecting cell adhesion and spreading. We suggest that Ajuba regulates cell motility by activating Rac through regulating the recruitment of p130Cas to nascent adhesion sites.

## Results

### Primary fibroblasts from Ajuba null mice exhibit reduced migration

In mouse embryonic stem (ES) cells, exon 1 of the Ajuba gene was replaced with a Neomycin cassette (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200406083/DC1>). Two different ES clones were used to generate chimeric mice and mice heterozygous for the targeted allele were recovered. Interbreeding between heterozygous mice resulted in the birth of mice of all three genotypes in the expected Mendelian ratios. PCR analysis and Southern blot analysis of genomic DNA (Fig. S1 B) from adult tails revealed that Ajuba null mice were viable and reached adulthood without any obvious phenotypes. Ajuba null mice were fertile. Western blot analyses of cell lysates from primary fibroblasts (MEFs) derived from E13.5-E15.5 Ajuba null or littermate wild-type (wt) control embryos demonstrated that there was no detectable Ajuba protein made





**Figure 3. Ajuba null MEFs adhere to ECM proteins, spread, and activate integrin receptors normally.** (A) Cell adhesion. WT (◆) and Ajuba null (□) MEFs were allowed to adhere for 1 h to plates coated with increasing concentration of the indicated ECM protein. (B) Cell spreading. WT (◆) and Ajuba null (□) MEFs were allowed to adhere and spread on fibronectin-coated coverslips over 2 h. Videos of multiple cells were obtained and data extrapolated at the indicated time points. Results are the mean values plus the SD about the mean. (C) Serum-starved wt (+/+) and Ajuba null (-/-) cells were allowed to adhere to fibronectin-coated plates for 1 h. (D) FAK and p130Cas were immunoprecipitated from cells in suspension (Sus) or bound to fibronectin (Fib) and bound products Western blotted with anti-pY397FAK or anti-phosphotyrosine antibodies, respectively. Actin Western blots are loading controls.

in Ajuba null cells, and that the expression of related Ajuba/Zyxin family members were not significantly altered (Fig. 1). Stable reintroduction of Ajuba into Ajuba null cells also did not affect the levels of related LIM proteins (Fig. 1).

To determine whether Ajuba played a role in cell migration, early passage MEFs from Ajuba null embryos and wt littermates were analyzed. Scratch-wound repair assays of MEFs in culture were performed. Ajuba null MEFs displayed a marked inhibition of wound repair compared with litter-matched wt MEFs (Fig. 2 A). By 10 h after wounding, wt MEFs had virtually closed the wound, whereas MEFs from Ajuba null mice had only migrated to cover 20–30% of the wound area (Fig. 2 A). By 24 h, Ajuba null MEFs closed the wound, indicating that migration of Ajuba null cells was inhibited not blocked. In a second approach, classic Boyden chamber migration assays were performed. As in the scratch-wound assay the number of Ajuba null MEFs that migrated across the membrane was reduced by 40%, compared with wt MEFs (unpublished data).

To demonstrate that the cell migration defect was specifically due to loss of Ajuba, rescue experiments were performed. Ajuba null MEFs were transiently transfected with plasmids expressing either full-length Ajuba, the PreLIM region of Ajuba, or the LIM region of Ajuba (all fused to RFP; Fig. 2 B), or control RFP alone. Scratch-wound assays were then performed. Reintroduction of Ajuba completely reversed the migration defect in Ajuba null MEFs as RFP-Ajuba positive cells migrated at a rate similar to wt MEFs, whereas RFP negative cells exhibited the expected migratory defect (Fig. 2 B; Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200406083/DC1>). Neither PreLIM region alone, LIM region alone, or PreLIM

and LIM regions expressed together as separate plasmids were able to rescue the motility defect (Fig. 2 B). These rescue experiments demonstrated that the loss of Ajuba protein was directly responsible for the reduction in cell motility, and that the function of each major region of Ajuba, in the context of the full-length protein, was required for normal migration.

### Ajuba null MEFs adhered to ECM proteins, spread, and activate integrin receptors normally

Cell migration defects are commonly associated with abnormalities in cell–ECM adhesion and spreading (Ilic et al., 1995; Honda et al., 1998; Xu et al., 1998; Monkley et al., 2000; Hagel et al., 2002). Adhesion assays with Ajuba null MEFs on collagen, fibronectin, vitronectin, or superfibronectin were therefore performed. The number of Ajuba null MEFs adhering to the various ECM protein-coated plates was indistinguishable from wt controls (Fig. 3 A). To determine if Ajuba null cells spread normally after adhesion to ECM the rate of cell spreading, on fibronectin, was determined. Both wt and Ajuba null MEFs showed the same rate of cell spreading (Fig. 3 B; Video 2, available at <http://www.jcb.org/cgi/content/full/jcb.200406083/DC1>).

Integrin-mediated adhesion to ECM proteins activates signaling cascades that strengthen cell adhesion and influence cell migration. Tyrosine phosphorylation of FAK and p130Cas are early signals of integrin activation (Sieg et al., 1999). Phosphorylation of FAK and p130Cas in Ajuba null and wt MEFs were assessed following adherence to fibronectin. Ajuba null and wt cells exhibited the same extent of both FAK and p130Cas tyrosine phosphorylation upon integrin activation (Fig. 3, C and D).

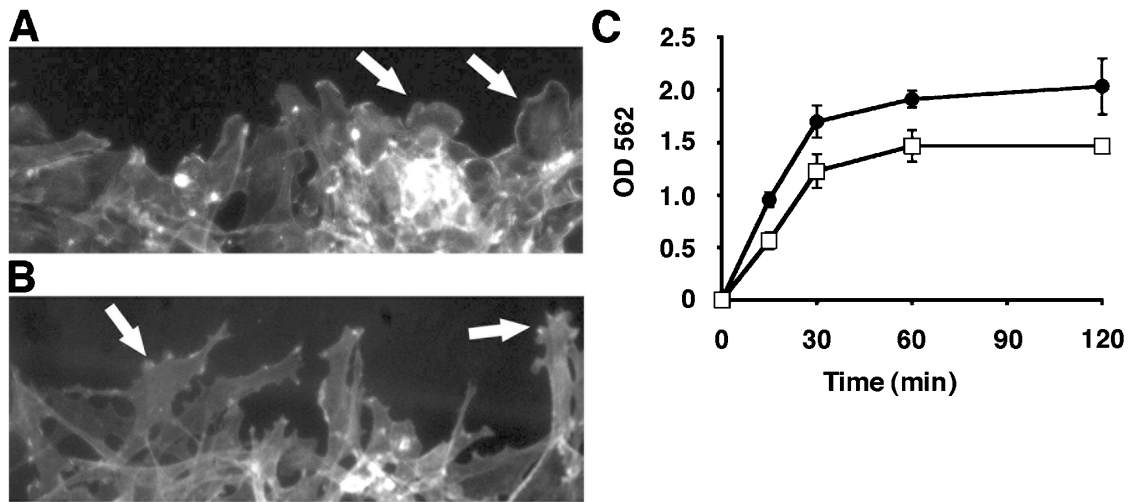


Figure 4. **Lamellipodia production by migrating Ajuba null MEFs was abnormal.** Phalloidin staining of multiple wt (A) and Ajuba null (–/–) (B) MEFs at the wound edge. The direction of migration is toward the top of the page. Arrows identify ruffles/lamellipodia. Both images were obtained using a 20× lens NA 1.4. (C) Quantitative lamellipodia assay as described in Materials and methods. To control for random lamellipodia production control filters were coated with BSA at each time point. Pseudopodia/lamellipodia production versus time of serum stimulation is presented as protein produced (OD<sub>562</sub>). Error bars represent the SD from the mean of multiple experiments.

There were minimal differences in overall protein tyrosine phosphorylation in Ajuba null and wt MEFs following adherence to fibronectin (unpublished data). These analyses indicated that migration-defective Ajuba null MEFs had no defect in their capacity to adhere to various ECM proteins, to spread on fibronectin, or to activate integrins in response to fibronectin binding.

#### Lamellipodia production by migrating Ajuba null MEFs was abnormal

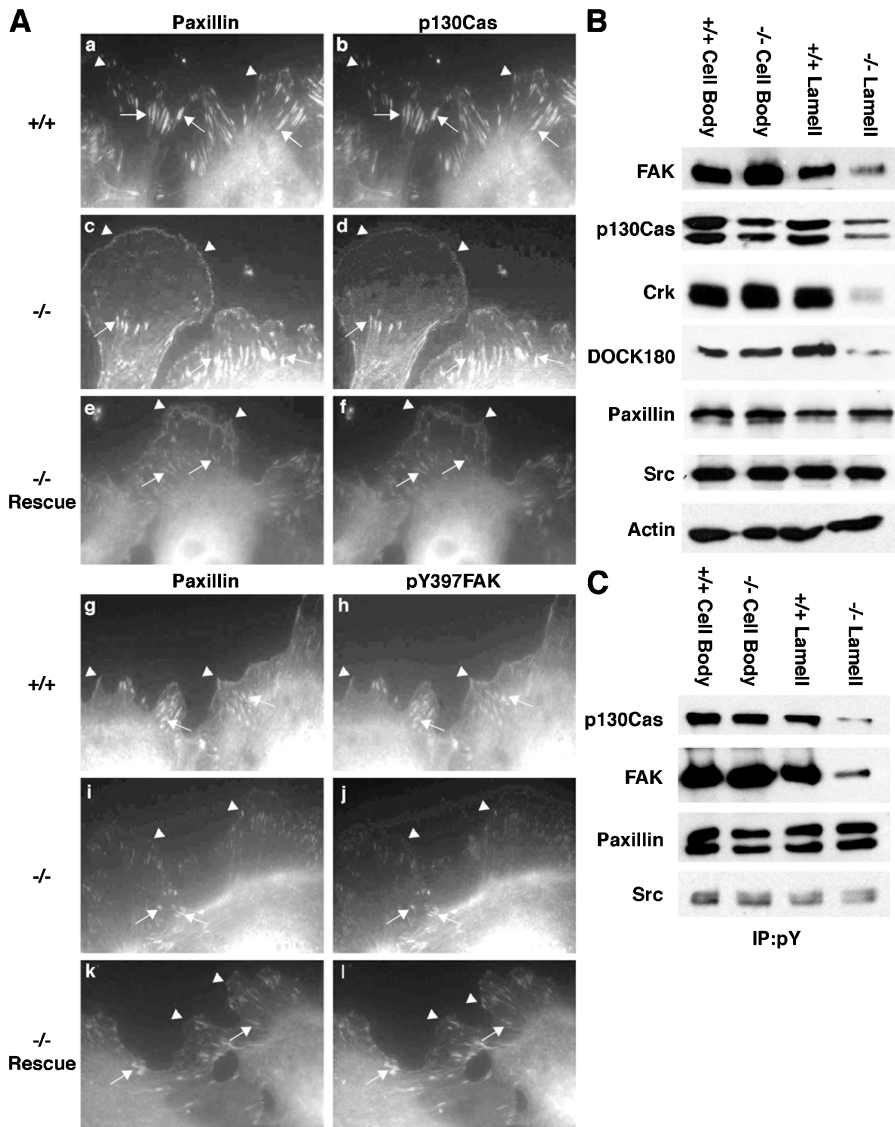
A hallmark of migrating cells is the production of lamellipodia at the forward leading edge. To assess the possibility that the migration defect in Ajuba null cells could be due to altered lamellipodia production, cells were induced to migrate, by scratch wounding, fixed, and stained with rhodamine-phalloidin to visualize filamentous actin. Migrating wt MEFs at the wound front produced broad lamellipodia and ruffles at their leading edge, as expected (Fig. 4 A, arrows; Video 3, available at <http://www.jcb.org/cgi/content/full/jcb.200406083/DC1>), whereas Ajuba null MEFs produced smaller lamellipodia and little ruffling (Fig. 4 B, arrows; Video 3).

To quantify lamellipodia production we used a recently described technique isolating pseudopodia by live migrating cells (Cho and Klemke, 2002). Pseudopodia production by wt and Ajuba null MEFs over increasing time of serum stimulation was determined. After 90 min, wt MEFs extended the maximum amount of lamellipodia/pseudopodia (Fig. 4 C). Ajuba null lamellipodia protrusion followed a similar kinetic profile as wt, but the total lamellipodia protein content was 25% less than wt cells at all time points (Fig. 4 C). For both cell types, lamellipodia production was maximal by 60 min. Even when this was extended to 4 h, Ajuba null cells did not produce any more lamellipodia than that found after 2 h. Results from actin immunofluorescence, video imaging, and pseudopodia quantification suggested that production of lamellipodia by migrating Ajuba null MEFs was abnormal.

#### FAK and p130Cas, but not paxillin, protein levels and activation were decreased at nascent adhesive complexes in lamellipodia of migrating Ajuba null MEFs

Because cell adhesion at the leading edge of migrating cells can influence lamellipodia dynamics and cell motility we asked whether cell–ECM adhesive complexes in migrating Ajuba null MEFs were altered. To do so cells were induced to migrate, by scratch wounding, then indirect immunofluorescence for select focal adhesion proteins (p130Cas, pY397-FAK, and paxillin) was performed on fixed cells at the wound edge. Compared with wt MEFs, migrating Ajuba null cells were found to have reduced staining of pY397-FAK at leading edge nascent focal complexes (Fig. 5 A, arrowheads), whereas staining at mature focal adhesions, present at the base of lamellipodia protrusions, appeared to be unchanged (Fig. 5 A, arrows). p130Cas staining at the leading edge nascent focal complexes in Ajuba null lamellipodia was also decreased and disordered (Fig. 5 A, arrowheads), whereas focal adhesion staining at the lamellipodia base was unchanged from wt (Fig. 5 A, arrows). In contrast, paxillin staining at both leading edge nascent focal complexes and focal adhesions was not altered in Ajuba null MEFs (Fig. 5 A). There did not appear to be any significant difference in total number of focal complexes and focal adhesions present in migrating Ajuba null MEFs compared with wt MEFs. Importantly reintroduction of Ajuba into Ajuba null cells normalized the staining patterns for pY397-FAK and p130Cas at nascent focal complexes, whereas paxillin staining remained unchanged (Fig. 5 A). The total cellular level of each of these three proteins was not different between wt and Ajuba null MEFs (Fig. S2 A, available at <http://www.jcb.org/cgi/content/full/jcb.200406083/DC1>).

In a second approach, we isolated lamellipodia (pseudopodia) from cell bodies, and determined the relative protein level and protein tyrosine phosphorylation (i.e., activation) of various



**Figure 5. FAK and p130Cas, but not paxillin, protein levels and tyrosine phosphorylation were decreased at focal complexes in migrating Ajuba null lamellipodia.** (A) Indirect immunofluorescence of migrating wt (+/+; a, b, g, h), Ajuba null (-/-; c, d, i, j), and Ajuba rescued Ajuba null MEFs (-/- Rescue; e, f, k, l) at the wound edge. Cells were costained for p130Cas (b, d, f) or pY397-FAK (h, j, l), and paxillin (a, c, e, g, i, k). Secondary antibodies conjugated with FITC (p130Cas and pY397FAK) or Alexa Fluor 568. Arrowheads identify leading edge focal complexes whereas arrows identify focal adhesions present at the base of the lamellipodia. Cells were imaged using a 60 $\times$  oil immersion lens (NA 1.4). (B) Western blot analysis for the indicated proteins was performed on lysates of cell bodies and lamellipodia from wt (+/+) and Ajuba null (-/-) MEFs. Samples were normalized so that an equal amount of protein was loaded in each lane. (C) Phosphotyrosine-containing proteins were immunoprecipitated from lysates of wt (+/+) and Ajuba null (-/-) cell bodies and lamellipodia and bound products Western blotted for the indicated proteins. Each sample was normalized to equal amounts of protein before immunoprecipitation.

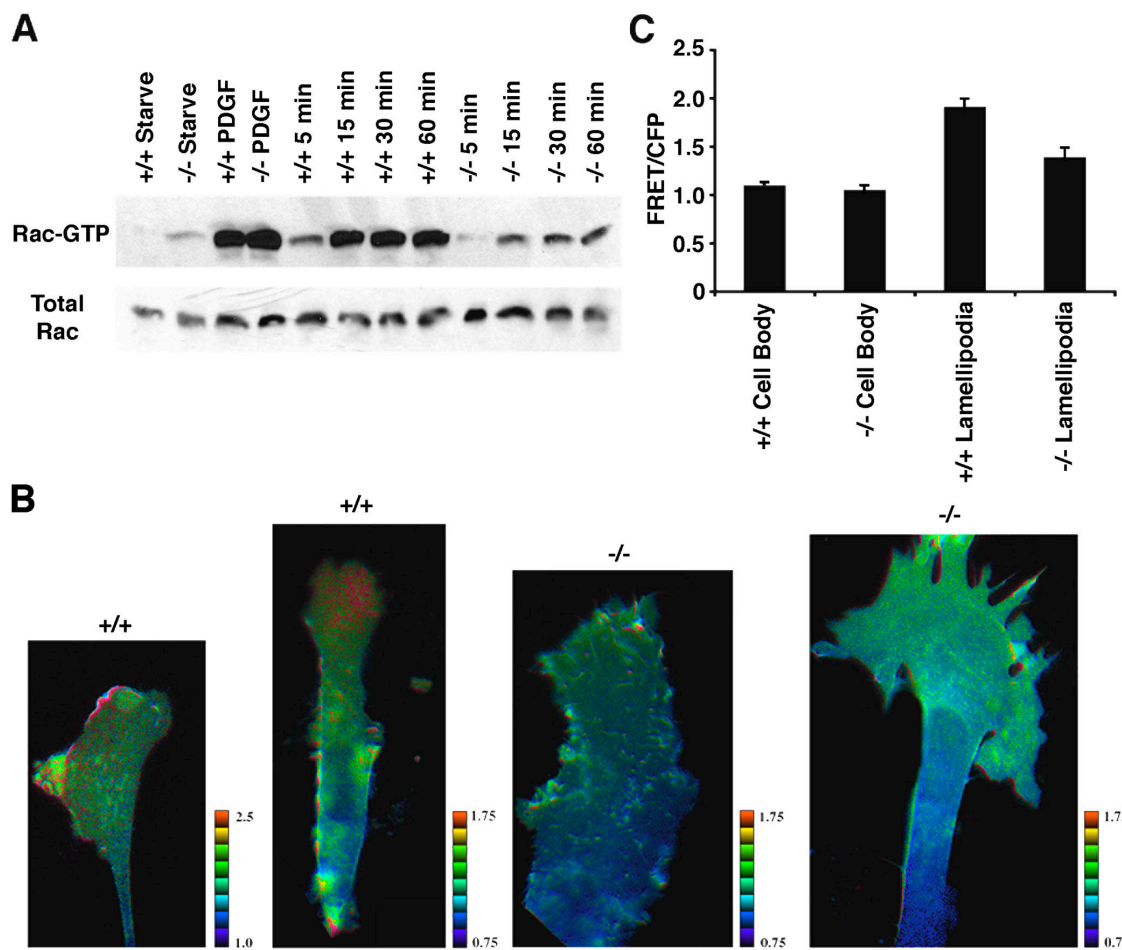
adhesion complex proteins in Ajuba null versus wt MEFs. In agreement with immunofluorescence analysis (Fig. 5 A) no difference in the protein level or tyrosine phosphorylation of paxillin was apparent (Fig. 5, B and C; Fig. S2 B). But the protein level and tyrosine phosphorylation of both p130Cas and FAK protein were reduced in Ajuba null lamellipodia (Fig. 5, B and C; Fig. S2 B). The levels of Crk and DOCK180 were also reduced in lamellipodia from Ajuba null MEFs, whereas Src and Zyxin levels were not changed (Fig. 5 B). When the ratio of pY-p130Cas-p130Cas, pY-FAK-FAK, and pY-paxillin-paxillin in lamellipodia were determined, there was a lamellipodia specific reduction in the activation of p130Cas (0.85) and FAK (0.42), but not paxillin (0.98; Fig. S2). This indicated that not only is there less total p130Cas and FAK protein in lamellipodia of Ajuba null cells, but that the protein there is less tyrosine phosphorylated.

These immunofluorescent and biochemical results both indicated that during cell migration the protein level and activation of both FAK and p130Cas, but not paxillin, are reduced in Ajuba null lamellipodia, particularly at nascent focal complexes present at the leading edge.

### Migration-induced Rac1 activation was reduced in Ajuba null MEFs

The Rho family of GTPases is a central regulator of cell migration. Rac1 activity, in particular, is important for lamellipodia production, and the formation of new focal complexes at the leading edge. Because Ajuba null cells exhibited abnormalities in both processes we asked whether Rac1 activation, in response to migratory cues, was defective in these cells. To determine Rac1 activity in migrating Ajuba null cells both biochemical and imaging analyses were performed. First, multiple scratch wounds were made in a confluent plate of cells, and resultant active GTP-bound Rac1 levels in cell lysates determined by pull-down assay (Etienne-Manneville and Hall, 2001). Although PDGF stimulation of starved MEFs resulted in equivalent activation of Rac1 in wt and Ajuba null cells (Fig. 6 A), when MEFs were induced to migrate wt cells exhibited a robust activation of Rac1, whereas Rac1 activation in Ajuba null MEFs was significantly blunted (Fig. 6 A).

To visualize Rac1 activity in live migrating cells, we used a FRET-based assay. Ajuba null and wt MEFs were transfected



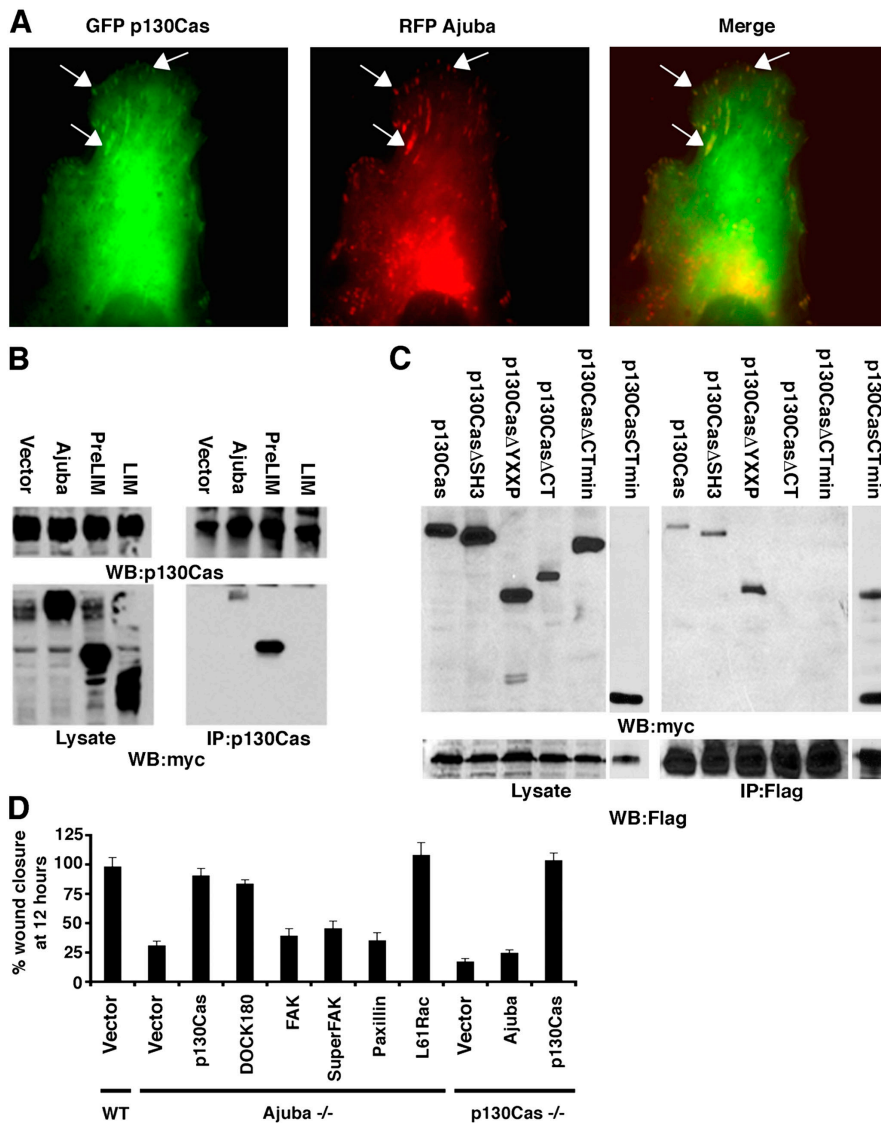
**Figure 6. Migration induced Rac activation is reduced in Ajuba null MEFs.** (A) WT (+/+, lanes 1, 3, 5–8) or Ajuba null (-/-, lanes 2, 4, 9–12) MEFs were starved overnight (lanes 1 and 2), stimulated with PDGF (lanes 3 and 4) or scratch wounded for the indicated times (lanes 5–12). Rac Western blots for GTP-bound Rac pull downs (top) and total Rac (bottom). (B) Rac-FRET analysis of migrating wt (+/+) and Ajuba null (-/-) MEFs. Cells are migrating toward the top of the page. Red and blue indicate high and low FRET activity, respectively. Scales for each image are shown to the right. (C) Quantification of FRET activity. Average FRET/CFP pixel intensity of the leading edge versus cell body in wt (+/+) versus Ajuba null (-/-) MEFs are shown. 30 or more cells were analyzed for each sample.

with the single molecule Raichu-Rac1 construct (Itoh et al., 2002), scratch wounds made, and dual-emission ratio FRET analyses of migrating cells at the wound front performed. Although total cell Rac FRET activity was similar between wt and Ajuba null MEFs, Rac1 activity at the leading edge of migrating Ajuba null MEFs was significantly reduced (Fig. 6 B). To quantify these observations, we compared the average pixel intensity of FRET and CFP channels at the leading edge of migrating cells to the pixel intensity within the cell body. Although wt and Ajuba null cell bodies had similar average FRET/CFP ratios, the leading edge Rac1 activation in Ajuba null cells was reduced by 25% compared with wt cells (Fig. 6 C), indicating that there were lower levels of Rac1 activity specifically in the lamellipodia of Ajuba null MEFs. Stable reintroduction of Ajuba into Ajuba null cells normalized Rac FRET activity at the leading edge in lamellipodia of migrating cells (unpublished data). When MEFs were stimulated with PDGF, which results in equivalent activation of Rac in wt and Ajuba null cells, there was no difference in FRET activity between cells (unpublished data). Therefore, imaging and biochemical

analyses indicated that Ajuba was required for the appropriate activation of Rac1 in response to migration stimuli, but not in response to PDGF stimulation.

#### Ajuba associated with p130Cas in cells

A major signaling pathway leading to Rac activation in migrating cells is the assembly of the p130Cas–Crk–DOCK180–ELMO Rac GEF signaling complex at activated integrin complexes present in nascent adhesive complexes (Kiyokawa et al., 1998b). Lamellipodia of migrating Ajuba null MEFs had selective deficiency in p130Cas, Crk, and DOCK180 levels, particularly at nascent adhesive complexes (Fig. 5 and Fig. S2 B). FAK levels were also reduced in Ajuba null lamellipodia, and p130Cas is recruited to adhesive complex through an interaction with FAK (Bouton et al., 2001). Furthermore, the tyrosine phosphorylation of p130Cas and FAK was also reduced in Ajuba null lamellipodia. These results suggested that Ajuba might affect Rac activity by influencing the recruitment of these proteins into adhesive complexes in the lamellipodia of migrating cells. In a yeast 2-hybrid protein–protein interactive



**Figure 7. Ajuba colocalized with p130Cas at focal complexes and focal adhesions in lamellipodia, and associated with p130Cas in cells.** (A) WT MEFs were cotransfected with GFP-p130Cas (green) and RFP-Ajuba (red) and induced to migrate (toward top of page) by scratch wounding. Arrows indicate localization of p130Cas and Ajuba (colocalization, yellow in merged image). (B) COS-7 cells were transfected with myc-tagged Ajuba plasmids, endogenous p130Cas was immunoprecipitated (right) and bound products Western blotted for the presence of Ajuba (anti-myc; bottom) and p130Cas (top). Left panels are loading controls of cell lysates. (C) COS-7 cells were cotransfected with Flag-tagged Ajuba and various myc-tagged p130Cas mutants. Ajuba was immunoprecipitated (anti-Flag; right) and bound products Western blotted for the presence of p130Cas (anti-myc; top) and Ajuba (anti-Flag; bottom). Loading controls of cell lysates (left). (D) Migration rescue experiments. WT, Ajuba  $-/-$ , and p130Cas  $-/-$  MEFs were transfected with control empty GFP vector, RFP-Ajuba, GFP-p130Cas, GFP-DOCK180, GFP-paxillin, SuperFAK-IRES.GFP, or myc-Rac1(L61) as indicated. Confluent cell layers were scratch wounded, and videos of wound repair obtained, as described in Fig. 2 B. Shown is the percent wound closure at 12 h. Multiple wounds were analyzed and the data presented as the mean and the SD about the mean.

screen we identified HEF1–p130Cas as an Ajuba-interacting protein (unpublished data). When wt MEFs were cotransfected with RFP-Ajuba and GFP-p130Cas, p130Cas and Ajuba were both present at focal adhesions at the base of lamellipodia as well as toward the cell periphery (Fig. 7 A, arrows; Videos 4 and 5, available at <http://www.jcb.org/cgi/content/full/jcb.200406083/DC1>). Ajuba and p130Cas also colocalized with pY397-FAK at these sites (not depicted).

Ajuba and p130Cas also interacted in cells. Myc-tagged Ajuba, PreLIM region, or LIM region of Ajuba were transfected into COS cells, and endogenous p130Cas was immunoprecipitated and bound products Western blotted for the presence of Ajuba. Full-length Ajuba and the PreLIM region of Ajuba, but not the LIM region, associated with endogenous p130Cas in cells (Fig. 7 B). In contrast, we did not detect an association between Ajuba and FAK (unpublished data). To determine which domain of p130Cas mediated its association with Ajuba p130Cas mapping studies were performed. Flag-tagged full-length Ajuba and various myc-tagged p130Cas deletion mutants were coexpressed in COS cells. Ajuba was

found to coimmunoprecipitate with all p130Cas mutants except those lacking a 180–amino acid sequence at the COOH terminus ( $\Delta$ CTmin; Fig. 7 C), and when Ajuba and the CTmin 180–amino acid piece of p130Cas were coexpressed they also coimmunoprecipitated (Fig. 7 C).

#### p130Cas, DOCK180, Rac1(L61), but not FAK or paxillin, rescued the Ajuba migration defect

Based on the above results we hypothesized that Ajuba functioned upstream of p130Cas to activate Rac1 and regulate cell migration. If so, then overexpression of p130Cas or DOCK180 (downstream of Ajuba) might ameliorate the migration defect of Ajuba null cells. To test this possibility, wt and Ajuba null MEFs were transfected with GFP-p130Cas, GFP-DOCK180, GFP-FAK, or myc-Rac1(L61) then scratch wounded and migration of GFP (or myc) positive cells relative to untransfected (GFP negative) cells at the wound edge were determined. GFP-p130Cas, GFP-DOCK180, and myc-Rac1(L61) all rescued the migration defect in Ajuba null cells, whereas overexpression of

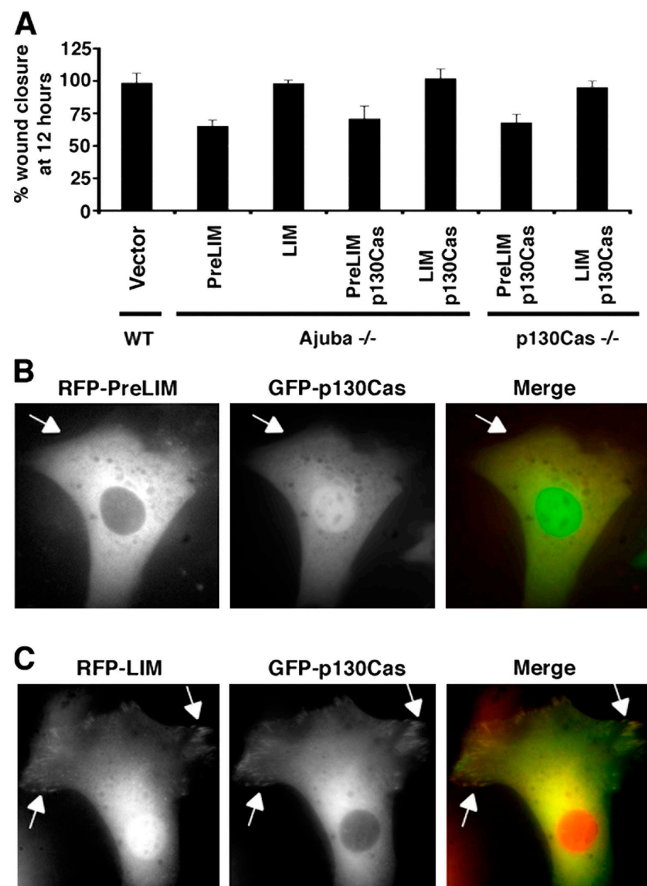
GFP-FAK did not (Fig. 7 D). Importantly, expression of either p130Cas or DOCK180 plasmid alone in wt cells did not accelerate migration, although myc-Rac1(L61) did (unpublished data). Rescue of the Ajuba migration defect was specific to p130Cas and DOCK180, as overexpression of GFP-paxillin (protein level, localization, and tyrosine phosphorylation not changed in Ajuba null lamellipodia) in Ajuba null MEFs did not rescue the motility defect (Fig. 7 D). Because FAK activation was decreased in Ajuba null lamellipodia we also tested whether a form of “constitutively activated” FAK, SuperFAK (Gabarra-Niecko et al., 2002) would rescue. Expression of SuperFAK in Ajuba null cells did not rescue the migratory defect (Fig. 7 D). Because SuperFAK expression increases phosphorylation of paxillin and tensin, but not p130Cas (Gabarra-Niecko et al., 2002), and because p130Cas, but not paxillin, rescued the migration defect in Ajuba null cells, it is perhaps not surprising that SuperFAK did not rescue the migration defect in Ajuba null cells.

MEFs from p130Cas null mice also exhibit reduced migration (Honda et al., 1998). If p130Cas acts downstream of Ajuba, then overexpression of RFP-Ajuba in p130Cas null cells would be predicted to not rescue their migration defect. Indeed, RFP-Ajuba overexpression in p130Cas null cells did not rescue the motility defect (Fig. 7 D). In control experiments, GFP-p130Cas was able to rescue the motility defect in p130Cas null fibroblasts (Fig. 7 D). In sum these results suggested that Ajuba acts upstream of p130Cas–DOCK180–Rac1 in regulating cell migration.

**The PreLIM region of Ajuba acts in a “dominant inhibitory” manner to block p130Cas localization to focal complexes and its capacity to rescue the migration defect of Ajuba- and p130Cas-null cells**

The COOH-terminal region of p130Cas (region that interacts with Ajuba) contains the major focal adhesion targeting sequence in p130Cas (Harte et al., 2000), yet is distinct from other regions in p130Cas that mediate its association with Crk and DOCK180 (Bouton et al., 2001). This suggested the possibility that Ajuba may influence the recruitment of p130Cas to focal adhesion sites. Because the PreLIM region of Ajuba directed its association with p130Cas and does not, itself, localize to adhesive complexes (Fig. 8 B) we asked whether overexpression of the PreLIM region of Ajuba in wt MEFs could act in a “dominant inhibitory” manner to prevent p130Cas recruitment to nascent focal complexes, and thereby inhibit cell migration. When overexpression in wt cells the PreLIM region of Ajuba did indeed inhibit cell migration (Fig. 8 A), but not to the same extent as observed for Ajuba null MEFs (Fig. 8 A). PreLIM Ajuba also reduced the amount of p130Cas present at focal complexes in these cells (Fig. 8 B). Overexpression of the Ajuba LIM region (does not interact with p130Cas, yet itself localizes to focal adhesions (Fig. 8 C) did not inhibit either wt MEF migration (Fig. 8 A) or p130Cas localization (Fig. 8 C).

When overexpressed in Ajuba- or p130Cas-null MEFs, the PreLIM region of Ajuba also blocked the ability of p130Cas to rescue the migration defect of both cells (Fig. 8 A).



**Figure 8. PreLIM Ajuba blocks p130Cas localization to adhesive sites and p130 rescue of migration.** (A) Migration rescue experiments. WT, Ajuba  $-/-$ , and p130Cas  $-/-$  MEFs were transfected with control empty GFP vector, RFP-PreLIM, RFP-LIM, both RFP-PreLIM and GFP-p130Cas, or both RFP-LIM and GFP-p130Cas as indicated. Confluent cell layers were scratch wounded, and videos of wound repair were obtained, as described in Fig. 2 B. Shown is the percent wound closure at 12 h. Multiple wounds were analyzed and the data presented as the mean and the SD about the mean. (B and C) Cells were cotransfected with RFP-PreLIM and GFP-p130Cas (B), or RFP-LIM and GFP-p130Cas (C), and immunofluorescence performed. Arrows identify focal adhesions.

As controls, the PreLIM region expressed alone in Ajuba null MEFs does not rescue the migration defect (Fig. 2 B), whereas p130Cas expressed alone does rescue the migration defect of both Ajuba null and p130Cas null cells (Fig. 7 D).

In sum these experiments indicated that the PreLIM region of Ajuba acts in a “dominant inhibitory” manner to block both localization of p130Cas to adhesive complexes and its capacity to rescue the cell motility defect of Ajuba and p130Cas null cells. This suggested that p130Cas is downstream of Ajuba in regulating cell motility and that Ajuba influences the localization of p130Cas to adhesive complexes in migrating cells.

## Discussion

Signaling at the leading edge of migrating cells must be tightly regulated so as to coordinate this process. For proteins recruited to the leading edge phosphorylation, dephosphorylation, and interaction with downstream signaling partners con-



trol the motility response. Ultimately, these signaling pathways converge upon the Rho family of GTPases, as these proteins are important regulators of cell migration. In particular, Rac1 activation leads to the formation of new focal complexes at the leading edge and lamellipodia production. In response to migratory cues multiple signaling pathways can influence Rac activity. For example, integrin activation can activate Rac through the formation of a p130Cas–Crk–DOCK180–ELMO Rac GEF (Brugnera et al., 2002) or a paxillin–Cool–PIX–p21PAK complex (Brown et al., 2002; Feng et al., 2002). Here, we show that primary MEFs lacking the LIM protein Ajuba are defective in cell migration, due to an inability to activate Rac1 as a result of defective localization, and thus, activation of the p130Cas–Crk–DOCK180-signaling complex.

Ajuba and p130Cas colocalize in cells, and coimmunoprecipitate. Immunofluorescent and biochemical evidence show that Ajuba null MEFs have reduced p130Cas (total protein and tyrosine phosphorylated p130Cas) at focal complexes of migrating cells, which is normalized following reexpression of Ajuba. Overexpression of p130Cas rescued the migration defect of Ajuba null cells. Mapping studies demonstrate that the PreLIM region of Ajuba mediates Ajuba's interaction with p130Cas, via the COOH-terminal 180-amino acid, major focal adhesion targeting domain, of p130Cas. When overexpressed in wt MEFs Ajuba's PreLIM region acted in a "dominant inhibitory" manner to block p130Cas localization to nascent focal complexes and inhibit cell migration. Furthermore, the PreLIM region of Ajuba also blocked the ability of p130Cas to rescue the migration defect in Ajuba null and p130Cas null cells. Together, these data suggest that Ajuba acts upstream of p130Cas in regulating Rac activation and thus cell motility.

Alternatively, overexpression of p130Cas in Ajuba null cells rescues migration through an Ajuba-independent pathway because p130Cas has more than one focal adhesion targeting domain (Harte et al., 2000), however, only when overexpressed. But because the PreLIM region of Ajuba blocked endogenous p130Cas localization and function (migration regulation) in wt cells, and blocked the ability of p130Cas to rescue the motility defect of p130Cas null cells, we would argue that the Ajuba–p130Cas interaction is physiologically relevant. However, we cannot exclude that there are other pathways regulating p130Cas to adhesive sites in migrating cells: Ajuba-dependent (primary) and a secondary Ajuba-independent pathway, possibly through a direct interaction with FAK or other Ajuba-related LIM proteins.

In epithelial cells, Ajuba's recruitment from the cytosol to cell–cell junctions is regulated by the formation of new cell–cell adhesion (Marie et al., 2003). Video imaging of live fibroblasts revealed that Ajuba also shuttles from cytosol to focal adhesions (unpublished data). This, and the fact that Ajuba interacts with the focal adhesion targeting domain in p130Cas, suggests that Ajuba might regulate recruitment p130Cas to the leading edge of migrating cells. Because the PreLIM region of Ajuba does not localize to adhesive sites, rather it is diffusely cytosolic (Marie et al., 2003), yet blocks p130Cas localization suggests that Ajuba and p130Cas may associate in the cytosol before recruitment to adhesive complexes.

The Ajuba-related LIM proteins, Zyxin and TRIP6, were recently shown to also interact with HEF1–p130Cas proteins (Yi et al., 2002). The functional consequences of these interactions have not been determined, however. Interestingly, the domains of HEF1 and Zyxin required for their interaction may be distinct from those of p130Cas and Ajuba. Why this difference in how these related proteins interact is not clear, but, overexpression of TRIP6 in fibroblasts inhibits cell migration, similar to knocking out Ajuba (Yi et al., 2002), suggesting that Zyxin and TRIP6 may exert opposing effects to that of Ajuba upon migration, possibly through effects on p130Cas family members.

The p130Cas–Crk–DOCK180–ELMO-signaling complex is highly conserved across species. In both *C. elegans* and *Drosophila*, mutations in the DOCK180 homologues result in phenotypes with defects in cell migration and phagocytosis (Nolan et al., 1998; Reddien and Horvitz, 2000). In mice, there are four other proteins related to Ajuba, with broad overlap in their cellular expression pattern. Probable functional redundancy between these proteins may explain the viability of Ajuba null mice. Database searches indicate that *C. elegans* and *Drosophila* have one and two LIM proteins similar to Ajuba or Zyxin, respectively. Whether these LIM protein genes regulate Rac activity and cell migration in these organisms through the conserved Crk–DOCK180 pathway is a provocative possibility that remains to be determined.

FAK levels are also reduced in the lamellipodia of Ajuba null MEFs. Because p130Cas is recruited to FAK present at integrin complexes (Bouton et al., 2001), the decreased FAK levels in lamellipodia of migrating Ajuba null MEFs could also account for the decrease in p130Cas. However, overexpression of FAK or a constitutively activated isoform of FAK (SuperFAK) did not rescue the migration defect of Ajuba null cells, or restore proper localization of p130Cas (unpublished data). The inability of SuperFAK to rescue could be explained by the signaling pathways selectively activated by this mutant. SuperFAK expression increased phosphorylation of paxillin and tensin, but not p130Cas (Gabarra-Niecko et al., 2002). Because p130Cas, but not paxillin, rescue the migration defect in Ajuba null cells, it is perhaps not surprising that SuperFAK did not rescue the migration defect in Ajuba null cells. Ajuba did not interact with FAK in cells, so why FAK levels are reduced in the lamellipodia of Ajuba null cells is not clear.

That Ajuba was found to interact with the mitotic kinase Aurora A, and regulate its kinase activity (Hirota et al., 2003), suggests that Ajuba may influence the activation (i.e., tyrosine phosphorylation) of FAK and p130Cas by cellular kinases (e.g., Src). Inherent to this argument is that the activation status of each protein is important for its appropriate, stable localization, however.

Focal complexes that form at the leading edge have a short half-life (Zaidel-Bar et al., 2003). The reduction in pY397-FAK and p130Cas at focal complexes at the leading edge of Ajuba null cells suggests a potential role for Ajuba in focal complex assembly, maintenance, or turnover. Ajuba may anchor p130Cas to focal complexes, thereby strengthening the focal complex. In the absence of Ajuba the strength of adhesion to the ECM may be impaired resulting in decreased size or

amount of lamellipodia produced. Alternatively, it is simply the reduction in activation of Rac1 in migrating Ajuba null cells that impairs actin polymerization and, or focal complex formation. Recent work by Bear et al. (2002), showed that the relation between net cell translocation and lamellipodia production are based on the persistence of lamellipodia protrusion.

Finally, paxillin, another LIM domain containing focal adhesion protein (distinct from Zyxin/Ajuba), also regulates migration (Turner, 1991). Through an association with CoolPIX and p21PAK, paxillin has been shown to activate Rac during migration (Brown et al., 2002; Feng et al., 2002). Ajuba null cells did not show any reduction in either the amount or activity of paxillin at the leading edge. This suggests that, although both p130Cas and paxillin activation at the leading edge of migrating cells leads to Rac activation they are not redundant pathways. The persistence of the paxillin–Rac signaling pathway in Ajuba null cells may also explain why the inhibition of cell migration in Ajuba null cells, *ex vivo*, did not result in a more dramatic whole animal phenotype.

## Materials and methods

### Knockout generation and analysis

A genomic fragment corresponding to Exon 1, which includes the 5' UTR and intron 1, was isolated from a mouse cosmid library. A neomycin resistance cassette was inserted in the 3' to 5' orientation. 129/SvJ ES cells were electroporated with the targeting sequence and selected with G418. Correctly targeted ES cells were confirmed by Southern blot analysis and genomic PCR. Correctly targeted ES cells were injected into C57BL/6 blastocysts. Chimeric males were mated with C57BL/6 females and heterozygous offspring were intercrossed to produce homozygous mutants. Genotyping was performed on tail DNA by either Southern blot or PCR analysis.

### Cell lines, transfection, and antibodies

To isolate MEFs, E13.5–E15.5 embryos were collected from Ajuba heterozygous matings and genotyped. Internal organs removed and the remaining embryo dissociated and plated in 10% FCS DME. Experiments were done on cells within the first 12 passages and before senescence. p130Cas null MEFs were a gift from the H. Hirai (University of Tokyo, Tokyo, Japan; Honda et al., 1998). Primary MEFs and COS cells were transfected by Nucleofection (Amaxa, Inc.) or Trans-IT LT-1 (Mirus), respectively.

Ajuba antibody has been described previously (Kanungo et al., 2000). Rabbit polyclonal FAK, p130Cas, Src, goat polyclonal actin, DOCK180, mouse monoclonal Crk and anti-phosphotyrosine antibodies were obtained from Santa Cruz Biotechnology. Mouse monoclonal paxillin was obtained from BD Biosciences. Mouse monoclonal talin, vinculin, and  $\alpha$ -actinin were obtained from Sigma-Aldrich. Rabbit polyclonal pY397-FAK was obtained from BioSource International, Inc. Mouse monoclonal Rac antibody was obtained from Upstate Biotechnology. Rabbit polyclonal Zyxin (B71) and TRIP6 (B65) were a gift from M. Beckerle (University of Utah, Salt Lake City, UT). Rabbit polyclonal LPP antiserum was a gift from L. Petit (University of Leuven, Leuven, Belgium). Mouse monoclonal anti-p130Cas was a gift from A. Bouton (University of Virginia, Charlottesville, VA).

### Immunofluorescence

Cells were fixed and permeabilized at the same time in 4% PFA/0.5% Triton X-100/PBS for 15 min. After washing cells were further permeabilized in 0.1% Triton X-100/10% FCS/PBS for 10 min. Incubation with primary antibody was in 10% FCS/PBS for 1 h. Fluorescently tagged secondary antibodies (Molecular Probes) were incubated for 1 h in 10% FCS/PBS. After washing cells were incubated with DAPI or fluorescently labeled phalloidin (Molecular Probes) in PBS for 10 min. Cells were then mounted and analyzed on a microscope (Nikon) with Optronics Camera and analyzed using Magnafire software (Optronics Co.). Temperature and objective strength are listed in figure legends.

### Plasmids

pCDNA3-RFP-Ajuba, RFP-PreLIM, and RFP-LIM constructs were generated using the monomeric form of RFP (Campbell et al., 2002). GFPp130Cas,

GFP-Crk, GFP-DOCK180, and GFP-paxillin were generated by subcloning into pEGFP-C2 (CLONTECH Laboratories, Inc.). pRK5myc-p130Cas and p130Cas deletion mutants were a gift from A. Bouton. SuperFAK.IRES-GFP plasmid was a gift from M. Schaller (University of North Carolina, Chapel Hill, NC).

### Cell motility, adhesion assay, and video microscopy

MEFs were grown to confluence, starved overnight, and scratch wounded using a P200 pipette tip. Cells were allowed to recover for 30 min before recording migration rates. The total area of the wound covered by migrating cells 12 h after wounding was measured. The value obtained for wt MEFs is arbitrarily set at 100%. Multiple wounds per plate are assessed, as well as different sets of cells so as to obtain statistical measurements. Adhesion assays on fibronectin, superfibronectin, vitronectin, and collagen (Sigma-Aldrich) were performed as described previously (Xu et al., 1998). Nonspecific cell adhesion was measured on BSA-coated wells and subtracted from each value point. For fluorescent video microscopy, a CoolSnap camera on a microscope (Nikon) was used. Videos were compiled using Metamorph software (Universal Imaging Corp.). Temperature and objective strength are listed in figure legends.

### Lamellipodia/Pseudopodia purification

Pseudopodia purification was performed as described previously (Cho and Klemke, 2002). In brief,  $10^6$  MEFs were starved overnight, plated on fibronectin coated (both sides) 3- $\mu$ m porous transwells (Fisher Scientific) in serum-free media. To control for random lamellipodia production control filters were coated with BSA. Serum was added to the bottom chamber to induce pseudopodia formation. Cell bodies or pseudopodia were isolated from the top and bottom of the membrane, respectively, directly into lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, 1% NP-40, plus protease inhibitors) and quantified using the BCA protein determination kit (Pierce Chemical Co.).

### Rac activity assays

Rac pull-down assays were performed as described previously (Etienne-Manneville and Hall, 2001). To confluent cultures of WT and Ajuba null MEFs scratch wounds were made with P200 tips using a multi-channel pipette such that 50% of the cells in the plate were within three rows of a wound edge. At specific times, cells were washed with PBS and lysed in cold Rac assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 1% Triton X-100, 0.5% sodium deoxycholate, plus protease inhibitors). Lysates were clarified by centrifugation. Lysates were then incubated with purified GST-PAK-CRIB beads (Ren et al., 1999) for 30 min. The beads were washed three times with Rac Assay buffer and bound material analyzed by Western blotting.

Raichu-Rac single molecule FRET construct (Itoh et al., 2002; we received the Raichu-Rac FRET probe from M. Matsuda, Osaka University, Osaka, Japan) was also used to assess GTPase activity. Cells were transfected, grown to confluence, scratch wounded, and placed in a heated chamber (Delta T; Bioprotech) containing L-15/10% FCS. Dual-emission ratio imaging was performed using an upright BX61WI microscope (Olympus), a xenon illumination source (Lambda LS; Sutter Instrument Co.), and a water-immersion lens (0.9 NA). Images were taken at the times indicated in the figures by using a cooled charge-coupled device camera (CoolSnap HQ, Roper Scientific) at  $2 \times 2$  binning. Sequential CFP and CFP–YFP emission imaging was conducted with an ultrafast filter wheel (Lambda 10–2; Sutter Instrument Co.). Filter combinations (Chroma Technology Corp.) were as follows: dichroic: 86008bs, (excitation:emission): CFP:CFP (S430\_25, S465\_30), CFP:YFP (S430\_25, S550\_50), and YFP:YFP for photobleaching experiments (S510\_20, S465\_30). After time-lapse imaging was complete, background-subtracted images were used to generate CFP and CFP–YFP images by using the ratio feature of Metamorph software. Temperature and objective strength are listed in figure legends.

### Online supplemental material

Video 1 shows that re-expression of Ajuba rescues migration defect of Ajuba null cells. Ajuba null MEFs transfected with RFP alone (left) or with RFP-Ajuba (right) were scratch wounded and wound repair observed over 12 h with a frame every 45 min at 37°C. See Fig. 2 B. Video 2 shows cell spreading. WT MEFs (left) and Ajuba null MEFs (right) cells were allowed to spread on fibronectin-coated dishes for 1 h at a frame every 3 min at 37°C. See Fig. 3 B. Video 3 shows that WT (left) and Ajuba null (right) MEFs were subjected to scratch wounding and their migration into the wound monitored at 37°C. Time of migration is indicated at bottom in hours and minutes at a frame every 35 min. See Fig. 2 A. Videos 4 and 5 show Ajuba and p130Cas localization in migrating wt MEFs. Cells were

cotransfected with GFP-p130Cas (Video 4, green) and RFP-Ajuba (Video 5, red). Video 4 is the GFP-p130Cas image and Video 5 the RFP-Ajuba image of the same cells at 37°C. Videos are 5 min with a frame every 16 s. See Fig. 7 A. Fig. S1 illustrates gene targeting strategy. (A) Exon 1 was replaced with a Neomycin cassette. 5' and 3' probes for Southern blot (black boxes) and PCR primer locations (black arrows) are indicated. (B) Genomic PCR from tail DNA: wt (+/+), heterozygous (+/-), and null (-/-) mice. 1-kb product identifying the Neo cassette and 1.2-kb product the wt allele are indicated by arrows. Fig. S2 illustrates that select adhesive receptor complex proteins are reduced in lamellipodia of Ajuba null MEFs. (A) Western blot analyses of total cell protein levels of FAK, p130Cas, Crk, and paxillin in wt (+/+), Ajuba null (-/-), and Ajuba rescued Ajuba null (-/- Rescue) MEFs. Equal amounts of protein were loaded in each lane. Densitometry was performed and values are listed below each lane (wt = 1). (B) p130Cas, FAK, paxillin, and Crk proteins were immunoprecipitated from cell bodies and lamellipodia and bound products Western blotted with a phosphotyrosine antibody, then stripped and reprobbed with antibodies to the specific proteins. Levels of total and active proteins relative to wt cell body were determined by densitometry measurements and are indicated below each lane. (C) The ratios of tyrosine-phosphorylated protein to total protein for p130Cas, FAK, and paxillin in cell bodies and lamellipodia from wt versus Ajuba null MEFs were calculated from densitometric quantification of B. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200406083/DC1>.

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