The endocytic adapter E-Syt2 recruits the p21 GTPase activated kinase PAK1 to mediate actin dynamics and FGF signalling

Steve Jean*, Michel G. Tremblay, Chelsea Herdman, François Guillou and Tom Moss‡

Laboratory of Growth and Development, Cancer Research Centre and Department of Molecular Biology, Medical Biochemistry and Pathology of Laval University, Cancer Axis of the CHUQ Research Centre, Pavillon St Patrick, 9 rue McMahon, Québec QC G1R 3S3, Canada

*Present address: Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093-0380, USA
‡Author for correspondence (Tom.Moss@crhdq.ulaval.ca)

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Summary

Fibroblast growth factor (FGF) signalling plays an essential role in early vertebrate development. However, the response to FGF requires endocytosis of the activated FGF receptor (FGFR) that is in part dependent on remodelling of the actin cytoskeleton. Recently we showed that the extended synaptotagmin family plasma membrane protein, E-Syt2, is an essential endocytic adapter for FGFR1. Here we show E-Syt2 is also an interaction partner for the p21-GTPase Activated Kinase PAK1. The phospholipid binding C2C domain of E-Syt2 specifically binds a site adjacent to the CRIB/GBD of PAK1. PAK1 and E-Syt2 selectively complex with FGFR1 and functionally cooperate in the FGF signalling. E-Syt2 binding suppresses actin polymerization and inhibits the activation of PAK1 by the GTPases Cdc42 and Rac. Interestingly, the E-Syt2 binding site on PAK1 extensively overlaps a site recently suggested to bind phospholipids. Our data suggest that PAK1 interacts with phospholipid membrane domains via E-Syt2, where it may cooperate in the E-Syt2-dependent endocytosis of activated FGFR1 by modulating cortical actin stability.

Key words: Extended synaptotagmin, E-Syt2, p21-GTPase activated kinase, PAK1, Cortical actin, FGF receptor, FGF signalling, Endocytosis

Introduction

Fibroblast growth factors (FGFs) are part of a large family of growth factors modulating cellular processes (Böttcher and Niehrs, 2005) as diverse as cell survival, differentiation, adhesion, migration and disease. They were the first growth factors shown to possess inductive properties (Kimelman and Kirschner, 1987; Slack et al., 1987) and in *Xenopus* are essential for mesoderm induction (Kimelman, 2006) and cell migration. Endocytosis is an important aspect of growth factor signalling, and controls both signal strength and specificity (Le Roy and Wrana, 2005). We recently identified the Extended Synaptotagmin-like membrane protein E-Syt2 as an essential endocytic adapter for FGF signalling in *Xenopus* and human (Jean et al., 2010). E-Syt2 was shown to interact specifically with the activated FGF receptors and with Adaptin-2 (AP-2), and it was suggested to play a role in targeting the receptor to the clathrin-mediated endocytic pathway. Here we show that E-Syt2 also interacts with the p21-GTPase Activated Kinase PAK1 and regulates the actin cytoskeleton, a function implicated in endocytosis.

The PAK family of protein kinases plays key roles in cytoskeletal regulation (Bokoch, 2003), phosphorylating important cytoskeletal regulators like LIM kinase (Edwards et al., 1999), MLCK (Sanders et al., 1999) and MLC (Bisson et al., 2003). PAK has also been shown to play a non-catalytic role in driving actin depolymerization by locally sequestering the active (GTP-bound) GTPases Cdc42 and Rac (Bisson et al., 2007). The role of actin dynamics in endocytosis has been the subject of conflicting reports, but most recently it has been shown that it may play a role at a late stage of clathrin pit internalization (McMahon and Boucrot, 2011). Hence, the recruitment of PAK1 to an endocytic adapter could logically be of functional significance.

Results

To better understand the *in vivo* functions of *Xenopus* PAK1 we sought to identify novel PAK1 interacting proteins. The regulatory N-terminal domain and the C-terminal kinase domain of PAK1 were used as bait in two-hybrid screens of a *Xenopus laevis* cDNA library. 4.7×10^6 independent clones were screened and eight isolates proved positive after retransformation. One of these, N126, interacted with the N-terminal domain of PAK1 and encoded the last C2 (C2C) domain of *Xenopus* E-Syt2, a member of a small sub-family of synaptotagmin-like membrane proteins (Min et al., 2007) (Fig. 1a). E-Syt2 is essential for the induction of mesoderm by FGF in early embryos, and E-Syt2 interacts directly and specifically with the activated form of the FGF Receptor (FGFR) (Jean et al., 2010). Further, E-Syt2 loss or overexpression inhibits receptor endocytosis and functional signal transduction via

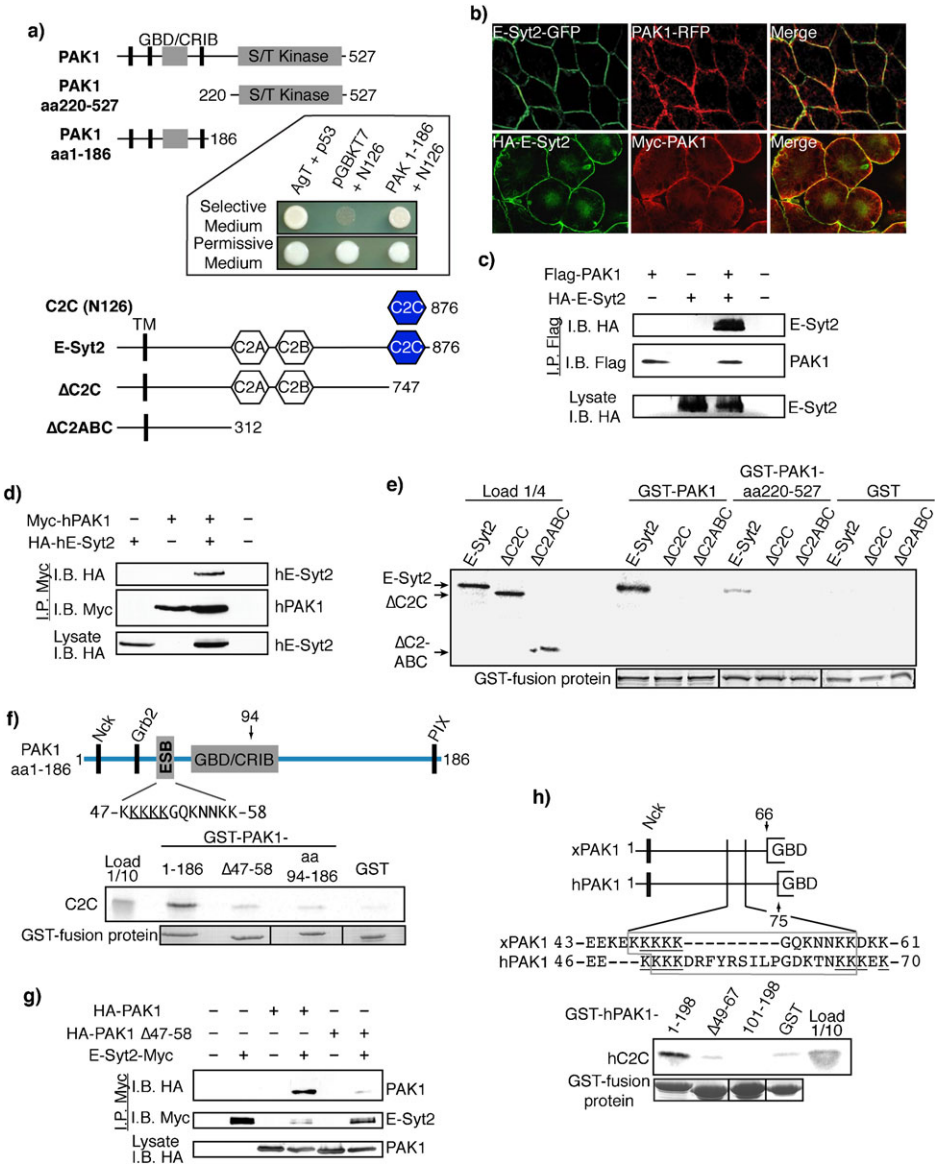

Fig. 1. E-Syt2 and PAK1 interact and co-localize. a) Schematic of PAK1, E-Syt2 and mutant constructs. Inset shows two-hybrid retransformation of the N126 isolate with *Xenopus* PAK a.a. 1–186 or the empty vector (pGBKT7). The SV40 T antigen (AgT) and p53 vectors (Clontech) provide a positive control. **b**) Fluorescent E-Syt2-GFP and PAK1-RFP fusion or the corresponding epitope tagged proteins were expressed in *Xenopus* animal caps and visualized respectively by direct or indirect epifluorescence. Upper panel shows images taken from the outer surface of the animal pole cortex, and lower panel from the inner surface. **c**) HA-E-Syt2 and/or Flag-PAK1 were expressed in HEK293T cells and Flag co-immunoprecipitated (I.P.) complexes were immunoblotted (I.B.) with HA and Flag antibodies. **d**) As in (c) but human PAK1 (Myc-hPAK1) and human E-Syt2 (HA-hE-Syt2) replaced the *Xenopus* forms. **e**) Pull-down assays of the interactions between full length PAK1 (GST-PAK1), its kinase domain (GST-PAK1 a.a.220–527) or GST and *in vitro* translated E-Syt2, or E-Syt2 deletion mutants (see (a)). **f**) Pull-down assays as in (e) between the *in vitro* translated C2C domain of E-Syt2 and the N-terminal regulatory domain (a.a. 1–186) or corresponding deletion mutants lacking a.a. 47–58 (Δ47–58) or a.a. 1 to 93 (a.a. 94–186) of PAK1. **g**) E-Syt2-Myc and HA-PAK1 or the E-Syt2 Box (ESB) HA-PAK1 deletion mutant (Δ47–58) were expressed in HEK293T cells and Myc co-immunoprecipitated (I.P.) complexes were immunoblotted (I.B.) with HA and Myc antibodies. **h**) Pull-down assays as in (f), but using the C2C domain of human E-Syt2 (hC2C) and human PAK1 (hPAK1) N-terminal regulatory domain a.a. 1–198 or corresponding deletion mutants lacking a.a. 49–67 (Δ49–67) and a.a. 1 to 100 (101–198). The *Xenopus* PAK1 amino acids shown in this study to be required for the E-Syt2 interaction (supplementary material Fig. S1c), and those of human PAK1 shown to be required for phospholipid binding (Strochlic et al., 2010), are shown underlined.

the ERK pathway, suggesting that PAK1 might functionally cooperate with E-Syt2 in mediating FGF signalling. Thus, we first sought to validate the interaction of PAK1 with E-Syt2.

E-Syt2 colocalizes and interacts with PAK1 *in embryo*

Full length E-Syt2 was found to co-localize with PAK1 at the plasma membrane of *Xenopus* animal cap (AC) cells (Fig. 1b) and to specifically co-immunoprecipitate with PAK1 (Fig. 1c). Further, we found that the human E-Syt2 orthologue specifically co-immunoprecipitated with human PAK1, demonstrating that this interaction was conserved in evolution (Fig. 1d).

The C2C of E-Syt2 recruits PAK1 via a novel regulatory domain sequence

As predicted from the two-hybrid data, interaction with E-Syt2 occurred within the N-terminal control region of PAK1. Full-length PAK1 pulled-down E-Syt2 very effectively while the C-terminal kinase domain (a.a. 220–527) showed only a weak interaction (Fig. 1e) (Fig. 1a for mutant structures). Deletion of the C-terminal C2 domain (C2C) of E-Syt2 eliminated this interaction, while deletion of all three C2 domains had no further effect (Fig. 1e). The N-terminal domain of PAK1 and the C2C domain of E-Syt2 were also sufficient for the interaction (supplementary material Fig. S1a). The site of interaction between the C2C domain of E-Syt2 and the regulatory N-terminus of PAK1 was further delimited to a.a. 47 to 58 of PAK1 using N- and C-terminal deletion mutants (supplementary material Fig. S1b) and deletion of this site, termed the E-Syt2 Box (ESB), essentially eliminated the interaction as determined both *in vitro* by pull-down and *in vivo* by immunoprecipitation (Fig. 1f,g). Deletion of the corresponding amino acids (a.a. 49–67) from human PAK1 also eliminated its interaction with human E-Syt2, showing that despite significant sequence divergence in this region, the ESB site was conserved in human (Fig. 1h). Alanine and glutamic acid scanning of the ESB of *Xenopus* PAK1 did not reveal any single crucial amino acid, but combined mutation of a.a. 48 and 50 (K48/50E) or a.a. 48 to 51 to A (K48-51A) or to E (K48-51E) strongly decreased the interaction (supplementary material Fig. S1c). Thus, the short blocks of lysine

residues within the ESB form an essential part of the PAK1 ESB. This said, the solution structure of the C2C domain of E-Syt2 (Protein Data Bank entry, updated in 2009 by Nagashima et al. [http://www.rcsb.org/pdb/explore/explore.do?structureId=2DMG]) does not reveal any obvious matching acid patch for these adjacent lysines, suggesting that the full ESB probably includes adjacent amino acids.

Interestingly, recent work identified the basic residues a.a. 48–51, 66–68 and 70 of human PAK1 that overlap the ESB (Fig. 1h), as being required for PAK1 recruitment to phospholipids (Strochlic et al., 2010). These authors suggested that in the cellular context activation of human PAK1 by PDGF depended on these basic amino acids. Given that E-Syt2 binds phospholipids via its C2C domain (Jean et al., 2010; Min et al., 2007), its interaction with PAK1 suggested a different scenario. We, therefore, endeavoured to determine whether E-Syt2 would enhance or would compete for PDGF-dependent activation of PAK1. However, despite strong activation of ERK, in our hands *Xenopus* and human PAK1 were only weakly if at all activated by PDGF (supplementary material Fig. S1d,e), and we note that others have made similar observations (Deacon et al., 2008; Yuan et al., 2010). The recruitment of PAK1 to the plasma membrane via phospholipids could, however, enhance its subsequent recruitment by E-Syt2. We have found that recruitment of the C2C domain of E-Syt2 to the plasma membrane is independent of the PAK1 interaction site (F.G., unpublished). Thus, E-Syt2 forms a bridge between PAK1 and the plasma membrane.

PAK1 is recruited to an E-Syt2/FGFR1 complex and its activation partially rescues FGF signalling

We found that PAK1 was recruited to the FGFR1/E-Syt2 complex *in vivo* and, as previously observed for E-Syt2, this recruitment was stimulated by receptor activation (Fig. 2a), suggesting that PAK1 might cooperate in FGF signalling. Overexpression of E-Syt2 was found to function as a dominant negative mutation, inhibiting FGF receptor endocytosis, FGF signalling to ERK and the induction of mesoderm (Jean et al., 2010). We therefore asked if expression of activated forms of PAK1 might rescue the dominant negative effect of E-Syt2. The mildly activated form of PAK1 (L98F) did indeed partially rescued FGFR1-induced mesoderm induction in ACs overexpressing E-Syt2, as judged by expression of the mesodermal marker Xbra (Fig. 2b), and this rescue was somewhat enhanced by the more strongly activated N-terminally deleted PAK1. (Rescue was limited by the fact that higher levels of PAK1 activation lead to a fragmentation of blastomeres caused by its direct activation of myosin II (Bisson et al., 2003)). In contrast, expression of wild type PAK1, or the catalytically inactive forms PAK-K281A or PAK-L98F/K281A, all of which bind E-Syt2, were all unable to rescue Xbra expression in E-Syt2 expressing AC (Fig. 2c). Thus, rescue required the kinase activity of PAK1 and was not simply due to sequestration of E-Syt2. Indeed, these dominant negative forms as well as the dominant negative PAK1 auto inhibitory domain (AID) (Parrini et al., 2002) all mildly suppressed Xbra induction by activated FGFR1 (supplementary material Fig. S2).

Gastrulation defects caused by ectopic E-Syt2 and dominant negative PAK1 resemble inhibition of FGF signalling

A defect in FGF signalling first becomes evident at gastrulation as a delay or inhibition of cell migration and involution of

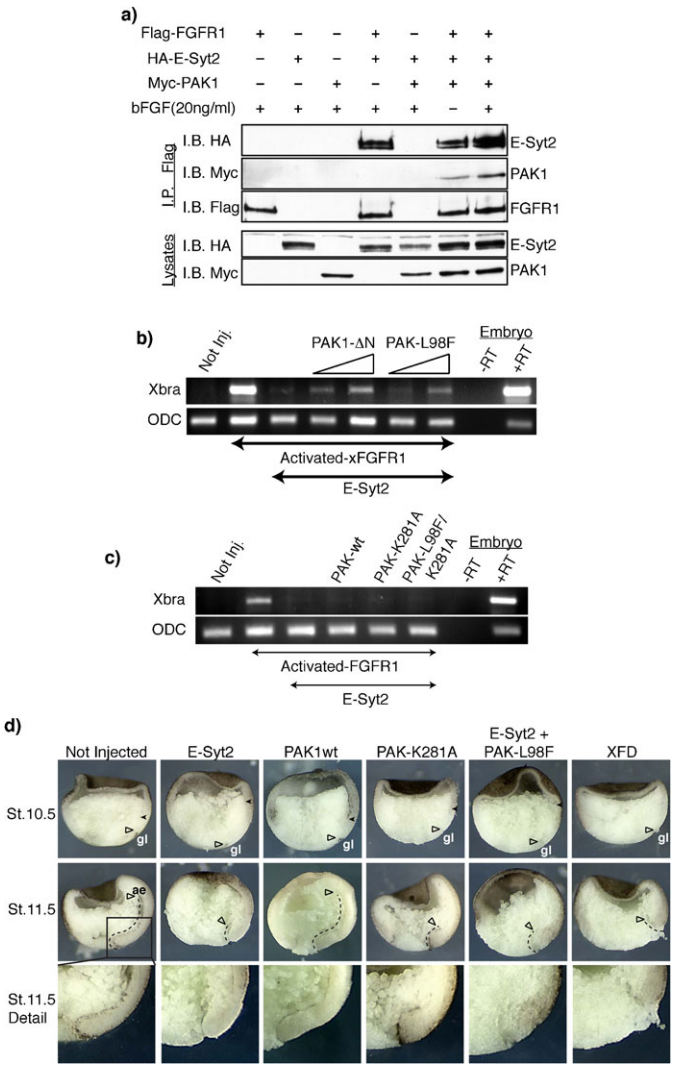

Fig. 2. PAK is implicated along with E-Syt2 in FGF signalling. a) PAK1 is recruited to an E-Syt2/FGFR1 complex. Flag-FGFR1, HA-E-Syt2 and/or Myc-PAK1 were expressed in HEK293T cells and Flag co-immunoprecipitated (I.P.) complexes were immunoblotted (I. B.) with HA, Myc and Flag antibodies. Where indicated cells were stimulated with bFGF. **b**) Activated PAK1 partially rescues E-Syt2 inhibited Xbra induction in AC. Activated FGFR1 was co-expressed with E-Syt2 and activated PAK (PAK-L98F) *in embryo* and ACs analyzed for Xbra induction by RT-PCR. ODC was used as a control. "Embryo" refers to analysis of uninjected whole embryo RNA before (−RT) and after (+RT) reverse transcription. **c**) As in (b) but co-expression was either with wild type PAK1 (PAKwt), or the catalytically inactive PAK1 mutants PAK1-K281A or PAK1-L98F/K281A. **d**) Embryos injected with the indicated constructs were fixed at the equivalent of stage 10.5 or 11.5 and manually sectioned. Dorsal is to the right side of each image. The visible extent of Brachet's cleft is indicated by a solid arrowhead and the mes-endodermal cleft by an open arrowhead and by a dashed line. Lower panels show an enlarged view of the mes-endodermal cleft region.

mesoderm, as observed for the dominant negative receptor XFD (Isaacs et al., 1994), (Fig. 2d). Since E-Syt2 and to a lesser extent catalytically inactive PAK-K281A both inhibited FGF signalling when over-expressed, we argued that they should also affect gastrulation movements. Indeed, dominant negative forms of PAK1 were previously shown to inhibit these movements (Nagel et al., 2009). Ectopic expression of both E-Syt2 and PAK-K281A, but not of wild type PAK1, delayed involution in a similar manner to dominant negative XFD, whereas wild type

PAK1 had no effect on gastrulation (Fig. 2d). At stage 10.5, involution at the dorsal gastropore lip and the formation of Brachet's cleft were delayed in E-Syt2 and PAK-K281A injected embryos, while by stage 11.5 involution was very limited and no archenteron, clearly evident in the control embryos, had formed. Activated PAK-L98F, shown to partially rescue Xbra expression when co-injected with E-Syt2, did not detectably rescue gastrulation movements. However, this was consistent with the limited rescue of FGF signalling afforded by this mutant.

E-Syt2 modulates activation of PAK1 by Cdc42 and Rac

We have shown that FGFR1 internalization via the clathrin-dependent endocytic pathway is essential for prolonged ERK activation and the induction of mesodermal markers in AC cells (Jean et al., 2010). However, clathrin-dependent receptor internalization requires the dynamic modulation of the actin cytoskeleton, both during clathrin pit formation and subsequently to drive endocytosis (Yarar et al., 2005). PAK1 is both a key downstream effector and upstream regulator of the p21 GTPases Cdc42 and Rac and together with these GTPases is in large part responsible for the regulation of cytoplasmic and cortical actin (Bisson et al., 2007; Bokoch, 2003; Obermeier et al., 1998). In particular, we previously showed that sequestration of activated Cdc42 by PAK regulated cortical actin in a manner independent of PAK catalytic activity (Bisson et al., 2007). Thus, one way that PAK1 might facilitate FGF signalling is by controlling the dynamic changes in the cytoskeleton required for endocytosis.

Since E-Syt2 recruited PAK1 via the ESB, a site immediately adjacent to the GBD/CRIB (a.a. 66–77) (Fig. 1f), it was possible that this recruitment might interfere with the activation of PAK1 either by preventing GTPase binding or the subsequent release of the activated GTPase. Deletion of the ESB (Δ47–58) had no effect on the interaction of PAK1 with Cdc42 (Fig. 3a), while deletion (Δ66–77) or mutation (H74,77L) of the GBD/CRIB GTPase binding site completely eliminated the interaction. Thus, the ESB and GBD/CRIB sites do not overlap to any detectable degree. Further, binding of the C2C domain of E-Syt2 (Fig. 1a) to the ESB of PAK1 was completely unaffected by competition with a large excess of activated Cdc42 or Rac (Fig. 3b). A similar result was obtained for Filamin, another GBD/CRIB interacting protein (Vadlamudi et al., 2002) (supplementary material Fig. S3a). Thus, E-Syt2 and Cdc42 or Rac did not interfere with each other's reaction with their proximal sites on PAK1. To functionally test the significance of E-Syt2 binding, we asked if E-Syt2 affected the activation of PAK1 by constitutively active Cdc42 or Rac *in vivo*. Increased levels of E-Syt2 led to a significant inhibition of Pak1 activation by Cdc42(L61) or Rac(L61), as judged by autophosphorylation of PAK1 (upshift in figure) and by PAK1 catalytic activity *in trans* (Fig. 3c,d; supplementary material Fig. S3b,c). This inhibition was not observed for PAK1Δ47–58 in which the E-Syt2 binding (ESB) was deleted (supplementary material Fig. S3c). Thus, E-Syt2 was able to limit the ability of both Rac and Cdc42 to activate PAK1. Since preventing PAK1 activation in turn sequesters the activated GTPases, inhibiting their function and disrupting cortical F-actin (Bisson et al., 2007), we hypothesized that E-Syt2 might in this way locally regulate cortical actin polymerization.

E-Syt2 modulates the actin cytoskeleton

To functionally test the effects of E-Syt2 on actin polymerization we first used the embryo wound-healing assay. In this assay the

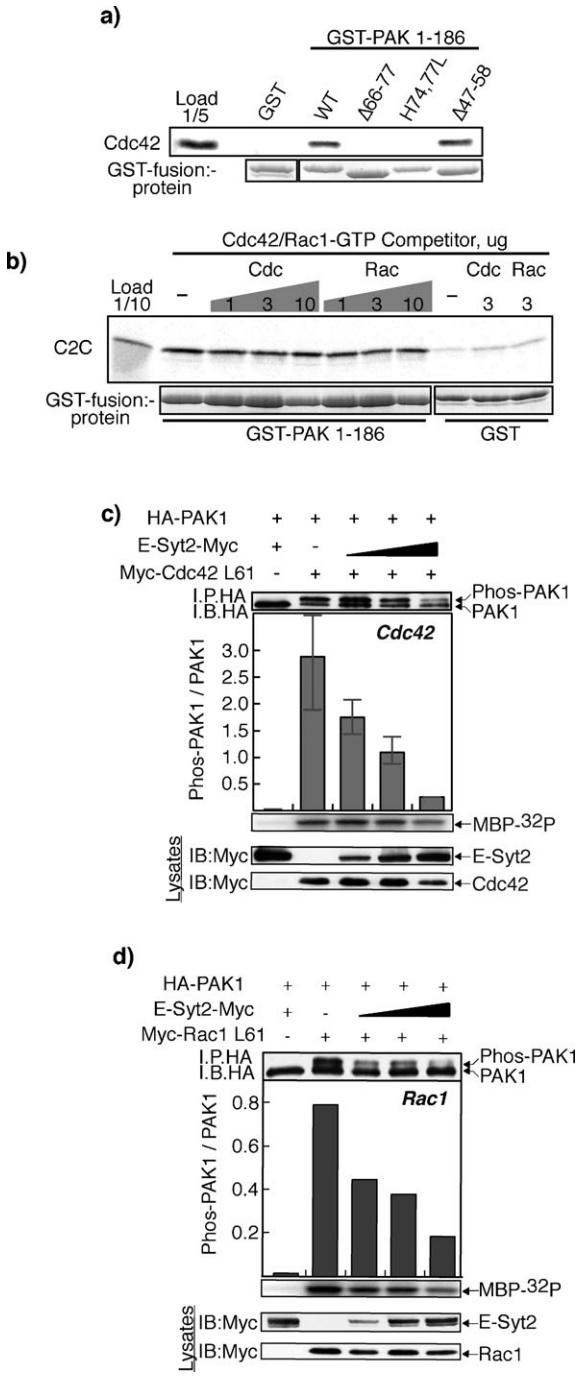

Fig. 3. E-Syt2 regulates GTPase activation of PAK1. a) Pull-down assays of the interaction between *in vitro* translated Cdc42-GTP and deletion mutants of PAK1 a.a. 1 to 186 fused to GST (GST-PAK 1–186) or GST alone. **b**) Pull-down competition assays of the interaction between the *in vitro* translated C2C domain of E-Syt2 and the GST-PAK 1–186 fusion or GST control in competition with increasing amounts of Cdc42-GTP or Rac-GTP. **c**,**d**) *In vivo* PAK1 activation by Cdc42-L61 and Rac-L61 in the presence of increasing amounts of E-Syt2. The indicated constructs were co-expressed in HEK293T cells and assays performed as described. PAK1 activation was quantified from the ratio of the upshifted, phosphorylated form (Phos-PAK1) to the unshifted, unphosphorylated form. The average data from two independent experiments are shown in (c) and a single experiment in (d). Error bars indicate the full extent of variation between the experiments. The kinase activities of PAK1 and PAK1Δ47-58 were also determined by the *in vitro* phosphorylation of MBP by the immunoprecipitated PAK1 (MBP-^{32}P). These data are shown in supplementary material Fig. S3b,c.

rapidity of wound-healing depends on the ability to polymerize a ring or purse-string of actin at the leading-edge of the wound, a process dependent on Cdc42 function (Benink and Bement, 2005; Kofron et al., 2002b). Morpholino depletion of E-Syt2 enhanced embryo wound-healing, as well as enhancing actin polymerization in the ACs removed from these embryos (Fig. 4a). In contrast, E-Syt2 overexpression slowed this wound healing, and inhibited actin polymerization in the corresponding ACs.

In agreement with the *Xenopus* wound healing data, *in vitro* wound healing (Scratch Test) assays (Coomber and Gotlieb, 1990) (Fig. 4b,c) also showed that in response to FGF stimulation NIH3T3 cells expressing E-Syt2 migrated less rapidly than mock transfected cells. This effect was particularly apparent at 16 h after FGF addition, and was dependent on the presence of the C2C domain of E-Syt2 shown to recruit PAK1.

Consistent with these macroscopic effects on cytoskeletal function, expression of E-Syt2 induced the cell-autonomous depletion of cortical F-actin (Fig. 5a). Analysis of many fields of E-Syt2 expressing and non-expressing cells showed that cortical actin staining was quantitatively reduced at regions positive for E-Syt2. Further, the induction of actin stress fibres in NIH3T3 fibroblasts was strongly affected by E-Syt2 expression, which caused the formation of large F-actin plaques at the expense of fibre formation (Fig. 5b). In contrast, deletion of the C2C domain of E-Syt2 eliminated this effect, allowing normal stress fibre formation. Thus, E-Syt2 suppressed normal F-actin polymerization both functionally in wound-healing assays, and visibly at the plasma membrane and during stress fibre formation.

Discussion

E-Syt2 is a novel endocytic adapter that is specifically recruited to activated FGFR1 and stimulates a rapid phase of receptor endocytosis required for activation of the ERK signalling pathway (Jean et al., 2010). E-Syt2 also recruits Adaptin-2 (AP-2) to the receptor, suggesting that it plays a role in the assembly of clathrin-coated pits and in this way targets the activated receptor to the clathrin-mediated endocytic pathway.

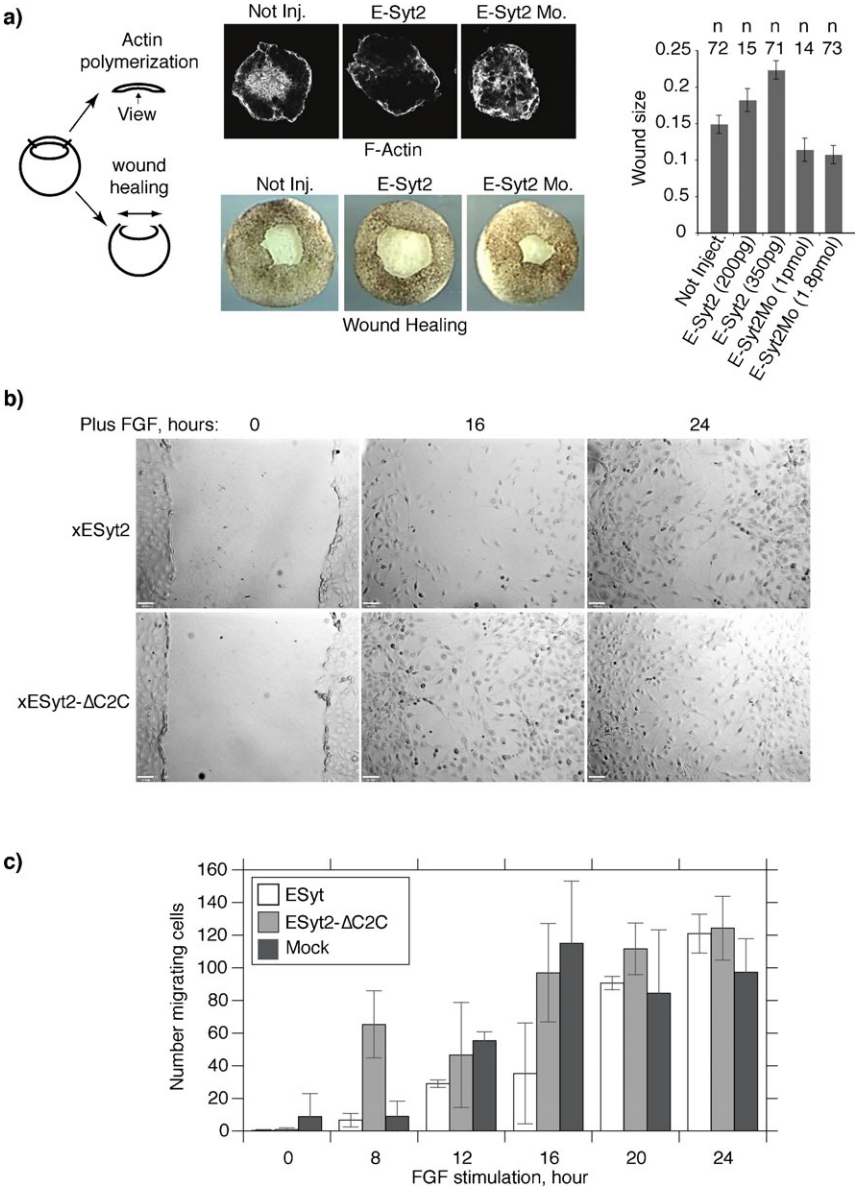

Fig. 4. E-Syt2 slows wound healing and FGF stimulated cell migration. a) Effects of loss and gain of E-Syt2 function on actin polymerization and wound healing in *Xenopus* embryos. The lower panel shows the effects of over-expression and Morpholino knock-down of E-Syt2 on wound healing 1 h after removal of animal caps. The upper panel shows fluorescent F-actin staining in the corresponding animal caps. The histogram to the right presents the quantification of the wound-healing assay. "n" indicates the number of embryos scored and the standard error is indicated by the vertical bars. An unpaired t-test showed that differences between embryo treatments were all significant. **b**) Scratch test assays of migration of NIH3T3 cells expressing E-Syt2 or E-Syt2-ΔC2C, or mock transfected cells. Examples of migration fields are shown for the E-Syt2 constructions at 0, 16 and 24 h after FGF stimulation. Scale bars indicate 100 μm. **c**) Quantitation of cell migration in the scratch test assays. The boundaries of the scratch are clearly visible at 0 h. The mean number of cell migrating into the scratch was determined from counts of three to six independent fields and is shown along with the standard deviation of these counts.

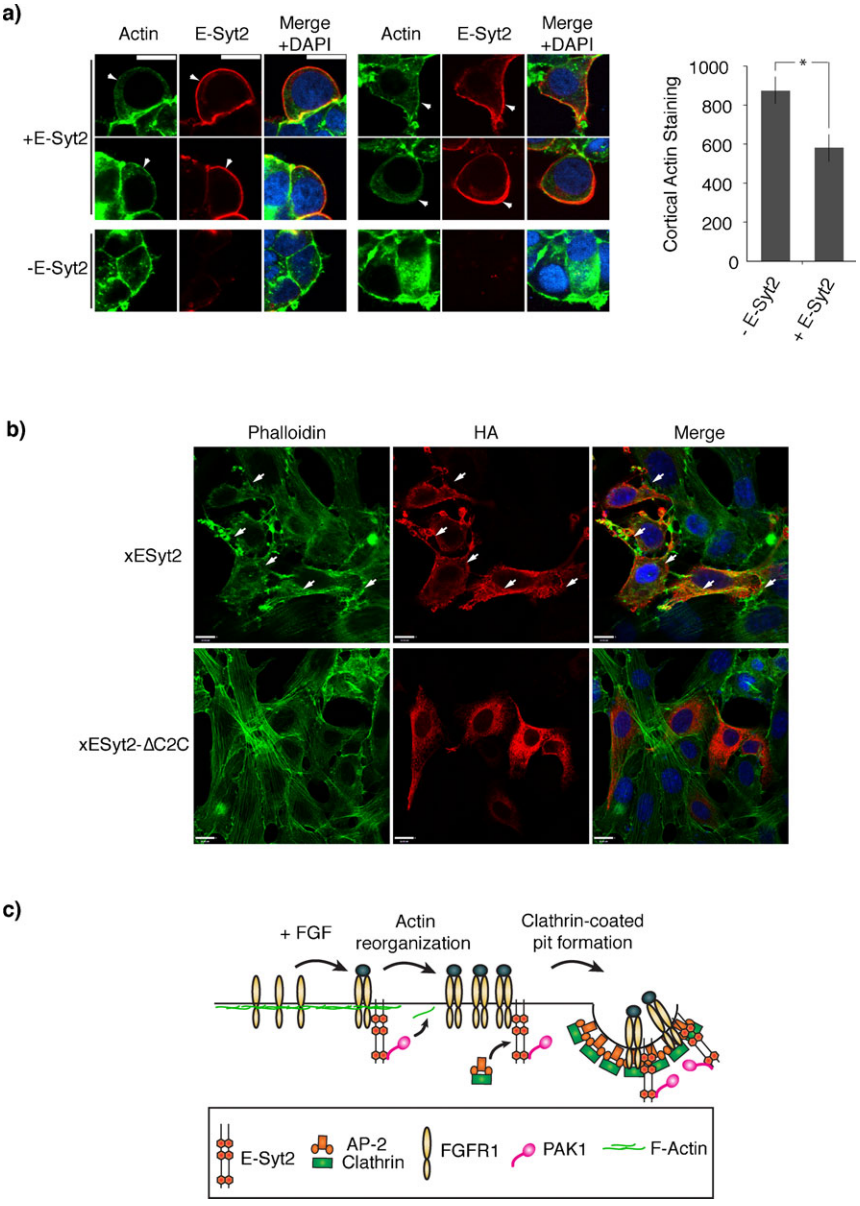

Fig. 5. Regulation of actin cytoskeleton by E-Syt2 its possible role in FGF receptor endocytosis. a) Visualization of cortical F-actin (Alexa488-phalloidin, green) and HA-E-Syt2 (Alexa568, red) in HEK293T cells expressing E-Syt2 (+E-Syt2) compared to mock transfected controls (−E-Syt2). Arrowheads indicate regions of reduced actin staining correspond to regions of E-Syt2 expression. The scale bar represents 15 μm. The histogram shows the average intensity of actin staining at the plasma membrane. Actin staining was quantified at 56 membrane regions of constant shape and size for cells expressing and cells not expressing E-Syt2 using the measurement functions in "Volocity" (Perkin-Elmer). The difference in average intensities was shown to be statistically significant (* $P<0.0001$). The standard error is indicated. **b**) Effects of E-syt2 and E-Syt2-ΔC2C (HA-tag, Alexa568, red) on actin stress fibre formation (Alexa488-phalloidin, green) in H_2O_2 treated NIH3T3 cells. Arrows indicate the large F-actin plaques forming in the E-Syt2 expressing cells. The scale bar in the upper and lower panels represents respectively 10 and 16 μm. **c**) A hypothetical model of the chain of events occurring at the plasma membrane during FGF receptor activation.

Here we show that E-Syt2 is also a novel interaction partner for the cytoskeletal kinase PAK1. The third C2 domain (C2C) of E-Syt2 binds to a CRIB/GBD adjacent site within the regulatory N-terminal domain of PAK1. This E-Syt2 binding site (ESB) was initially mapped on *Xenopus* PAK1, but was shown to be functionally conserved in human PAK1. Homologies between the various PAK isoforms suggest that an ESB may be present in the PAK3 and possibly the PAK2 orthologs (supplementary material Fig. S4), but probably not in PAKs 4 to 6.

E-Syt2 can bind PAK1, and both are recruited to the FGF receptor on its activation. E-Syt2 inhibits wound healing possibly by suppressing F-actin polymerization. This may be related to the ability of E-Syt2 to suppress the activation of PAK1 by Cdc42 and Rac small GTPases. We previously found that inactive forms of PAK1 locally sequester these GTPases and prevent their action in catalysing actin polymerization (Bisson et al., 2007). Since, regulation of the actin cytoskeleton has been implicated in endocytosis (Boulant et al., 2011; Liu et al., 2010; Taylor et al., 2011; Yarar et al., 2005). Our data suggest a possible role for E-Syt2 binding of PAK1 in this process. We hypothesize that PAK1 recruitment to E-Syt2 could cause local cortical actin depolymerisation or displacement and open the way for the assembly of a clathrin-coated pit (Fig. 5c). E-Syt2 is not present in early endosomes (Jean et al., 2010) and (F.G., unpublished), and thus probably leaves the coated pit before its endocytosis or even early in its formation. Though a thorough test of this hypothetical series of events awaits the elaboration of an adequate experimental system, it is interesting to note that PAK1 was previously implicated in both clathrin and non-clathrin mediated endocytosis (Pelkmans et al., 2005).

During the course of this study, human PAK1 was shown to bind phospholipids. It was suggested that this property enhanced its recruitment to the plasma membrane as well as its catalytic activation (Strochlic et al., 2010). It is interesting that the phospholipid binding site mapped by these authors overlaps the E-Syt2 binding site mapped here. Unfortunately, we were unable

to reproduce the experimental data supporting a role for phospholipid binding in the activation of either human or *Xenopus* PAK1. On the other hand, using alanine-scanning mutation we have shown that E-Syt2 recruitment to the plasma membrane and its interaction with PAK1 are mediated by distinct binding sites (F.G., unpublished). This suggests that PAK1 could interact with phospholipids via E-Syt2. However, if PAK1 does interact directly with phospholipids *in vivo*, this might be expected to enhance its subsequent recruitment to E-Syt2 by concentrating it to the plasma membrane.

Materials and Methods

Yeast two-hybrid screening, plasmids and constructs

Two-hybrid baits were constructed by amplifying sequences encoding a.a. 1–186, 240–528 and 1–528 of kinase dead *Xenopus laevis* PAK1 using cloned PFU polymerase. The fragments were subcloned in pGBKT7-AD (Clontech) and used to screen a stage 30 *Xenopus laevis* cDNA library derived from head and tail mRNA, using the Matchmaker Library Contruction and Screening Kit (Clontech) according to the manufacturer instructions. An average of 4.7×10^6 independent clones were screened using each bait and positive clones verified by retransformation. Mutant and GST constructs were created by PCR amplification where appropriate using the QuikChange protocol (Stratagene) and fully sequenced. The PAK1 and E-Syt2 constructs and antisense reagents have been described previously (Bisson et al., 2003; Jean et al., 2010). Human PAK1 was obtained from J. Chernoff. The coding region for a.a. 1795 to 2647 of Filamin was subcloned by PCR from I.M.A.G.E. clone 4800733 and verified by sequencing. Embryo expression constructs for *Xenopus* FGFR1 and its activated form (K562E) were obtained from R. E. Friesel and Flag-tagged FGFR1 was obtained from C. Niehrs (Böttcher et al., 2004). Both were cloned in the pCS2+ vector. Wherever the source of E-Syt2, PAK1 and FGFR1 is not specified the *Xenopus* forms were used. Human Cdc42 and Rac1 GTPase T/C expression constructs were provided by N. Lamarche-Vane and the corresponding bacterial expression vectors were provided by A. Hall. The position of epitope tags is indicated as N- or C-terminal by prefixing or suffixing the epitope indicator, e.g. HA, Myc, etc.

Embryo manipulation, injection and explants

Embryos were staged using the Nieuwkoop and Faber tables (Nieuwkoop and Faber, 1967) and injection performed as described elsewhere (Jean et al., 2010). To assay expression of mesodermal marker Xbra Animal Caps AC), explants were removed from injected embryos at stage 8–9 using forceps on agarose coated dishes in 1× Barth's medium and cultured in 0.5× Barth's medium in parallel with control embryos and stored at −80℃ before gene expression analysis. For actin staining ACs were cultured in 1× MMR for 10 minutes before staining with Alexa488-coupled phalloidin (Invitrogen) (Tao et al., 2007), while the vegetal region of the embryo was culture for 1 h in 1× MMR and then fixed in MEMFA. The diameter of the wound was measured using a graticule mounted on binocular microscope and normalized to the embryo diameter. The wound size is indicated as the relative surface area of a circle corresponding to the normalized diameter. For blastula and gastrula sections, embryos at the indicated stage were fixed in MEMFA overnight at 4℃ and cut sagittally with a scalpel blade.

Cell culture

HEK293T and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Wisent).

Transfection, immunoprecipitation and pull-down assays

Transfection, immunoprecipitation and pull-down assays were carried out as in (Jean et al., 2010; Vadlamudi et al., 2002). In the case of competition pull-down assays with Cdc42, Rac and Filamin, interacting components were added simultaneously.

Kinase assay

HEK293T cells were transfected with the indicated constructs and lysed in MGE, (20 mM MOPS pH 7.0, 10% glycerol, 0.5 mM EDTA, 5 mM EGTA, 1 mM $NaVO_4$, 5 mM NaPPi, 50 mM NaF, 80 mM β-glycerophosphate, 1% Triton X-100, 1 mM benzamidine, 1 mM DTT, 1 mM PMSF). Otherwise the procedure was as previously described (Bisson et al., 2003).

Immunofluorescence microscopy

Immunofluorecence and GFP/RFP imaging were carried out essentially as previously described (Jean et al., 2010). Stage 8 animal caps (AC) were processed for actin staining with Alexa 488-conjugated phalloidin as described by (Kofron et al., 2002a; Lloyd et al., 2005). HEK293T and NIH3T3 cells expressing HA tagged E-Syt2 or E-Syt2 mutant were fixed in 4% PFA in PBS, 20 min R/T and permeabilized with 0.5% Triton X-100 in PBS, 5 min. R/T. E-Syt2 revealed with anti-HA mouse (12CA5) and Alexa568 conjugated anti-mouse second antibody (Invitrogen) and Alexa 488-conjugated phalloidin used to reveal F-actin. Stress fibres were induced in NIH3T3 cells by 5 min. treatment at 37℃ with 250 μM H_2O_2 in PBS before fixing (Huot et al., 1998).

Migration assay

The effects of E-Syt2 and mutants on NIH3T3 cell migration were determined in a wound healing assay (scratch test) (Coomber and Gotlieb, 1990). Cells were tranfected with E-Syt2 constructs or mock transfected, 12 h later serum was withdrawn and cells incubated for a further 12 h before the assay. After scratching with a 2 μl pipette tip cells were incubated for the indicated times in the presence or absence of 20 $ng.ml^{-1}$ bFGF (Sigma-Aldrich) and 5 $\mu g.ml^{-1}$ heparin (Sigma-Aldrich).

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Competing Interests

The authors declare that there are no competing interests.

References

Benink, H. A. and Bement, W. M. (2005). Concentric zones of active RhoA and Cdc42 around single cell wounds. *J. Cell Biol.* **168**, 429-439.

Bisson, N., Islam, N., Poitras, L., Jean, S., Bresnick, A. and Moss, T. (2003). The catalytic domain of xPAK1 is sufficient to induce myosin II dependent in vivo cell fragmentation independently of other apoptotic events. *Dev. Biol.* **263**, 264-281.

Bisson, N., Poitras, L., Mikryukov, A., Tremblay, M. and Moss, T. (2007). EphA4 signaling regulates blastomere adhesion in the Xenopus embryo by recruiting Pak1 to suppress Cdc42 function. *Mol. Biol. Cell* **18**, 1030-1043.

Bokoch, G. M. (2003). Biology of the p21-activated kinases. *Annu. Rev. Biochem.* **72**, 743-781.

Böttcher, R. T. and Niehrs, C. (2005). Fibroblast growth factor signaling during early vertebrate development. *Endocr. Rev.* **26**, 63-77.

Böttcher, R. T., Pollet, N., Delius, H. and Niehrs, C. (2004). The transmembrane protein XFLRT3 forms a complex with FGF receptors and promotes FGF signalling. *Nat. Cell Biol.* **6**, 38-44.

Boulant, S., Kural, C., Zeeh, J. C., Ubelmann, F. and Kirchhausen, T. (2011). Actin dynamics counteract membrane tension during clathrin-mediated endocytosis. *Nat. Cell Biol.* **13**, 1124-1131.

Coomber, B. L. and Gotlieb, A. I. (1990). In vitro endothelial wound repair. Interaction of cell migration and proliferation. *Arteriosclerosis* **10**, 215-222.

Deacon, S. W., Beeser, A., Fukui, J. A., Rennefahrt, U. E., Myers, C., Chernoff, J. and Peterson, J. R. (2008). An isoform-selective, small-molecule inhibitor targets the autoregulatory mechanism of p21-activated kinase. *Chem. Biol.* **15**, 322-331.

Edwards, D. C., Sanders, L. C., Bokoch, G. M. and Gill, G. N. (1999). Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. *Nat. Cell Biol.* **1**, 253-259.

Huot, J., Houle, F., Rousseau, S., Deschesnes, R. G., Shah, G. M. and Landry, J. (1998). SAPK2/p38-dependent F-actin reorganization regulates early membrane blebbing during stress-induced apoptosis. *J. Cell Biol.* **143**, 1361-1373.

Isaacs, H. V., Pownall, M. E. and Slack, J. M. (1994). eFGF regulates Xbra expression during Xenopus gastrulation. *EMBO J.* **13**, 4469-4481.

Jean, S., Mikryukov, A., Tremblay, M. G., Baril, J., Guillou, F., Bellenfant, S. and Moss, T. (2010). Extended-synaptotagmin-2 mediates FGF receptor endocytosis and ERK activation in vivo. *Dev. Cell* **19**, 426-439.

Kimelman, D. (2006). Mesoderm induction: from caps to chips. *Nat. Rev. Genet.* **7**, 360-372.

Kimelman, D. and Kirschner, M. (1987). Synergistic induction of mesoderm by FGF and TGF-beta and the identification of an mRNA coding for FGF in the early Xenopus embryo. *Cell* **51**, 869-877.

Kofron, M., Heasman, J., Lang, S. A. and Wylie, C. C. (2002a). Plakoglobin is required for maintenance of the cortical actin skeleton in early Xenopus embryos and for cdc42-mediated wound healing. *J. Cell Biol.* **158**, 695-708.

Kofron, M., Heasman, J., Lang, S. A. and Wylie, C. C. (2002b). Plakoglobin is required for maintenance of the cortical actin skeleton in early Xenopus embryos and for cdc42-mediated wound healing. *J. Cell Biol.* **158**, 695-708.

Le Roy, C. and Wrana, J. L. (2005). Signaling and endocytosis: a team effort for cell migration. *Dev. Cell* **9**, 167-168.

Liu, J., Sun, Y., Oster, G. F. and Drubin, D. G. (2010). Mechanochemical crosstalk during endocytic vesicle formation. *Curr. Opin. Cell Biol.* **22**, 36-43.

Lloyd, B., Tao, Q., Lang, S. and Wylie, C. (2005). Lysophosphatidic acid signaling controls cortical actin assembly and cytoarchitecture in Xenopus embryos. *Development* **132**, 805-816.

McMahon, H. T. and Boucrot, E. (2011). Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nat. Rev. Mol. Cell Biol.* **12**, 517-533.

Min, S. W., Chang, W. P. and Südhof, T. C. (2007). E-Syts, a family of membranous Ca2+-sensor proteins with multiple C2 domains. *Proc. Natl. Acad. Sci. USA* **104**, 3823-3828.

Nagel, M., Luu, O., Bisson, N., Macanovic, B., Moss, T. and Winklbauer, R. (2009). Role of p21-activated kinase in cell polarity and directional mesendoderm migration in the Xenopus gastrula. *Dev. Dyn.* **238**, 1709-1726.

Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table Of Xenopus Laevis (Daudin): A Systematical And Chronological Survey Of The Development From The Fertilized Egg Till The End Of Metamorphosis.* Amsterdam: North-Holland Publishing Company.

Obermeier, A., Ahmed, S., Manser, E., Yen, S. C., Hall, C. and Lim, L. (1998). PAK promotes morphological changes by acting upstream of Rac. *EMBO J.* **17**, 4328-4339.

Parrini, M. C., Lei, M., Harrison, S. C. and Mayer, B. J. (2002). Pak1 kinase homodimers are autoinhibited in trans and dissociated upon activation by Cdc42 and Rac1. *Mol. Cell* **9**, 73-83.

Pelkmans, L., Fava, E., Grabner, H., Hannus, M., Habermann, B., Krausz, E. and Zerial, M. (2005). Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. *Nature* **436**, 78-86.

Sanders, L. C., Matsumura, F., Bokoch, G. M. and de Lanerolle, P. (1999). Inhibition of myosin light chain kinase by p21-activated kinase. *Science* **283**, 2083-2085.

Slack, J. M., Darlington, B. G., Heath, J. K. and Godsave, S. F. (1987). Mesoderm induction in early Xenopus embryos by heparin-binding growth factors. *Nature* **326**, 197-200.

Strochlic, T. I., Viaud, J., Rennefahrt, U. E., Anastassiadis, T. and Peterson, J. R. (2010). Phosphoinositides are essential coactivators for p21-activated kinase 1. *Mol. Cell* **40**, 493-500.

Tao, Q., Nandadasa, S., McCrea, P. D., Heasman, J. and Wylie, C. (2007). G-protein-coupled signals control cortical actin assembly by controlling cadherin expression in the early Xenopus embryo. *Development* **134**, 2651-2661.

Taylor, M. J., Perrais, D. and Merrifield, C. J. (2011). A high precision survey of the molecular dynamics of mammalian clathrin-mediated endocytosis. *PLoS Biol.* **9**, e1000604.

Vadlamudi, R. K., Li, F., Adam, L., Nguyen, D., Ohta, Y., Stossel, T. P. and Kumar, R. (2002). Filamin is essential in actin cytoskeletal assembly mediated by p21-activated kinase 1. *Nat. Cell Biol.* **4**, 681-690.

Yarar, D., Waterman-Storer, C. M. and Schmid, S. L. (2005). A dynamic actin cytoskeleton functions at multiple stages of clathrin-mediated endocytosis. *Mol. Biol. Cell* **16**, 964-975.

Yuan, L., Santi, M., Rushing, E. J., Cornelison, R. and MacDonald, T. J. (2010). ERK activation of p21 activated kinase-1 (Pak1) is critical for medulloblastoma cell migration. *Clin. Exp. Metastasis* **27**, 481-491.