Spatial recruitment and activation of the Fes kinase by ezrin promotes HGF-induced cell scattering

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The remodeling of epithelial monolayers induced by hepatocyte growth factor (HGF) results in the reorganization of actin cytoskeleton and cellular junctions. We previously showed that the membrane–cytoskeleton linker ezrin plays a major role in HGF-induced morphogenic effects. Here we identified a novel partner of phosphorylated ezrin, the Fes kinase, that acts downstream of ezrin in HGF-mediated cell scattering. We found that Fes interacts directly, through its SH2 domain, with ezrin phosphorylated at tyrosine 477. We show that in epithelial cells, activated Fes localizes either to focal adhesions or cell–cell contacts depending on cell confluency. The recruitment and the activation of Fes to the cell–cell contacts in confluent cells depend on its interaction with ezrin. When this interaction is impaired, Fes remains in focal adhesions and as a consequence the cells show defective spreading and scattering in response to HGF stimulation. Altogether, these results provide a novel mechanism whereby ezrin/Fes interaction at cell–cell contacts plays an essential role in HGF-induced cell scattering and implicates Fes in the cross-talk between cell–cell and cell–matrix adhesion.

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Introduction

Ezrin is a member of the highly conserved ezrin/radixin/ moesin (ERM) protein family. The ERM proteins provide a regulated linkage between cell-surface proteins and the actin cytoskeleton (Bretscher *et al*, 2002). In its membrane–cytoskeleton-associated form, ezrin participates in several cortical actin-based processes such as cell adhesion (Takeuchi *et al*, 1994; Hiscox and Jiang, 1999; Pujuguet *et al*, 2003), cell motility (Crepaldi *et al*, 1997; Ng *et al*, 2001) and cell

(Yonemura and Tsukita, 1999; Gautreau *et al*, 2000) and tissue (Fiévet *et al*, 2007) morphogenesis.

The hepatocyte growth factor (HGF) acting through its receptor Met induces a variety of biological events in epithelial cells (Stella and Comoglio, 1999). Ezrin was shown to be an essential component in HGF-induced cell scattering (Crepaldi *et al*, 1997; Orian-Rousseau *et al*, 2002, 2007) and morphogenesis (Crepaldi *et al*, 1997; Gautreau *et al*, 1999). Ezrin phosphorylation at tyrosine residues is required at different steps of epithelial cell morphogenesis in a three-dimensional matrix. Thus, tyrosine 353 signals cell survival through the activation of the PI3K pathway, whereas tyrosine 145 controls cell proliferation (Gautreau *et al*, 1999; Srivastava *et al*, 2005).

The above HGF-triggered events are also mediated by cytoplasmic effectors from the Src family kinases (Rahimi *et al*, 1998; Tsukamoto and Nigam, 1999; Cutrupi *et al*, 2000). We and others have shown that ezrin is phosphorylated by Src family kinases at either tyrosine 145 or 477 (Autero *et al*, 2003; Heiska and Carpen, 2005; Srivastava *et al*, 2005). Src-dependent ezrin phosphorylation influences cell adhesion-based signaling. The spreading of epithelial cells expressing Y145F ezrin is impaired when cells are grown on fibronectin and this defect can be reverted by overexpressing activated Src (Srivastava *et al*, 2005). In studies performed with mammary carcinoma cells, a cooperative effect of Src and ezrin in the regulation of cell–cell contacts has been observed (Elliott *et al*, 2004). These findings establish a functional link between ezrin and the Src family kinases in the control of cell adhesion.

To identify the signaling components involved in adhesion-mediated events downstream of ezrin, we sought to identify proteins that interact with ezrin phosphorylated by Src family kinases using a modified two-hybrid screen. We found that the kinase Fes interacts specifically with phosphorylated ezrin. The *fes* protooncogene encodes a 93-kDa non-receptor protein-tyrosine kinase and is a member, along with the ubiquitous kinase Fer and the testis-specific form FerT, of the *fes/fps* non-receptor protein kinase subfamily (Greer, 2002). Fes displays a modular structure that consists of an N-terminus Fes/CIP4 homology (FCH) domain, followed by two coiled-coil domains and an SH2 (Src homology 2) domain (Figure 1A). The C-terminus kinase domain of the protein contains the major autophosphorylation site at tyrosine 713 (Rogers *et al*, 1996).

Although Fes was mainly studied in hematopoietic cells of the myeloid lineage and in endothelial cells, this protein is also present in epithelial cells (Haigh *et al*, 1996; Delfino *et al*, 2006). Here we report that in its activated form Fes has a dual localization in epithelial cells, in focal adhesions or at cell–cell contacts, depending on cell confluency. We demonstrate that ezrin plays a crucial role in the spatial recruitment and activation of Fes at the membrane, and that the correct

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Figure 1 Fes interacts with ezrin phosphorylated at tyrosine 477. (A) Structural organization of the Fes kinase. Fes contains an N-terminus Fes/CIP4 homology domain (FCH), two coiled-coil domains, an SH2 domain followed by the kinase domain. Tyrosine 713 is the major autophosphorylation site in the kinase domain. The ezrin-binding domain found in the two-hybrid screen comprises amino acids 255–593. (B) GST or GST-Fes_{255–593} immobilized on glutathione beads was incubated with lysates from cells expressing WT ezrin and treated with pervanadate alone or with PP2 or SU6656. Immunoblot was performed with anti-VSVG antibody. For each condition, lysates (inputs) were divided in two parts to achieve incubation with the GST alone or with GST-Fes_{255–593} (right panel). The interaction of WT ezrin with GST-Fes_{255–593} is abolished when the cells were treated with Src kinase inhibitors. Lower panels: Coomassie staining of GST proteins. (C) GST or GST-Fes_{255–593} immobilized on glutathione beads was incubated with lysates from cells expressing WT, Y145F or Y477F ezrin. Immunoblot was performed with anti-VSVG antibody. WT and Y145F ezrin interact with GST-Fes_{255–593}, whereas Y477F ezrin does not. Lower panels: Coomassie staining of GST proteins. (D) Tyrosine 477 in ezrin is a substrate of Src. Immunoblot with anti-ezrin or anti-pY477 ezrin antibodies was performed on lysates of LLC-PK1 cells treated with pervanadate alone or with PP2 or SU6656. (E) GST-Fes_{255–593} binds to a peptide comprising phosphorylated tyrosine 477 of ezrin. Purified GST (lower lane) or GST-Fes_{255–593} (upper lane) proteins was incubated with beads alone, the ezrin peptide (PV⁴⁷⁷YEPV), the phosphopeptide (PVp⁴⁷⁷YEPV) or a control phosphopeptide immobilized on beads. Coomassie staining of the input GST proteins and bound protein are shown.

localization of Fes is essential for the transmission of signals elicited by both HGF and extracellular matrix components. Therefore, these experiments place the ezrin/Fes interaction at the crossroad of growth factors and extracellular matrix signaling for the control of HGF-induced epithelial cell scattering.

Results

The SH2 domain of Fes interacts with ezrin phosphorylated at tyrosine 477

To identify partners of ezrin phosphorylated by Src family kinases, we conducted a modified two-hybrid screen with yeast strains transformed or not with Lyn kinase. As bait, we used a truncated form of ezrin lacking a 52-amino-acid sequence at the C-terminal end, since in the full-length protein most of the binding sites for ezrin partners are masked. This masking is due to a strong intramolecular interaction between the N- and C-terminal domains of the protein (Gary and Bretscher, 1995). Among the preys that were found to interact specifically with phosphorylated ezrin and not found in the screen performed in the absence of Lyn kinase, we focused our study on Fes. The region that was found in the screen to interact with ezrin corresponds to the second coiled-coil domain and the SH2 domain of Fes (Figure 1A), this comprises amino acids 255–593 (Fes_{255–593}).

Different approaches were used to confirm the interaction between ezrin and Fes and its dependence on ezrin phosphorylation by Src family kinases. First, we performed pull-down experiments with GST-Fes_{255–593}, using extracts from LLC-PK1 cells overexpressing wild-type (WT) ezrin treated with pervanadate alone or in combination with the Src family kinases inhibitors, PP2 or SU6656. GST-Fes_{255–593} but not GST alone interacted specifically with WT ezrin and this interaction was disrupted when the cells were treated with either PP2 or SU6656 (Figure 1B). Two ezrin residues have been described to be phosphorylated by Src family kinases, namely tyrosines 145 and 477. To determine if the phosphorylation of one of these two residues was required for the interaction between Fes and ezrin, we performed pull-down experiments with GST-Fes_{255–593} on extracts from cells expressing WT ezrin, or ezrin carrying the point mutations Y145F or Y477F. GST-Fes_{255–593} interacted specifically with WT ezrin and Y145F ezrin but not with Y477F ezrin (Figure 1C). Altogether, these results indicated that the interaction between Fes and ezrin occurs through phosphorylated tyrosine 477 of ezrin. To confirm that in our model system the tyrosine 477 of ezrin is a substrate of Src family kinases, we have generated a phosphospecific antibody directed against the phosphotyrosine 477 of ezrin (Supplementary Figure S1). As shown in Figure 1D, this antibody recognizes a band corresponding to ezrin in lysates of cells treated with pervanadate, and failed to recognize it when the cells were treated with PP2 or SU6656, indicating that the tyrosine 477 of ezrin is a substrate of Src in LLC-PK1 cells. We next wanted to determine if the interaction between the two proteins was direct and if it required the SH2 domain of Fes. Two peptides surrounding tyrosine 477 in a phosphorylated or non-phosphorylated form were synthesized (PVY⁴⁷⁷EPV or PVpY⁴⁷⁷EPV) and incubated with purified GST-Fes_{255–593}. A peptide comprising phosphorylated tyrosine 190 of ezrin was used as control. Whereas no interaction of either peptide was

detected with GST alone, we observed an interaction of GST-Fes_{255–593} with the peptide phosphorylated at tyrosine 477 (Figure 1E) but not with the control phosphopeptide. These data indicate that the interaction between ezrin and Fes is direct and occurs through phosphorylated tyrosine 477 of ezrin and the SH2 domain of Fes.

Activated Fes shuttles between focal adhesions and cell–cell contacts

We next wanted to examine where the interaction between ezrin and Fes takes place in epithelial cells. Immunofluorescence was performed on LLC-PK1 cells with an antibody generated against a peptide corresponding to the last 15 amino acids of Fes (Supplementary Figures S2 and S3). LLC-PK1 cells derive from proximal kidney tubules and form a polarized monolayer in culture. In these cells, ezrin is mainly present in the apical microvilli but is also observed at the lateral membrane. As shown in Figure 2A, Fes displays a punctate cytoplasmic staining concentrated at the basal region of LLC-PK1 cell (Figure 2A, xz section). No obvious colocalization with ezrin was observed.

The autophosphorylation of Fes at tyrosine 713 reflects its transition from an inactive to a catalytically active kinase. We then asked where in epithelial cells the active form of Fes was localized. Using an antibody against phosphorylated tyrosine 713 of Fes (Supplementary Figures S2 and S3) we observed that the major pool of activated Fes was concentrated in two different subcellular compartments. In sparse cells, activated Fes was mainly present in focal adhesions, where it colocalized with vinculin (Figure 2B) paxillin and FAK (data not shown). However, in confluent monolayers, the localization of activated Fes was restricted to the lateral membrane. This Fes staining appeared discontinuous and overlapped with the staining of the adherens junction marker E-cadherin (Figure 2C, upper panel). A colocalization was also observed with ezrin present at the lateral surface (Figure 2C, lower panel). Given the colocalization between activated Fes and ezrin, we performed an immunoprecipitation using the anti-pY713 Fes antibody. As shown in Figure 2D, we detected an interaction between activated Fes and ezrin. Consistent with the immunofluorescence data that showed no colocalization between ezrin and Fes, we could not co-immunoprecipitate these two proteins using an anti-Fes antibody. Altogether, these results indicate that only the pool of activated Fes interacts with ezrin and this interaction occurs at the cell–cell contacts.

Ezrin recruits activated Fes to the lateral membrane

The localization of activated Fes at the lateral membrane and its interaction with ezrin suggested that ezrin might be responsible for the recruitment of Fes to the plasma

membrane. To test this hypothesis, we used stable LLC-PK1 cell lines expressing either vesicular stomatitis virus glycoprotein (VSVG)-tagged WT (Crepaldi *et al*, 1997) or Y477F ezrin. Two clones (F1 and F2) that showed a fourfold increase in Y477F ezrin expression over the endogenous protein were used in all experiments and gave identical results. In these cells, the localization of Y477F ezrin was similar to that of WT ezrin (Figure 3A, right panels). Although no difference in the localization of Fes was observed in cells expressing WT or Y477F ezrin (data not shown), the distribution of activated Fes was dramatically perturbed in cells expressing Y477F ezrin. Indeed, in sparse cells it localized to the focal adhesions, which appeared enlarged (Figure 3A, left panels). In confluent cells expressing Y477F ezrin, activated Fes failed to localize at the site of cell–cell contacts and was observed in numerous focal adhesions that were not observed in cells expressing WT ezrin (Figure 3A, right panels). In line with these data, we did not observe an interaction between activated Fes and Y477F ezrin, unlike what was observed with WT ezrin (Figure 3B). These results demonstrate that the inhibition of ezrin/Fes interaction prevents the recruitment of activated Fes to the cell–cell contacts and leads to its accumulation in focal adhesions.

To confirm the requirement of ezrin for the recruitment of Fes to the lateral plasma membrane, we knocked down ezrin by transiently transfecting LLC-PK1 cells with a plasmid encoding short-hairpin RNA-targeting ezrin (shEz) and green fluorescent protein (GFP), which allowed us to monitor transfected cells. Expression of two different shRNA-targeting ezrin gave the same results and led to an efficient knock down (Supplementary Figure S4). In cells transfected with a non-targeting sequence (Scr), activated Fes was observed at cell–cell contacts. In contrast, the localization of activated Fes in ezrin knocked down cells was perturbed, with activated Fes only present at the sites of focal adhesions and nearly absent from cell–cell contacts (Figure 3C). Altogether, these observations demonstrate that the presence of ezrin is crucial for the recruitment of Fes at the cell–cell contacts.

The ezrin/Fes interaction is required for cell spreading

Since cells expressing Y477F ezrin displayed aberrant focal adhesions, we tested the ability of cells expressing WT or Y477F ezrin cells to spread on two substrates, collagen I and fibronectin. Whereas 89% of the cells expressing WT ezrin were spread 1.5 h after seeding on collagen I-coated surfaces, 81% of cells expressing Y477F ezrin remained round and failed to spread (Figure 4A). These cells showed a disorganization of the actin cytoskeleton and enlarged focal adhesion at the cell periphery that contained activated Fes and vinculin (Figure 4B). The average area of cells expressing Y477F ezrin

Figure 2 Activated Fes localizes to adhesion sites in epithelial cells. (A) Fes displays a punctate cytoplasmic staining. Double staining was performed on confluent LLC-PK1 cells with anti-Fes (green) and anti-ezrin (red) antibodies. Images were taken with a 3D optical sectioning wide-field microscope and restored using the iterative constrained modified Gold algorithm. A 3D maximum-intensity projection (MIP) along the z-axis of the whole volume (5- μ m thick) and an xz cross-section are shown. No colocalization between ezrin and Fes is observed (bar, 5 μ m). (B) In non-confluent cells, activated Fes (pY713 Fes, green) is observed in focal adhesions, where it colocalizes with vinculin (red) and actin stress fibers as shown by the phalloidin staining (blue) (bar, 10 μ m). (C) In confluent cells, activated Fes localizes at cell–cell contacts. pY713 Fes (green) colocalizes with E-cadherin (red) (upper panels) or ezrin (red) (lower panels). Images were taken with a 3D wide-field optical sectioning microscope and restored by deconvolution. 3D MIP along the z-axis at the level of adherens junctions (0.5 μ m thick) are shown (bar, 10 μ m). (D) Endogenous ezrin co-immunoprecipitates with activated Fes. Immunoprecipitation was performed on LLC-PK1 cell lysates with anti-pY713 Fes antibody or non-immune rabbit IgG. Immunoprecipitated proteins and total cell lysates (inputs) were immunoblotted with the anti-pY713 Fes or anti-ezrin antibodies.

was about 30% that of cells expressing WT ezrin (Figure 4C). Three hours after seeding, the spreading of cells expressing Y477F ezrin was still impaired as compared with cells expressing WT ezrin (data not shown); however, after 24 h, cell

spreading was similar in both cell types, indicating that the lack of tyrosine 477 phosphorylation dramatically delayed the ability of cells to spread on collagen (Figure 4A) or on fibronectin (data not shown). The spreading delay resulting

Figure 3 Ezrin recruits Fes to cell-cell contacts. (A) pY713 Fes does not localize at the cell-cell contacts in cells expressing Y477F ezrin. Left panels: double labeling was performed with anti-pY713 Fes and anti-vinculin antibodies on non-confluent cells stably expressing WT or Y477F ezrin. Right panels: double labeling was performed with the anti-pY713 Fes and anti-VSVG antibodies on confluent cells stably expressing WT or Y477F ezrin (bar, 10 μ m). (B) The ezrin/Fes interaction is abolished by the point mutation Y477F in ezrin. Immunoprecipitation with the anti-pY713 Fes antibody or non-immune rabbit IgG was performed on lysates from cells expressing VSVG-tagged WT or Y477F ezrin. Immunoblot was performed using the anti-pY713 Fes or anti-VSVG antibodies. An interaction was observed between pY713 Fes and WT ezrin but not with Y477F ezrin. (C) Activated Fes is relocated from cell-cell contacts to focal adhesions in ezrin knocked down cells. Immunostaining was performed with the anti-pY713 Fes antibody. Cells expressing scrambled (Scr) or ezrin-targeting (shEz1) shRNA are also GFP positive (bar, 10 μ m).

Figure 4 Delayed spreading of LLC-PK1 cells expressing Y477F ezrin. **(A)** Phase-contrast images of cells expressing WT or Y477F ezrin (clone F1) 1.5 h after plating on type I-collagen (bar, 20 μ m). The percentage of spread cells is shown in the bar charts and results from the counting of cells from 10 random fields in three independent experiments for each clone (F1 and F2). Data are means $\pm$ s.e. Asterisk indicates $P < 0.05$. **(B)** Cells were stained 1.5 h after seeding for pY713 Fes, vinculin and actin. Cells expressing Y477F ezrin remained round, displayed enlarged and only peripheral focal adhesions, and actin bundles were nearly absent (bar, 10 μ m). **(C)** Comparison of the surface of cells expressing WT or Y477F ezrin. For each clones the area of five representative cells was measured in three independent experiments using Metamorph software. Area of cells expressing WT ezrin was taken as 100%. The data are means $\pm$ standard error (s.e.). **(D)** Cells expressing Y477F ezrin do not display an adhesion defect. Bar charts represent the percentage of adherent cells in clones F1 and F2 as compared with cells expressing WT ezrin. Data are means $\pm$ s.e. Asterisk indicates $P < 0.05$. **(E)** Expression of the constitutively active form of Fes rescues the spreading defect of cells expressing Y477F ezrin. Cells were stained for pY713 Fes, vinculin and actin (bar, 10 μ m). Bar charts represent the percentage of P1 and P2 cells spread 1.5 h after seeding on collagen-coated coverslips as compared with cells transfected with the vector alone (Hygro). The values in the histogram are means $\pm$ s.e. Asterisk indicates $P < 0.05$.

from the expression of Y477F ezrin was observed whether the cells were grown in media with low serum or containing 10% serum indicating that signaling from extracellular matrix requires ezrin/Fes interaction. We next determined whether this spreading defect was primarily due to an adhesion defect. We observed that cells expressing WT or Y477F ezrin adhere to the same extent on collagen I (Figure 4D) or fibronectin (data not shown), indicating that the delayed spreading of cells expressing Y477F ezrin was not due to an adhesion defect.

To confirm that the inability of cells expressing Y477F ezrin to spread was due to the lack of ezrin/Fes interaction, we sought to rescue this spreading defect by overexpressing Fes. We stably transfected the F1 clone with the hygromycin vector alone or with a plasmid containing the cDNA encoding WT or a constitutively active form of Fes. This form is obtained by a point mutation in the first coiled-coil motif (L145P) that results in increased Fes tyrosine kinase activity (Cheng *et al*, 2001). Two different clones for each cell lines, showing a six- to an eightfold increase in Fes level over the endogenous protein, were subsequently used and gave the same results. Interestingly, the spreading defect was not rescued in cells expressing Y477F ezrin and WT Fes (data not shown). In contrast, 44% of the clones (P1 or P2) overexpressing the active form of Fes (L145P Fes) were spread (Figure 4E). This rescue is only partial as compared with cells expressing WT ezrin, but significant as compared with cells expressing Y477F ezrin and the vector alone (Hygro). As shown in Figure 4E, the overexpression of L145P Fes restored the formation of focal adhesions, comparable to that observed in LLC-PK1 cells. The same results were observed when cells were plated on fibronectin (data not shown). Therefore, these experiments indicate that Fes acts downstream of ezrin and that Fes activation is necessary to transmit signals from extracellular matrix receptors for the control of cell spreading.

Ezrin and Fes cooperates to promote cell scattering

Ezrin has previously been involved in HGF-induced cell scattering (Crepaldi *et al*, 1997; Orian-Rousseau *et al*, 2002, 2007). Given the colocalization of ezrin and Fes at the cell-cell contacts, we reasoned that these proteins might cooperate to promote cell scattering. To test this hypothesis, LLC-PK1 cells expressing WT or Y477F ezrin were grown either on glass or on collagen-coated surfaces. Whereas 80% of cells expressing WT ezrin scattered in response to HGF when plated either on glass (data not shown) or on collagen, 80% of cells expressing Y477F ezrin did not scatter in response to HGF either on glass or on collagen and remained as islets (Figure 5A). To rule out that this scattering defect might be due to a global effect of ezrin mutation on HGF signaling, we determined if the activation of the HGF receptor or of the MAPK pathway was perturbed in cells expressing

Y477F ezrin. Indeed, it has been shown that ERM proteins are required for the activation of the Ras/MAPK pathway downstream of HGF/Met signaling (Orian-Rousseau *et al*, 2002, 2007). As shown in Supplementary Figure S5, the activation of HGF receptor and ERK1/2 was similar in cells expressing WT or Y477F ezrin. These results indicate that the lack of ezrin/Fes interaction impairs the HGF-induced cell scattering but does not affect the MAPK pathway.

Ezrin-dependent localization of Fes to the lateral membrane is required for cell scattering

To better understand how ezrin and Fes cooperate in HGF-induced scattering, we examined the localization of ezrin and activated Fes in cells stimulated or not with HGF. As shown in Figure 5B, HGF stimulation leads to a breakdown of the microvilli and to the recruitment of ezrin to the lateral membrane and to the leading edge of the cells. Similar localization was observed with Y477F ezrin (data not shown). Interestingly, upon HGF stimulation, activated Fes was also recruited to the leading edge and to cell-cell contacts, where it colocalized with ezrin, whereas in non-stimulated cells, Fes was only present in focal adhesions (Figure 5C, upper panels). Strikingly, in cells expressing Y477F ezrin, activated Fes remained in focal adhesions upon HGF treatment and was not recruited to the leading edge or to cell-cell contacts (Figure 5C, lower panels). This indicates that HGF stimulation is not sufficient to localize Fes at the membrane and that the interaction between ezrin and Fes is critical for the recruitment of Fes to the lateral membrane.

The activation of Fes at the membrane is required for cell scattering

Our results suggested that Fes acts downstream of ezrin to promote HGF-induced cell scattering. We therefore determined if the scattering defect observed in cells expressing Y477F ezrin could be reversed by expressing either WT or L145P Fes in cells expressing Y477F ezrin. Cells coexpressing Y477F ezrin and WT Fes were unable to scatter upon HGF treatment either on glass or on collagen (data not shown). However, 50% of the cells expressing Y477F ezrin and L145P Fes scattered in response to HGF only when seeded on a collagen-coated surface (Figure 6A) but not on glass (data not shown). These results demonstrate that Fes must be activated to trigger cell scattering. Moreover, since the reversion is only observed when cells are grown on collagen, it indicates that scattering also requires signals from extracellular matrix.

We next asked how the constitutively active form of Fes can rescue the scattering defect of cells expressing Y477F ezrin. Since L145P Fes cannot interact with Y477F (Supplementary Figure S6) and because we showed that cell scattering requires the interaction of ezrin and Fes at the membrane, we examined the localization of L145P Fes in cells expressing Y477F ezrin treated or not with HGF. As

Figure 5 Ezrin/Fes interaction is required to promote HGF-induced cell scattering. (A) Cells expressing Y477F ezrin do not scatter upon HGF treatment. Cells stably expressing WT or Y477F ezrin were treated overnight with HGF and analyzed by phase-contrast microscopy (bar, 10 μ m). Quantification was performed on islets of at least four cells in five random fields and in three independent experiments. Bar charts show the percentage of scattered islets. Data are means $\pm$ s.e. Asterisk indicates $P < 0.001$. (B) Ezrin is recruited to the plasma membrane upon HGF stimulation. Immunofluorescence was performed with the anti-ezrin antibody. Overlay pictures show ezrin (green) and actin (red) stainings. Arrowheads show ezrin localization at the leading edge of the cells (bar, 10 μ m). (C) Fluorescence was performed with the anti-pY713 Fes antibody (green) and phalloidin (red). Upon HGF stimulation, activated Fes is recruited to cell-cell contacts and to the leading edge of cells expressing WT ezrin (arrowheads). In cells expressing Y477F ezrin, pY713 Fes remains in focal adhesions (bar, 10 μ m).

shown in Figure 6B, L145P Fes was present in both focal adhesions and at the leading edge of the cells, whereas WT Fes was only present in focal adhesions (data not shown). This indicates that the constitutively active form of Fes is able

to bypass the need for ezrin to be recruited to the plasma membrane.

The observation that only a constitutively active form of Fes but not the WT form was able to rescue the phenotypic

defects displayed by cells expressing Y477F ezrin suggested that the ezrin/Fes interaction was required for the activation of Fes. We therefore analyzed the level of Fes phosphorylation in cells seeded on collagen and treated or not with HGF. As shown in Figure 6C, Fes was phosphorylated at tyrosine 713 in cells expressing WT ezrin seeded on collagen and this phosphorylation level was highly increased upon HGF treatment. In contrast, no phosphorylation of Fes was observed in cells expressing Y477F ezrin even after HGF treatment. This result indicates that ezrin/Fes interaction is required for Fes activation as reflected by its phosphorylation at tyrosine 713.

Discussion

The ezrin/Fes interaction we have uncovered provides a molecular link between receptor and non-receptor tyrosine kinases and extracellular matrix receptors for the control of cell scattering. This interaction occurs through phosphorylated tyrosine 477 in ezrin with the SH2 domain of Fes. Interestingly, the sequence surrounding tyrosine 477, pYEPV, matches exactly the consensus sequence defined for the SH2 domain of Fes, pYExV (Songyang *et al*, 1994). Moreover, phosphorylated tyrosine 477 is likely associated with functions that are specific to ezrin since this residue is not present in radixin or moesin, two proteins that are highly homologous to ezrin.

In this study we provide evidence that in epithelial cells, ezrin/Fes interaction is important for the localization of Fes at cell-cell contacts and for its activation. We show that a significant proportion of endogenous Fes is localized in punctate cytoplasmic structures. This observation is in agreement with previous reports showing that overexpressed GFP-tagged Fes is present in vesicular structures (Zirngibl *et al*, 2001; Takahashi *et al*, 2003; Laurent *et al*, 2004a). However, we report here that activated Fes is present in two different compartments in epithelial cells, depending on cell confluency. At low confluency, activated Fes is mainly observed in focal adhesions. In line with this observation, Kanda *et al* (2006) reported the localization of Fes in focal adhesions of endothelial cells following fibroblast growth factor-2 stimulation. In contrast, in confluent cells, activated Fes is localized mainly at cell-cell contacts.

The presence of activated Fes in two different subcellular compartments implies that this protein interacts with specific partners. We have identified ezrin as the protein that recruits Fes at the cell-cell contacts. Although ezrin is mainly present in the apical microvilli, our data indicate that only the pool of ezrin present at the cell-cell contacts can recruit Fes. This pool is likely increased following HGF treatment, since we observed a relocation of ezrin from the microvilli to the

lateral surface. Since receptor tyrosine kinases are present at the lateral surface of the cells, it is possible that only this pool of ezrin is phosphorylated at tyrosine 477 by Src family kinases and in response to growth factor. However, we were not able to detect an increase in ezrin phosphorylation at tyrosine 477 upon HGF stimulation either because a small fraction of ezrin is phosphorylated or because the turnover of this phosphorylation is too rapid.

The localization of Fes in focal adhesions is independent of ezrin, since this protein is not detected in these structures. Moreover, in ezrin knocked down cells, activated Fes is still present in focal adhesions. Thus, the protein that targets Fes to the focal adhesions remains to be identified. One candidate may be p130 Cas, the Crk-associated substrate present in focal adhesions, as Fes has been reported to interact with this protein in macrophages (Jucker *et al*, 1997). Another candidate may be the focal adhesion kinase (FAK). Indeed Arregui *et al* (2000) have shown that abolishing the interaction between Fer kinase, another member of the *fes/fps* family and N-cadherin increases the association of Fer with FAK.

Here we show that in addition to recruiting Fes to the membrane, ezrin also participates in its activation. Indeed, the lack of ezrin/Fes interaction leads to a strong decrease in the level of activated Fes. How ezrin triggers Fes activation is not clear. Greer *et al* (1994) have shown that addition of a myristylation sequence at the N-terminus of Fes targets the protein to the membrane and leads to a constitutively active form. Our results suggest that the recruitment of Fes by ezrin to the membrane may represent the first step in the activation process. This binding may induce changes in the conformation of Fes that would allow its oligomerization and activation.

The recruitment of Fes and its activation in a specific compartment are a prerequisite for HGF-induced cell scattering. Whereas Y477F ezrin is still recruited to the membrane when cells are stimulated with HGF, Fes is not; it remains in focal adhesions and as a consequence, cells do not scatter. We can exclude that in cells expressing Y477F ezrin, the scattering defect is due to an impaired activation of Met or of the Ras pathway. Rather, our observations indicate that the scattering defect is due to the fact that Fes is not activated in the proper compartment. Indeed, a constitutively active form of Fes is recruited to the membrane independently of ezrin and can rescue the scattering defect of Y477F ezrin-expressing cells. This observation further illustrates that Fes must be recruited to the membrane to promote cell scattering. How Fes does participate to cell scattering? One hypothesis is that Fes phosphorylates specific targets at cell-cell contacts that are important for cell scattering.

In agreement with previous work indicating that the extracellular matrix potentiates the effects of HGF on cell

Figure 6 The recruitment and the activation of Fes at the membrane are required for cell scattering. (A) Activated Fes rescues the scattering defect of cells expressing Y477F ezrin. Phase-contrast images of cells seeded on collagen-coated coverslips and treated or not with HGF. Bar charts represent the percentage of P1 and P2 cell islets scattered as compared with cells expressing Y477F ezrin and the vector alone (Hygro). The values in the histogram are means $\pm$ s.e. and asterisk indicates $P < 0.001$. (B) L145P Fes is recruited to the membrane independently of ezrin. Fluorescence was performed with the anti-pY713 Fes antibody (green) and phalloidin (red) on cells expressing Y477F ezrin and transfected with either the vector alone (Hygro) or the cDNA coding L145P Fes. Arrowheads show activated Fes localization at the leading edge of the cells (bar, 10 μ m). (C) Ezrin/Fes interaction leads to Fes activation. Cells stably expressing WT or Y477F ezrin were transfected with Myc-tagged Fes, seeded on collagen-coated dishes and treated or not for 10 min with 120 ng/ml HGF. Immunoprecipitation was performed on cell lysates with the anti-Myc antibody. Immunoprecipitated proteins and total cell extracts (inputs) were immunoblotted with anti-Myc (lower panels) or anti-pY713 Fes antibodies (upper panel). Fes is phosphorylated at tyrosine 713 in cells expressing WT ezrin but not in cells expressing Y477F ezrin.

scattering (Clark, 1994; de Rooij *et al*, 2005), our results further indicate that the ability of the ezrin/Fes interaction to mediate cell scattering also requires signals from the extra-cellular matrix. Cell scattering requires both an increase in

cell spreading/motility and the disruption of cell-cell contacts. The coordination of these two events is regulated by a cross-talk between integrin- and cadherin-based adhesion (Clark, 1994; Yano *et al*, 2004; Avizienyte and Frame, 2005;

de Rooij *et al*, 2005). We can hypothesize that the scattering defect observed when the ezrin/Fes interaction is abolished likely results from both the lack of recruitment of Fes to the membrane and from the accumulation of Fes in the focal adhesions that may perturb integrin signaling and actin cytoskeleton organization, thus preventing the dissociation of cell-cell contacts. Interestingly, it has been observed that an increased association of Fes with integrin complexes inhibits β 1-integrin functions (Arregui *et al*, 2000).

We show here for the first time that through its localized activation, Fes coordinates the cross-talk between cell-cell and cell-matrix adhesion. Dissociation of cell-cell contacts and increased cell motility and invasion are key events in the formation of metastases. Furthermore, increased Src kinase activity and constitutive activation of Met have been detected in many invasive cancer types (Frame, 2002; Corso *et al*, 2005). Likewise, the analysis of the kinome of colon cancer cells has revealed the presence of activating mutations in Fes (Bardelli *et al*, 2003). In view of recent findings that ezrin is required for metastasis of breast carcinoma, osteosarcoma and HGF-induced rhabdomyosarcoma (Khanna *et al*, 2004; Yu *et al*, 2004; Elliott *et al*, 2005), it would be interesting to address the ezrin/Fes interaction as a mechanism connecting Src activation with changes in the adhesive properties of tumor cells and metastasis progression.

Materials and methods

Reagents and antibodies

Reagents used were as follows: PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) and SU6656 (2-oxo-3-(4,5,6,7-tetrahydro-1H-indol-2-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide) (Calbiochem, LaJolla, CA), human recombinant HGF (Upstate, Lake Placid, NY), type I collagen (BD Biosciences, Palo Alto, CA) and lambda protein-phosphatase (New England Biolabs, Ipswich, MA). Characterization of anti-pY477 ezrin, anti-Fes and anti-pY713 Fes antibodies is provided in Supplementary data and Supplementary Figures. The antibodies used were as follows: polyclonal anti-VSVG and anti-ezrin, (Algrain *et al*, 1993), monoclonal anti-VSVG (P5D4) (Kreis, 1986), polyclonal anti-Myc antibody was generated in the laboratory, monoclonal anti-ezrin antibody clone 4A7A6C1 was a kind gift from Dr Choquet-Kastylevsky (New Markers Department, BioMérieux, Marcy l'Etoile, France), monoclonal anti- α -tubulin antibody (Sigma, St Louis, MO), monoclonal anti-vinculin antibody (clone V11F9) (Glukhova *et al*, 1990), polyclonal anti-Erk1/2 and anti-phospho-Met (tyrosines 1234/1235) antibodies (Upstate, Lake Placid, NY), monoclonal anti-E-cadherin (BD Transduction Laboratories, Lexington, KY), anti-phospho Erk1/2 and anti-Met (clone 25H2) antibodies (Cell Signalling, Beverly, MA). Phalloidin coupled to rhodamin or to AlexaFluor 350 was purchased from Invitrogen (Carlsbad, CA).

Plasmid constructs

The pCB6 vector containing the cDNA coding for WT ezrin fused to the VSVG tag was previously described (Algrain *et al*, 1993). The cDNA encoding human Fes (clone IMAGE 5170548) was subcloned in fusion with the Myc tag into pcDNA3.1 vector (Invitrogen, Carlsbad, CA). The cDNA fragment of Fes encoding amino acids 255–593 was subcloned into the pGEX-CS vector. The point mutants Y477F ezrin and L145P Fes were obtained using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA).

Cells and transfections

LLC-PK1 cells (CCL 101; American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum.

The stable LLC-PK1 cell line expressing VSVG tagged Y477F ezrin was generated as previously described (Crepaldi *et al*, 1997). To generate the double stable cell lines, the clone F1 was subsequently

transfected by electroporation with the empty plasmid (pcDNA3-Hygro) or with the plasmid containing the cDNA encoding either Myc-tagged WT or the constitutively active form of Fes (L145P Fes). Selection of the clones was carried out with 0.6 g/l of hygromycin in the presence of 0.6 g/l geneticin.

Yeast two-hybrid analysis

Lyn cDNA was cloned in the pASZ16 vector derived from pASZ10 (Stotz and Linder, 1990) and integrated into CG1945-ade2 (Mata, ade2-101, his3- Δ 200, leu2-3112, trp1-901, lys2-801, ura3-52, URA3::Gal4 17mers(X3)-CyC1TATA-LacZ, LYS2::GAL1UAS-GAL1TAT A-HIS3) to generate the yeast strain CG1945-Lyn. The baits were cloned in the pB6 plasmid derived from the original pAS2 $\Delta\Delta$ (Fromont-Racine *et al*, 1997) and transferred into the yeast strains carrying or not an integrated copy of Lyn. A random-primed cDNA library from human placenta poly(A⁺) RNA was constructed into the pP6 plasmid derived from the original pGADGH (Bartel *et al*, 1993). Y187 yeast strain was transformed with the library and 10⁷ independent colonies were collected and mated with the bait-transformed strains as described (Rain *et al*, 2001). Each screen was performed to ensure a minimum of 50 million interactions tested.

GST pull down and immunoprecipitation

For GST pull-down experiments, cells were lysed in RIPA buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl₂, 10% glycerol, 0.1% SDS, 1% Triton, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma). After preclearing, supernatants were incubated for 2 h at 4°C with glutathione beads coupled to GST fusion proteins. For immunoprecipitation, cells were lysed in RIPA lysis buffer (50 mM Hepes (pH 7.5), 150 mM NaCl, 10 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 1 mM sodium orthovanadate, and protease inhibitor cocktail). After preclearing, supernatants were incubated for 2 h at 4°C with antibody and protein G-Sepharose beads. Where indicated, cells were pretreated with 0.1 mM pervanadate for 10 min at 37°C, 10 μ M PP2 for 20 min at 37°C or 10 μ M SU6656 for 15 min at 37°C.

Affinity-binding assay with phosphorylated peptides

Peptides comprising ezrin amino acids 475–480 (PV(p)⁴⁷⁷YEPV) in the phosphorylated or non-phosphorylated forms, and the phosphopeptide surrounding the tyrosine 190 in ezrin (CLKDNAMLE-p¹⁹⁰YLKIA) were synthesized by Covalab (Lyon, France). The peptides were coupled to AminoLink beads using the AminoLink kit (Pierce, Rockford, IL). Beads were incubated with GST-Fes (amino acids 255–593) in 0.5 ml of a buffer consisting of 50 mM Hepes (pH 7.4), 100 mM NaCl, 2 mM EDTA and 1% Triton X100 for 1 h at 4°C.

Cell spreading and cell adhesion assays

Cell spreading was performed as previously described (Srivastava *et al*, 2005). Cell area measurement was performed using Metamorph software (Universal Imaging). For the cell adhesion assay, cells were plated in 96-well tissue culture plates coated with 10 μ g/ml of collagen and were allowed to adhere for 1.5 h at 37°C. After washing, adherent cells were fixed and stained with crystal violet for 10 min at room temperature and counted in a microplate reader (Molecular Device, Sunnyvale, CA) at OD 595 nm. For statistical analyses, Student's *t*-test was performed and the *P*-value calculated.

Cell scattering assay

Cells were plated in 12-well tissue culture dishes coated or not with type I collagen and allowed to grow as small islets. Cells were placed in a low serum containing medium (0.5% of fetal bovine serum) for 5 h and then treated for 16 h with 45 ng/ml HGF. Cells were fixed and photographed under phase-contrast using a Leica microscope. Islets were considered as scattered when the cells were dispersed and displayed an elongated shape.

Supplementary data

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

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References

- Algrain M, Turunen O, Vaheri A, Louvard D, Arpin M (1993) Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. *J Cell Biol* **120**: 129–139
- Arregui C, Pathre P, Lilien J, Balsamo J (2000) The nonreceptor tyrosine kinase fer mediates cross-talk between N-cadherin and beta1-integrins. *J Cell Biol* **149**: 1263–1274
- Autero M, Heiska L, Ronnstrand L, Vaheri A, Gahmberg CG, Carpen O (2003) Ezrin is a substrate for Lck in T cells. *FEBS Lett* **535**: 82–86
- Avizienyte E, Frame MC (2005) Src and FAK signalling controls adhesion fate and the epithelial-to-mesenchymal transition. *Curr Opin Cell Biol* **17**: 542–547
- Bardelli A, Parsons DW, Silliman N, Ptak J, Szabo S, Saha S, Markowitz S, Willson JK, Parmigiani G, Kinzler KW, Vogelstein B, Velculescu VE (2003) Mutational analysis of the tyrosine kinome in colorectal cancers. *Science* **300**: 949
- Bartel P, Chien CT, Sternglanz R, Fields S (1993) Elimination of false positives that arise in using the two-hybrid system. *Biotechniques* **14**: 920–924
- Bretscher A, Edwards K, Fehon RG (2002) ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol* **3**: 586–599
- Cheng HY, Schiavone AP, Smithgall TE (2001) A point mutation in the N-terminal coiled-coil domain releases c-Fes tyrosine kinase activity and survival signaling in myeloid leukemia cells. *Mol Cell Biol* **21**: 6170–6180
- Clark P (1994) Modulation of scatter factor/hepatocyte growth factor activity by cell-substratum adhesion. *J Cell Sci* **107** (Part 5): 1265–1275
- Corso S, Comoglio PM, Giordano S (2005) Cancer therapy: can the challenge be MET? *Trends Mol Med* **11**: 284–292
- Crepaldi T, Gautreau A, Comoglio PM, Louvard D, Arpin M (1997) Ezrin is an effector of hepatocyte growth factor-mediated migration and morphogenesis in epithelial cells. *J Cell Biol* **138**: 423–434
- Cutrupi S, Baldanzi G, Gramaglia D, Maffe A, Schaap D, Giraudo E, van Blitterswijk W, Bussolino F, Comoglio PM, Graziani A (2000) Src-mediated activation of alpha-diacylglycerol kinase is required for hepatocyte growth factor-induced cell motility. *EMBO J* **19**: 4614–4622
- de Rooij J, Kerstens A, Danuser G, Schwartz MA, Waterman-Storer CM (2005) Integrin-dependent actomyosin contraction regulates epithelial cell scattering. *J Cell Biol* **171**: 153–164
- Delfino FJ, Stevenson H, Smithgall TE (2006) A growth-suppressive function for the c-fes protein-tyrosine kinase in colorectal cancer. *J Biol Chem* **281**: 8829–8835
- Elliott BE, Meens JA, SenGupta SK, Louvard D, Arpin M (2005) The membrane cytoskeletal crosslinker ezrin is required for metastasis of breast carcinoma cells. *Breast Cancer Res* **7**: R365–R373
- Elliott BE, Qiao H, Louvard D, Arpin M (2004) Co-operative effect of c-Src and ezrin in deregulation of cell-cell contacts and scattering of mammary carcinoma cells. *J Cell Biochem* **92**: 16–28
- Fiévet B, Louvard D, Arpin M (2007) ERM proteins in epithelial cell organization and functions. *Biochim Biophys Acta* **1773**: 653–660
- Frame MC (2002) Src in cancer: deregulation and consequences for cell behaviour. *Biochim Biophys Acta* **1602**: 114–130
- Fromont-Racine M, Rain JC, Legrain P (1997) Toward a functional analysis of the yeast genome through exhaustive two-hybrid screens. *Nat Genet* **16**: 277–282
- Gary R, Bretscher A (1995) Ezrin self-association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site. *Mol Biol Cell* **6**: 1061–1075
- Gautreau A, Louvard D, Arpin M (2000) Morphogenic effects of ezrin require a phosphorylation-induced transition from oligomers to monomers at the plasma membrane. *J Cell Biol* **150**: 193–203
- Gautreau A, Pouillet P, Louvard D, Arpin M (1999) Ezrin, a plasma membrane-microfilament linker, signals cell survival through the phosphatidylinositol 3-kinase/Akt pathway. *Proc Natl Acad Sci USA* **96**: 7300–7305
- Glukhova MA, Frid MG, Kotliansky VE (1990) Developmental changes in expression of contractile and cytoskeletal proteins in human aortic smooth muscle. *J Biol Chem* **265**: 13042–13046
- Greer P (2002) Closing in on the biological functions of Fps/Fes and Fer. *Nat Rev Mol Cell Biol* **3**: 278–289
- Greer P, Haigh J, Mbamalu G, Khoo W, Bernstein A, Pawson T (1994) The Fps/Fes protein-tyrosine kinase promotes angiogenesis in transgenic mice. *Mol Cell Biol* **14**: 6755–6763
- Haigh J, McVeigh J, Greer P (1996) The fps/fes tyrosine kinase is expressed in myeloid, vascular endothelial, epithelial, and neuronal cells and is localized in the trans-Golgi network. *Cell Growth Differ* **7**: 931–944
- Heiska L, Carpen O (2005) Src phosphorylates ezrin at tyrosine 477 and induces a phosphospecific association between ezrin and a kelch-repeat protein family member. *J Biol Chem* **280**: 10244–10252
- Hiscox S, Jiang WG (1999) Ezrin regulates cell-cell and cell-matrix adhesion, a possible role with E-cadherin/beta-catenin. *J Cell Sci* **112** (Part 18): 3081–3090
- Jucker M, McKenna K, da Silva AJ, Rudd CE, Feldman RA (1997) The Fes protein-tyrosine kinase phosphorylates a subset of macrophage proteins that are involved in cell adhesion and cell-cell signaling. *J Biol Chem* **272**: 2104–2109
- Kanda S, Miyata Y, Kanetake H, Smithgall TE (2006) Fibroblast growth factor-2 induces the activation of Src through Fes, which regulates focal adhesion disassembly. *Exp Cell Res* **312**: 3015–3022
- Khanna C, Wan X, Bose S, Cassaday R, Olomu O, Mendoza A, Yeung C, Gorlick R, Hewitt SM, Helman LJ (2004) The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis. *Nat Med* **10**: 182–186
- Kreis TE (1986) Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. *EMBO J* **5**: 931–941
- Laurent CE, Delfino FJ, Cheng HY, Smithgall TE (2004a) The human c-Fes tyrosine kinase binds tubulin and microtubules through separate domains and promotes microtubule assembly. *Mol Cell Biol* **24**: 9351–9358
- Ng T, Parsons M, Hughes WE, Monypenny J, Zicha D, Gautreau A, Arpin M, Gschmeissner S, Verwee PJ, Bastiaens PI, Parker PJ (2001) Ezrin is a downstream effector of trafficking PKC-integrin complexes involved in the control of cell motility. *EMBO J* **20**: 2723–2741
- Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P, Ponta H (2002) CD44 is required for two consecutive steps in HGF/c-Met signaling. *Genes Dev* **16**: 3074–3086
- Orian-Rousseau V, Morrison H, Matzke A, Kastilan T, Pace G, Herrlich P, Ponta H (2007) Hepatocyte growth factor-induced Ras activation requires ERM proteins linked to both CD44v6 and F-actin. *Mol Biol Cell* **18**: 76–83
- Pujuguet P, Del Maestro L, Gautreau A, Louvard D, Arpin M (2003) Ezrin regulates E-cadherin-dependent adherens

- junction assembly through Rac1 activation. *Mol Biol Cell* **14**: 2181–2191
- Rahimi N, Hung W, Tremblay E, Saulnier R, Elliott B (1998) c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells. *J Biol Chem* **273**: 33714–33721
- Rain JC, Selig L, De Reuse H, Battaglia V, Reverdy C, Simon S, Lenzen G, Petel F, Wojcik J, Schachter V, Chemama Y, Labigne A, Legrain P (2001) The protein–protein interaction map of *Helicobacter pylori*. *Nature* **409**: 211–215
- Rogers JA, Read RD, Li J, Peters KL, Smithgall TE (1996) Autophosphorylation of the Fes tyrosine kinase. Evidence for an intermolecular mechanism involving two kinase domain tyrosine residues. *J Biol Chem* **271**: 17519–17525
- Songyang Z, Shoelson SE, McGlade J, Olivier P, Pawson T, Bustelo XR, Barbacid M, Sabe H, Hanafusa H, Yi T, Ren R, Baltimore D, Ratnofsky S, Feldman RA, Cantley LC (1994) Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol Cell Biol* **14**: 2777–2785
- Srivastava J, Elliott BE, Louvard D, Arpin M (2005) Src-dependent ezrin phosphorylation in adhesion-mediated signaling. *Mol Biol Cell* **16**: 1481–1490
- Stella MC, Comoglio PM (1999) HGF: a multifunctional growth factor controlling cell scattering. *Int J Biochem Cell Biol* **31**: 1357–1362
- Stotz A, Linder P (1990) The ADE2 gene from *Saccharomyces cerevisiae*: sequence and new vectors. *Gene* **95**: 91–98
- Takahashi S, Inatome R, Hotta A, Qin Q, Hackenmiller R, Simon MC, Yamamura H, Yanagi S (2003) Role for Fes/Fps tyrosine kinase in microtubule nucleation through is Fes/CIP4 homology domain. *J Biol Chem* **278**: 49129–49133
- Takeuchi K, Sato N, Kasahara H, Funayama N, Nagafuchi A, Yonemura S, Tsukita S (1994) Perturbation of cell adhesion and microvilli formation by antisense oligonucleotides to ERM family members. *J Cell Biol* **125**: 1371–1384
- Tsukamoto T, Nigam SK (1999) Cell–cell dissociation upon epithelial cell scattering requires a step mediated by the proteasome. *J Biol Chem* **274**: 24579–24584
- Yano H, Mazaki Y, Kurokawa K, Hanks SK, Matsuda M, Sabe H (2004) Roles played by a subset of integrin signaling molecules in cadherin-based cell–cell adhesion. *J Cell Biol* **166**: 283–295
- Yonemura S, Tsukita S (1999) Direct involvement of ezrin/radixin/moesin (ERM)-binding membrane proteins in the organization of microvilli in collaboration with activated ERM proteins. *J Cell Biol* **145**: 1497–1509
- Yu Y, Khan J, Khanna C, Helman L, Meltzer PS, Merlino G (2004) Expression profiling identifies the cytoskeletal organizer ezrin and the developmental homeoprotein Six-1 as key metastatic regulators. *Nat Med* **10**: 175–181
- Zirngibl R, Schulze D, Mirski SE, Cole SP, Greer PA (2001) Subcellular localization analysis of the closely related Fps/Fes and Fer protein-tyrosine kinases suggests a distinct role for Fps/Fes in vesicular trafficking. *Exp Cell Res* **266**: 87–94