Ezrin is required for efficient Rap1-induced cell spreading

Sarah H. Ross*, Anneke Post‡, Judith H. Raaijmakers‡, Ingrid Verlaan, Martijn Gloerich and Johannes L. Bos

Molecular Cancer Research, Centre for Biomedical Genetics and Cancer Genomics Centre, University Medical Centre Utrecht, 3584 CG Utrecht, The Netherlands

*Author for correspondence (S.Ross@umcutrecht.nl)
‡These authors contributed equally to this work

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Summary

The Rap family of small GTPases regulate the adhesion of cells to extracellular matrices. Several Rap-binding proteins have been shown to function as effectors that mediate Rap-induced adhesion. However, little is known regarding the relationships between these effectors, or about other proteins that are downstream of or act in parallel to the effectors. To establish whether an array of effectors was required for Rap-induced cell adhesion and spreading, and to find new components involved in Rap-signal transduction, we performed a small-scale siRNA screen in A549 lung epithelial cells. Of the Rap effectors tested, only Radil blocked Rap-induced spreading. Additionally, we identified a novel role for Ezrin downstream of Rap1. Ezrin was necessary for Rap-induced cell spreading, but not Rap-induced cell adhesion or basal adhesion processes. Furthermore, Ezrin depletion inhibited Rap-induced cell spreading in several cell lines, including primary human umbilical vein endothelial cells. Interestingly, Radixin and Moesin, two proteins with high homology to Ezrin, are not required for Rap-induced cell spreading and cannot compensate for loss of Ezrin to rescue Rap-induced cell spreading. Here, we present a novel function for Ezrin in Rap1-induced cell spreading and evidence of a non-redundant role of an ERM family member.

Key words: Cell spreading, Ezrin, Focal adhesions, Rap1

Introduction

The response of cells following contact with an extracellular matrix, either for spreading or for migration, requires the tightly controlled regulation of cell adhesion, actin polymerisation and force generation (Maheshwari et al., 2000). Co-ordination of these processes is ensured by signals that derive from inside the cell (inside-out signalling) and those received through interactions with the extracellular environment (outside-in signalling) (Geiger et al., 2001; Ginsberg et al., 2005; Luo et al., 2007).

The small GTPase, Rap1, is strongly implicated in the regulation of inside-out signalling of cell-matrix adhesion (Bos et al., 2003; Caron, 2003). Within cells, levels of active Rap–GTP are spatially and temporally controlled through the activity of GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), such as the cAMP-regulated Epac proteins. Studies in which Rap has been selectively activated in cells, either by overexpression of constitutively active Rap proteins, or by the specific activation of endogenous Rap using the Epac-specific cAMP analogue, 8-pCPT-2′-O-Me-cAMP (also called 007), have demonstrated that Rap activation has the capacity both to increase the affinity of integrins for their ligand (Caron et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000) and to promote integrin clustering (Boettner and Van Aelst, 2009; Bos, 2005; Caron, 2003; Kinbara et al., 2003). Along with this, Rap can regulate the cytoskeleton, as exemplified by the induction of cortical actin formation in endothelial cells (Cullere et al., 2005; Fukuhara et al., 2005; Kooistra et al., 2005; Kooistra et al., 2007) and the inhibition of membrane protrusion in migrating cells (Lyle et al., 2008). These functions of Rap are fundamental for the control of basal adhesion (Tsukamoto et al., 1999) as well as for activation of integrins in response to a number of external stimuli, including Mn^{2+} (de Bruyn et al., 2002).

Although the outcome of Rap activation on cell adhesion and actin remodelling is well established, the mechanisms by which these occur are less well understood, but are beginning to be elucidated. A number of proteins, including Riam (Lafuente and Boussiotis, 2006; Lafuente et al., 2004; Lee et al., 2009; Watanabe et al., 2008), RapL (Ebisuno et al., 2010; Katagiri et al., 2006; Katagiri et al., 2003; Kinashi and Katagiri, 2004; Miertzschke et al., 2007), Arap3 (Krugmann et al., 2004; Raaijmakers et al., 2007), Tiam1 (Arthur et al., 2004), AF6 (Boettner et al., 2000; Zhang et al., 2005), and Radil (Ahmed et al., 2010; Smolen et al., 2007) have been proposed to be Rap effector proteins that mediate aspects of Rap-induced changes to integrins or the cytoskeleton (Bos, 2005; Raaijmakers and Bos, 2009). Most of these proteins have been identified by their ability to bind to Rap–GTP via RA domains (Frische and Zwartkruis, 2010). However, with the exception of Riam and RapL, which can regulate integrin complexes (Ebisuno et al., 2010; Katagiri et al., 2006; Katagiri et al., 2003; Kinashi and Katagiri, 2004; Lafuente and Boussiotis, 2006; Lafuente et al., 2004; Lee et al., 2009; Miertzschke et al., 2007; Watanabe et al., 2008), it is unclear how most of these different proteins bring about Rap-induced changes. Furthermore, it is not yet understood how the functions of these individual proteins relate to one another. In a particular cell type, Rap-regulated adhesion and cytoskeletal changes might require activation of a single effector protein. Alternatively, a number of proteins might be regulated by Rap concurrently or sequentially, performing separate functions, which are all required downstream of Rap.

In order to determine whether an array of proteins is required for Rap-induced focal adhesion formation and cell spreading, we performed a limited siRNA screen in A549 cells, using a small library of siRNAs against putative Rap effectors and other proteins indirectly implicated in Rap-mediated adhesion. As expected, core regulators of focal adhesions and cellular tension were required for both basal and Rap-stimulated cell spreading. In the Rap signalling pathway, we found that, although both Rap1A and Rap1B are expressed in these cells, only siRNA against Rap1A blocked the changes in cellular morphology induced by activation of the Epac–Rap signalling pathway. Moreover, of the Rap effectors investigated in the screen, depletion of Radil had the biggest inhibitory effect on Rap-induced spreading. Interestingly, we identified that Ezrin, one of the Ezrin–Radixin–Moesin (ERM) family proteins, played a specific role in Rap1-induced spreading changes. Our results indicate that Ezrin is not crucial for the initial phase of Rap-induced adhesion, but rather functions during later stages of the Rap-dependent spreading processes. Moreover, this function of Ezrin does not appear to be shared by Radixin and Moesin. Here, we report on our findings, which demonstrate a specific role of an ERM family protein in Rap-regulated cellular spreading.

Results

Knockdown of Ezrin and of core components of cell adhesion inhibits cell spreading following activation of the Rap signal transduction pathway

To analyse the activation of the Rap signalling pathway, we used A549-Epac1 cells, and activated the endogenous Rap proteins through the exchange factor, Epac1, using the cAMP analogue, 007. We then used immunofluorescence to monitor changes to focal adhesions, the cytoskeleton and cell shape. Following 3 hours of adhesion and spreading on fibronectin with Rap activation, cells showed a marked difference in overall morphology compared to cells that had spread under control conditions. Treatment of cells with 007 stimulated the spreading of cells, and promoted an increase in the number of focal adhesions around the periphery of individual cells (Fig. 1A and supplementary material Fig. S1). This increase in focal adhesions resulted in the Rap-stimulated cells taking on a pronounced rounded morphology, quite unlike the angularly shaped control cells (Fig. 1A and supplementary material Fig. S1). This rounded cell morphology was not shown by mock-treated cells, which were allowed to spread on fibronectin for longer than 3 hours. This suggests that prolonged activation of the Rap signalling pathway induced a distinctive type of cell spreading.

To confirm that the 007-phenotype was due to stimulation of the Epac–Rap signalling pathway, we blocked activation of the pathway using siRNA against Rap and Epac proteins. We found that siRNA against Rap1A (Fig. 1B and supplementary material Fig. S2) and Epac1 (supplementary material Table S1) inhibited the basal spreading of cells and blocked the response of cells to 007. Interestingly, siRNA against Rap1B (Fig. 1C and supplementary material Fig. S2) and the Rap2 proteins (supplementary material Table S1) did not block 007-induced spreading, suggesting that the changes in cell morphology that we observed were induced specifically by Rap1A. These data validated the conclusion that the distinctive phenotype induced by 007 resulted from activation of the Epac–Rap pathway, and that monitoring these morphological changes could be used to screen for components and mediators of 007-induced adhesion.

Our screen used a custom siRNA library that targeted proteins that have been previously described to be Rap effectors, as well as other proteins linked to Rap function, regulation of the cytoskeleton, focal adhesions and cell polarity. We found that siRNA against most of the proteins targeted in our screen did not block the 007 effect (supplementary material Table S1). However, knockdown of both Talin (Fig. 2A and supplementary material Fig. S2) and RockII (supplementary material Table S1) inhibited basal and 007-induced adhesion and spreading, consistent with their essential function in regulating integrins and cell spreading. Of the reported Rap effectors, only knockdown of Radil (also known as AF6-L) caused an inhibition of 007-induced cell spreading in approximately 70% of the replated cells (Fig. 2B and supplementary material Fig. S2).

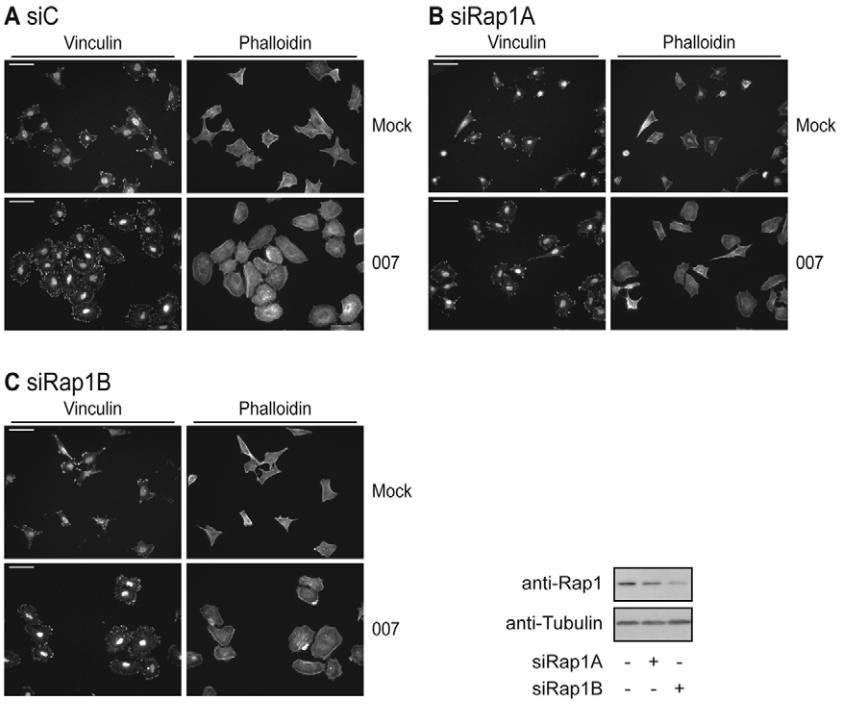

Fig. 1. Activation of the Epac1-Rap signalling pathway by the cAMP analogue, 8-pCPT-2′-O-Me-cAMP, stimulates cell spreading, rounding and focal adhesion formation. A549-Epac1 cells were treated with (**A**) scrambled siRNA (siC), (**B**) siRNA against Rap1A (siRap1A) or (**C**) siRNA against Rap1B (siRap1B) for 48 hours. Cells were then replated and allowed to adhere to fibronectin for 3 hours with 100 μM 007 or without 007 (mock). Following adhesion and spreading, cells were fixed. Anti-vinculin antibodies were used to detect focal adhesions and phalloidin was used to detect F-actin by immunofluorescence. Western blot analysis to confirm knockdown of Rap1A and Rap1B is shown alongside. The antibody used recognises both Rap1A and Rap1B isoforms. Scale bars: 50 μm.

This result was corroborated by two single siRNAs against Radil, and confirms previous reports of a role for Radil in Rap-induced cell adhesion (Ahmed et al., 2010; Smolen et al., 2007). In comparison, other Rap effector proteins, such as Riam (Fig. 2C and supplementary material Fig. S2), did not block the 007 effect. This suggests that, in the A549-Epac1 cells, Radil plays a more crucial role in 007-induced spreading and focal adhesion formation than other Rap effectors. Knockdown of AF6 (Fig. 2D and supplementary material Fig. S2) increased the spreading of A549-Epac1 cells in response to 007, which is consistent with its previously described role as a negative regulator of Rap function (Zhang et al., 2005). Although Radil is known as AF6-L, because, like AF6, it contains RA and DIL domains, the divergence of these two genes predates the emergence of animals (T. J. P. van Dam, personal communication). Such a long evolutionary distance between AF6 and Radil might account for their different functions downstream of Rap activation.

Work from our group has demonstrated that ERM proteins play a redundant role as anchors that localise Epac1 at the plasma membrane. This interaction regulates cell adhesion because simultaneous knockdown of all three ERM proteins decreased, but did not block completely, Epac1-induced cell adhesion (Gloerich et al., 2010). Interestingly, although all three ERM family members are expressed in A549-Epac1 cells, we found that depletion of Ezrin blocked the 007 effect in more than 70% of cells (Fig. 3A), similar to siRNA targeting Rap1A and Radil. However, knockdown of Radixin (Fig. 3B and supplementary material Fig. S2) and Moesin (Fig. 3C and supplementary material Fig. S2) did not block cell spreading. Importantly, siRNA against Ezrin neither inhibited the spreading of cells under basal conditions nor depleted the levels of the Radixin and Moesin protein (Fig. 3A). To confirm our visual observations of the immunofluorescence studies, correct regulation of Rap signalling was analysed by quantifying the area of A549-Epac1 cells under different conditions, using ImageJ

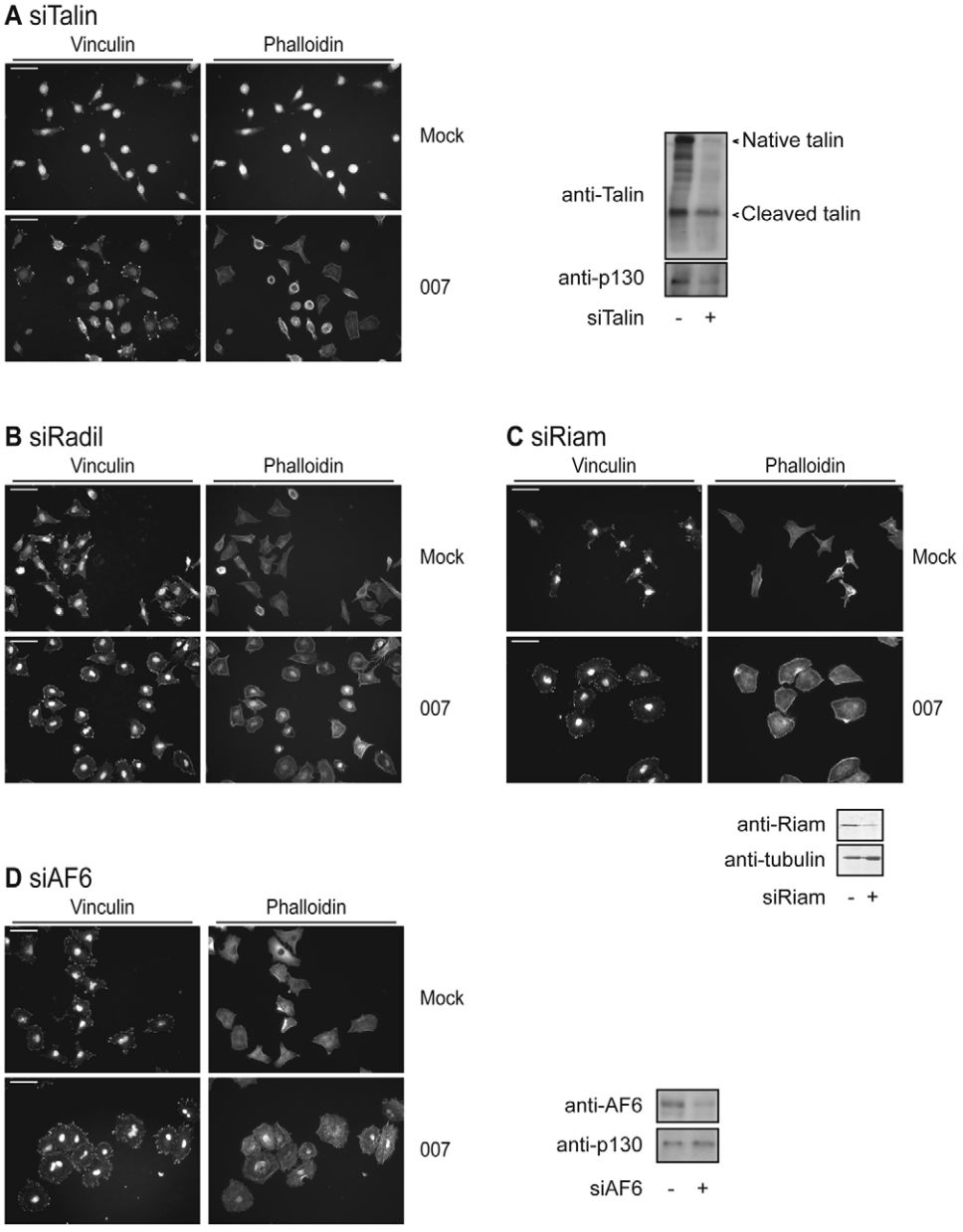

Fig. 2. Knockdown of Talin and the Rap effector, Radil, inhibits cell spreading stimulated by activation of Epac. A549-Epac1 cells treated with siRNA against (**A**) Talin (siTalin), (**B**) Radil (siRadil), (**C**) Riam (siRiam) or (**D**) AF6 (siAF6) were replated and allowed to adhere to fibronectin for 3 hours with 100 µM 007 or without 007 (mock). Following adhesion and spreading, cells were fixed. Focal adhesions and the cytoskeleton were visualised by immunofluorescence. Western blot analysis of protein levels to confirm knockdown of the proteins by siRNA is shown alongside. Scale bars: 50 µm.

software, as an indication of cell spreading. Using this method, we confirmed that Ezrin knockdown caused a significant decrease in the Rap-induced spreading response (Fig. 3D). To validate that the siRNA was, indeed, targeting Ezrin specifically, we used single siRNAs against Ezrin (Fig. 3E). We found that the level of depletion of Ezrin protein by each siRNA corresponded to the inhibition of 007-induced spreading.

The block in the 007 response could be consistent with Ezrin being required to localise Epac1 for signal transduction. Western blot analysis of the pool of active ERM proteins in A549-Epac1 cells revealed that siRNA against Ezrin and Moesin had the biggest effect on depletion of the phospho-ERM levels (Fig. 3F). However, as depletion of Moesin had a similar effect on the levels of phospho-ERM proteins, but did not block Rap-induced spreading, our findings suggested that Ezrin had another function downstream of Rap.

Overexpression of an Ezrin resistant to a single siRNA can rescue the inhibition of cell spreading caused by Ezrin depletion

To confirm that depletion of Ezrin itself caused the observed defects in 007-induced spreading, we made a GFP–Ezrin wild-type construct, GFP–WT Ezrin (rescue), which was resistant to one of the single siRNAs that depleted Ezrin protein levels and blocked 007-induced spreading (Fig. 3E). A549-Epac1 cells, which were transfected with scrambled siRNA and expressed

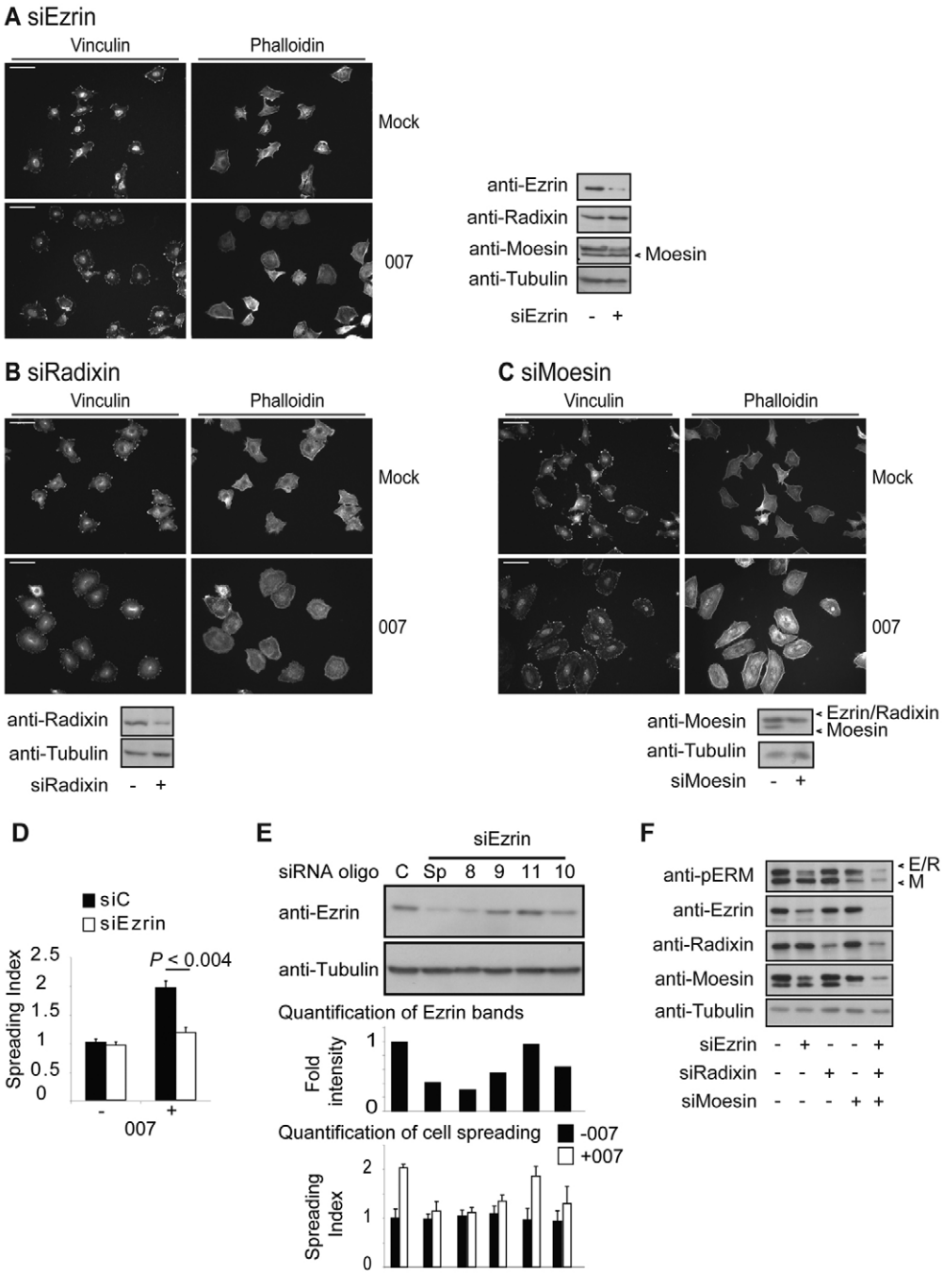

Fig. 3. Knockdown of Ezrin, but not Radixin and Moesin, inhibits Epac-induced cell spreading. A549-Epac1 cells treated with siRNA against (**A**) Ezrin (siEzrin), (**B**) Radixin (siRadixin) or (**C**) Moesin (siMoesin) were replated and allowed to spread on fibronectin for 3 hours with 100 μM 007 or without 007 (mock). Focal adhesions and the actin cytoskeleton were visualised by immunofluorescence. Knockdown of each ERM protein was confirmed by western blot. The anti-Moesin antibody also recognises Ezrin and Radixin, which have a higher molecular weight, as indicated. (**D**) Spreading of at least 25 cells from each condition from four separate experiments was measured and quantified using ImageJ. Spreading of cells treated as in A was compared with that of cells treated with scrambled siRNA (siC). The spreading index was calculated by using the mean size of the control cells, which had spread in the absence of 007, as the standard. The graph shows the means + s.e.m. of four experiments. The *P* value was obtained by performing a paired Student's *t*-test. (**E**) Spreading was quantified from a single representative experiment using cells subjected to non-targeting siRNA (C), the SMARTpool (Sp), siRNA against Ezrin (siEzrin), and the single siRNAs from the SMARTpool (oligos 8–11). The means + s.d. of the average spreading index per field of view are shown (lower panel). Five fields of view were analysed per condition. The ability of these siRNAs to decrease the cellular levels of the Ezrin protein is shown in the western blot (upper panel) and the corresponding quantification of the Ezrin bands is shown below (middle panel). (**F**) Cells transfected with siRNA against Ezrin, Radixin, Moesin or all three ERM proteins together were lysed and the knockdown of each ERM protein and their contribution to the active pool of ERM proteins were analysed by western blot. Scale bars: 50 μm.

GFP–empty vector control or GFP–WT Ezrin (rescue), spread normally under mock and 007-stimulated conditions (Fig. 4A,D). Importantly, GFP–WT Ezrin (rescue) did not seem to alter the basal spreading of the cells. In cells that had been transfected with siRNA against Ezrin, the GFP–empty vector-expressing cells did not respond to 007 (Fig. 4B,D), whereas cells expressing the GFP–WT Ezrin (rescue) protein showed an increase in their spreading comparable to the spreading observed in the control cells (Fig. 4C,D).

The recovery of the 007-induced phenotype by re-introducing Ezrin into cells treated with siRNA against Ezrin confirmed that the effects on spreading were due to the depletion of the endogenous Ezrin protein, and were not due to off-target effects of the siRNA. Depletion of Ezrin caused a substantial loss of the active pool of ERM proteins (Fig. 3F). To determine whether overexpression of another ERM protein could restore 007-induced spreading in Ezrin-depleted cells, we performed similar rescue experiments, but overexpressed YFP–Radixin (Fig. 4E) or YFP–Moesin (Fig. 4F). In order to approximate for equal protein expression, cells of equal fluorescence to those expressing GFP–WT Ezrin (rescue) were imaged and quantified (Fig. 4G). Cells expressing YFP–Radixin or YFP–Moesin were not sufficient to recover the 007-induced spreading (Fig. 4E–G). Hence, our data strongly suggest that the Ezrin protein, specifically, is required for 007-induced cell spreading.

Ezrin is required downstream of Rap activation to regulate cellular spreading

Our group has demonstrated that active ERM family proteins can localise Epac1, and hence can regulate Epac-mediated cell adhesion (Gloerich et al., 2010), therefore it is possible that the major defect caused by Ezrin depletion occurs via disruption or mislocalisation of Epac1–Rap signalling complexes. To investigate whether Epac1–Rap1 signal transduction was still intact in cells depleted of Ezrin, we analysed the levels of Rap1–GTP, which were stimulated in response to 007 in cells transfected with siRNA against Ezrin. We found that Rap1 was activated robustly in the presence of Ezrin knockdown (Fig. 5A).

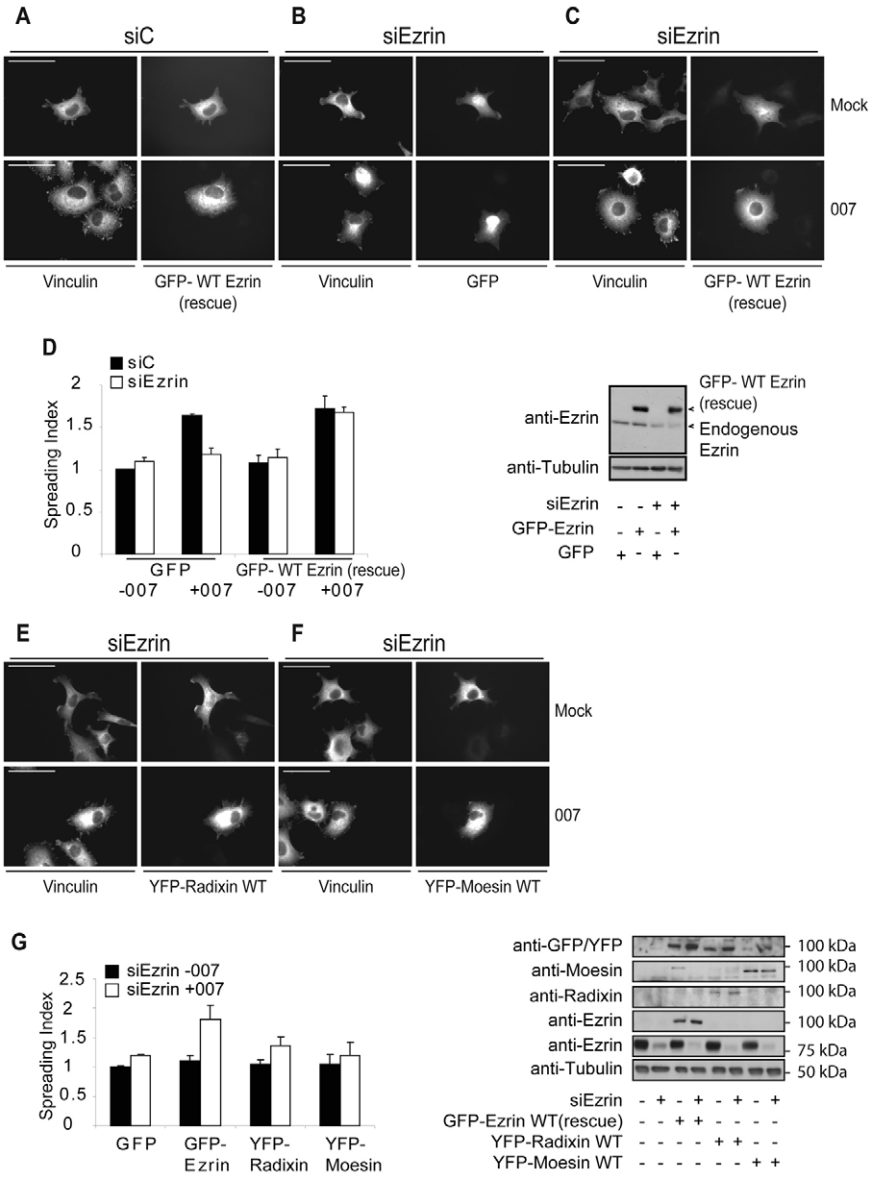

Fig. 4. Inhibition of the 007 phenotype in cells with knockdown of Ezrin can be recovered by a protein overexpressed using an siRNA-resistant Ezrin construct. To rescue the effects of Ezrin knockdown, cells were transfected with either (**A**) scrambled siRNA (siC) or (**B**,**C**,**E**,**F**) siRNA against Ezrin (siEzrin) for 8 hours before being transfected with GFP–empty vector (GFP), GFP–WT Ezrin (rescue), YFP–Radixin or YFP–Moesin. Images of representative cells were captured. (**D**,**G**) The spreading of individual cells expressing GFP–empty vector, GFP–WT Ezrin (rescue), YFP–Radixin or YFP–Moesin transfected with either scrambled siRNA or siRNA against Ezrin was quantified using ImageJ. For each experiment, 20 cells were analysed per condition. In order to take into account differences in transfection efficiencies and to try to standardise for equal overexpression of each of the ERM proteins in individual cells, cells of equal YFP fluorescence were selected for quantification. (D) Average spreading + s.e.m. for three individual experiments are shown. Western blot analysis from a representative experiment confirming knockdown of endogenous Ezrin and overexpression of the GFP–WT Ezrin (rescue) construct is shown alongside. (G) Median spreading + range of the means for two individual experiments are shown. Western blot analysis to confirm depletion of Ezrin and overexpression of YFP–Radixin and YFP–Moesin is shown alongside. Scale bars: 50 μm.

Then, to determine whether the effects of Ezrin depletion on Rap-induced cell spreading were independent from its function as an anchor for Epac, we used the constitutively active RapV12 protein to induce cell spreading in the A549-Epac1 cells. Indeed, in cells that had been transfected with scrambled siRNA, overexpression of the haemagglutinin (HA)–RapV12 protein caused an increase in the number of focal adhesions and promoted spreading of these cells in the absence of 007 (Fig. 5B,C), which was comparable to the spreading induced by 007 (Fig. 5C). In comparison, in cells transfected with siRNA against Ezrin, the focal adhesion formation and cell spreading induced by RapV12 overexpression was inhibited (Fig. 5B,C). Quantification of the cells expressing HA–RapV12 confirmed that Ezrin depletion caused a significant reduction in RapV12-induced spreading (Fig. 5C) and demonstrated that Ezrin is required for the focal adhesion formation and cell spreading induced by Rap activation, and would suggest that its role in spreading is not entirely upstream, via localisation of Epac1 (Gloerich et al., 2010), but also downstream of Rap1.

To strengthen our conclusion that Ezrin was performing a function independent of its ability to bind to Epac1, we investigated whether depletion of Ezrin could block spreading regulated by YFP–Epac1 Δ49. In this protein, the first 49 amino acids that mediate the interaction with ERM proteins are deleted, producing a protein that is still activated by 007 and can induce cell adhesion, although to a lesser extent than the wild-type protein, but with any upstream regulation of Epac1–Rap1 signalling by Ezrin being abolished (Gloerich et al., 2010). Therefore, if Ezrin regulated cell spreading only by localising the Epac–Rap signalling complex, depletion of Ezrin would have no effect on the spreading induced by activation of YFP–Epac1 Δ49 with 007. In A549 cells, which lack endogenous Epac proteins (Lyle et al., 2008), overexpression of YFP–Epac1 Δ49 stimulated the formation of Rap1–GTP (Fig. 5D) and permitted cells to spread in response to 007 (Fig. 5D,F). In cells treated with siRNA against Ezrin, the basal spreading of cells expressing YFP–Epac1 Δ49 was not repressed, but the 007-induced spreading was inhibited (Fig. 5E,F). Together, these data

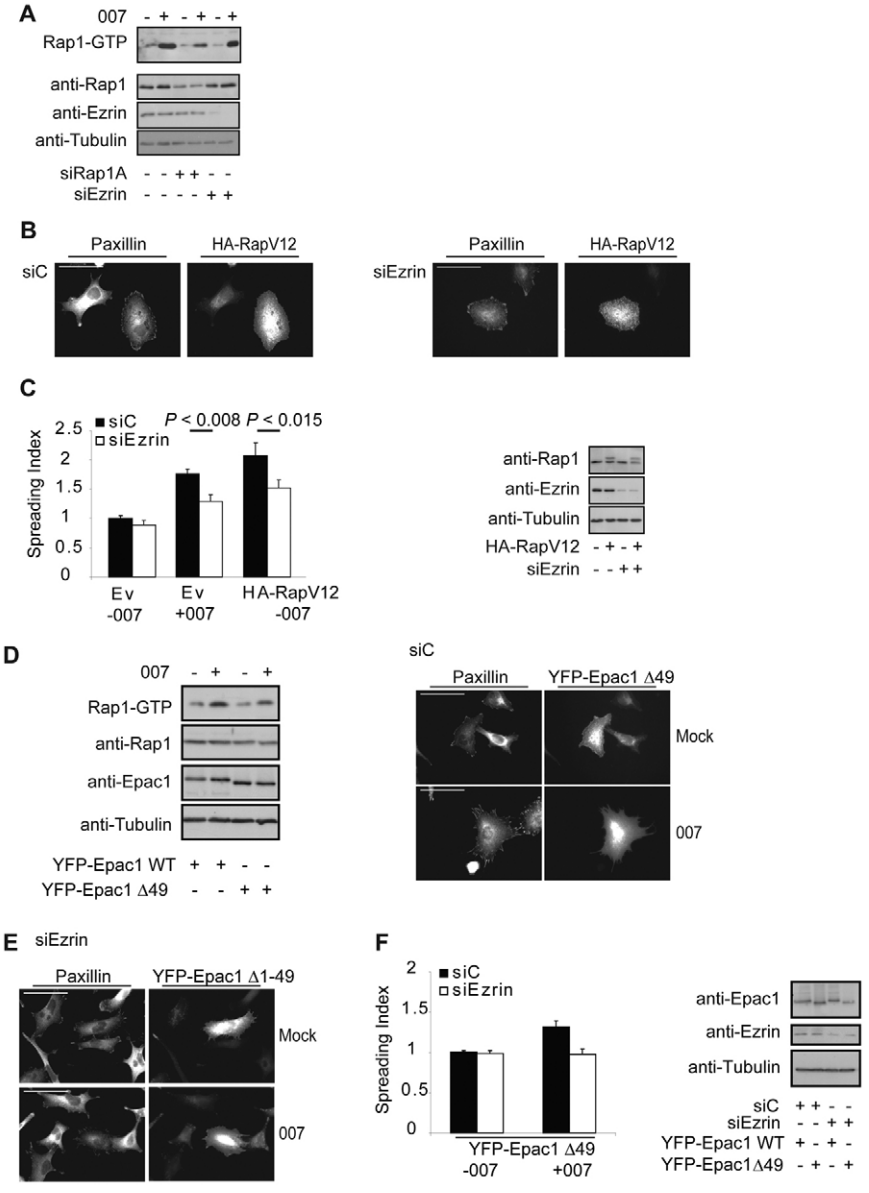

Fig. 5. Ezrin functions downstream of Rap in the signal transduction pathway. (**A**) A549-Epac1 cells were transfected with scrambled siRNA or siRNA against Rap and Ezrin for 48 hours before being exposed to either 100 µM 007 or being mock stimulated (–007) for 15 minutes. Pull down of Rap–GTP, by the RalGDS Rap-binding domain, and knockdown of Rap1A and Ezrin were detected by western blot. (**B**) Cells were transfected with scrambled siRNA (siC) or Ezrin-specific siRNA (siEzrin) for 8 hours before being transfected with HA-RapV12. After 48 hours, cells were replated on fibronectin and allowed to adhere and spread for 3 hours. Cells were fixed and stained as described in Materials and Methods. Images are representative cells. (**C**) Spreading of cells transfected with empty vector (Ev) or expressing HA-RapV12 were quantified using ImageJ. The graph shows the mean spreading index + s.e.m. of cells from four separate experiments. In each experiment, at least 50 cells were analysed per condition. The *P* values were calculated using a paired Student's *t*-test. Western blots confirming the overexpression of HA-Rap1A and knockdown of Ezrin are shown alongside. (**D**,**E**) A549 cells that had been subjected to scrambled siRNA or siRNA against Ezrin for 8 hours were then transfected with YFP–Epac1 Δ49 for 48 hours before being allowed to adhere under mock or 007-stimulated conditions for 3 hours. The ability of YFP–Epac1 Δ49 to increase Rap–GTP levels in response to 007 in A549 cells, in the absence of siRNA transfection, is shown alongside. (**F**) The spreading of the YFP–Epac1 Δ49 expressing cells was measured using ImageJ and the graph shows the mean spreading + s.e.m. of cells from three experiments At least 20 cells were analysed per condition in each experiment. Western blot analysis, shown alongside, confirmed the overexpression of the truncated YFP–Epac1 Δ49 protein and the knockdown of Ezrin. Scale bars: 50 µm.

demonstrate that the Ezrin protein functions downstream of Rap activation to regulate 007-induced spreading.

Depletion of Ezrin specifically blocks the spreading of A549-Epac1 cells in response to 007, without inhibiting the increase in Rap-stimulated initial adhesion

Because cell spreading requires the coordinated regulation of both integrin activity and cytoskeletal changes, it is possible that depletion of Ezrin blocked the initial adhesion of cells to fibronectin, which resulted in the cells not being able to spread properly. To gain understanding of how Ezrin might be functioning during spreading, we used a short-term adhesion assay to analyse whether the ability of cells to adhere to fibronectin was reduced by Ezrin knockdown.

Over a 30-minute period, 007 induced an approximately twofold increase in cell adhesion in cells treated with scrambled siRNA (Fig. 6). Depletion of Rap1A from the cells decreased basal adhesion and inhibited 007-induced adhesion. Interestingly, depletion of Ezrin did not affect initial adhesion of cells, with or without 007 treatment. This suggests that Ezrin does not play a role in the initial adhesion of A549-Epac1 cells to fibronectin, but is involved in the cellular changes that occur to produce the increase in focal adhesions and spreading that we observed following adhesion of cells in the presence of 007.

Depletion of Ezrin using a validated siRNA blocks 007-induced spreading in a number of cell lines

To determine whether Ezrin blocks 007-induced spreading in cells other than the A549-Epac1 cell line, we performed the spreading assay with MDA MB231 breast cancer cell line (Fig. 7A), which stably overexpresses Epac1, and with human umbilical vein endothelial cells (Fig. 7B), which have endogenous Epac. We found that depletion of Ezrin in these cells blocked 007-induced spreading changes (Fig. 7A,B). This suggests that the role of Ezrin is not limited to cells overexpressing Epac1, or restricted to a particular cell type. Ezrin, therefore, might have a widespread role in Rap-induced cellular changes.

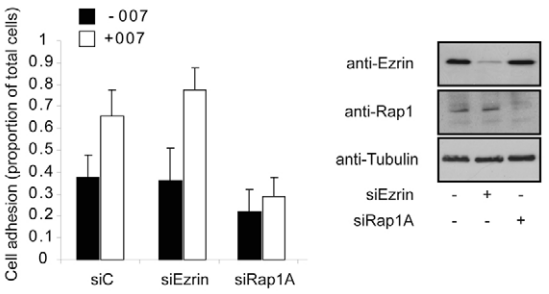

Fig. 6. Knockdown of Ezrin does not inhibit either the basal or 007-stimulated initial adhesion of cells to fibronectin. Cells were transfected with scrambled siRNA (siC) or with siRNAs against Ezrin (siEzrin) or Rap1A (siRap1A) for 48 hours before their adhesion to fibronectin for 30 minutes was measured. The adhesion of cells was determined by alkaline phosphatase activity. Background adhesion to uncoated wells was subtracted from the basal and 007-induced adhesion and expressed as a proportion of the total cells added per well. For each experiment, each condition was performed in quadruplicate. The graph shows the mean adhesion + s.e.m. for three experiments. A representative western blot analysis, to confirm the knockdown of Rap1A and Ezrin in the cells used in the alkaline phosphatase adhesion assay, is shown alongside.

Ezrin is not sufficient to produce the spreading and morphology changes associated with Rap activation in A549 cells

To determine whether Ezrin alone is sufficient to induce the 007 effect in A549-Epac1 cells, we overexpressed either GFP–Ezrin WT or the open, constitutively active GFP–Ezrin T567D mutant protein (Gautreau et al., 2000; Matsui et al., 1998) in A549-Epac1 cells. Using a short-term adhesion assay to measure the ability of cells to adhere to fibronectin, we observed that overexpression of wild-type Ezrin did not stimulate initial cell adhesion. However, overexpression of the Ezrin T567D mutant caused an increase in the adhesion of the A549-Epac1 cells comparable to the effects of 007 (Fig. 8A). This overexpression study suggests that although Ezrin is not required in the initial adhesion induced by Rap, Ezrin can regulate adhesion processes, which might be required by Rap during the spreading response.

To observe whether this ability to regulate adhesion caused an increase in cell spreading, we used immunofluorescence to monitor cells overexpressing GFP–Ezrin. As observed with the Ezrin rescue experiments, GFP–Ezrin WT did not stimulate the spreading of A549-Epac1 cells (Fig. 8B,C). By contrast, cells expressing GFP–Ezrin T567D were significantly more spread in the absence of 007 (Fig. 8B,C). Crucially, the spreading that was induced by GFP–Ezrin T567D was different from the spreading induced by 007, as the cells did not show an increase in focal adhesions compared to GFP–empty vector mock-treated cells (Fig. 8B,D) and they did not display the rounded morphology characteristic of Rap-induced spreading. To analyse this morphology difference, we quantified the shapes of the cells by determining the convex hull of individual cells (Fig. 8E). Then, to give an indication of the shape, the total area between the cell and the edges that joined up the outermost parts of the cell was measured. We found that, consistent with being round in shape, GFP-expressing cells treated with 007 had less area outside of the cell (Fig. 8E). However, in

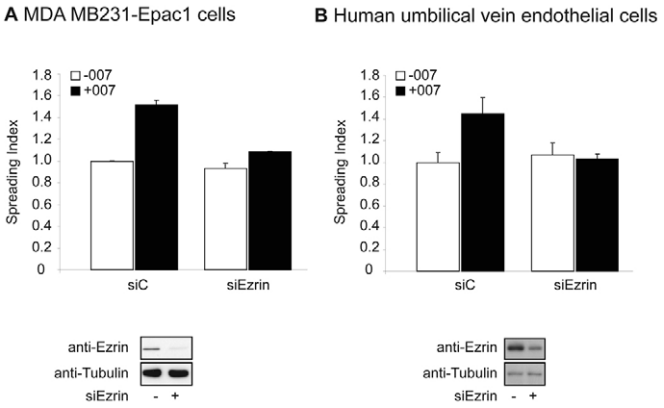

Fig. 7. Knockdown of Ezrin blocks Rap-induced spreading of a number of cell lines. (**A**) MDA MB321-Epac1 cells or (**B**) human umbilical vein endothelial cells transfected with scrambled siRNA (siC) or siRNA against Ezrin (siEzrin) for 48 hours were replated and allowed to adhere to fibronectin for 3 hours with or without 100 μM 007. Following adhesion and spreading, cells were fixed and visualised by immunofluorescence, and the spreading of the cells was quantified using ImageJ. The graph shows the mean spreading + range of the means for two experiments. Twenty cells were analysed per condition in each experiment. Western blot analyses to confirm the knockdown of Ezrin in each cell type are shown alongside.

accordance with being different in shape, cells overexpressing GFP–Ezrin T567D without 007 stimulation had consistently more area outside of the cell than 007-treated cells (Fig. 8E). This difference in shape corresponded to the cells overexpressing GFP–Ezrin T567D having fewer focal adhesions than cells that had spread to a similar size with 007 (Fig. 8D). This suggests that, although GFP–Ezrin T567D regulates spreading adhesion processes, it cannot initiate the focal adhesion formation and cellular morphology changes that are sufficient to produce the 007 phenotype.

Because the open form of Ezrin can mediate these effects, we performed a time course of 007 treatment on adherent cells to determine whether Rap activation stimulates phosphorylation of the ERM pool at the threonine residue responsible for stabilising the open conformation of the proteins. We found that Rap activation does not stimulate the phosphorylation of the total pool of ERM proteins in the cell (Fig. 8F).

Discussion

The small GTPase Rap is involved in inside-out control of cell adhesion processes, which can be monitored as changes to cell spreading and focal adhesion formation. This paper presents evidence that the ERM family member, Ezrin, is crucial for spreading and morphology changes induced by Rap activation. Our screen identified that knockdown of Ezrin had a similar effect on 007-induced spreading as depletion of either Rap1A or the Rap effector, Radil. Furthermore, we have identified that Ezrin is required for Rap-induced cellular spreading in both epithelial and endothelial cells.

Interestingly, although the homologous ERM proteins, Radixin and Moesin, are coexpressed in the A549-Epac1 cells, their depletion did not block Rap-induced spreading. Previous work from our laboratory has found that ERM proteins can localise Epac1 at the plasma membrane. In this case, the function of ERM proteins did seem to be redundant, because all three ERM proteins

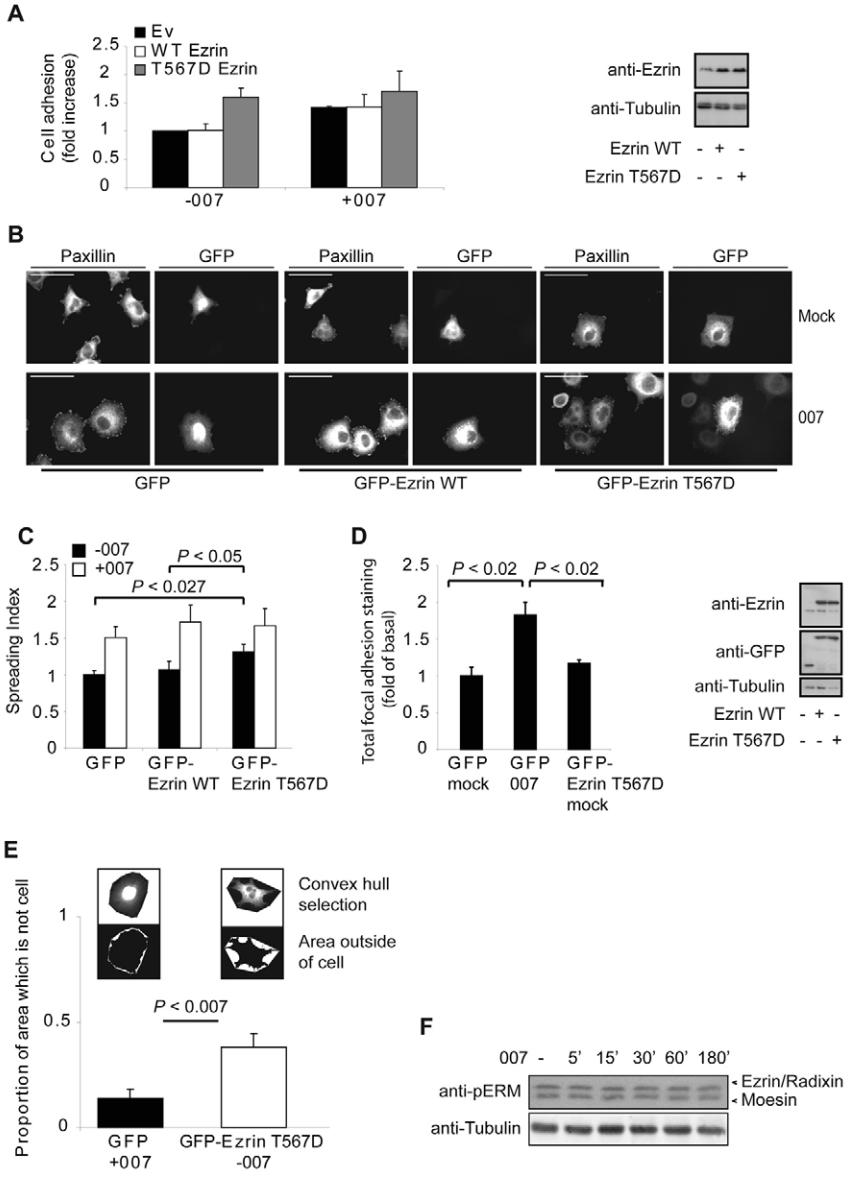

Fig. 8. Overexpression of Ezrin is not sufficient to produce the 007 phenotype. (**A**) Empty vector (Ev), FLAG-His-Ezrin WT or VSVG-Ezrin T567D were co-transfected with a luciferase construct into A549-Epac1 cells for 48 hours prior to cells being replated on fibronectin for 30 minutes. Cells that had adhered were measured by luciferase assay. The background adhesion of cells to uncoated wells was subtracted from the basal and 007-induced adhesion. Each condition was standardised to the basal adhesion of empty vector-expressing cells. The graph shows the mean + s.e.m. of four separate experiments. Overexpression of Ezrin was confirmed by western blot. (**B**) GFP–Ev (GFP), GFP–Ezrin WT or GFP–T567D Ezrin were overexpressed in the A549-Epac1 cells for 48 hours before the cells were replated and allowed to adhere and spread on fibronectin for 3 hours; they were then fixed, and focal adhesions were detected using an antibody against paxillin. (**C**,**D**) The spreading of cells (C) and the total area of cells that stained for paxillin (D) in cells overexpressing GFP–Ev, GFP–Ezrin WT or GFP–Ezrin T567D were quantified using ImageJ. The graphs show the average size + s.e.m. of cells from three experiments. At least 20 cells were analysed per condition in each experiment. Western blot analysis confirmed overexpression of Ezrin. (**E**) To analyse the shape of GFP–Ev cells treated with 007 and cells overexpressing Ezrin T567D, the convex hull of individual cells was determined using ImageJ. The outermost points of the cell were joined up to create a shape, and the size of the shape was measured. Then, the area between the outside of the cell and the edge of the shape was measured, as indicated by the white areas in the lower panel. The proportion of the shape that was outside the cell was calculated. The graph shows the mean+ s.e.m. for three experiments; 10 cells were analysed per experiment. A rounder cell gives rise to a lower proportion of the shape outside of the cell. (**F**) To determine whether Rap activation could regulate the activity of ERM proteins, adherent cells were stimulated with 007 for different times, lysed, and then the levels of the open, active conformation of Ezrin were detected using a phospho-ERM antibody by western blot. Scale bars: 50 μm.

had to be depleted in to order block Epac-induced cell adhesion (Gloerich et al., 2010). In this study, through our analysis of the spreading induced by HA–RapV12 and YFP–Epac1 Δ49, we have shown that Ezrin has an additional role downstream of Rap activation, independent from its ability to bind to Epac1. Moreover, overexpression of YFP–Radixin or YFP–Moesin could not rescue the 007-induced spreading of Ezrin knockdown cells. Therefore, the role of Ezrin in cell spreading downstream of active Rap appears to be a novel example of a non-redundant function of a single ERM protein.

Our data suggest that Ezrin is crucial during Rap-dependent cell spreading of replated cells, without being necessary for the initial cell adhesion steps. Interestingly, we found that knockdown of the Rap effector, Radil, also inhibited Rap-induced cell spreading, but not adhesion. Together, these data indicate that Rap1 regulates two independent steps during adhesion and spreading, with both Ezrin and Radil being required during the second spreading step. In a similar way, the Rap effector protein, RapL, does not mediate the Rap-dependent rolling arrest of T cells, but is required for their Rap-dependent stable interaction with ICAM-1 (Ebisuno et al., 2010).

The activity of Ezrin is regulated by phosphorylation of Thr567 (Gautreau et al., 2000; Matsui et al., 1998), with phosphorylation of other tyrosine residues also being implicated in the function of the protein (Gautreau et al., 1999; Naba et al., 2008; Srivastava et al., 2005). Currently, we do not have evidence that Rap1 can induce activation of Ezrin. For instance, treatment of cells with 007 did not result in an increase in Thr567 phosphorylation. Although overexpression of the open, active form of Ezrin, Ezrin T567D, did induce cell adhesion and spreading, it did not mimic the activation of Rap1 in cells. Specifically, Ezrin did not increase the number of focal adhesions to the same extent as activation of Rap1 and did not induce the rounded cell morphology. This implies that although Ezrin is required for Rap1-induced spreading, other Rap1-mediated changes are necessary as well. Indeed, from our screen, we found that knockdown of the previously identified Rap-effector, Radil, blocked Rap-induced spreading of A549-Epac1 cells. The function of Radil, therefore, might be needed along with Ezrin to induce the 007-phenotype in cells. Recently, it has been shown that Gβγ subunits coordinate and localise the complex of active Rap and Radil, which is crucial for cell-matrix adhesion following activation of G-protein coupled receptors via fMLP (Ahmed et al., 2010). Therefore, downstream of Rap activation, Ezrin might be a crucially important signalling scaffold, localising complexes of proteins, including Epac1, active Rap1A and effectors such as Radil. However, thus far we have been unable to demonstrate an interaction of Radil with Ezrin or a synergism between Ezrin and Radil in cell spreading.

Like the other ERM family members, Ezrin acts to link the cytoskeleton to the plasma membrane by binding to actin and PtdIns(4,5)P_2 (Algrain et al., 1993) or to membrane proteins like ICAM-1, ICAM-2, CD44 and syndecan-2 (Granes et al., 2003; Granes et al., 2000; Heiska et al., 1998; Helander et al., 1996; Hirao et al., 1996; Tsukita et al., 1994; Yonemura et al., 1998). By creating such a linkage, ERM proteins can perform both structural and regulatory functions in cells. For instance, ERM proteins are able to control cell shape by organising actin filaments and regulating cortical stiffness (Fehon et al., 2010; Fievet et al., 2007; Louvet-Vallee, 2000). Regulation of the interaction of ERM proteins with the actin cytoskeleton is required to alter T cell morphology (Delon et al., 2001; Faure et al., 2004) and to control the membrane diffusion and signalling of the B cell receptor (Treanor et al., 2010). Therefore, Ezrin might be able to create discrete and dynamically regulated compartments within the plasma membrane, in order to organise and localise transmembrane proteins, such as adhesion molecules. Indeed, Ezrin has been implicated in cell–cell and cell–substrate adhesion (Hiscox and Jiang, 1999; Martin et al., 1995; Osawa et al., 2009; Takeuchi et al., 1994), stabilisation of β4 integrins (Wan et al., 2009) and modulation of cell spreading (Hiscox and Jiang, 1999; Martin et al., 1995). However, these studies give conflicting reports on the effects of Ezrin on cell adhesion. It is possible that by regulating the diffusion of adhesion molecules and by controlling cellular shape changes, Ezrin might either increase or limit adhesion and spreading in different cell types. In addition, by linking the co-receptor, CD44v6, to the actin cytoskeleton, Ezrin can organise signalling complexes that enable signal transduction from the c-Met receptor (Orian-Rousseau et al., 2007) and the VEGFR-2 receptor (Tremmel et al., 2009). Ezrin might function downstream of Rap as a multipurpose molecule, controlling cell architecture, by regulating the tension changes in actin and the localisation of adhesion molecules, as well as by coordinating signalling processes.

Our finding that Ezrin is required for Rap-induced cell spreading suggests that, in combination with the modulation of the integrin–actin linkage, Rap might regulate a link between the plasma membrane and the cytoskeleton. Thus, during cell spreading, in parallel to changes to focal adhesions, Rap might modulate cellular rigidity through signalling complexes controlled by Ezrin. Mapping the protein–protein interactions of Rap-induced Ezrin signalling complexes might give insights into why Ezrin, specifically, is required in Rap signal transduction. Such studies could lead to the discovery of other unique roles for ERM family proteins in cells.

Materials and Methods

Cell lines and culture

The lung A549 cell line and the derivative monoclonal Epac1-expressing A549-Epac1 cell line, which has been described previously (Lyle et al., 2008), were cultured in RPMI supplemented with L-glutamine, antibiotics and 10% FCS. Stable expression of Epac1 in the MDA MB231 breast cancer cell line was achieved using amphotropic retroviruses. Single Epac1-expressing colonies were isolated following zeocin selection, and cultured in DMEM supplemented with L-glutamine, antibiotics and 10% FCS. Human umbilical vein endothelial cells were obtained from Clonetics and were cultured using standard procedures (Kooistra et al., 2005).

Reagents and antibodies

8-pCPT-2′-O-Me-cAMP (007) was obtained from BioLog Life Sciences (Bremen, Germany). Antibodies were from Santa Cruz Biotechnology (Rap1, p130), BD Biosciences (Paxillin, Ezrin, Radixin, Moesin, AF6), Cell Signaling Technology (phospho-Ezrin[Thr567]/Radixin[Thr564]/Moesin[588]), Roche (GFP), Chemicon (α-tubulin), Sigma-Aldrich (Vinculin, Talin) and Covance (monoclonal HA antibody, HA11). Anti-Riam antibody was a gift from Matthias Krause (MIT, Cambridge, MA). The monoclonal anti-Epac1 5D3 antibody has been described before (Price et al., 2004). siRNAs were obtained from Dharmacon (Lafayette, CO). The initial screen was performed using ON-targetplus SMARTpool siRNA oligos, with Ezrin, Radil, Riam, AF6 and Radixin knockdowns being confirmed with single siRNAs.

DNA constructs

The HA-RapV12 construct has been described previously (Zwartkruis et al., 1998). cDNA for Ezrin (EZR, *Homo sapiens*, GI: 161702985), Radixin (RDX, *Homo sapiens*, GI: 62244047) and Moesin (MSN, *Homo sapiens*, GI: 53729335), were obtained from RZPD (Berlin, Germany). The ERM proteins were cloned to express an N-terminal FLAG-His tag or VSVG tag in a pCDNA3 vector, or GFP or YFP in a modified pLV CMVbc vector, or pCDNA3.1, respectively, using the Gateway system (Invitrogen) as described previously (Gloerich et al., 2010). Site-directed mutagenesis was used to make the Ezrin T567D mutant, and a rescue construct producing a wild-type protein sequence not targeted by the single Dharmacon siRNA (sequence GCGCAAGGAGGAUGAAGUU) was made using the primers 5′-CCTGGAAATGTATGGCATAAATTACTTTGAAATAAAAAACAAG-3′ and 5′-CTTGTTTTTTATTTCAAAGTAATTTATGCCATACATTTCCAGG-3′.

Epac1 (RapGEF3, *Homo sapiens*, GI: 3978530) was cloned N-terminally to a YFP tag in a pCDNA3 vector. Epac1 Δ1-49 was generated by site-directed mutagenesis as described previously (Gloerich et al., 2010).

Cell transfection

For knockdown experiments, A549-Epac1 and MDA MB231 cells were seeded sparsely for 24 hours prior to transfection with 50 nM ON-Targetplus SMARTpool siRNA oligos (Dharmacon) using oligofectamine (Invitrogen) according to the manufacturer's protocol. HUVEC cells with transfected with 50 nM siRNAs using Dharmafect 1 reagent. For overexpression of proteins, sparsely seeded A549-Epac1 cells were transfected with RapV12 or Ezrin constructs using FuGENE 6 (Roche) according to the manufacturer's instructions. For rescue experiments or experiments where cells were subjected to siRNA and overexpression constructs, A549-Epac1 cells were seeded sparsely and transfected with siRNA for 8 hours before transfection of the RapV12, Ezrin or Epac1 constructs.

Immunofluorescence and spreading assay

Cells were trypsinised 48 hours after transfection, washed once with the appropriate media with 10% FCS and then kept in suspension for 1.5 hours at 37°C in media containing 0.5% FCS, glutamine, antibiotics and 20 mM HEPES, to allow surface proteins to recover. The cells were maintained in 0.5% serum because the A549 cells failed to adhere and spread adequately in the absence of serum. For the spreading assay using human umbilical vein endothelial cells, all steps were carried out in full media. Following recovery, cells (2×10^4 cells/ml) were plated onto coverslips that had been coated with fibronectin overnight at 4°C. The cells were allowed to adhere and spread for 3 hours in the presence or absence of 100 μM 007. For mock treatments, without 007, cells were treated with equal volumes of the vehicle for 007 (final concentration, 100 μM Tris-HCl pH 7.5). In the knockdown experiments, after 3 hours, the coverslips were washed in PBS, then cytoskeletal buffer (0.5% Triton X-100, 10 mM PIPES, pH 7, 50 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 300 mM sucrose) and then cells were fixed in 4% (v/v) paraformaldehyde for 15 minutes. In experiments where cytosolic proteins were overexpressed, after cells had been allowed to adhere, the coverslips were washed in PBS, fixed with 4% (v/v) paraformaldehyde and permeabilised with 0.1% Triton X-100 for 5 minutes. In all experiments, the coverslips were blocked overnight in 2% (w/v) BSA in PBS at 4°C. Focal adhesions were detected using a monoclonal anti-vinculin antibody or anti-paxillin antibody, and the Alexa-Fluor-568-coupled secondary antibody. Actin was visualized using phalloidin coupled to Alexa Fluor 488 (Invitrogen) or Alexa Fluor 568 (for experiments where GFP/YFP-tagged proteins had been overexpressed). Images were acquired using a Zeiss Axioskop 2 microscope fitted with a Zeiss Axiocam CCD camera and 40× and 100× Plan APO objective lenses. ImageJ (NIH) was used to quantify cell spreading and focal adhesion staining. For the siRNA screening experiments, at least 20 cells per condition were analysed. To avoid bias, all of the cells imaged in a single field of view taken through the 40× objective were analysed. Measurements were taken from at least three different fields of view with a minimum of six cells per field. Cells were selected using the threshold function, to measure either the whole area of the cell, or the areas stained for focal adhesion proteins. Areas were measured in pixels and the spreading index for individual experiments was determined by standardising the spreading of cells under each condition to the mean size of cells under control conditions. Focal adhesions were analysed by measuring the total area per cell that stained for vinculin or paxillin, as individual focal adhesions could not always be easily distinguished. Focal adhesion staining was standardised to the mean area of focal adhesion staining measured under basal conditions. To analyse the cell shape, ImageJ was used to determine the convex hull of the cells. To do this, the outermost points of the cell were manually selected then joined up using the convex hull selection tool of ImageJ. The area of the resulting shape, the size of cell, and the size of the region outside the cell, between the cell edges and the edge of the shape, were measured in pixels. To standardise results, the proportion of the shape filled with cell and the proportion of the shape that was outside of the cell was calculated. By this method, the more circular the cell, the lower the proportion of the shape is present outside the edges of the cell. Statistical differences in cell size, to calculate the *P* values, were determined by carrying out paired, two-tailed Student's *t*-tests.

Short-term adhesion assays

A549-Epac1 cells were transfected with siRNA or co-transfected with Ezrin and luciferase expression vectors for 48 hours before cells were trypsinised, washed once in RPMI containing 10% FCS, and kept in suspension for 1.5 hours in RPMI containing 0.5% FCS, glutamine, antibiotics, and 10 mM HEPES, pH 7.4, at 37°C to allow recovery of surface proteins. 96-well plates were coated with fibronectin overnight at 4°C, followed by a blocking step with heat-denatured BSA for 1 hour at 37°C. Following recovery, cells were plated into the fibronectin-coated wells and allowed to adhere for 30 minutes at 37°C in the presence or absence of 100 μM 007. Every condition was done in quadruplicate. After 30 minutes, non-adherent cells were removed and adherent cells were washed once with pre-warmed PBS. For siRNA transfections, cells were lysed in alkaline phosphatase buffer [0.4% Triton X-100, 50 mM sodium citrate, and 10 mg/ml phosphatase substrate (Sigma-Aldrich)]. In cells where Ezrin constructs were coexpressed with a luciferase construct, cells were lysed in luciferase assay buffer (25 mM Tris phosphate pH 7.8, 10% glycerol, 2% Triton X-100, 8 mM $MgCl_2$ and 1 mM DTT). Total cells added per well were determined by taking aliquots of the cell suspension added to each well, then pelleting the cells before lysis in the appropriate buffer. The total amount of cells adhering was determined by either phosphatase assay (Schwartz and Denninghoff, 1994), or luciferase assay (Medema et al., 1992).

Isolation and analysis of Rap–GTP

Cells were transfected with siRNA for 48 hours and then stimulated with mock or 100 μM 007 for 15 minutes. Cells were lysed in Ral buffer [50 mM Tris.HCl pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 1% (v/v) NP40, 10% (v/v) glycerol, 1 mM PMSF, 1 μM leupeptin, 0.1 μM aprotinin], and the lysates were pre-cleared by centrifugation. The Rap–GTP was captured using the RalGDS-RBD, pre-bound to glutathione agarose, for 45 minutes as described previously (Franke et al., 1997). Endogenous Rap was detected following western blotting with polyclonal anti-Rap1 antibody.

Western blotting

Cells were lysed using Laemmli sample buffer and proteins were separated using SDS-PAGE. Proteins were transferred to PVDF membranes (Immobilon) and blocked in 2% milk containing 2% BSA for 1 hour and then probed with the appropriate primary antibody. The antibodies were detected by anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase, and proteins were detected by ECL.

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