Development 140, 4266-4276 (2013) doi:10.1242/dev.096073

A dominant-negative provides new insights into FAK regulation and function in early embryonic morphogenesis

Nicoletta I. Petridou, Panayiota Stylianou and Paris A. Skourides*

SUMMARY

FAK is a non-receptor tyrosine kinase involved in a wide variety of biological processes and crucial for embryonic development. In this manuscript, we report the generation of a new FAK dominant negative (FF), composed of the C terminus (FRNK) and the FERM domain of the protein. FF, unlike FRNK and FERM, mimics the localization of active FAK in the embryo, demonstrating that both domains are necessary to target FAK to its complexes *in vivo*. We show that the FERM domain has a role in the recruitment of FAK on focal adhesions and controls the dynamics of the protein on these complexes. Expression of FF blocks focal adhesion turnover and, unlike FRNK, acts as a dominant negative *in vivo*. FF expression in *Xenopus* results in an overall phenotype remarkably similar to the FAK knockout in mice, including loss of mesodermal tissues. Expression of FF in the animal cap revealed a previously unidentified role of FAK in early morphogenesis and specifically epiboly. We show that a fibronectin-derived signal transduced by FAK governs polarity and cell intercalation. Finally, failure of epiboly results in severe gastrulation problems that can be rescued by either mechanical or pharmacological relief of tension within the animal cap, demonstrating that epiboly is permissive for gastrulation. Overall, this work introduces a powerful new tool for the study of FAK, uncovers new roles for FAK in morphogenesis and reveals new mechanisms through which the FERM domain regulates the localization and dynamics of FAK.

KEY WORDS: FAK, FERM, *Xenopus*, Dominant negative, Epiboly

INTRODUCTION

The focal adhesion kinase (FAK) is a 125 kDa non-receptor tyrosine kinase shown to be activated by integrin signaling and act as a phosphorylation-regulated signaling scaffold to control adhesion turnover, cell migration, proliferation and survival (Mitra et al., 2005). FAK is composed of three major domains, the N-terminal FERM (4.1-band, ezrin, radixin, moesin) domain, followed by the central catalytic kinase domain and the C-terminal focal adhesion targeting (FAT) domain. The FERM domain of FAK is an important regulator of FAK activity as deletion or overexpression of the FERM domain leads to enhanced or suppressed tyrosine phosphorylation status, respectively (Cooper et al., 2003; Jácamo and Rozengurt, 2005). Specifically, the FERM domain promotes FAK interactions that cause conformational changes on the FAK molecule: from a closed inactive form where the FERM domain interacts with the kinase domain, to an open active form where this intramolecular interaction is relieved and Y397 becomes phosphorylated (Ceccarelli et al., 2006). This phosphorylation leads to exposure of the FAK activation loop and full catalytic activation, through binding of Src and subsequent phosphorylation on Y576 and Y577 (Calalb et al., 1995). The FERM domain was also found to bind to peptides of the β1-integrin cytoplasmic tail, the Arp2/3 complex, PIP_2 [PtdIns(4,5)P_2] and growth factor receptors (GFRs) (Cai et al., 2008; Chen and Chen, 2006; Schaller et al., 1995; Serrels et al., 2007; Sieg et al., 2000). However, exogenously expressed FERM domain does not localize to focal adhesions (FAs) and no direct *in vivo* binding has been demonstrated between FAK and integrins (Lawson and Schlaepfer, 2012). By contrast, the C terminus of FAK contains the FAT domain, a four-helix bundle that has been shown to be both necessary and sufficient for FA targeting (Chen et al., 1995; Hayashi et al., 2002; Hildebrand et al., 1993; Hildebrand et al., 1995). FRNK (FAK-related non kinase), which encompasses the FAK C-terminus, has been shown to act as a dominant-negative, possibly through competition with endogenous FAK at FAs (Richardson et al., 1997; Sieg et al., 1999).

Cells isolated from E8.0 FAK-null embryos display reduced mobility *in vitro* and an increased number of FAs, indicating that FAK is involved in the turnover of FAs (Ilić et al., 1995). Several lines of evidence have since confirmed a crucial role for FAK in FA turnover through a variety of mechanisms, including a spatially and temporally resolved role in Rho activation and inhibition, and interactions between the FERM domain of FAK and dynamin 2, which promote microtubule-dependent FA disassembly (Ezratty et al., 2005; Lim et al., 2008b; Ren et al., 2000; Tomar et al., 2009).

FAK has also been shown to play a role in cell survival and apoptosis through p53 and PI3K-dependent signaling (Golubovskaya et al., 2005; Lim et al., 2008a; Xia et al., 2004). FAK-null endothelial cells exhibit cell proliferation problems and high levels of apoptosis (Ilic et al., 2003). In addition, FAK was found to be phosphorylated on several serine residues during mitosis (Yamakita et al., 1999), and Ser732 specifically was shown to be phosphorylated by Cdk5 and to regulate centrosome function during mitosis and neuronal migration (Park et al., 2009; Xie et al., 2003).

Disruption of FAK in mice leads to early embryonic lethality by E8.5 due to generalized mesodermal defects (Furuta et al., 1995). The phenotype of the FAK null is similar to the fibronectin (FN) and integrin α5 knockouts, which also exhibit early embryonic lethality due to defects in mesodermally derived tissues, shortening of the anterior-posterior (A-P) axis and abnormal vascular development (George et al., 1993; Yang et al., 1993). The similarity of the FAK, FN and integrin knockouts provides strong evidence that FAK mediates the FN-integrin signaling required for early embryonic development. Studies in *Xenopus* have shown that FN-integrin interactions are essential for proper embryonic development, regulating major morphogenetic movements during

Department of Biological Sciences, University of Cyprus, Nicosia 2109, Cyprus.

*Author for correspondence (skourip@ucy.ac.cy)

Accepted 8 August 2013

gastrulation and neurulation. Disruption of these interactions leads to radial intercalation failure and loss of polarity in the deep ectodermal cells of the animal cap (AC) (Davidson et al., 2006; Marsden and DeSimone, 2001; Rozario et al., 2009). Both processes are required for epiboly, the morphogenetic movement that drives the thinning and expansion that allow the ectoderm to encompass the entire embryo by the end of gastrulation (Keller, 1980). However, studies of FAK in *Xenopus* with the use of morpholino (MO)-based knockdown have failed to identify any early morphogenetic roles for FAK. One study showed that FAK regulates Wnt3a expression and cell fate specification during neurulation (Fonar et al., 2011), and another revealed that FAK also plays an important role in *Xenopus* cardiogenesis (Doherty et al., 2010). However, we have shown that FAK-MO reduces FAK protein levels by about 50% at gastrula stages (Petridou et al., 2012); given the fact that the mouse $FAK^{+/-}$, which also display a 50% reduction of FAK protein levels, develops normally, it is likely that this reduction in not sufficient to reveal early roles of FAK in morphogenesis (Kostourou et al., 2013).

In this study, we have generated a new FAK construct that contains the N and C termini of FAK, termed FF. In the embryo, FF mimics the localization of active endogenous FAK, primarily localizing at sites of cell-cell contact. We show that the FERM domain is important for both the recruitment of FAK to nascent adhesions, as well as for the affinity and dynamics of FAK on FAs. Finally, we show that FF acts as a strong dominant-negative both *in vivo* and *in vitro*, and present data suggesting that FAK has a role in the transduction of a FN-integrin signal required for epiboly.

MATERIALS AND METHODS

Cell culture and transfections

XL177 cells were grown in 70% L-15, 15%FBS and 100 mM L-Glutamine at room temperature. Electroporation was carried out according to the manufacturer's protocol (Invitrogen).

Embryos, microinjections and explants

Xenopus laevis embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Embryos were fertilized *in vitro* and dejellied using 1.8% L-cysteine (pH 7.8), then maintained in 0.1× Marc's Modified Ringer's (MMR). Microinjections were performed in 4% Ficoll in 0.3×MMR. Capped mRNAs were *in vitro* transcribed using mMessage machine (Ambion). Rho kinase (ROCK) inhibitor (Sigma) (7 mM) was injected in the blastocoel at stage 10 as described previously (Woolner and Papalopulu, 2012).

Radial intercalation explants and induced AC explants were performed as described (Alfandari et al., 2001; Marsden and DeSimone, 2001) but with the use of glass bridges to hold the AC in place. AC elongation assays have been described previously (Stylianou and Skourides, 2009).

DNA constructs and morpholinos

All plasmids generated were verified by sequencing. Primers used are listed in supplementary material Table S1. HA-FF construct in pCS108 vector was generated by amplifying the FERM domain up to 402 amino acids from the HA-FAK pKH3 plasmid (Zhao et al., 1998) using the primers F/HA and R/FERM. FRNK was amplified from the same plasmid using F/FRNK and R/FAK with the inserted linker segment GGTAGCGGCAGCGGTAGC in the forward primer and ligated to the FERM domain. GFP sequence from pEGFP-N1 or mCherry sequence from pShuttle mCherry-tubulin (Addgene) was inserted at the *Xba*I site to generate the FF-GFP or FF-mCherry, respectively. GFP-FRNK and HA-FERM have been described previously (Petridou et al., 2012). HA-FRNK and HA-FAK K38A were subcloned from pKH3 (Zhao et al., 1998) using F/HA and R/FAK into pCS108 or pCS2++, respectively. HA-FF S732A was generated by site-directed mutagenesis of the HA-FF pCS108 plasmid with the primer FF732. All FAK mutants were generated from chicken FAK (GenBank AAA48765.1).

The sequence of FAK-MO is TTGGGTCCAGGTAAGCCGCAGCCAT (Fonar et al., 2011) and of Vinculin-MO is TATGGAAGACCGGCAT-CTTGGCAAT.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization of *Xenopus* embryos has been described previously (Smith and Harland, 1991).

RT-PCR

cDNA was prepared via reverse transcription (SuperScriptIII First strand synthesis, Invitrogen) from RNA extracted from FF-injected and control embryos. PCR was carried out using specific primer pairs for each marker.

TUNEL assay

TUNEL assay of *Xenopus* embryos was performed according to the Harland protocol (Conlon laboratory, North Carolina). Apoptotic nuclei were quantified by using the ImageJ ICTN plug-in.

Immunofluorescence

Immunofluorescence on XL177 cells and whole embryos has been described (Petridou et al., 2012). Primary antibodies used were: anti-vinculin (Hybridoma Bank), GFP (Invitrogen), P-Y576FAK (Santa Cruz), P-S732FAK (Invitrogen), HA (Santa Cruz), β-tubulin (Hybridoma Bank), FN (4H2, kindly provided by Dr Douglas DeSimone, Virginia, USA), β-catenin (Santa Cruz), P-MLC (Abcam) and H3 histone (Novus).

Western blot and analysis

Protein lysate preparation and western blotting have been described previously (Petridou et al., 2012). Antibodies used were: N-FAK (Millipore), P-S732FAK, HA, FN (4H2), actin (Santa Cruz) and P-Y20 (Santa Cruz). Densitometry analysis was carried out using the Vision Works LS Software. The analysis of the results in Fig. 6 included normalization of the intensity values of phospho-FAK signal against total FAK and averaging values from three independent experiments.

Immunoprecipitation

Immunoprecipitation was performed as described previously (Klymkowsky Lab Methods, see http://klymkowskylab.colorado.edu/Methods/Precipitation.htm) using anti-GFP antibody (Invitrogen).

Imaging analysis

Embryos were imaged either under a Zeiss AxioImager Z1 microscope, using a Zeiss Axiocam MR3 and Axiovision 4.8 or a Zeiss Lumar V12 stereomicroscope, or an LSM710 (Zeiss). The generation of the intensity profiles and the data analysis of FRAP and FLIP experiments were performed using the ZEN2010 software. FRAP experiments were conducted using a Plan-Apochromat 63×/1.40 oil. Relative recovery rates were compared using half time for recovery of fluorescence towards the asymptote. The fluorescence recovery curve was fitted by single exponential function, given by $F(t)=A(1-e^{-R})+B$; where F(t) is the intensity at time t, A and B are the amplitudes of the time-dependent and time-independent terms, respectively; τ is the lifetime of the exponential term; and the recovery rate is given by $R=1/\tau$. Immobile fractions were calculated by comparing the intensity ratio in the bleached area just before bleaching and after recovery.

RESULTS

The FERM and FAT domains cooperate to target FAK at the plasma membrane in the embryo, and at FAs in cultured cells

In the embryo, FAK can be detected in the cytosol, the nucleus and the plasma membrane; however, phosphorylated FAK is found almost exclusively on the plasma membrane (Petridou et al., 2012). The FAT domain has been shown to be both necessary and sufficient for FA targeting of FAK (Hildebrand et al., 1993). However, we have recently shown that neither the FAT nor the FERM domain alone can place FAK on the plasma membrane in the embryo (Petridou et al., 2012). We wanted to explore the possibility that the FERM and FAT

domains cooperate to target active FAK at the plasma membrane. We thus generated FERM-FRNK (FF), a construct which contains the N and C terminus sequences of FAK (supplementary material Fig. S1A). By removing the kinase domain, we wanted to mimic the active conformation of FAK in which the FERM domain is free to bind PIP_2 and GFRs (Cai et al., 2008; Chen and Chen, 2006; Sieg et al., 2000). Combining the two regions resulted in a dramatic change in localization, as shown in Fig. 1A. FF is almost exclusively localized at the plasma membrane, unlike FRNK and FERM, mimicking the localization of endogenous phosphorylated FAK. To explore the role of the FERM domain in the localization of FAK further, we compared the localization of FF and FRNK in cultured cells after generating GFP fusions of the two constructs. As shown in Fig. 1B, FRNK localizes in the cytosol and FAs, whereas FF is predominantly localized on FAs with little signal in the cytosol. In addition, FF expressors have FAs throughout the cell, whereas, in FRNK expressors, FAs are primarily found in the cell periphery (Fig. 1B; supplementary material Fig. S1B). To preclude the possibility that FF appeared to have stronger localization on FAs, due to the fact that FF expression induces stronger and more abundant FAs, we co-transfected GFP-FRNK with FF-mCherry. As shown in supplementary material Fig. S1C, FF displays lower cytoplasmic signal than FRNK, suggesting a role for the FERM domain in the localization of FAK at FA complexes. In addition, FF expression displaces full-length FAK from FAs and nearly eliminates FAK phosphorylation on these complexes, demonstrating a strong dominant-negative activity (Fig. 1C,D).

To better understand the mechanism underlying the higher affinity of FF for FAs, we carried out FRAP and FLIP experiments on cells expressing GFP-FRNK and FF-GFP (Fig. 2A,B). As shown in Fig. 2A, FF displays a slower recovery and has a larger immobile fraction compared with FRNK, suggesting slower turnover on FAs (FF t1/2, 11.01±1.74 seconds; FRNK t1/2, 2.6±0.4 seconds; Fig. 2A′; immobile fraction FF, 30.3±4.5%; FRNK, 9.78±1.4%; Fig. 2A″). To preclude the possibility that these differences arise due to differences induced by FF on the FA complexes, we carried out FRAP experiments in cells co-transfected with FF-mCherry and GFP-FRNK that gave similar results (supplementary material Fig. S1D). FLIP experiments on these cells showed that loss of FF from FAs is slower than loss of FRNK, confirming that FF has longer residence times on FAs (supplementary material Fig. S1E). These data show that the FERM domain is required for the localization of FAK in the embryo and has an important role for the affinity and dynamics of FAK on FA complexes in cultured cells.

FF expression blocks cell migration by blocking FA turnover

Fibroblasts from FAK-null mice show a significant increase in FA number and intensity and decreased migration rates (Ilić et al., 1995), which can be restored by re-expression of FAK, suggesting a role in FA turnover (Sieg et al., 1999). Overexpression of FRNK has been shown to reduce the rates of cell migration and induce the formation of enlarged FA complexes (Richardson et al., 1997; Sieg et al., 1999; Taylor et al., 2001).

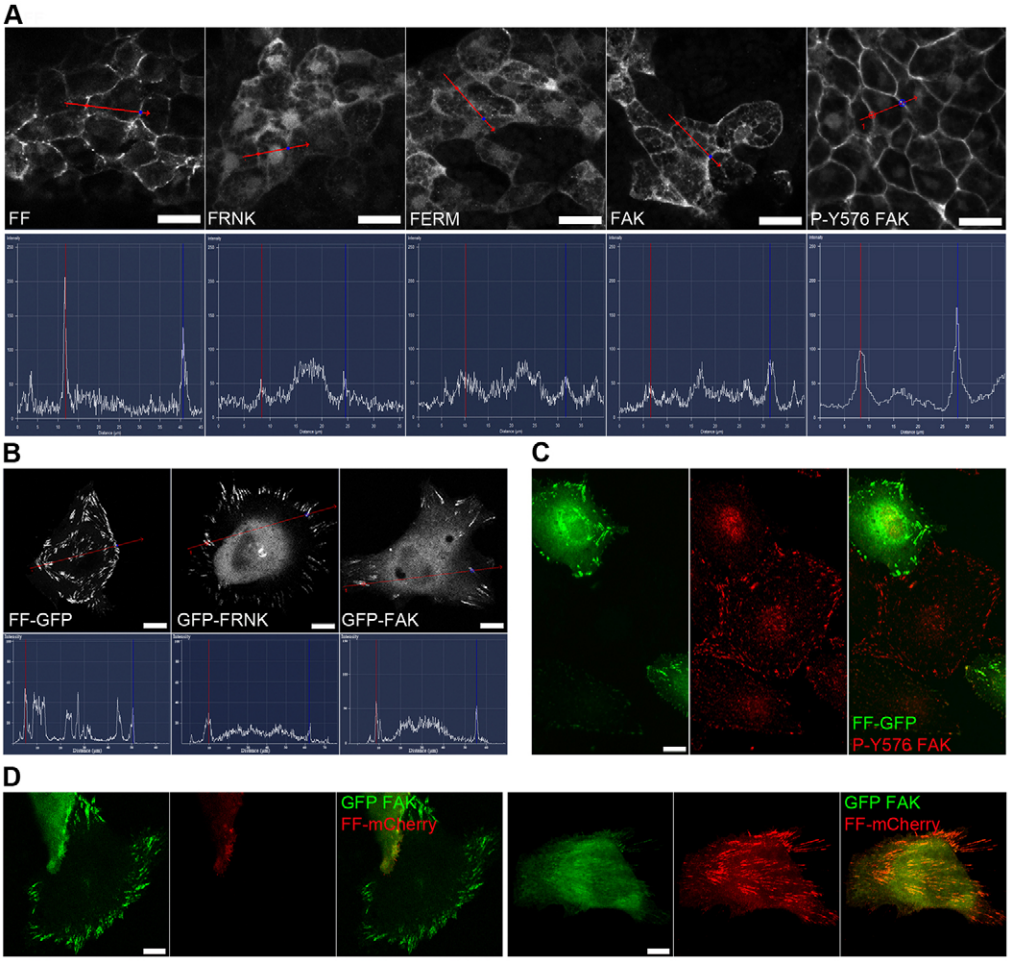

Fig. 1. The FERM and FAT domains cooperate to target FAK at the plasma membrane in the embryo and at FAs in cultured cells. (**A-A″**) Confocal images and intensity profiles of gastrula-stage embryos injected with 300 pg of wild-type FAK; of the DMZs of HA-tagged FAK mutants (FF, FRNK and FERM) stained with anti-HA antibody; or of control embryos stained with anti P-Y576 FAK. (**B**) Confocal images and intensity profiles of live XL177 cells transfected with FF-GFP, GFP-FRNK or GFP-FAK. (**C**) Wide-field images of XL177 cells transfected with FF-GFP and stained with P-Y576 FAK. (**D**) Confocal images of XL177 cells co-transfected with FF-mCherry and GFP-FAK, showing FAK displacement from FF-positive FAs. Scale bars: 20 μm in A,C; 10 μm in B,D.

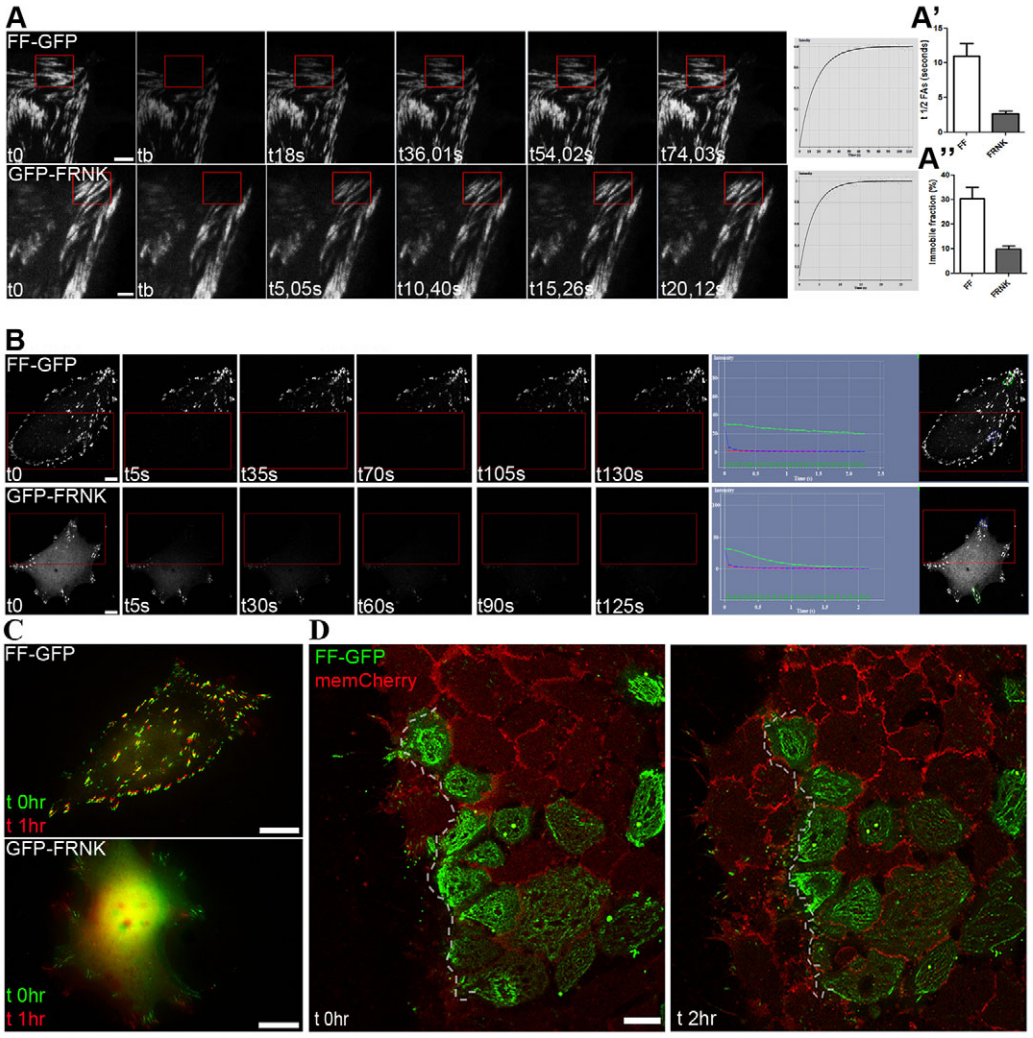

Fig. 2. FF exhibits longer residence times on FAs compared with FRNK, and blocks FA turnover and cell migration. (**A**) FRAP experiments comparing FF-GFP and GFP-FRNK. FF displays slower recovery and larger immobile fraction than FRNK. Data are mean+s.e.m. (**A′**) FF t1/2: 11.01±1.74 seconds; FRNK t1/2: 2.6±0.4 seconds, *n*=50. (**A″**) FF immobile fraction: 30.3±4.5%. FRNK immobile fraction: 9.78±1.4%, *n*=50. (**B**) FLIP experiments comparing loss of FF-GFP and GFP-FRNK. Rate of loss of FF is slower than FRNK. (**C**) High-magnification wide-field live imaging of FA turnover in FF-GFP or GFP-FRNK transfected XL177 cells. Cells were imaged for 1 hour. The merged image is composed from the first (green) and last time point (red). (**D**) The first (t 0hr) and last (t 2hr) time point of a 2-hour time-lapse movie of a mesodermal explant placed on FN-coated coverslips injected with memCherry (red) and FF-GFP (green), showing block of cell migration in FF-expressing cells. Scale bars: 2 μm in A; 10 μm in B; 20 μm in C,D.

As shown above, FF-expressing cells display a marked increase in the numbers and intensity of FAs compared with FRNK and control cells, suggesting that FF leads to a more dramatic reduction of FA turnover compared with FRNK. To explore this possibility further, FF- and FRNK-expressing cells were imaged using time-lapse fluorescence microscopy. As shown in Fig. 2C, a typical FF-expressing cell completely fails to disassemble its FAs while a FRNK-expressing cell has completely disassembled all its FAs and generated new ones within the same time frame.

FF displayed different dynamics on FAs and higher affinity compared with FRNK, and it has been suggested in previous studies that the FERM domain may have a role in the recruitment of FAK at the sites of FA formation, possibly via binding of locally generated PIP_2 (Cai et al., 2008). If this hypothesis is correct, one would expect FF to be recruited to nascent FAs prior to FRNK. To test this hypothesis, we co-transfected and imaged FF-mCherry and GFP-FRNK. As shown in the images from supplementary material Fig. S2A, FF (red) enrichment appears first on two nascent adhesions and FRNK enrichment follows (green). These results suggest that the FERM domain plays a role in the recruitment of FAK to nascent adhesions.

To further explore the effects of FF expression on cell migration, we expressed FF in mesodermal explants. As shown in Fig. 2D, FF-GFP-expressing cells (green) spread and form very strong FAs, normally absent from these cells (Stylianou and Skourides, 2009; Wacker et al., 1998). FF expression blocks FA turnover, preventing these cells from migrating (Fig. 2D; supplementary material Movie 1). In addition, expression of FF in activin-induced ACs, which normally spread and migrate as a cohesive sheet in all directions when plated on FN (supplementary material Movie 2), blocks spreading, and migration is reduced in the GFP-positive areas (as shown in supplementary material Movie 3).

FAK signals cell polarity during *Xenopus* epiboly

FAK is a major transducer of integrin signaling, and the earliest integrin-dependent process described in *Xenopus* is the FN

fibrillogenesis taking place on the blastocoel roof (BCR). In addition, FAK is expressed in the AC of *Xenopus* embryos and is specifically expressed in the deep cells of the AC that colocalize with FN, suggesting a possible role in this process (Hens and DeSimone, 1995).

In an effort to address the role of FAK in *Xenopus* early morphogenesis, we decided to test whether FF expression could block endogenous FAK function in the embryo. As shown in Fig. 3A, expression of 500 pg of FF in the AC leads to severe gastrulation defects, including failure of blastopore closure. These defects are partially rescued by expression of a constitutively active FAK K38A point mutant, which speeds up blastopore closure but not FAK Δ375, stressing the requirement of the FERM domain for the function of FAK in this context (Fig. 3A; supplementary material Fig. S3A). In addition, in FF-expressing embryos, the blastocoel is displaced vegetally, the archenteron fails to form and the mesoderm (despite proper patterning) only involutes partially (Fig. 3B-D). In agreement with the failure of the Δ375 construct to rescue the FF phenotype, expression of FRNK, which lacks the FERM domain, does not induce any appreciable gastrulation defects, confirming the requirement of the FERM domain for the dominant-negative function in this context (Fig. 3B).

FF expression also leads to the visible thickening of the BCR, suggesting that epiboly and radial intercalation are blocked (Fig. 3E,

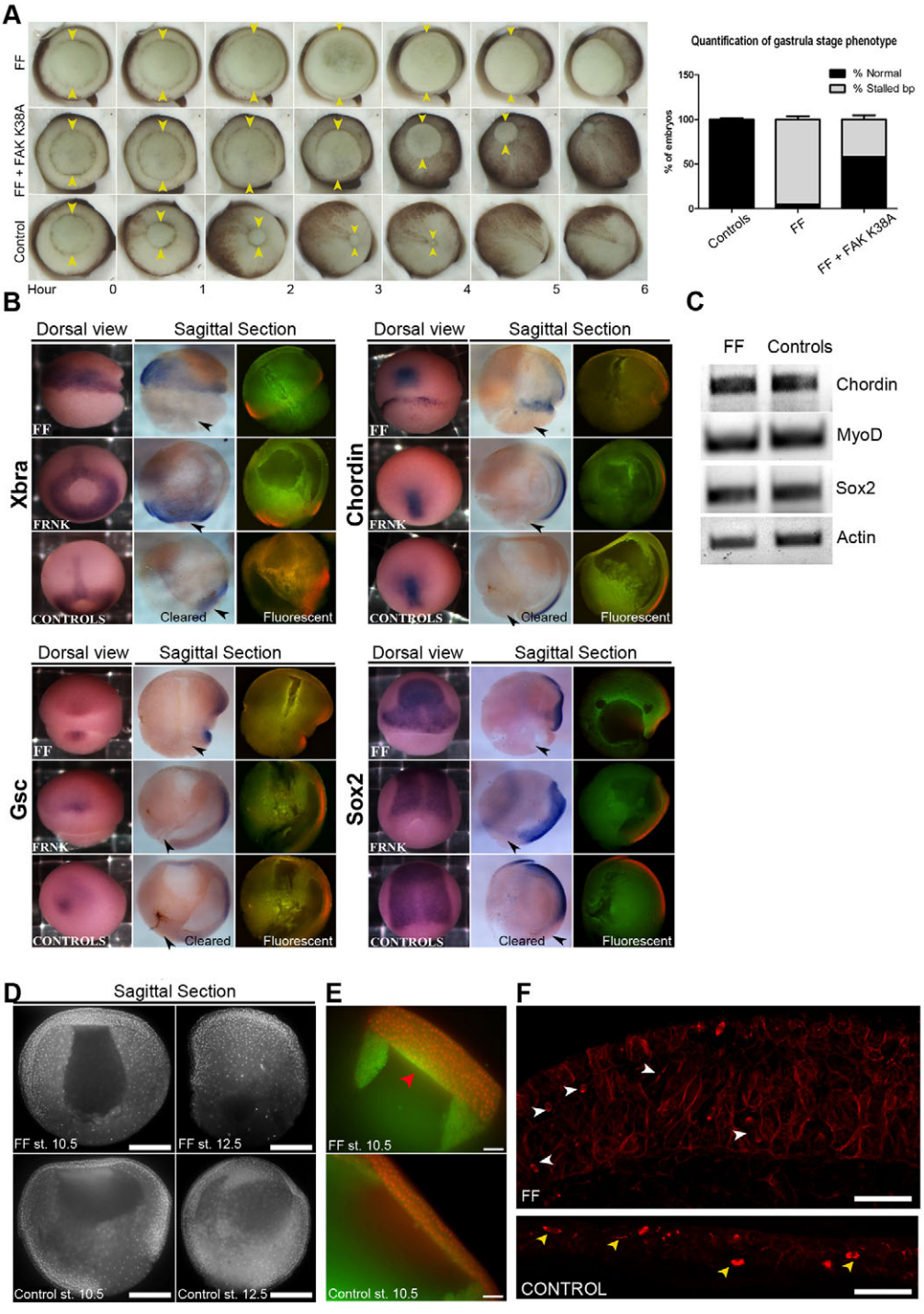

Fig. 3. FF expression in the AC leads to epiboly failure and gastrulation arrest. (**A**) Stills from a movie showing blastopore closure in controls and in embryos injected with 500 pg HA-FF (95.05±3.7% stalled blastopore, *n*=211) or with 500 pg HA-FF + 150 pg FAK K38A (41.97±4.7% stalled blastopore, *n*=128) from three independent experiments. Yellow arrowheads indicate the size of the blastopore. Data are mean±s.e.m. (**B**) Whole-mount *in situ* hybridization of control and embryos injected with HA-FF and HA-FRNK for the mesodermal markers Xbra, chordin and MyoD, and the neural marker Sox2. Black arrowheads show the blastopore. (**C**) RT-PCR for the markers chordin, MyoD and Sox2 and for actin as a loading control from control and FF-injected mid-gastrula stage embryos. (**D**) Wide-field images of sagittal sections of early and late gastrula stage control and HA-FF-injected embryos stained with Hoechst, showing thick AC in FF-expressing embryos. (**E**) Wide-field images of the AC of the same embryos showing epiboly failure after HA-FF injection (red arrowhead). (**F**) HA-FF-injected and control stage 11 embryos stained with β-tubulin antibody to image spindle orientation (78.7% misoriented spindles, *n*=61, three independent experiments). Yellow and white arrowheads indicate oriented and unoriented spindles, respectively. Scale bars: 500 μm in D; 100 μm in E; 50 μm in F.

red arrowhead). In addition, although normally cell divisions in the BCR occur in the horizontal plane of the epithelium, spindle orientation in FF expressors is randomized, showing loss of polarity (Fig. 3F, white arrowheads). Dose response of a previously characterized FAK-MO showed no early gastrulation defects (Fonar et al., 2011). However, sectioning of morphant embryos at gastrula stages revealed epiboly defects, albeit milder than those induced by FF expression (supplementary material Fig. S3B). Epiboly occurs in two distinct phases, beginning in the AC and spreading to the marginal zones as gastrulation proceeds (Keller, 1980); and morphants display more prominent thickening in the marginal zone, suggesting that the MO is more effective in preventing later intercalative activities as the maternal pool of FAK is slowly depleted. This is in agreement with previous work showing that the FAK-MO only reduces FAK protein levels by 50% at gastrula stages (Petridou et al., 2012).

The gastrulation defects observed in FF expressors are quite similar to what was observed when FN fibrillogenesis is blocked (Davidson et al., 2006; Marsden and DeSimone, 2001), suggesting that FF may be eliciting these by preventing FN fibrillogenesis. FN staining of stage 11 FF-expressing embryos shows that a dense fibrillar matrix is present (Fig. 4A) and FN protein levels are unaffected (Fig. 4A′). Sagittal sections reveal a dense network of FN on the BCR and ectopic fibrils between the cells of the AC (Fig. 4A, red arrowhead), suggesting that FF does not elicit its effect through the loss of the FN matrix. Another possible mechanism through which FF may be blocking epiboly is through the inhibition of adhesive complex turnover. To explore this possibility, we used MOs against *Xenopus* vinculin. Vinculin-null cells adhere poorly and have been shown to display increased migration rates and faster FA turnover (Coll et al., 1995). FRAP experiments comparing FF-GFP recovery in FF alone and FF + vinculin-MO AC explants show

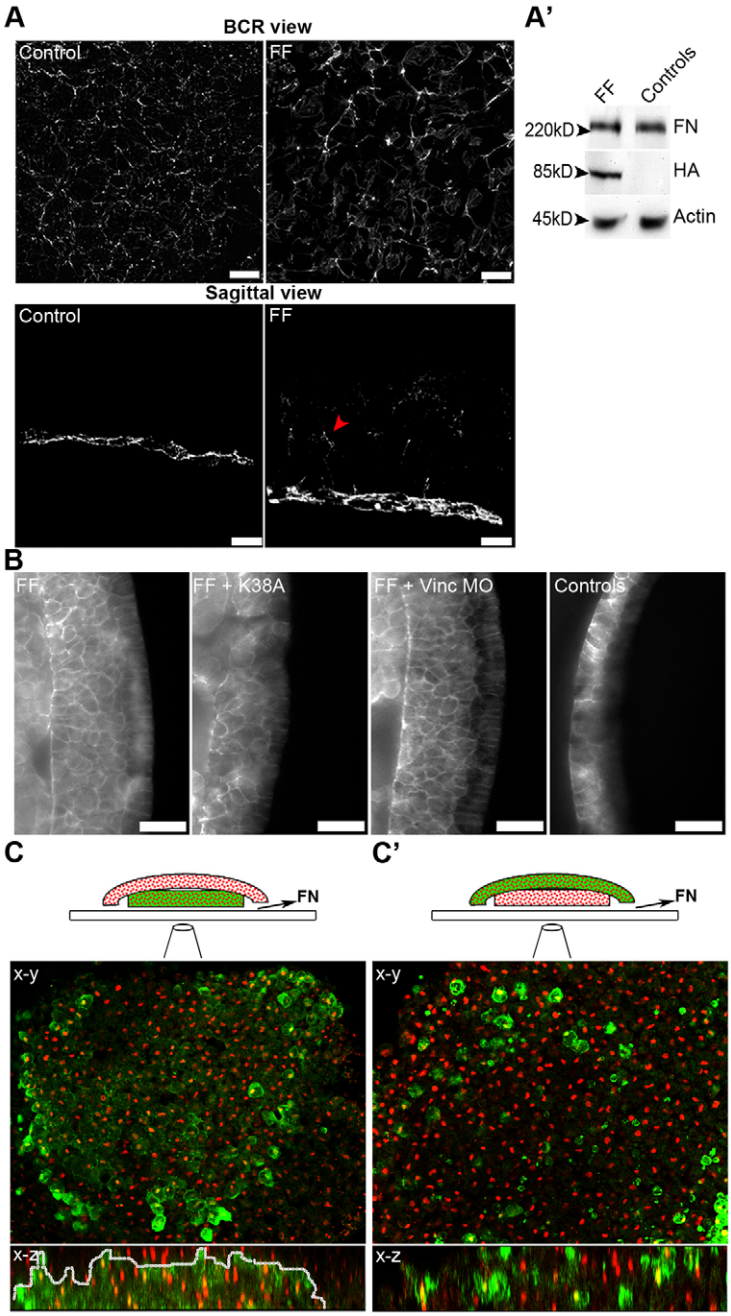

Fig. 4. FF expression blocks a FN-dependent polarizing signal during *Xenopus* epiboly. (**A**) Confocal images of the FN matrix on the BCR of control and FF-GFP-injected embryos at stage 11 and sagittal sections from embryos of the same experiment showing ectopic FN fibril formation between the inner cells of the AC in FF-injected embryos (red arrowhead). (**A′**) Western blot analysis shows similar expression levels of FN between FF and control embryos. (**B**) Wide-field images of gastrula stage control and embryos injected with FF, FF + FAK K38A, FF + vinculin-MO stained with β-catenin antibody to visualize the cell boundaries. (**C**) Radial intercalation explants from control and FF-GFP-injected embryos. The deep cell layer explant dissected from an FF-GFP-injected embryo (green) was placed on a FN-coated coverslip and another explant from an unlabeled control embryo was placed on top. Explants were cultured for 3 hours, fixed and stained with anti-histone H3 (nuclei) and anti-GFP antibodies. They were subsequently cleared and *z*-stacks were acquired on a confocal microscope. A single optical section at the level of the FN (*x-y*) and a vertical reconstruction of the *z*-stack (*x-z*) are shown. The FF-GFP-expressing explant (green with red dots) remains coherent and no GFP-negative cells have intercalated into it. A clear boundary is visible between the two explants (dashed line). (**C′**) In the reverse explant with control tissue placed on FN (red dots) and FF-GFP-injected explant on top (green with red dots), radial intercalation takes place normally and there is extensive cell mixing without any visible boundaries between the two explants. Scale bars: 20 μm in A; 50 μm in B.

that vinculin knockdown leads to increased turnover of FF in addition to fewer and smaller adhesions in a similar fashion to FAK K38A (supplementary material Fig. S4A). However, unlike FAK K38A, which rescues epiboly, vinculin knockdown fails to do so (Fig. 4B) suggesting that the effects of FF on FA turnover are not responsible for the phenotype. To confirm this, we generated an FF with a point mutation which abolishes binding of FAK to paxillin (FF-L1034S) (Tachibana et al., 1995). We postulated that as paxillin binding is one of the major determinants for FAK FA localization, this mutation would weaken the FF FA interaction and diminish its effects on FA turnover. FF-L1034S displays significantly faster turnover on FAs and fails to block FA disassembly; however, it blocks epiboly effectively uncoupling FA turnover from the epiboly defects (supplementary material Fig. S4B-D).

Previous studies in *Xenopus* have demonstrated that FN can provide signals that instruct cells to intercalate in the plane perpendicular to the matrix, and that these signals can act at a distance (Marsden and DeSimone, 2001). Our results suggest that FF expression leads to loss of polarity in the cells of the BCR by blocking these signals. If this is true one would expect that restricted FF expression in the cells in contact with the FN matrix would be sufficient to block directional intercalative behavior. To test this hypothesis, we took advantage of the deep layer explant developed by Marsden and DeSimone (Marsden and DeSimone, 2001). In this explant, dorsal marginal zone (DMZ) tissue is cut from one embryo and layers of deep cells lining the blastocoel are shaved and placed on a FN-coated coverslip. Another DMZ fragment is cut, the majority of deep cells removed and then positioned over the deep explant. As shown in Fig. 4C, when the deep explant in contact with the FN is expressing FF-GFP, little or no intercalation takes place from the overlying tissue and the GFP-positive FF explant remains coherent (dashed line). However, when a control deep explant is overlaid with an FF-GFP expressing superficial explant, FF cells intercalate into the control and GFP-positive cells can be seen interspersed between the controls (Fig. 4C′). This shows that FF expression specifically blocks the FN-dependent signal originating from the cells in direct contact with the substrate, and that FF-expressing cells can in fact polarize and intercalate if the signal is provided by control cells.

Epiboly plays an essential but permissive role during *Xenopus* gastrulation

The block of epiboly in FF expressors leads to failure of blastopore closure, as well as to partial block of involution. We postulated that this is due to the fact that in the absence of radial intercalation and BCR thinning, the mesodermal belt and the blastopore are held back, unable to move vegetally. This, coupled with the fact that mediolateral intercalation proceeds normally, leads to constriction of the embryo at the equatorial region and produces the characteristic mushroom-shaped embryos also seen in the 70 kDa FN experiments (Rozario et al., 2009). If this explanation is correct, one would expect increased tension in FF ACs. Phalloidin staining, in combination with β-catenin staining, shows that cells in FF-expressing ACs display stronger actin staining and have many protrusions compared with controls, suggesting they are under increased mechanical tension (Fig. 5A). The levels of β-catenin and C-cadherin are similar between FF and controls, suggesting that FF does not elicit its effect via strengthening of cell-cell adhesions (Fig. 5A; supplementary material Fig. S4E). Staining of FF and control ACs with a phospho-specific myosin light chain (MLC) antibody shows that in FF-expressing ACs, the apical surface of the deep cells displays elevated levels of phosphorylated MLC (Fig. 5B, white arrow), suggesting that FF BCRs are under increased mechanical tension and respond with increased contractility. In addition, levels of phosphorylated MLC are elevated both in expressing and in control cells, suggesting that FF expression does not cell autonomously lead to Rho activation, but rather the increased tension in the AC leads to elevated phosphorylation of MLC (Fig. 5B′, yellow box, white arrowhead). This result is consistent with the above postulated explanation with regards to the failure of blastopore closure being a result of the lack of epiboly and mechanical linkage of the mesodermal belt to the AC. To confirm this, we decided to relieve the tension within the FF ACs. To do so, we generated an incision on the BCR of FF-expressing embryos and monitored the progress of blastopore closure (Fig. 5C). Generating an incision on the BCR of an FF embryo (Fig. 5Ca) leads to the abrupt speeding up of blastopore closure compared with the FF control, which remains stalled (Fig. 5Cb). In agreement with previous reports, such small incisions only affect blastopore closure rates in control embryos marginally (data not shown) (Keller and Jansa, 1992). The incision on the BCR presumably relieves tension within the AC and allows the mesodermal belt to move vegetally, suggesting that epiboly plays an essential but permissive role during *Xenopus* gastrulation. Interestingly, injection of an inhibitor of ROCK in the blastocoel at stage 10 to chemically relive tension within the AC of FF-expressing embryos has the same effect, rescuing blastopore closure and dissection of ROCK inhibitor-treated FF-expressing embryos revealed a thinned AC (Fig. 5D,E). This result, in combination with the non-cell autonomy of MLC phosphorylation elevation, suggests that loss of polarity by FF expression leads to defective epiboly, which in turn leads to increased tension and stiffness in the AC. Inhibition of ROCK leads to a reduction of tissue stiffness and improved tissue rheology, allowing the AC tissue to deform in response to the pulling forces of the mesodermal belt, leading to thinning of the AC and rescuing blastopore closure.

FF expression in *Xenopus* leads to loss of mesodermal tissues

The major defect reported in FAK knockout mice is loss of mesodermally derived tissues. We, thus, decided to target FF to the dorsal mesoderm via DMZ injection at the four-cell stage. FF expression lead to smaller, curved and severely shortened embryos, the majority of which died by tadpole stages (Fig. 6A). The phenotype could be rescued via co-injection of 150 pg FAK K38A, indicating that the phenotype is specific (Fig. 6A). Interestingly, injection of 500 pg of FRNK has a very mild impact on development (Fig. 6A) and examination of the phosphorylation status of FAK shows that FF is more effective in blocking FAK phosphorylation *in vivo* (supplementary material Fig. S5A). To further address the specificity of the FF phenotype, we combined FAK-MO injections with FF expression and, as shown in supplementary material Fig. S5B, FF and FAK-MO act synergistically, suggesting that FF elicits the phenotype via inhibition of FAK activity. *In situ* hybridization and RT-PCRs showed that FF-expressing embryos, despite normal mesoderm induction and marker expression at gastrula and neurula stages (Xbra and MyoD), display extensive loss of somitic mesoderm (MyoD) at tadpole stages whereas no effect is seen in the expression of Sox2 (Fig. 6B-D). Despite normal patterning at gastrula stages, Xbra staining of the notochord appeared wider (Fig. 6B), whereas the MyoD expression domain at neurula stages was wider and positioned at the posterior (Fig. 6D). This, coupled with the dorsally bent tadpole-stage embryos, suggested that convergent extension

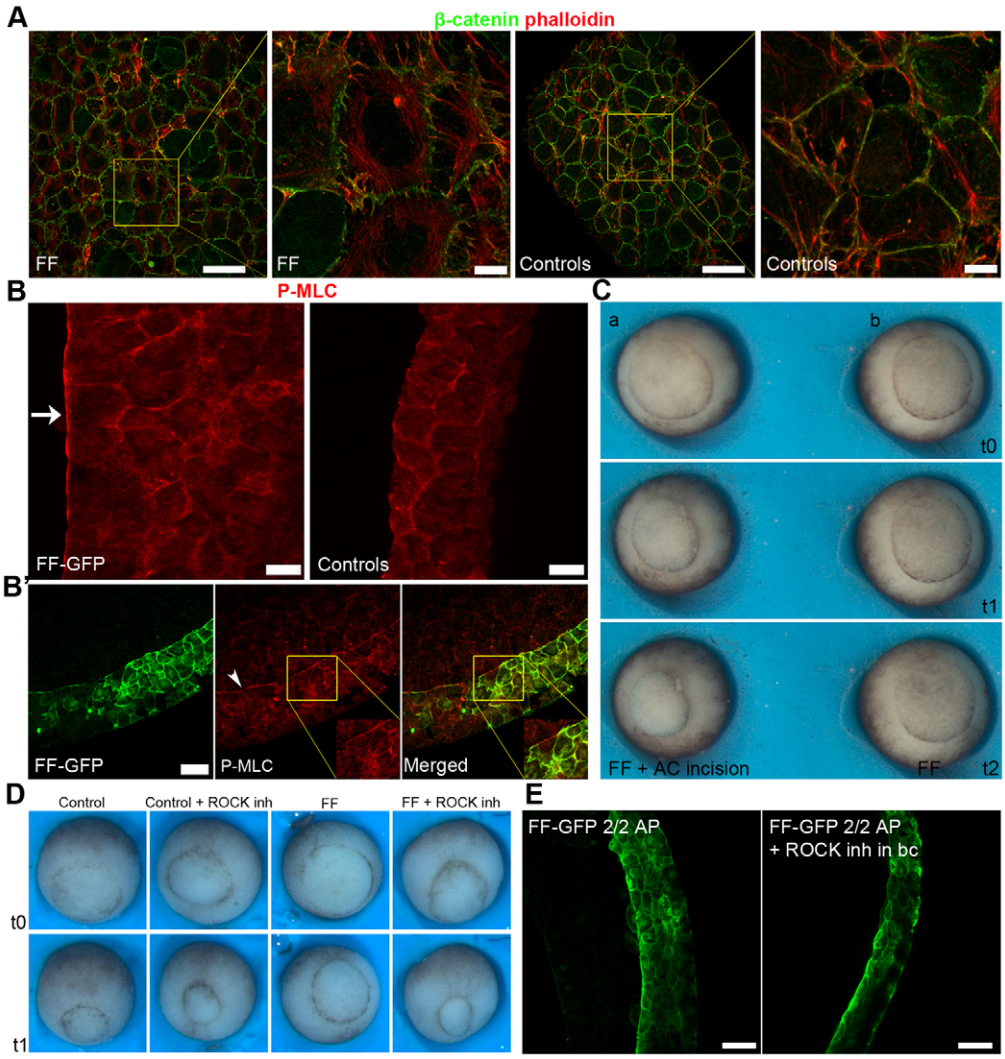

Fig. 5. FF ACs are under increased tension and epiboly has a permissive role during *Xenopus* gastrulation. (**A**) Low- and high-magnification confocal images and maximum intensity projections of the inner cell layer of the AC in FF-injected and control gastrula embryos stained with β-catenin (green) and phalloidin-488 (red). (**B**) Confocal images of FF-GFP-injected and control embryos stained for P-MLC (red) showing elevated P-MLC at the inner apical surface facing the blastocoel in FF-injected embryos (white arrow). (B′) Elevation of P-MLC is not cell-autonomous as elevation is seen in both FF-expressing (green) and non-expressing cells of the BCR (yellow box, white arrowhead). (**C**) FF blastopore closure failure is rescued by a small incision on the BCR (a). t0, FF-injected embryos before AC incision; t1, first time point immediately after incision; t2, second time point after incision. (b) FF injected embryo without any manipulation. (**D**) FF blastopore closure failure is also rescued by pharmacological release of tension by injecting the ROCK inhibitor in the blastocoel at stage 10. (**E**) Sagittal sections of FF-GFP-injected embryos, non-treated or treated with the ROCK inhibitor, showing thinning of the AC and epiboly rescue. Scale bars: 10 µm in A; 50 µm in A,B′,E; 20 µm in B.

movements may be affected; however, AC elongation assays showed that FF-expressing ACs elongate to the same extent as controls, suggesting that the mild early mesodermal morphogenesis problems may be due to defective mesoderm migration and possibly radial intercalation of the mesoderm (supplementary material Fig. S5C). Immunofluorescence experiments of FF-expressing embryos co-injected with histone-GFP revealed a large number of anaphase bridges from late gastrula stages onwards (Fig. 6F), and TUNEL staining of FF-expressing embryos showed increased apoptosis beginning at neurula stages (Fig. 6E), suggesting that FF expression induces mitotic defects and apoptosis. As Ser732 phosphorylation of FAK has been shown to play a role in centrosome function during mitosis (Park et al., 2009), we examined the effects of FF expression on the levels of endogenous P-Ser732. FF expression leads to a reduction of P-Ser732 levels and FF itself is heavily phosphorylated on Ser732 (Fig. 6G). As phosphorylation of Ser732 has been shown to be essential for the association of endogenous FAK with dynein, we postulated that FFs dominant-negative activity with respect to centrosomal function would require this residue. We thus generated a Ser732 point mutant that substituted serine to alanine (FF-S732A), and compared the effects of its expression to that of FF. As shown in Fig. 6F, FF-S732A expression fails to induce mitotic defects, resulting in a much milder phenotype compared with FF (Fig. 6H), suggesting that at least part of the FF phenotype is due to effects of FF on centrosome function through Ser732. Interestingly, FF-S732A expression induced identical phenotypes, as FF both in terms of FA turnover *in vitro* as well as block of epiboly *in vivo* (data not shown, supplementary material Fig. S5D). These results suggest that FFs effects on FA turnover and epiboly are related to the function of FAK at FAs and strengthen the conclusion that the defects elicited in the mesoderm are primarily dependent on the role of FAK in cell division and survival.

DISCUSSION

The FERM domain has been shown to bind several molecules that are important for FAK activation, as well as downstream effectors, including PIP_2, integrins, p53, Arp2/3 and GFRs (Cai et al., 2008; Chen and Chen, 2006; Schaller et al., 1994; Serrels et al., 2007; Sieg et al., 2000). Here, we show that despite the fact that the FAT domain is both necessary and sufficient for FA localization, the FERM domain is also required for correct localization *in vivo*. We go on to show that FF has a higher affinity for FA complexes, displaying significantly longer residence times compared with FRNK and demonstrating that the FERM domain regulates the dynamics of FAK on FAs. In addition, we show that FF becomes enriched on nascent adhesions prior to FRNK, suggesting a role of the FERM domain in the recruitment of FAK to these complexes. This may be due to the ability of the FERM domain to bind locally

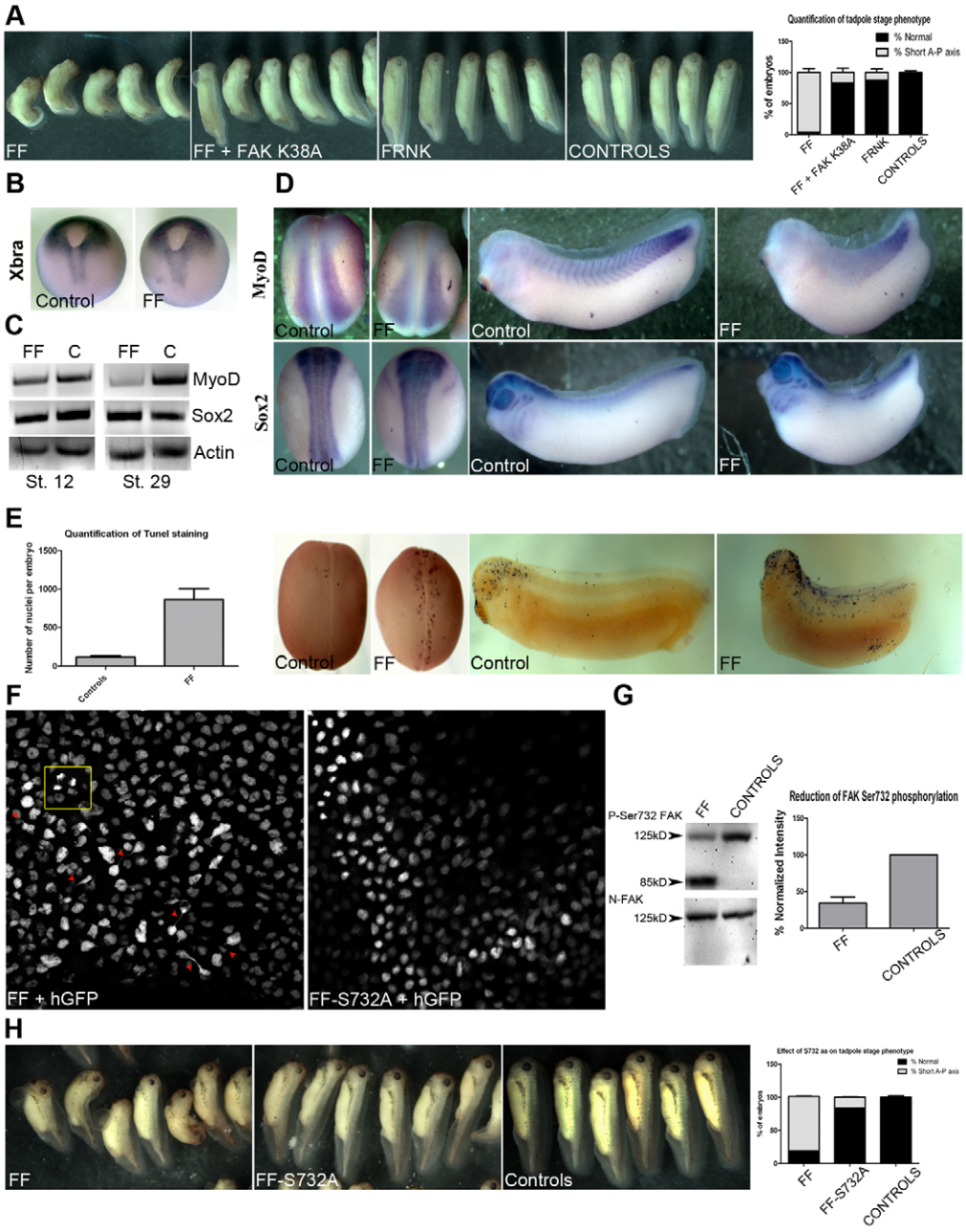

Fig. 6. FF expression leads to loss of mesoderm in a Ser732-dependent manner. (**A**) Injection of 500 pg FF into the DMZ of four-cell stage embryos leads to severe shortening of the A-P axis when compared with control (95.83±6.45%, *n*=149) and with FRNK-injected (12.45±5.87%, *n*=136) embryos. This phenotype is rescued by co-injection of 150 pg of FAK K38A (16.41±7.1%, *n*=136). (**B**) Whole-mount *in situ* hybridization for Xbra of gastrula stage control embryos or embryos injected with 500 pg FF into two dorsal blastomeres at the four-cell stage. (**C**) RT-PCR of gastrula (St. 12) and tadpole (St. 29) stages of control embryos or embryos injected with 500 pg FF into two out of four blastomeres at the DMZ. MyoD, Sox2 and actin levels were analyzed, showing loss of MyoD in FF-injected embryos. (**D**) Whole-mount *in situ* hybridization for MyoD and Sox2 of neurula- and tadpole-stage control embryos and embryos injected with FF as described above. (**E**) TUNEL assay of control and DMZ FF-injected embryos at the four-cell stage. Quantification of the apoptotic nuclei at the tadpole stages shows a sevenfold increase in apoptosis in FF expressors (861.67±140.96 apoptotic nuclei compared with 114.67±17.79 apoptotic nuclei in controls, *n*=6, two independent experiments). (**F**) Confocal images of embryos co-injected with histone-GFP and HA-FF or HA-FF S732A to visualize mitotic cells showing the formation of anaphase bridges (red arrowheads) and multipolar spindles (yellow box) in FF expressors. (**G**) Western blot and densitometry analysis of the effect of HA-FF expression on the reduction of the phosphorylation levels of P-Ser732 endogenous FAK and Ser732 phosphorylation of FF (lower band in FF lane). (**H**) Control embryos and embryos in which FF or FF-S732A has been injected into the DMZ at the four-cell stage. Graph shows statistical analysis of the shortened A-P axis phenotype. Data are mean+s.e.m.

generated PIP_2, keeping FF in close proximity to nascent adhesions; however, another possibility is that the FERM domain is transiently binding integrins at the early stages of FA formation (Cai et al., 2008; Schaller et al., 1995).

FF displaces FAK from FAs, resulting in the loss of FAK phosphorylation. This leads to a dramatic reduction of FA turnover and an increase in size and number of FAs. When compared with FRNK, FF elicits a much stronger phenotype with expressors

resembling FAK-null fibroblasts. The significant differences between FRNK and FF expressors with respect to their ability to turnover FAs, may be attributed to the higher affinity that FF displays for FAs. The long residence times of FF suggest a slower off rate from these complexes and, as a result, at steady state and at given fixed levels of expression, FF would be more effective in displacing endogenous FAK than would FRNK. In addition, although FRNK can compete FAK from a subset of target proteins that bind the C terminus, FF can presumably compete with endogenous FAK for a wider array of FAK partners.

Expression of FF in the AC leads to BCR thickening and severe gastrulation defects, whereas expression of FF in the mesoderm via DMZ injections leads to smaller curved embryos, in part due to loss of mesodermal tissues, similar to FAK knockout mice (Furuta et al., 1995). The mild defects induced by FF in mesodermal morphogenesis are in contrast to the severe defects induced in the AC and in agreement with the requirement of fibrillar FN for epiboly but not for convergent extension (Davidson et al., 2006; Marsden and DeSimone, 2001; Rozario et al., 2009). Here, we show that, despite the presence of a fibrillar matrix, both radial intercalation and cell polarity are lost in FF expressors, suggesting that an integrin-dependent polarizing signal is required for both processes. We also show that FF expression in the cells in direct contact with the FN blocks intercalative behavior, whereas expression in the overlying cells does not, confirming that a FN-integrin signal can direct intercalative behaviors at a distance and that FAK has a crucial role in its transduction.

Block of epiboly through a variety of experimental approaches leads to gastrulation defects in the mesoderm, including partial block of involution and delay or failure of blastopore closure. However, it has been shown that BCR-less embryos close their blastopores without any problems. In fact, in these embryos, a speeding up of blastopore closure is observed, suggesting that the mesoderm is actually exerting forces on the AC and is held back by this interaction (Keller and Jansa, 1992). In FF expressors, generation of a small incision on the AC leads to the abrupt speeding up of blastopore closure, suggesting that the failure of blastopore closure is due to the fact that the mesoderm is held back by the AC. The AC in FFs not only displays loss of polarity but in addition, displays increased stiffness, as evidenced by the elevation of phosphorylation of MLC and increased F-actin. This would suggest that the inability of FF-expressing ACs to thin and allow vegetal translocation of the blastopore results in increased tension imposed on this tissue, which responds by increasing its stiffness. In agreement with this interpretation, use of a ROCK inhibitor rescues blastopore closure and BCR thinning. It appears that under conditions of low contractility, improved tissue rheology allows the AC to deform in response to the forces generated by the mesoderm. This suggests that epiboly requires the forces generated by the mesoderm in order to take place.

Overall, our work identifies new roles for the FERM domain in the regulation of the dynamics of FAK on its signaling complexes *in vivo* and *in vitro*, and identifies epiboly as the earliest developmental process in which FAK plays a crucial role during *Xenopus* development. The new dominant negative that we have generated and characterized will be a valuable tool, through targeted injections and inducible expression, for future studies of the involvement of FAK in a variety of developmental processes.

Acknowledgments
We thank Dr Douglas DeSimone for kindly providing the fibronectin 4H2 antibody, Dr Jun-Lin Guan for providing the pKH3 FAK and pKH3 FRNK plasmids, and Drs Chenbei Chang and Chao Yun Irene Yan for comments on the paper.

Funding
Funding was provided by the Cyprus Research Promotion Foundation [ΥΓΕΙΑ/ΒΙΟΣ/0609(ΒΕ)/14]

Competing interests statement
The authors declare no competing financial interests.

Author contributions
N.I.P., P.S. and P.A.S. conceived and designed the experiments. N.I.P. and P.S. performed the experiments. N.I.P., P.S. and P.A.S. analyzed the data. N.I.P. and P.A.S. wrote the paper.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.096073/-/DC1

References

Alfandari, D., Cousin, H., Gaultier, A., Smith, K., White, J. M., Darribère, T. and DeSimone, D. W. (2001). Xenopus ADAM 13 is a metalloprotease required for cranial neural crest-cell migration. *Curr. Biol.* **11**, 918-930.
Cai, X., Lietha, D., Ceccarelli, D. F., Karginov, A. V., Rajfur, Z., Jacobson, K., Hahn, K. M., Eck, M. J. and Schaller, M. D. (2008). Spatial and temporal regulation of focal adhesion kinase activity in living cells. *Mol. Cell. Biol.* **28**, 201-214.
Calalb, M. B., Polte, T. R. and Hanks, S. K. (1995). Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Mol. Cell. Biol.* **15**, 954-963.
Ceccarelli, D. F., Song, H. K., Poy, F., Schaller, M. D. and Eck, M. J. (2006). Crystal structure of the FERM domain of focal adhesion kinase. *J. Biol. Chem.* **281**, 252-259.
Chen, S. Y. and Chen, H. C. (2006). Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. *Mol. Cell. Biol.* **26**, 5155-5167.
Chen, H. C., Appeddu, P. A., Parsons, J. T., Hildebrand, J. D., Schaller, M. D. and Guan, J. L. (1995). Interaction of focal adhesion kinase with cytoskeletal protein talin. *J. Biol. Chem.* **270**, 16995-16999.
Coll, J. L., Ben-Ze'ev, A., Ezzell, R. M., Rodríguez Fernández, J. L., Baribault, H., Oshima, R. G. and Adamson, E. D. (1995). Targeted disruption of vinculin genes in F9 and embryonic stem cells changes cell morphology, adhesion, and locomotion. *Proc. Natl. Acad. Sci. USA* **92**, 9161-9165.
Cooper, L. A., Shen, T. L. and Guan, J. L. (2003). Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction. *Mol. Cell. Biol.* **23**, 8030-8041.
Davidson, L. A., Marsden, M., Keller, R. and Desimone, D. W. (2006). Integrin alpha5beta1 and fibronectin regulate polarized cell protrusions required for Xenopus convergence and extension. *Curr. Biol.* **16**, 833-844.
Doherty, J. T., Conlon, F. L., Mack, C. P. and Taylor, J. M. (2010). Focal adhesion kinase is essential for cardiac looping and multichamber heart formation. *Genesis* **48**, 492-504.
Ezratty, E. J., Partridge, M. A. and Gundersen, G. G. (2005). Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat. Cell Biol.* **7**, 581-590.
Fonar, Y., Gutkovich, Y. E., Root, H., Malyarova, A., Aamar, E., Golubovskaya, V. M., Elias, S., Elkouby, Y. M. and Frank, D. (2011). Focal adhesion kinase protein regulates Wnt3a gene expression to control cell fate specification in the developing neural plate. *Mol. Biol. Cell* **22**, 2409-2421.
Furuta, Y., Ilić, D., Kanazawa, S., Takeda, N., Yamamoto, T. and Aizawa, S. (1995). Mesodermal defect in late phase of gastrulation by a targeted mutation of focal adhesion kinase, FAK. *Oncogene* **11**, 1989-1995.
George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H. and Hynes, R. O. (1993). Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* **119**, 1079-1091.
Golubovskaya, V. M., Finch, R. and Cance, W. G. (2005). Direct interaction of the N-terminal domain of focal adhesion kinase with the N-terminal transactivation domain of p53. *J. Biol. Chem.* **280**, 25008-25021.
Hayashi, I., Vuori, K. and Liddington, R. C. (2002). The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin. *Nat. Struct. Biol.* **9**, 101-106.
Hens, M. D. and DeSimone, D. W. (1995). Molecular analysis and developmental expression of the focal adhesion kinase pp125FAK in Xenopus laevis. *Dev. Biol.* **170**, 274-288.
Hildebrand, J. D., Schaller, M. D. and Parsons, J. T. (1993). Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. *J. Cell Biol.* **123**, 993-1005.
Hildebrand, J. D., Schaller, M. D. and Parsons, J. T. (1995). Paxillin, a tyrosine phosphorylated focal adhesion-associated protein binds to the carboxyl terminal domain of focal adhesion kinase. *Mol. Biol. Cell* **6**, 637-647.

Ilić, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M. and Yamamoto, T. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* **377**, 539-544.
Ilic, D., Kovacic, B., McDonagh, S., Jin, F., Baumbusch, C., Gardner, D. G. and Damsky, C. H. (2003). Focal adhesion kinase is required for blood vessel morphogenesis. *Circ. Res.* **92**, 300-307.
Jácamo, R. O. and Rozengurt, E. (2005). A truncated FAK lacking the FERM domain displays high catalytic activity but retains responsiveness to adhesion-mediated signals. *Biochem. Biophys. Res. Commun.* **334**, 1299-1304.
Keller, R. E. (1980). The cellular basis of epiboly: an SEM study of deep-cell rearrangement during gastrulation in Xenopus laevis. *J. Embryol. Exp. Morphol.* **60**, 201-234.
Keller, R. and Jansa, S. (1992). Xenopus Gastrulation without a blastocoel roof. *Dev. Dyn.* **195**, 162-176.
Kostourou, V., Lechertier, T., Reynolds, L. E., Lees, D. M., Baker, M., Jones, D. T., Tavora, B., Ramjaun, A. R., Birdsey, G. M., Robinson, S. D. et al. (2013). FAK-heterozygous mice display enhanced tumour angiogenesis. *Nat Commun* **4**, 2020.
Lawson, C. and Schlaepfer, D. D. (2012). Integrin adhesions: who's on first? What's on second? Connections between FAK and talin. *Cell Adh. Migr.* **6**, 302-306.
Lim, S. T., Chen, X. L., Lim, Y., Hanson, D. A., Vo, T. T., Howerton, K., Larocque, N., Fisher, S. J., Schlaepfer, D. D. and Ilic, D. (2008a). Nuclear FAK promotes cell proliferation and survival through FERM-enhanced p53 degradation. *Mol. Cell* **29**, 9-22.
Lim, Y., Lim, S. T., Tomar, A., Gardel, M., Bernard-Trifilo, J. A., Chen, X. L., Uryu, S. A., Canete-Soler, R., Zhai, J., Lin, H. et al. (2008b). PyK2 and FAK connections to p190Rho guanine nucleotide exchange factor regulate RhoA activity, focal adhesion formation, and cell motility. *J. Cell Biol.* **180**, 187-203.
Marsden, M. and DeSimone, D. W. (2001). Regulation of cell polarity, radial intercalation and epiboly in Xenopus: novel roles for integrin and fibronectin. *Development* **128**, 3635-3647.
Mitra, S. K., Hanson, D. A. and Schlaepfer, D. D. (2005). Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell Biol.* **6**, 56-68.
Park, A. Y., Shen, T. L., Chien, S. and Guan, J. L. (2009). Role of focal adhesion kinase Ser-732 phosphorylation in centrosome function during mitosis. *J. Biol. Chem.* **284**, 9418-9425.
Petridou, N. I., Stylianou, P., Christodoulou, N., Rhoads, D., Guan, J. L. and Skourides, P. A. (2012). Activation of endogenous FAK via expression of its amino terminal domain in Xenopus embryos. *PLoS ONE* **7**, e42577.
Ren, X. D., Kiosses, W. B., Sieg, D. J., Otey, C. A., Schlaepfer, D. D. and Schwartz, M. A. (2000). Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J. Cell Sci.* **113**, 3673-3678.
Richardson, A., Malik, R. K., Hildebrand, J. D. and Parsons, J. T. (1997). Inhibition of cell spreading by expression of the C-terminal domain of focal adhesion kinase (FAK) is rescued by coexpression of Src or catalytically inactive FAK: a role for paxillin tyrosine phosphorylation. *Mol. Cell. Biol.* **17**, 6906-6914.
Rozario, T., Dzamba, B., Weber, G. F., Davidson, L. A. and DeSimone, D. W. (2009). The physical state of fibronectin matrix differentially regulates morphogenetic movements in vivo. *Dev. Biol.* **327**, 386-398.
Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R. and Parsons, J. T. (1994). Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol. Cell. Biol.* **14**, 1680-1688.
Schaller, M. D., Otey, C. A., Hildebrand, J. D. and Parsons, J. T. (1995). Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. *J. Cell Biol.* **130**, 1181-1187.
Serrels, B., Serrels, A., Brunton, V. G., Holt, M., McLean, G. W., Gray, C. H., Jones, G. E. and Frame, M. C. (2007). Focal adhesion kinase controls actin assembly via a FERM-mediated interaction with the Arp2/3 complex. *Nat. Cell Biol.* **9**, 1046-1056.
Sieg, D. J., Hauck, C. R. and Schlaepfer, D. D. (1999). Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J. Cell Sci.* **112**, 2677-2691.
Sieg, D. J., Hauck, C. R., Ilic, D., Klingbeil, C. K., Schaefer, E., Damsky, C. H. and Schlaepfer, D. D. (2000). FAK integrates growth-factor and integrin signals to promote cell migration. *Nat. Cell Biol.* **2**, 249-256.
Smith, W. C. and Harland, R. M. (1991). Injected Xwnt-8 RNA acts early in Xenopus embryos to promote formation of a vegetal dorsalizing center. *Cell* **67**, 753-765.
Stylianou, P. and Skourides, P. A. (2009). Imaging morphogenesis, in Xenopus with Quantum Dot nanocrystals. *Mech. Dev.* **126**, 828-841.
Tachibana, K., Sato, T., D'Avirro, N. and Morimoto, C. (1995). Direct association of pp125FAK with paxillin, the focal adhesion-targeting mechanism of pp125FAK. *J. Exp. Med.* **182**, 1089-1099.
Taylor, J. M., Mack, C. P., Nolan, K., Regan, C. P., Owens, G. K. and Parsons, J. T. (2001). Selective expression of an endogenous inhibitor of FAK regulates proliferation and migration of vascular smooth muscle cells. *Mol. Cell. Biol.* **21**, 1565-1572.
Tomar, A., Lim, S. T., Lim, Y. and Schlaepfer, D. D. (2009). A FAK-p120RasGAP-p190RhoGAP complex regulates polarity in migrating cells. *J. Cell Sci.* **122**, 1852-1862.
Wacker, S., Brodbeck, A., Lemaire, P., Niehrs, C. and Winklbauer, R. (1998). Patterns and control of cell motility in the Xenopus gastrula. *Development* **125**, 1931-1942.
Woolner, S. and Papalopulu, N. (2012). Spindle position in symmetric cell divisions during epiboly is controlled by opposing and dynamic apicobasal forces. *Dev. Cell* **22**, 775-787.
Xia, H., Nho, R. S., Kahm, J., Kleidon, J. and Henke, C. A. (2004). Focal adhesion kinase is upstream of phosphatidylinositol 3-kinase/Akt in regulating fibroblast survival in response to contraction of type I collagen matrices via a beta 1 integrin viability signaling pathway. *J. Biol. Chem.* **279**, 33024-33034.
Xie, Z., Sanada, K., Samuels, B. A., Shih, H. and Tsai, L. H. (2003). Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. *Cell* **114**, 469-482.
Yamakita, Y., Totsukawa, G., Yamashiro, S., Fry, D., Zhang, X., Hanks, S. K. and Matsumura, F. (1999). Dissociation of FAK/p130(CAS)/c-Src complex during mitosis: role of mitosis-specific serine phosphorylation of FAK. *J. Cell Biol.* **144**, 315-324.
Yang, J. T., Rayburn, H. and Hynes, R. O. (1993). Embryonic mesodermal defects in alpha 5 integrin-deficient mice. *Development* **119**, 1093-1105.
Zhao, J. H., Reiske, H. and Guan, J. L. (1998). Regulation of the cell cycle by focal adhesion kinase. *J. Cell Biol.* **143**, 1997-2008.