Integrin adhesion and force coupling are independently regulated by localized PtdIns(4,5)₂ synthesis

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The 90-kDa isoform of the lipid kinase PIP kinase Type I γ (PIPKIγ) localizes to focal adhesions (FAs), where it provides a local source of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂). Although PtdIns(4,5)P₂ regulates the function of several FA-associated molecules, the role of the FA-specific pool of PtdIns(4,5)P₂ is not known. We report that the genetic ablation of PIPKIγ specifically from FAs results in defective integrin-mediated adhesion and force coupling. Adhesion defects in cells deficient in FA-PtdIns(4,5)P₂ synthesis are corrected within minutes while integrin–actin force coupling remains defective over a longer period. Talin and vinculin, but not kindlin, are less efficiently recruited to new adhesions in these cells. These data demonstrate that the specific depletion of PtdIns(4,5)P₂ from FAs temporally separates integrin–ligand binding from integrin–actin force coupling by regulating talin and vinculin recruitment. Furthermore, it suggests that force coupling relies heavily on locally generated PtdIns(4,5)P₂ rather than bulk membrane PtdIns(4,5)P₂.

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Introduction

Cell adhesion to extracellular matrix (ECM) is mediated by integrins, which regulate their affinity for ligand (also termed integrin inside-out signalling or integrin activation), cluster and couple the ECM to the actin cytoskeleton (Hynes, 2002). Integrin-mediated coupling of the ECM to F-actin is important to transduce forces from the cytoskeleton to the extracellular environment and to translate external forces into biochemical signals (Friedland et al, 2009; Legate et al, 2009). The mechanisms that regulate the divergent signalling properties of integrins are poorly understood. However, it is clear that signalling is controlled by hundreds of different molecules that are recruited to integrin cytoplasmic domains (Zaidel-Bar et al, 2007; Legate and Fässler, 2009).

The initiation of integrin-mediated adhesion consists of two main steps. The first is the initial activation of integrins, which enables them to bind ligand. The second is the generation of force on the integrin to reinforce this interaction. β1 integrin exhibits catch bond behaviour; that is, the application of force increases the lifetime of the integrin–ligand bond (Kong et al, 2009). Since the bond lifetime in the absence of force is quite short (<2 s), reinforcement of the integrin–ligand bond should occur rapidly if it is to persist, and the signal to initiate the application of force should originate from within the focal adhesions (FA).

Two molecules that have a key role in integrin activation and in forming a bridge between integrins and the cytoskeleton are talin and the kindlins, which both bind β-integrin cytoplasmic tails (reviewed in Moser et al, 2009). The β-integrin tail binding activity of talin is regulated through interactions with the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂). PtdIns(4,5)P₂ binds the talin head domain, which disrupts an autoinhibitory interaction between the rod domain and the integrin binding region on the talin head and orients talin on the plasma membrane to permit an interaction with β-integrin tails (Anthis et al, 2009; Goult et al, 2009, 2010; Saltel et al, 2009). These observations led to the suggestion that PtdIns(4,5)P₂ represents a regulatory signal for integrin activation. Kindlins contain bona fide PH domains that also bind phosphoinositides (Qu et al, 2011), but the functional consequences of lipid binding are unknown.

PtdIns(4,5)P₂ comprises ~2.5% of the plasma membrane phospholipid and is relatively evenly distributed throughout the membrane (Hilgemann, 2007). Therefore, it is thought that targeted synthesis and sequestration of PtdIns(4,5)P₂ dictate its temporal and spatial specificity with respect to function (McLaughlin and Murray, 2005). Most PtdIns(4,5)P₂ within the cell is synthesized by Type I phosphatidylinositol 4-phosphate 5-kinases (PIPKIs), which exist in three isoforms in mammals: PIPKIγ, PIPKIβ and PIPKIγ (Loijens and Anderson, 1996, 1998). PIPKIγ consists of up to five splice variants; the major variants are an 87-kDa molecule (PIPKIγ₁₁, also called PIPKIγ835 or PIPKIγ87) and a 90-kDa molecule (PIPKIγ₁₂, also called PIPKIγ661 or PIPKIγ90) (Ishihara et al, 1998; Giudici et al, 2004; Schill and Anderson, 2009). PIPKIγ₁₂ differs from PIPKIγ₁₁ by the addition of 26 C-terminal amino acids in mouse (28 in...
human) that contain a talin binding site and specifically localizes PIPK\(_{\gamma, 12}\) to FAs (Di Paolo et al., 2002; Ling et al., 2005). PIPK\(_{\gamma, 12}\) has been shown to be involved in neutrophil polarization (Lokuta et al., 2007; Xu et al., 2010) regulating LFA-1-mediated T-cell adhesion (Wernimont et al., 2010), and promotes the interaction between the cytoskeleton and the membrane in megakaryocytes (Wang et al., 2008). The precise role for PIPK\(_{\gamma, 12}\) in regulating FAs in non-haematopoietic cells is not established, but the high accumulation of proteins and concomitant exclusion of lipids from FAs means that the local production of PtdIns(4,5)\(_2\) is likely required to prevent PtdIns(4,5)\(_2\) from becoming rate limiting.

Structural analysis revealed that the distal talin-binding sequence of the \(\beta\)-integrin tail and of PIPK\(_{\gamma, 12}\) bind the same region on the talin head with nM affinity (Barsukov et al., 2003; de Pereda et al., 2005). Overexpression of PIPK\(_{\gamma, 12}\) or the talin-binding peptide causes defects in cell spreading and FA formation suggestive of an integrin activation defect (Di Paolo et al., 2002; Ling et al., 2002). Therefore, in addition to providing a localized source of PtdIns(4,5)\(_2\) at FAs, PIPK\(_{\gamma, 12}\) may influence FA dynamics by competing directly with \(\beta\)-integrin tails for talin binding. Such a function for PIPK\(_{\gamma, 12}\), however, has never been shown.

Since PtdIns(4,5)\(_2\) has regulatory functions in many cellular processes, general depletion of PtdIns(4,5)\(_2\) cannot be used to study specific biological functions. Furthermore, genetic ablation of all PIPK\(_{\gamma}\) isoforms leads to perinatal or embryonic lethality in mice (Di Paolo et al., 2002; Xu et al., 2005). Overexpression of PIPK\(_{\gamma, 12}\) or the talin-binding peptide causes defects in cell spreading and FA formation suggestive of an integrin activation defect (Di Paolo et al., 2002; Ling et al., 2002). Therefore, to define the role for PIPK\(_{\gamma, 12}\) in regulating FAs we selectively ablated PIPK\(_{\gamma, 12}\) from FAs by deleting the exon encoding the FA targeting signal. This approach provided a unique opportunity to examine the role of a specific pool of PtdIns(4,5)\(_2\) on integrin function. As predicted, the consequences of disrupting localized PtdIns(4,5)\(_2\) synthesis resulted in small and transient defects in integrin-mediated cell adhesion and force coupling due to compensation by the bulk membrane diffusion of PtdIns(4,5)\(_2\). It also demonstrated that talin and vinculin are less efficiently recruited to new adhesions in the absence of FA-localized PtdIns(4,5)\(_2\). Thus, in addition to demonstrating the significant role of localized PtdIns(4,5)\(_2\) in integrin function, this study emphasizes the importance of performing sophisticated sensitive measures to define subtle alterations in cell function.

**Results**

**Generation of PIPK\(_{\gamma, 12}\)-deficient fibroblasts**

To obtain cells in which PIPK\(_{\gamma}\) is excluded from FAs, embryonic stem (ES) cells were generated, in which the exon encoding the talin-binding sequence of PIPK\(_{\gamma}\) (exon 17) was flanked by loxP sites (Supplementary Figure S1). The mutant ES cells were used to establish a mouse strain in which the frt-flanked neo cassette was removed with a transgenic line expressing a deleter flipase. Subsequent Cre-mediated deletion of exon 17 produced mice expressing only the talin binding-deficient PIPK\(_{\gamma, 11}\) whose phenotype is currently being analysed and will be described elsewhere. Mixed mouse embryonic fibroblasts (MEFs) and fibroblasts cloned from the kidney of a mouse carrying two floxed exon 17 alleles were generated and exon 17 was deleted in vitro using transient adenoviral Cre transduction. The phenotype of the MEFs and the cloned knockout kidney-derived fibroblasts (PIPK\(_{\gamma}AE17\), hereafter referred to as AE17 cells) was compared with parental floxed cells (referred to as WT cells) to avoid differences that could arise from using cells of different origins. Ablation of exon 17 led to the same defects in MEFs and cloned kidney fibroblasts; we display results from the latter cell lines throughout the manuscript, and present confirmation of some of the results in primary MEFs in the supplement.

Western blot analysis revealed that the predominant PIPK\(_{\gamma}\) splice variant expressed in fibroblasts was PIPK\(_{\gamma, 11}\), which lacks the talin-binding sequence. PIPK\(_{\gamma, 12}\) was also expressed, albeit at lower levels in wild-type (WT) cells (Figure 1A), and could be easily detected following enrichment of FA proteins using fibronectin (FN)-coated beads (Figure 1B). Although our antisera did not work efficiently in immunostaining we confirmed prior

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**Figure 1** PIPK\(_{\gamma, 12}\) is the primary PtdIns(4,5)\(_2\)-generating enzyme at FAs. (A) Western blot analysis of fibroblast protein lysates from WT and AE17 cells. In all, 50\(\mu\)g of lysate was loaded to detect PIPK\(_{\gamma, 12}\), 20\(\mu\)g of lysate was loaded to detect PIPK\(_{\gamma}\) and talin, and 5\(\mu\)g of lysate was loaded to detect actin. (B) Western blot analysis of cytoskeletal extract (CSK) from WT cells, and enriched FA fractions from WT and AE17 cells. (C) Kinase assays using equal protein amounts of CSK from WT cells, and enriched FA fractions from WT and AE17 cells, analysed by thin layer chromatography. The migration positions of PtdIns(4,5)\(_2\) (PIP\(_2\)) and an unidentified lipid contaminant from the FA preparation (asterisk) are identified. Ori, origin. Figure source data can be found in Supplementary data.
FA-specific PtdIns(4,5)P2 increases the rate of talin accumulation in FAs

To investigate how early stages of adhesion formation are altered in the absence of local PtdIns(4,5)P2 synthesis, we monitored the incorporation rates of specific molecules into FAs using TIRF microscopy (Figure 2A–E). Talin1, vinculin and kindlin 2 were selected for this analysis because talin and vinculin bind PtdIns(4,5)P2 (Goksoy et al., 2008; Palmer et al., 2009), and kindlin 2 contains a PH domain and also binds phosphoinositides (Qu et al., 2011). Paxillin incorporation was monitored as it is believed not to be influenced by PtdIns(4,5)P2. Each protein was tagged with GFP and incorporation rates were calculated by monitoring the increase in fluorescence in individual new FAs over time (Figure 2A). The incorporation rates of each of these proteins in WT cells were similar to a previously reported rate of paxillin accumulation into FAs in fibroblasts (Doan and Huttenlocher, 2007). In the absence of local PtdIns(4,5)P2 synthesis, the incorporation rate of talin1 into new FAs was diminished by half (Figure 2A and B; Supplementary Figure S3A; Supplementary Movie 1). The incorporation rate of vinculin was also decreased by half as expected for talin-dependent recruitment of vinculin to FAs (Crichley, 2004; Zhang et al., 2008; Figure 2C). In contrast, recruitment of kindlin 2 and paxillin to new FAs was insensitive to PIPKIγ_i2 (Figure 2D and E; Supplementary Figure S4A). The independence of kindlin 2 recruitment and increased local PtdIns(4,5)P2 synthesis raises the issue of why it was not affected despite the presence of a potential PtdIns(4,5)P2 binding domain. To explore the reason for this insensitivity, we used a vesicle sedimentation assay to determine the phosphoinositide binding profile of kindlin 2 in the context of a lipid bilayer. We found that both kindlin 2 and the talin head domain cosedimented with vesicles containing either PtdIns(4,5)P2 or PtdIns(3,4,5)P3, although kindlin 2 sedimented more efficiently with PtdIns(3,4,5)P3 (Supplementary Figure S4B). Therefore, although kindlin 2 bound to PtdIns(4,5)P2 in vitro, this lipid was not a recruitment signal for kindlin 2 in vivo.

To examine the possibility of a kinase-independent role for PIPKIγ_i2 in talin trafficking, we monitored talin recruitment to new adhesions in ΔE17 cells expressing kinase-dead PIPKIγ_i2 (PIPKIγ KD; Figure 2F). PIPKIγ_i2 KD failed to increase the rate of talin recruitment, thus eliminating the possibility that PIPKIγ_i2 functions as an adapter to direct talin to FAs. The recruitment was slightly decreased, probably owing to competition between PIPKIγ_i2 and the β-integrin tail for binding to the talin head, but the low expression level of PIPKIγ KD rendered the effect of competition insignificant. In contrast, expression of WT PIPKIγ_i2 in ΔE17 cells rescued the recruitment of talin to FAs. Therefore, the kinase activity of PIPKIγ_i2 is essential for the normal recruitment rate of talin to new FAs, and the advantage imparted by local synthesis of PtdIns(4,5)P2 more than compensates for any competition between PIPKIγ_i2 and β-integrin at the expression level achieved in this assay.

To dissect which of the talin-mediated PtdIns(4,5)P2-dependent functions are affected by the loss of localized PtdIns(4,5)P2 production, we created mutations within talin either to disrupt the autoinhibitory interaction or to disrupt the orientation of the talin head on the plasma membrane. The ‘unclamping’ mutations K318A/K320A within the F3 domain, or the E1770K mutation within the talin rod did not change the incorporation rate into new adhesions compared with WT talin, indicating that release of talin autoinhibition was not the rate-limiting step for incorporation into FAs (Figure 2G). To examine whether orientation of talin on the plasma membrane is disrupted, we made a mutation within a basic ridge of the talin F2 domain (K274E) which was shown to reduce the interaction of talin with acidic phospholipids (Anthis et al., 2009; Saltel et al., 2009). This mutation reduced the incorporation rate of talin into new FAs in WT cells to a similar extent as for WT talin in ΔE17 cells (Figure 2H). Taken together, these data show that PtdIns(4,5)P2 synthesized by PIPKIγ_i2 facilitates talin recruitment to FAs through an interaction between PtdIns(4,5)P2 and the basic ridge on the talin F2 subdomain, rather than through relieving the autoinhibitory interaction.

FA-specific PtdIns(4,5)P2 is critical for establishing cell adhesion

To obtain an approximate measure of initial adhesion in the presence or absence of local PtdIns(4,5)P2 synthesis, we conducted a ‘plate-and-wash’ adhesion assay on FN-coated dishes as a function of time (Figure 3A and B). This assay measures the rate that cells attach to a substrate, but does not provide an indication of the number of adhesive bonds or how strongly cells adhere (Boettiger and Wehrle-Haller, 2010). The rate of attachment of ΔE17 cells was significantly decreased compared with WT cells in magnesium-containing adhesion buffer (rate constant 0.067 ± 0.021/min (WT) versus 0.036 ± 0.018/min (ΔE17); Figure 3A); the adhesion was integrin-specific as incubation with 100 μM cilengitide, a concentration that blocks both β1- and β3-integrins (Frank et al., 2010), largely abolished binding to the FN-coated dish (Figure 3A). When manganese was used in place of magnesium to bypass inside-out signalling there was no difference in the rate constants for cell attachment (0.102 ± 0.044/min for WT versus 0.108 ± 0.057/min for ΔE17; Figure 3B). Thus,
PtdIns(4,5)P₂ synthesized by PIPKIγ_i2 contributes to the initial attachment of cells to FN. Since diffusion would supply PtdIns(4,5)P₂ synthesized by other PIPKI isoforms, we used single-cell atomic force microscopy (AFM) to measure formation of adhesive bonds during the first seconds of cell-substrate contact. Cells attached to the AFM cantilever were brought into contact with a FN-coated surface for 10–30 s and then the cantilever was retracted from the surface, generating a detachment force curve. Detachment force was calculated as the maximum deflection of the AFM cantilever from the

**Figure 2** Talin and vinculin recruitment to new FAs is impaired in PIPKIγ_i2ΔE17 fibroblasts. (A) TIRF time-lapse images were collected from the leading edge of GFP–talin-expressing WT or ΔE17 fibroblasts. The incorporation of talin into new adhesions (arrows) was monitored by quantifying the rate of increase in GFP epifluorescence. Heatmaps of pixel intensities from a representative example are shown. (B–E) Rate constants of incorporation were determined from the linear phase of GFP epifluorescence increase in cells expressing GFP-tagged (B) talin (n = 11 WT, 9 ΔE17), (C) vinculin (n = 7 WT, 7 ΔE17), (D) kindlin2 (n = 6 WT, 7 ΔE17) and (E) paxillin (n = 6 WT, 6 ΔE17). (F) Talin incorporation rate was calculated for ΔE17 cells expressing GFP (vector; 4 cells), kinase-dead GFP–PIPKIγ_i2 (PIPKIγ KD; 7 cells) or wild-type GFP–PIPKIγ_i2 (PIPKIγ; 6 cells). (G) The incorporation rates of the talin ‘unclasping’ mutants K318A/K320A (KK-AA) and E1770K were calculated in WT cells (n = 6 KK-AA, 8 E1770K) and ΔE17 cells (n = 9 KK-AA, 6 E1770K). (H) The incorporation rate of talinK274E was compared with the incorporation rate of WT talin in WT (n = 6 WT, 5 K274E) and ΔE17 cells (n = 5 WT, 5 K274E). All data are mean ± s.e.m. Mann–Whitney tests were used to establish statistical significance in (B–H); NS = not significant. Scale bar in (A) = 1 μm.
To distinguish between integrin-dependent and integrin-independent adhesion, we blocked integrin function by incubating cells with a linear RGD peptide (Supplementary Figure S5A and B). The difference between the force required to detach cells in the presence and absence of the peptide corresponds to integrin-dependent adhesion. At both time points tested, the integrin-dependent adhesion arising from DE17 cells was reduced compared with WT cells, indicating that the localized production of PtdIns(4,5)P2 at adhesion sites was important for the initial interaction of integrins with FN (Supplementary Figure S5B). As both α5β1- and αvβ3-integrins can contribute to FN binding, we used specific integrin blockers to test whether PIPKIγi2 activity affects the function of β1- and β3-integrins differently (Figure 3C). Antibody-mediated blockade of α5β1 activity significantly reduced the detachment force of WT cells, whereas ΔE17 cells, which had markedly reduced detachment force compared with WT cells in the absence of inhibitors, were not significantly affected by α5β1 blockade. The addition of 100 nM cilengitide, a low concentration that specifically blocks β3-integrins (Frank et al, 2010), reduced the detachment force of both cell lines by ~180 pN; furthermore, a plate-and-wash assay using 5 μg/ml vitronectin as an αvβ3-specific ligand showed comparable binding between the cell lines (Supplementary Figure S6; rate constant 0.034 ± 0.018 for WT and 0.029 ± 0.021 for ΔE17), indicating that the adhesive contribution of β3-integrins is unchanged in the absence of PIPKIγi2. Therefore, reduced PtdIns(4,5)P2 at FAs reduced α5β1-dependent cell adhesion.

To assess the effect of PIPKIγi2 on the increase in adhesive α5β1 bonds, we employed a spinning disc adhesion...
assay, in which the force required for cell detachment is proportional to the number of adhesive integrin–FN bonds (García et al., 1998; Boettiger, 2007). In this assay, cells are uniformly plated onto FN-coated coverslips and subjected to a linear hydrodynamic shear stress gradient in a buffer-filled chamber. Cells at the centre of the disc are exposed to negligible shear, whereas shear force increases linearly with distance from the centre (Figure 3D, diagonal line). The cell detachment profile fits a sigmoid curve from which the mean shear stress required to detach a cell (±S) is calculated (Figure 3D). This value is proportional to the number of adhesive bonds (in this case bonds between cell surface integrin and FN) (García et al., 1998; Shi and Boettiger, 2003; Boettiger, 2007). After 5 min of adhesion, few ΔE17 cells could be observed on the coverslip (Supplementary Figure S7A) supporting the plate-and-wash and AFM data, demonstrating that local PtdIns(4,5)P2 synthesis is important for the initial attachment event. Once the cells had adhered the rate of increase in the number of adhesive integrin–ligand bonds was similar for WT and ΔE17 (rate constant for 3 μg/ml FN coating 0.062 ± 0.013/min for WT versus 0.066 ± 0.018/min for ΔE17; Figure 3E), although the number of ΔE17 cells adhered to the disc remained lower than WT (Supplementary Figure S7A). A higher FN coating density (5 μg/ml) rendered differences between WT and ΔE17 cells insignificant (Supplementary Figure S7B and C). Consistent with an insensitivity of β3-integrins to PIPKIγ2 loss, adhesion to vitronectin (2 μg/ml) resulted in the same mean shear force required to detach both cell lines (Supplementary Figure S7D). However, low numbers of cells counted on the coverslip at early times prevented rate constants from being calculated (Supplementary Figure S7E). Incubation with 100 μM cilengitide greatly reduced the number of cells that adhered to the coverslip (Supplementary Figure S7F). Thus, local PtdIns(4,5)P2 affected the initial attachment as suggested above but had little or no effect on the rate of formation of α5β1–FN bonds once the cells attached.

Formation of new FAs in newly attached cells
To monitor the appearance of FAs in adherent cells over time, GFP–talin-transfected cells were plated onto FN for discrete time intervals and immunostained for paxillin (Figure 4A–C). Peripheral adhesions containing both paxillin and talin were well formed in WT cells after 20 min, whereas ΔE17 cells showed a punctate localization of paxillin throughout the basal cell membrane that was relatively deficient in talin (Figure 4A). Between the 20- and 40-min time points the peripheral adhesions structures matured into elongated FAs in WT cells and were fully matured after 60 min. In contrast, adhesion maturation appeared to be delayed in ΔE17 cells, with talin colocalization with paxillin apparent after 40 min (Figure 4A). Quantifying the cell area occupied by adhesion structures revealed a significant reduction in the paxillin-positive area in ΔE17 cells after 1 h of plating (Figure 4B). Phalloidin staining indicated that actin fibrils were able to anchor to FAs in both cell lines (Supplementary Figure S8). Morphometric analysis of FA length in cells plated for 1 h revealed that while 30% of WT adhesions were >3 μm in length, only 10% of ΔE17 adhesions were >3 μm long (Figure 4C), supporting delayed maturation of ΔE17 FAs.

Next, we plated cells on different concentrations of FN to assess their spreading behaviour. One hour after plating, ΔE17 cells were significantly less spread than their WT counterparts (Figure 4D and E; Supplementary Figure S3B); this difference was abolished following a longer incubation (Figure 4D), indicating that while ΔE17 cells are impaired at early time points, at steady state they perform as well as WT cells. During 1 h of spreading, ΔE17 cells plated on the lowest density of FN (coated at 2 μg/ml) achieved a two-fold increase in spread area, whereas WT cells increased their area four- to five-fold (Figure 4E); less dramatic differences were observed at higher densities of FN. Importantly, the adhesion and spreading defects in ΔE17 cells were not due to reduced surface expression levels of FN-binding integrins. FACS analysis showed similar levels of α5β1 and αvβ3 surface expression (Supplementary Figure S9A). Nor were the differences due to an intrinsic inability of integrins to respond to ligand. Incubation with soluble RGD ligand resulted in similar increases in 9EG7 antibody binding, a reporter for the ‘active’ β1-integrin conformation (Supplementary Figure S9B). One hour after plating, cell staining with the 9EG7 antibody was similar for WT and ΔE17 cells (Supplementary Figure S9C).

Altogether these data indicate that FA-localized PtdIns(4,5)P2 synthesis facilitates cell adhesion (the binding of integrins) by accelerating the recruitment of talin to new adhesions. PtdIns(4,5)P2 affects the early stages of cell attachment and FA maturation most profoundly but once cells become stably adherent the accumulation of additional bonds was not reduced by FA-localized PtdIns(4,5)P2 deficiency.

FA-specific PtdIns(4,5)P2 is important for development of force generation
Thus far our assays failed to demonstrate that the inside-out function of talin in promoting integrin activation is impaired in the absence of localized PtdIns(4,5)P2 synthesis. However, adherent ΔE17 cells exhibited a spreading defect despite a normal rate of accumulation of integrin–ligand bonds (as measured by the spinning disc assay). Therefore, FA-specific PtdIns(4,5)P2 synthesis likely serves additional functions. Since the generation of intracellular force is required for cells to spread we examined the ability of integrins to transduce force in the absence of localized PtdIns(4,5)P2 synthesis. Previous work has shown that the spinning disc assay measures the total number of bound integrins per cell independently of cytoskeletal tension, but only tensioned integrins can be crosslinked to ligand (Friedland et al., 2009). We, therefore, monitored β1-integrin crosslinking to a 5 μg/ml FN-coated surface as a function of time. During the first 2 h after cell plating, the amount of crosslinked β1-integrin was reduced by half in ΔE17 cells compared with WT cells (Figure 5A, 2.04 ± 0.11% for WT cells versus 1.11 ± 0.09% for ΔE17 cells), although spinning disc analysis showed that the total number of bonds was not reduced in the ΔE17 cells at this FN concentration (Supplementary Figure S7B). The reduced proportion of crosslinked β1-integrin indicates a reduction in the number of tensioned integrins (Friedland et al., 2009), suggesting that local PtdIns(4,5)P2 synthesis promotes force coupling of integrins to ligand. The ability of cells to exert force on their environment was also examined by monitoring the contraction of FN-impregnated collagen gels seeded with WT or ΔE17 cells. The data in Figure S5B and C clearly show that ΔE17 cells contracted...
the collagen gels less efficiently than WT control cells. Exponential decay curves fit to the data in Figure 5C revealed a four-fold reduction in the rate constant for collagen gel contraction by D_E17 cells (WT = 0.209 ± 0.027/h; D_E17 = 0.052 ± 0.042/h).

To obtain a direct measure of force, WT and D_E17 cells were plated onto 5 μg/ml FN-coated flexible polyacrylamide gels embedded with fluorescent beads (E = 12.8 kPa). The elastic strain energy stored in the gel as a result of cell traction was calculated as the product of local deformations of the gel (Figure 5D). When normalized for cell area the strain energy imparted by D_E17 cells was half of that imparted by WT cells (Figure 5E). In contrast, no differences were observed in phosphorylation of myosin light chain (MLC) as measured by glycerol-urea PAGE, a proxy measure of activation of myosin contraction (Figure 5F). Therefore, the defect probably lies at the level of the integrin–actin interaction.

The transition between lamellipodium and lamellum (characterized by fast actin retrograde flow and slow actin flow, respectively) is marked by the formation of focal complexes, which capture actin filaments and act as a clutch (Alexandrova et al., 2008; Giannone et al., 2009). We reasoned that if the integrin–actin clutch is defective in D_E17 cells the formation of the lamellipodium–lamellum border may be delayed, leading to a wider lamellipodium. Using enhanced

![Figure 4](https://example.com/figure4.png)

**Figure 4** FA formation and cell spreading are delayed in the absence of local PtdIns(4,5)P2 synthesis. (A) Immunofluorescence images of WT and D_E17 fibroblasts expressing GFP–talin (green) and immunostained for paxillin (red) after 20, 40 or 60 min of spreading on 5 μg/ml FN-coated glass coverslips. Boxed areas in the upper panels are magnified in the lower panels. (B) Quantification of the cell area occupied by FAs in 25 cells of each genotype. Values are mean ± s.d. A Mann–Whitney test was used to establish statistical significance. (C) FA lengths measured from cells immunostained for paxillin were measured and binned into 0.5 μm increments to obtain a comparative distribution of FA length in WT (black bars, 1128 FAs from 10 cells) and D_E17 (grey bars 1006 FAs from 10 cells) cells. (D) Representative bright field images of WT and D_E17 fibroblasts plated onto plastic dishes coated with the indicated concentrations of FN for 1 h, or overnight in the absence of serum. (E) Temporal analysis of spreading behaviour for the cells plated on 2 μg/ml fibronectin. Squares = wild type; triangles = D_E17. Values are mean ± s.e.m. for 15 cells of each genotype.
phase contrast microscopy to directly observe actin flow within the narrow lamellipodium (Verkhovsky et al., 2003; Alexandrova et al., 2008; Figure 6A and C; Supplementary Movie 2A and B) we could visualize fast retrograde actin flow that manifested as dark bands in kymographs (Figure 6B and D). The net forward movement of the cell front in the kymographs shows that protrusive lamellipodia were analysed in both WT and ΔE17 cells. By measuring the length and angle of the dark bands, we determined that the velocity of retrograde actin flow was unchanged between WT and ΔE17 cells (Figure 6E); however, the width of the lamellipodium was significantly increased in ΔE17 cells (Figure 6F). This indicates that the actin cytoskeleton was less coupled, and therefore not slowed as efficiently by the integrin clutch. Consistent with this, stable talin-rich adhesions were present at the lamellipodium-lamellum border in WT cells and were excluded from the lamellipodium (Figure 6G), whereas in ΔE17 cells stable adhesions extended beneath the cortactin-rich lamellipodium to the edge of the cell (Figure 6H).

Despite several lines of evidence supporting a force coupling defect in ΔE17 cells, it is interesting to note that migration defects were not apparent in these cells. Both a scratch assay and single-cell tracking assay failed to uncover differences in migration speed (Figure 7A; Supplementary Figures S3C and S10) or persistence (Euclidean distance/accumulated distance; WT = 0.44, ΔE17 = 0.46; Figure 7B) in two-dimensional culture compared with WT controls. Chemotactic migration towards a gradient of epidermal growth factor (EGF) was likewise not affected at all concentrations tested but migration towards platelet-derived growth factor (PDGF) was abolished in ΔE17 cells (Figure 7C), supporting a role for PIPKIγ2 in chemotactic response to certain growth factors (GFs; Sun et al., 2007).

Discussion
The assembly and disassembly of FAs and their linkage to the F-actin cytoskeleton are tightly controlled by complex combinations of post-translational modifications including phosphorylation, dephosphorylation, ubiquitination and proteolysis. Additionally, the proximity of the plasma membrane makes it likely that protein–phospholipid interactions also participate in the control of FA function. Several FA molecules including talin and vinculin can bind to the lipid PtdIns(4,5)P2 and PIPKIγ, one of several lipid kinase isoforms in the cell that make PtdIns(4,5)P2, localizes to FAs, raising the possibility that FA-localized PtdIns(4,5)P2 synthesis has a specific regulatory function within FAs. We sought to understand which aspects of FA function are controlled by PtdIns(4,5)P2 synthesis in FAs. To avoid a large, global perturbation of PtdIns(4,5)P2 within the cell, we ablated the FA localization of PIPKIγ to achieve a small but specific depletion of...
PtdIns(4,5)P2 at this location. We find that this specific pool of PtdIns(4,5)P2 regulates the rate of talin recruitment into FAs. Although talin binds to PtdIns(4,5)P2 at several locations within the FERM domain (Goksoy et al., 2008; Anthis et al., 2009; Saltel et al., 2009; Goult et al., 2010) mutational analysis shows that an interaction between PtdIns(4,5)P2 and the talin F2 subdomain is responsible for mediating the recruitment of talin to adhesions. Preventing the autoinhibitory conformation by mutating residues important for the talin head–tail interaction did not normalize talin recruitment in DE17 cells. Therefore, the reduced recruitment rate does not result from a failure to activate talin. Abnormal recruitment of talin in ΔE17 cells has two consequences. First, the early formation of integrin–ligand bonds is impaired, leading to a reduced rate of initial cell attachment to FN-coated surfaces. Second, the transduction of force through integrins to the environment is reduced as a result of abnormal coupling of integrins to the actin cytoskeleton.

The earliest detectable consequence of impaired PtdIns(4,5)P2 synthesis in FAs is a reduced rate of cell attachment. Our AFM experiments revealed a profound delay in integrin-mediated attachment to a FN-coated surface in ΔE17 cells, which is reflected in the reduced adhesion of ΔE17 cells measured by a plate-and-wash assay. Since this assay measures the proportion of cells that adhere, but not how strongly they adhere (Boettiger and Wehrle-Haller, 2010), we also determined the rate of bond formation using spinning disc analysis. These experiments indicate that adherent ΔE17 cells accumulate integrin–ligand bonds at the same rate as WT cells. This observation contrasts with the reduced rate of bond formation in ΔE17 cells. Since talin binding to β-integrin tails has been described as an essential step in integrin activation (Tadokoro et al., 2003; Simonson et al., 2006; Wegener et al., 2007; Bouauina et al., 2008) a similar reduction in the rate of bond formation ought to be expected, but it is not observed. Our data suggest that talin has an important role in initial cell matrix interactions but once a cell becomes adherent it does not contribute to additional integrin–ligand bonds. Extending this argument further, it may be postulated that talin facilitates a functional interaction between integrins and FN but is not essential for it to occur. Studies showing that depletion of both talin isoforms does

Figure 6 Enhanced phase contrast and fluorescence microscopy reveals an adhesion-actin coupling defect in PIPKIγΔE17 cells. (A) A single frame from a time-lapse series of WT cells. The black line marks the region from where a kymograph was generated. (B) The kymograph obtained from the wild-type cell depicted in (A). Red lines are examples of regions used to measure the distance and angle of the dark bands corresponding to retrograde actin flow. (C) A single frame from a time-lapse series of ΔE17 cells, showing the region from which the kymograph was derived (black line). (D) The kymograph obtained from the cell depicted in (C). Red lines are examples of regions used to calculate the length and angle of bands resulting from retrograde actin flow. (E, F) Lengths and angles of 50 actin flow lines from 9 cells of each genotype were used to calculate the rate of retrograde actin flow (E) and depth of penetration of the fast actin flow (as defined as lamellipodial width) (F). Plots are mean ± s.d. (G, H) GFP-talin-expressing WT (G) and ΔE17 cells (H) were immunostained for cortactin (blue) and localization of talin (green) relative to the lamellipodium was examined. Arrowheads denote adhesions that terminate at the lamella–lamellipodium border. Arrows in (H) highlight regions where the GFP–talin signal overlaps with the lamellipodium in ΔE17 cells.
not completely block adhesion or initial spreading in fibroblasts (Zhang et al., 2008), and does not impair the binding of β1-integrin to FN-coated beads (Roca-Cusachs et al., 2009) support this assertion. The recruitment of kindlin 2 into adhesions was not affected by the absence of FA-localized PtdIns(4,5)P2 synthesis. Therefore, PtdIns(4,5)P2 binding does not serve as the rate-limiting step for kindlin 2 recruitment. It remains unknown whether PtdIns(4,5)P2 is a true physiologic ligand for the kindlin 2 PH domain, but recent work suggests that it may bind PtdIns(3,4,5)P3 most strongly (Qu et al., 2011). Our vesicle sedimentation assay demonstrates that kindlin 2 can bind anionic phospholipid vesicles rather non-specifically but in a cellular context kindlin 2 would encounter a broad choice of phosphoinositides to bind, and an array of competitors for lipid binding. Lipid signals may still influence kindlin 2 dynamics at the FA, but our study rules out PIPKIγ2 as the relevant lipid kinase.

The incorporation rate for kindlin 2 was higher than for talin (0.317 ± 0.037/min for kindlin versus 0.233 ± 0.018/min for talin), which may reflect the complexity of the interactions necessary for recruitment. Whereas kindlin 2 interacts with membrane distal sequences on the β-integrin tail and does not appear to require an interaction with lipids for assembly into FAs (Montañez et al., 2008; Qu et al., 2011) talin binds to β-integrin membrane distal sequences as well as to membrane proximal sequences which may be in close proximity with the α-integrin tail (García-Alvarez et al., 2003; Wegener et al., 2007; Anthis et al., 2009). Furthermore, interactions with the plasma membrane properly orient the talin head for productive binding to the membrane proximal sequences (Anthis et al., 2009; Kalli et al., 2010). Each of these interactions may represent a distinct binding step that together give rise to a lower rate of incorporation compared with FA components that have simpler modes of interaction. The higher kindlin 2 incorporation rate also suggests that kindlin assembles onto integrins before talin, but this has not been formally tested.

The coalescence of kindlin 2 into distinct adhesion structures visible by TIRF indicates that kindlin–integrin clusters could still form. By selectively slowing the incorporation of talin into adhesions in ΔE17 cells, we have apparently uncoupled talin recruitment from integrin clustering. These data suggest that talin does not initiate integrin clustering, but it may stabilize clusters once they have formed. Depletion of both talin isoforms results in the absence of clustered integrins (Zhang et al., 2008) and disruption of talin–integrin binding destabilizes integrin clusters (Saltel et al., 2009), implying that talin is essential for the maintenance of these structures. We propose that talin achieves this by mediating the interaction between integrins and the cytoskeleton, thereby force coupling integrin to actin–myosin to maintain tension and to stabilize the integrin–FN bond.

Several lines of evidence also point to a force coupling defect in the absence of FA-localized PtdIns(4,5)P2 synthesis. First, β1-integrin crosslinking to extracellular ligand is reduced in ΔE17 cells, similar to what is observed when cells are treated with pharmacological inhibitors of myosin
function (Friedland et al., 2009). Second, the reduced paxillin-positive area and adhesion length in ΔE17 cells support a force coupling defect as adhesion growth and elongation are regulated by the application of mechanical force to these structures (Balaban et al., 2001; Bershadsky et al., 2006). Third, ΔE17 cells are impaired in their ability to contract collagen gels despite a normal level of MLC phosphorylation. Fourth, direct measurement of force using traction force microscopy demonstrates that ΔE17 cells are deficient in force generation. Fifth, direct visualization of actin flow and indirect immunofluorescence assays revealed wider lamellipodia in ΔE17 cells that contain underlying talin-rich adhesion structures. Careful analysis has shown that the rate of lamellipodial actin flow is abruptly slowed over peripheral FAs, consistent with a model whereby integrin-associated actin-binding proteins capture fast moving actin filaments (Alexandrova et al., 2008; Gardel et al., 2008; Shemesh et al., 2009). According to our data, it is this capture step that is regulated by the local production of PtdIns(4,5)P2 at FAs. Lamellipodial actin may be captured directly by talin (Hemmings et al., 1996; Lee et al., 2004; Gingras et al., 2008, 2010), or by talin-bound vinculin (Humphries et al., 2007), thus enabling a linkage between actin and integrins. The retarded recruitment of talin and vinculin in ΔE17 cells leads to a linkage that is slower to establish. A similar defect in coupling the cytoskeleton to the membrane has been reported in PIPKIγ-null megakaryocytes (Wang et al., 2008).

A force coupling defect manifested most strongly in gel contraction assays, but did not appear to play any role in non-directional cell migration or directed migration into a wound. In these assays, cells were adherent and spread for several days in the case of the scratch assay and the single-cell tracking assay also selected for adherent, spread cells. These data are consistent with reports that establish human PIPkinz (PIPKIβ in mice), and not PIPKIγ, as an important mediator of cell migration through activating the small Rho GTPase Rac at lamellipodia, which in turn stimulates N-WASP-dependent actin polymerization (Mao et al., 2009; Chao et al., 2010). Under conditions where cells are already adherent and spread, PIPKz-mediated PtdIns(4,5)P2 synthesis at the lamellipodium may overcome the deficiency arising from loss of PIPKIγ to allow remodelling of adhesions at the leading edge and permit cell migration. As most of the defects observed as a result of impaired FA-PtdIns(4,5)P2 synthesis occur at pre-steady-state conditions, and cell migration can be considered a rearrangement of the steady state, FA-localized PtdIns(4,5)P2 synthesis may be most important under pre-steady-state conditions where diffusion of bulk PtdIns(4,5)P2 is restricted or insufficient for the needs of the cell. Indeed, steady-state phenomena such as spreading area and adhesion area following prolonged incubation, or turnover of talin in mature FAs are not affected in ΔE17 cells (Figure 4D and unpublished data).

Chemotactic migration of ΔE17 fibroblasts towards a PDGF gradient was abolished, but migration towards EGF was not impaired. This contrasts with data showing that PIPKIγ to is required for migration of HeLa cells towards EGF and hepatocyte growth factor (Sun et al., 2007). In these cells, PIPKIγ to is required to assemble talin into FAs in the direction of the GF gradient; here, we demonstrate that talin recruitment is a general function of PIPKIγ to that also occurs in the absence of GFs. Our finding that migration defects arising from PIPKIγ to deficiency depend on which GF is used as chemoattractant suggests that PIPKIγ to may have an additional function downstream of GF signalling.

Significantly, the delay in integrin–ligand bond formation and failure to establish efficient force coupling are not corrected on a similar time scale. This suggests that the two functions of talin studied here show differential sensitivity to PtdIns(4,5)P2, with force transduction being more sensitive to FA-localized PtdIns(4,5)P2 synthesis. One intriguing possibility, suggested by the AFM data, is that β1-integrin and β3-integrin functions have different sensitivities to PtdIns(4,5)P2 generated by PIPKIγ to. By using specific inhibitors, we could show that β1-mediated binding is strongly influenced by PIPKIγ to, whereas β3-mediated binding is insensitive to the absence of PIPKIγ to. Magnetic tweezer experiments have shown that while both β1- and β3-integrins can bind to FN-coated beads, only β1-integrin mediates adhesion strength (Roca-Cusachs et al., 2009). The mechanism underlying different PtdIns(4,5)P2 requirements for β1- and β3-integrin function is not known, but may also involve talin. Whereas the talin F3 domain is sufficient to activate β3-integrin, β1 additionally requires the F0/F1 domains (Bouaouina et al., 2008). An interaction between PtdIns(4,5)P2 and sequences within the F1 domain may assist in aligning this part of the talin head with the membrane (Goult et al., 2010) and this orientation of the talin head may be important to facilitate force coupling by α5β1-integrin. Future work will be heavily focused on deciphering the differences between β1- and β3-integrins and their requirement for localized PtdIns(4,5)P2 production.

Alternatively, there may be additional PtdIns(4,5)P2-sensitif structural interactions that depend on FA-localized PtdIns(4,5)P2 synthesis that have not been examined here. Although the vinculin–talin interaction is critical for the induction of agonist-induced contraction of tracheal smooth muscle cells (Huang et al., 2010), vinculin function is unlikely to be impaired in our system as PtdIns(4,5)P2 has been reported to inhibit rather than to promote actin binding (Steimle et al., 1999). An integrin–actin interaction via tensin may be perturbed, since tensin possesses PtdIns(4,5)P2 binding activity (Leone et al., 2008), but the functional significance of phosphoinositide binding and a role for tensin in force coupling remain undefined. PtdIns(4,5)P2 facilitates oligomerization of the heparin sulphate proteoglycan syndecan-4, which is also found in FAs (Woods and Couchman, 1994; Lee et al., 1998). Oligomerization of syndecan-4 is required for an interaction with the actin crosslinker z-actinin (Choi et al., 2008) but while syndecan-4 is well known to induce cytoskeleton reorganization (Couchman, 2010), a direct role in force transduction has not been reported.

Clearly, the role of PtdIns(4,5)P2 in regulating FA dynamics is complex. By generating cell lines that are deficient in FA-localized PtdIns(4,5)P2 synthesis we now have an important tool by which we can begin to address the importance of local, transient changes in PtdIns(4,5)P2 concentration in a specific cellular process.

Materials and methods

Generation of a PIPKIγ to knockout mouse and cell lines

A 495-bp DNA fragment surrounding PIPKIγ exon 17 was used to screen a PAC library. The PIPKIγΔE17 construct consisted of a

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Glass-bottom dishes were prepared with a drop of 2 μg/ml FN and blocked with 1% BSA. Cells were trypsinized, resuspended in assay buffer (10 mM HEPES-NaOH, 150 mM NaCl, 1 mM MgCl₂, 2 mM CaCl₂, 3 mM KCl and 22 mM glucose, pH 7.4 containing the appropriate inhibitors, if applicable), and sparsely added to the dish. The plate was maintained at 37°C throughout the experiment with a heated stage. Individual cells were selected from a region of the dish containing no FN, and attached to the tip of a cantilever using visual guidance of a Zeiss Axiovert 200 M fitted with a ×20 objective. Cells were allowed to attach to the cantilever, fully retracted from the surface for 5 min before force measurements were taken. During force measurement runs, cells approached a FN-coated region of the dish at 5 μm/s and were brought into contact for the indicated times with a force of 200 pN, prior to retraction from the dish at 5 μm/s. Cells were allowed to rest in the fully retracted position for 20 s between repeat measurements on adjacent FN-coated regions. Where indicated, experiments were performed in the presence of 0.2 mM linear RGD peptide (GRGDPN; Biotom), 250 μg/ml FN (Roche), 100 nM wortmannin and sonicated lipid solutions of 350 μM C6-cinglide (Horst Kessler, LMU, Munich).

Spinning disc assay

The spinning disc adhesion assay was performed as described (Boettiger, 2007). Briefly, 1 × 10⁶ cells were plated onto FN- or VN-coated coverslips for the indicated times, prior to being affixed to the spinning disc apparatus and spun at 6000 r.p.m. in Dulbecco’s PBS for 5 min. Coverslips were fixed in 2% PFA and stained with DAPI for automated counting using the × 10 objective of a Zeiss Axiovert 200 M controlled by Metamorph software (version 7.7.0.0). Images were taken at defined positions of the coverslip, a threshold was applied and detected nuclei were assigned X and Y coordinates, which were recorded in an Excel file. Excel files were imported into Sigmaplot and data were graphed and fit to the equation f = 1/(1 + exp(b(τ−c))), where c = τ₀, the mean shear stress for cell detachment.

TIRF-based incorporation assays

Cells were transiently transfected with plasmids encoding GFP-tagged proteins and analysed 24 h after transfection. Cells were trypsinized and replated onto 5 μg/ml FN-coated glass-bottom dishes and allowed to adhere for at least 1 h before analysis. Prior to analysis, media was exchanged for fluorescence microscopy buffer (10 mM HEPES-NaOH, 150 mM NaCl, 1 mM MgCl₂, 2 mM CaCl₂, 3 mM KCl and 22 mM glucose, pH 7.4) and plates were transferred to a heated stage of a Zeiss Axiovert 200 M fitted with a Coolmap HQ camera and controlled by Metamorph software (Visirion Systems). 488 nm TIRF lasers were used to excite GFP. Images were collected every 5 s for 15 min. Calculation of incorporation rates from time-lapse series were determined as described (Franco et al., 2004).

Immunofluorescence assays

To examine coincidence of talin and paclixin in spreading cells, GFP-talin-expressing cells were plated onto 5 μg/ml FN-coated coverslips for the indicated time periods, fixed with 4% paraformaldehyde (PFA) at 37°C and probed with a mouse anti-paclixin antibody followed by anti-mouse Cy3. For analysis of adhesion area and morphology of FAs, cells were prepared as above and probed with a mouse anti-paclixin antibody followed by anti-mouse Cy3 and Alexa 488-conjugated phallidin. Images were collected with a Leica SP2 confocal microscope at ×100 magnification and a digital zoom of 1.95. FA length was calculated by linear region selection in ImageJ. To examine coincidence of talin-rich adhesions with the lamellodium, cells were transfected with GFP-talin and analysed 24 h post-transfection. Cells were trypsinized and transferred to 5 μg/ml FN-coated coverslips for 2 h, fixed with 2% PFA for 15 min at 37°C and stained with a mouse anti-cortactin antibody and anti-mouse Alexa 647 secondary antibody. Images were taken sequentially with a Leica SP2 confocal microscope at ×100 magnification and a digital zoom of 2.00, and merged.

Atomic force microscopy

AFM measurements were taken with a CellHesion 200 atomic force microscope (JPK Instruments). Cantilevers were calibrated using a thermal noise method provided by the JPK CellHesion 200 control software V3.3. and force curves were analysed using JPK Image Processing software. Tipless silicon cantilevers (Arrow-TTL-50; Nanoworld) were coated with concanavalin A according to an established protocol (Franz et al., 2007) and used within 3 days.
measured before and after cell detachment with 8 mM Cytochalasin D and Trypsin/EDTA (0.25/0.02%) in PBS (Butler et al., 2002). During the measurements, the cells were maintained at 37°C in humidified atmosphere containing 5% CO₂. Gel deformations were estimated using a Fourier-based difference-with-interpolation image analysis (Metzner et al., 2007). To characterize the contractile forces of each cell, the elastic strain energy stored in the polyacrylamide gel due to cell tractions was calculated as the product of local tractions and deformations, integrated over the spreading area of the cells (Butler et al., 2002).

**MLC phosphorylation**

In all, 10 cm dishes of subconfluent cell monolayers were harvested by washing with PBS and incubating on ice with 800 μl 10% TCA/10 mM DTT. Material was scraped into a tube, washed twice with diethyl ether and dried under vacuum. Cell pellets were solubilized at room temperature with saturating amounts of urea in 20 mM glycine/22 mM Tris—Cl, pH 8.6, 1 mM EDTA, 10 mM DTT and 5% glycerol. MLC was separated from phospho-MLC by glycerol-urea PAGE (Garcia et al., 1995; Mehta et al., 2002).

**Data analysis and statistics**

Analysis of microscopy images was carried out using 64-bit ImageJ 1.43 (http://rsb.info.nih.gov/ij/) with the MBF ‘ImageJ for Microscopy’ plugin (http://www.macbiophotonics.ca/downloads/MBF_ImageJ.zip), Image Stabilizer plugin (http://www.cs.cmu.edu/~kangli/code/Image_Stabilizer.html) and Chemotaxis and Migration Tool (http://www.ibidi.de/applications/ap_chemo.html) installed. Statistical significance was established using either Mann–Whitney tests using the R statistical software package, or using Sigmaplot. Non-linear best-fit lines and constants derived from them were generated in Sigmaplot.

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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**Author contributions:** The overall study was conceived and supervised by RF. KRL designed and performed and analysed most experiments. ST made the targeting construct enabling the generation of the cell lines. NB performed and analysed the traction force experiments, under the supervision of BF. DB contributed the spinning disc technology and participated in analysing the results. KRL, RF, DB and RZ wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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