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Regulation of somitogenesis by Ena/VASP proteins and FAK during *Xenopus* development

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The metameric organization of the vertebrate body plan is established during somitogenesis as somite pairs sequentially form along the anteroposterior axis. Coordinated regulation of cell shape, motility and adhesion are crucial for directing the morphological segmentation of somites. We show that members of the Ena/VASP family of actin regulatory proteins are required for somitogenesis in *Xenopus. Xenopus* Ena (Xena) localizes to the cell periphery in the presomitic mesoderm (PSM), and is enriched at intersomitic junctions and at myotendinous junctions in somites and the myotome, where it co-localizes with β1-integrin, vinculin and FAK. Inhibition of Ena/VASP function with dominant-negative mutants results in abnormal somite formation that correlates with later defects in intermyotomal junctions. Neutralization of Ena/VASP activity disrupts cell rearrangements during somite rotation and leads to defects in the fibronectin (FN) matrix surrounding somites. Furthermore, inhibition of Ena/VASP function impairs FN matrix assembly, spreading of somitic cells on FN and autophosphorylation of FAK, suggesting a role for Ena/VASP proteins in the modulation of integrin-mediated processes. We also show that inhibition of FAK results in defects in somite formation, blocks FN matrix deposition and alters Xena localization. Together, these results provide evidence that Ena/VASP proteins and FAK are required for somite formation in *Xenopus* and support the idea that Ena/VASP and FAK function in a common pathway to regulate integrin-dependent migration and adhesion during somitogenesis.

KEY WORDS: Somitogenesis, Morphogenesis, Mesoderm, Ena/VASP, FAK, Integrin, Cadherin, Adhesion, Migration

INTRODUCTION

The sequential subdivision of the paraxial mesoderm into somites is the initial manifestation of segmentation during vertebrate development. Somitogenesis is dependent on a molecular oscillator, or 'somite clock', and on gradients of signaling molecules, which interact to generate serially reiterated patterns of gene expression within the PSM (Aulehla and Herrmann, 2004; Dubrulle and Pourquie, 2004; Weinmaster and Kintner, 2003). One output of these signaling events is to initiate changes in cell shape, motility and adhesion that mediate morphological segmentation (Kalcheim and Ben-Yair, 2005). Although the molecular pathways involved in establishing segmental identity have been studied in some detail, the mechanisms responsible for morphological segmentation remain elusive.

Previous studies have described the cellular processes that accompany somite formation in *Xenopus* (Hamilton, 1969; Keller, 2000; Wilson et al., 1989; Youn and Malacinski, 1981). During gastrulation, paraxial mesoderm cells intercalate radially and mediolaterally to drive anteroposterior extension of the PSM and establish the notochord/somite boundary (Wilson et al., 1989). At the end of gastrulation, PSM cells change shape, lengthening along their mediolateral axis and narrowing along their anteroposterior axis (Wilson et al., 1989). Subsequently, blocks of cells in the rostral-most region of the PSM rotate 90° relative to the anteroposterior axis to generate somites (Keller, 2000), which primarily differentiate into mono-nucleate muscle cells of the tadpole (Chanoine and Hardy, 2003).

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Analysis of cell behaviors during segmentation suggest that cells rearrange independently during rotation (Wilson et al., 1989; Youn and Malacinski, 1981), indicating a role for directed migration in this process. Spatially and temporally coordinated regulation of cell adhesion is also essential for somitogenesis. Cell-cell adhesion during rotation is dependent on Type I cadherins (Giacomello et al., 2002) and somite boundary formation requires the function of paraxial protocadherin (PAPC) (Kim et al., 2000). The importance of integrins in *Xenopus* somitogenesis is suggested by the expression of several integrins, including $\alpha_3\beta_1$-, $\alpha_5\beta_1$- and $\alpha_6\beta_1$-integrins, in developing somites, disruption of somite formation by overexpression of α_3-integrin (Meng et al., 1997) or expression of a dominant negative form of β1-integrin (Marsden and DeSimone, 2003), and requirement for integrin function in somite formation in other vertebrates (Drake et al., 1992; Goh et al., 1997; Julich et al., 2005; Koshida et al., 2005; Krotoski and Bronner-Fraser, 1990; Yang et al., 1993; Zagris et al., 2004). In addition, presumptive somites become surrounded by a FN-rich matrix during somitogenesis (Davidson et al., 2004; Wedlich et al., 1989) and FN is required for somitogenesis in mice and zebrafish (George et al., 1993; Koshida et al., 2005). FAK, a crucial signaling molecule activated by integrin-ECM interactions, also accumulates at somite boundaries and is required for somitogenesis in mice (Crawford et al., 2003; Furuta et al., 1995; Henry et al., 2001; Hens and DeSimone, 1995), implicating a potential role for integrin signaling in this process.

Somite formation is dependent on tightly orchestrated morphogenetic processes, yet little is known about the molecular pathways that coordinate changes in cell shape, migration and adhesion during somitogenesis. The Ena/VASP family of actin regulatory proteins function in a variety of cell types to regulate cell migration and adhesion and these roles are borne out by the localization of Ena/VASP proteins to focal adhesions and sites of dynamic membrane reorganization (Krause et al., 2003; Kwiatkowski et al., 2003; Sechi and Wehland, 2004). The

vertebrate Ena/VASP family comprises three genes, Ena, vasodilator-stimulated phosphoprotein (VASP) and Ena/VASP-like (Evl). Ena/VASP proteins are characterized by several protein-protein interaction domains: a N-terminal EVH1 domain that binds tightly and specifically to a consensus motif (F/LPPPP) found in a number of proteins, including vinculin, zyxin, RIAM and lamellipodin (Brindle et al., 1996; Drees et al., 2000; Fedorov et al., 1999; Krause et al., 2004; Lafuente et al., 2004); a central, proline-rich domain that binds Profilin (Gertler et al., 1996; Reinhard et al., 1995) and SH3 domain proteins such as Abl and nSrc (Gertler et al., 1995; Lambrechts et al., 2000); and a C-terminal EVH2 domain that binds F-actin and mediates multimerization of Ena/VASP proteins (Bachmann et al., 1999; Harbeck et al., 2000; Huttelmaier et al., 1999). Knockout studies in mice show that Ena/VASP proteins are required for platelet aggregation, neural tube formation, craniofacial development and axon guidance (Aszodi et al., 1999; Hauser et al., 1999; Lanier et al., 1999; Menzies et al., 2004). However, these studies have been hindered by the functional redundancy of the highly related family members, making it likely that additional roles for Ena/VASP proteins remain to be uncovered. To overcome the problem of redundancy, several studies have used dominant-negative proteins to neutralize the function of all Ena/VASP proteins. This work has revealed additional roles for Ena/VASP proteins in formation of cell-cell junctions in epithelial cells (Vasioukhin et al., 2000), regulation of intercalated disc function in cardiac muscle (Eigenthaler et al., 2003) and migration of pyramidal neurons in the cerebral cortex (Goh et al., 2002). Furthermore, dominant-negative proteins have also been employed to examine the mechanism by which Ena/VASP proteins regulate actin dynamics and cell motility in cultured fibroblasts (Bear et al., 2000; Bear et al., 2002).

Previously, we have reported that *Xena* is expressed throughout the mesoderm during gastrulation, and that *Xena* and *Xenopus Evl* (*Xevl*) transcripts are present in the myotome of the tadpole (Wanner et al., 2005; Xanthos et al., 2005), suggesting that Ena/VASP proteins might play a role in somitogenesis and muscle development in *Xenopus*. Here, we show that Xena is localized to cell borders in the PSM and is later enriched at intersomitic and intermyotomal junctions. Using targeted expression of dominant-negative proteins that neutralize the function of all Ena/VASP family members, we demonstrate that Ena/VASP activity is required for somite rotation and boundary formation. Furthermore, these studies revealed a requirement for Ena/VASP proteins in FN matrix deposition, spreading of somitic cells on FN and autophosphorylation of FAK. Finally, we show that FAK is required for somite formation, FN matrix deposition and localization of Xena to the cell cortex. Together, these data provide evidence that Ena/VASP proteins and FAK coordinately regulate somite formation by modulating integrin-dependent processes during development.

MATERIALS AND METHODS

Plasmids

Plasmids containing FP_4-mito-GFP, AP_4-mito-GFP and FRNK were generated by subcloning parental constructs into pCS2+. EVH1-GFP was constructed by amplifying regions of Xena (amino acids 1-115) using PCR and subcloning the fragment into pCS2+ GFP-N1. Details of construction are available upon request.

Embryos and microinjections

Xenopus laevis embryos were obtained by fertilization of eggs from females injected with human chorionic gonadotrophin (Sigma). Eggs were dejellied in 2% cysteine, cultured in 0.33×MMR (Sive et al., 2000), and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Capped mRNA for microinjections was synthesized using the SP6 mMessage Machine kit (Ambion) and embryos were injected in 4% ficoll in 0.33×MMR.

FN spreading and adhesion assays

Somitic cell cultures were prepared as described (Gomez et al., 2003). Briefly, the dorsal region of stage 20 embryos were excised, transferred to Ca^{2+}/Mg^{2+}-free 0.3×MMR, and dissociated for 1 hour. Ectoderm was removed and remaining cells were transferred to FN-coated coverslips (0.5 μg/ml; Sigma) in 1×Steinberg's (Sive et al., 2000) and allowed to adhere for 30 minutes (adhesion assay) or overnight (spreading assay) at 20°C. Prior to collection, cells were washed three times with 0.3×MMR then fixed in Dent's fixative (Sive et al., 2000) for 2 hours at 4°C.

Immunofluorescence

Embryos were fixed in Dent's fixative overnight at 4°C. For imaging of FN, blastocoel roofs were fixed in 2% trichloroacetic acid in PBS overnight at 4°C. Immunostaining was performed with the following antibodies: anti-GFP (Santa Cruz Biotechnology), anti-Xena (Xanthos et al., 2005), anti-Mena (Lebrand et al., 2004), anti-tenascin (HB1, provided by H. R. Erickson), anti-FN (4H2) (Ramos and DeSimone, 1996) and anti-FAK (2A7, Upstate Biotechnology). The following monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank: vinculin (VN 3-24), β_1-integrin (8C8), β-tubulin (E7) and 12/101. Staining was visualized using Alexa568-conjugated (Molecular Probes) or Cy2-conjugated (Jackson ImmunoResearch) secondary antibodies. For imaging of cross-sections, immunostained embryos were incubated overnight in PBST, and slices were cut with a surgical scalpel. With the exception of BCR explants and FN adherent cells (mounted in 80% glycerol, 0.5% propylgallate), all samples were dehydrated, cleared in Murray's clear (Sive et al., 2000) and mounted in Sylgard (Dow Corning) wells. Images were captured using a Zeiss spinning disc microscope and merged images were produced using Adobe Photoshop. Quantitative analysis of somite area and Xena staining in BCRs was performed with ImageJ. For analysis if somite area, cross-sectional area of 12/101 positive cells was measured. Results are reported as a ratio of the area of injected versus uninjected side, or right versus left sides for controls. For analysis of Xena distribution in BCRs, average pixel intensity at the membrane (two peak intensities 0.3 μm apart) was compared with the average pixel intensity of the juxtamembrane region (3.3 μm adjacent to membrane).

In situ hybridization

In situ hybridization was carried out as described (Harland, 1991). Digoxigenin-labeled *MyoD* (Hopwood et al., 1989) and *PAPC* (Kim et al., 2000) probes were synthesized using a MAXIScript kit (Ambion). Probes were detected by alkaline phosphatase-conjugated anti-digoxigenin (Roche) using BM Purple substrate (Boehringer Mannheim).

Immunoblotting

Protein lysates were prepared by homogenizing explants or embryos in ice-cold lysis buffer [10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM $Na_4PO_2O_7$, 1% Triton-X100] supplemented with phosphatase and protease inhibitors. Homogenates were cleared by centrifugation at 8000 ***g*** for 10 minutes at 4°C. Proteins were blotted to PVDF membrane and blots were blocked in 5% milk in TBS + 0.1% Tween (TBSTw) or 5% BSA in TBSTw for phospho-FAK analysis. Blots were probed with anti-FAK (1:1000, Santa Cruz Biotechnology) or anti-α-fodrin antibodies (1:2000) (Giebelhaus et al., 1987) in 5% milk in TBSTw or anti-FAKpY397 antibodies (1:1000, BioSource) in 3% BSA in TBSTw overnight at 4°C. Visualization was performed using HRP-conjugated antibodies (Jackson ImmunoLabs) and enhanced chemiluminescence (Pierce).

RESULTS

Xena localization predicts a role in somitogenesis and muscle development

Our previous studies have revealed that *Xena* transcripts are expressed in mesodermal tissues including somites and myotome (Xanthos et al., 2005). To further these studies, we examined the

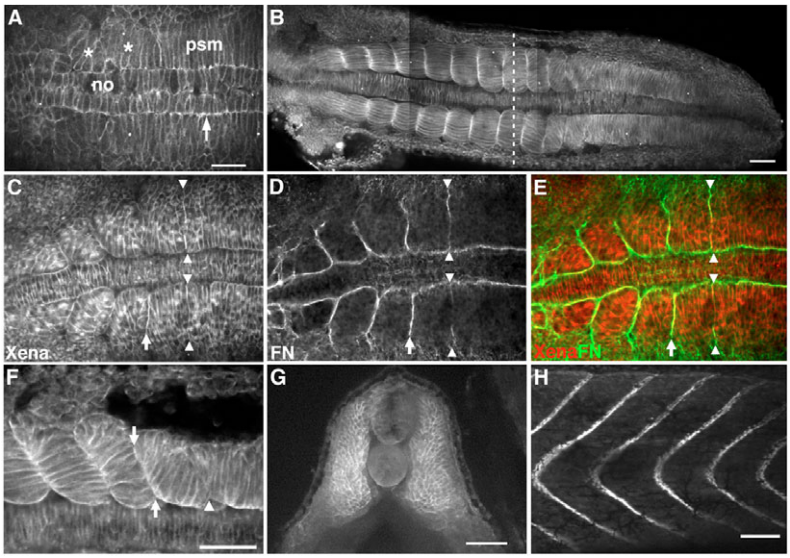

Fig. 1. Xena localization during somitogenesis. (**A**) At stage 17, Xena is enriched at the PSM/notochord (no) boundary (arrow) and at the cell periphery in the PSM and somitic mesoderm during somite formation (asterisks). (**B**) Longitudinal view of a stage 22 embryo showing accumulation of Xena at intersomitic boundaries. (**C-E**) In anterior regions (stage 18 embryo is shown), Xena and FN accumulate at presumptive somite boundaries prior to rotation. Arrow indicates accumulation at a newly formed boundary and arrowheads indicate accumulation at the next forming boundary. (**F**) In posterior regions (stage 22 embryo is shown), Xena accumulation at somite boundaries coincides with rotation. Arrow indicates accumulation at forming boundary; arrowhead shows lack of Xena enrichment in the PSM where the next boundary will form. (**G**) Cross-section of a stage 22 embryo showing Xena localization to cell-cell contacts within somites. (**H**) Xena is enriched at intermyotomal boundaries in stage 45 embryos. Anterior is towards the left in all images except G. Xena localization was visualized with antibodies raised against Mena (A,B) or Xena (C,E-H). Scale bars: 100 μm.

subcellular distribution of Xena using polyclonal antibodies raised against Xena (Xanthos et al., 2005) or monoclonal antibodies raised against mammalian Ena (Mena) (Lebrand et al., 2004) that crossreact with Xena (Xanthos et al., 2005). Both antibodies displayed identical staining patterns, and anti-Xena staining was blocked by pre-incubation with Xena peptide antigen, indicating that staining is specific for Xena (data not shown). In the PSM, Xena localizes to the cell periphery and is enriched at the PSM/notochord boundary (Fig. 1A). Xena persists at cell borders during somite rotation and becomes enriched at intersomitic junctions (Fig. 1B-F). Accumulation of Xena at presumptive intersomitic boundaries in anterior regions of the embryo is first observed prior to rotation at presumptive somite boundaries in the rostral-most region of the PSM. Enrichment of Xena appears coincident with the deposition of FN at these sites (Fig. 1C-E, arrow indicates formed boundary, arrowheads indicate newest forming boundary). Xena and FN often colocalize at nascent boundaries (Fig. 1E), although their staining patterns are sometimes discontinuous in a single focal plane. By contrast, Xena accumulation at intersomitic junctions in posterior regions appears to coincide with the formation of nascent boundaries during somite rotation (Fig. 1F). The differences in the timing of Xena accumulation at somite boundaries in anterior versus posterior regions suggests that different mechanisms may be at work to mediate somite formation in these two regions, as appears to be the case in zebrafish (Julich et al., 2005; Koshida et al., 2005). As development proceeds, Xena is found at cell-cell contacts within somites, continues to be enriched at intersomitic junctions, and later is enriched at intermyotomal junctions in tadpoles (Figs 1, 2). Xena colocalizes at intersomitic and intermyotomal junctions with components of cell-matrix adhesion complexes, including β_1-integrin (Fig. 2A-F), vinculin (Fig. 2G-L), FAK (Fig. 2M-O) and tenascin (data not shown).

With respect to the expression of other Ena/VASP family members in somites and muscle, *Xevl* is expressed in somites beginning at stage 25 (Wanner et al., 2005). *Xvasp* transcripts are provided maternally and are present throughout embryogenesis (data not shown). However, in situ hybridization analyses show that *Xvasp* mRNA does not appear to be expressed in the PSM, somites or myotome (data not shown). RT-PCR analysis of Ena/VASP expression in adult muscle reveals the presence of *Xena* transcripts in thigh and pectoral muscle, whereas *Xevl* and *Xvasp* transcripts are present at low levels in adult thigh and pectoral muscle (data not shown).

Dominant negative inhibition of Ena/VASP function during embryogenesis

The expression patterns of *Xena* and *Xevl* suggest that Ena/VASP proteins may play a role in somite and/or muscle development in *Xenopus*. To test this hypothesis, we neutralized the function of all Ena/VASP proteins using a dominant-negative construct containing four repeats of the EVH1-binding motif (FPPPP) linked to the ActA mitochondrial targeting sequence (FP_4-mito). This construct takes advantage of the highly specific binding of the EVH1 domain to the FPPPP ligand (Carl et al., 1999; Niebuhr et al., 1997) and has previously been shown to redirect Ena/VASP proteins from their normal localization to the surface of the mitochondria, effectively blocking the function of all Ena/VASP proteins in cultured cells (Bear et al., 2000; Bear et al., 2002) and mouse embryos (Goh et al., 2002). Importantly, this dominant-negative approach in conjunction with targeted injection of mRNAs into early *Xenopus* embryos allows for tissue-specific inhibition of all Ena/VASP family members, thereby alleviating potential problems with functional redundancy observed in mice. A similar construct containing a mutated binding motif (APPPP; AP_4-mito) shows a substantially lower affinity for EVH1 binding (Bear et al., 2000) and serves as a control. Both constructs are tagged with EGFP to allow visualization.

The efficacy of the FP_4-mito and AP_4-mito proteins in *Xenopus* was tested by injecting capped mRNA (500 pg) encoding these proteins unilaterally into four-cell stage *Xenopus* embryos just vegetal to the equator and ventral to the second cleavage furrow, which resulted in mosaic expression of the proteins almost exclusively in the somites and myotome. Injected embryos were raised to stage 22, fixed and co-stained for Xena and GFP. In cells expressing FP_4-mito, Xena was not visible at the cell periphery and instead was restricted to the cell body, where it co-localized with FP_4-mito-GFP (Fig. 3A-C). Adjacent cells that did not express FP_4-mito protein retained normal, cortical localization of Xena. AP_4-mito expression had little effect on Xena (Fig. 3D-F), causing only a mild and incomplete mis-localization of Xena when AP_4-mito was present at very high levels. Mis-localization of Xena by FP_4-mito was also observed in the blastocoel roof (BCR) of stage 12 embryos and the myotome (data not shown), and thus is predicted to neutralize Ena/VASP activity throughout embryonic development. These results, together with previously

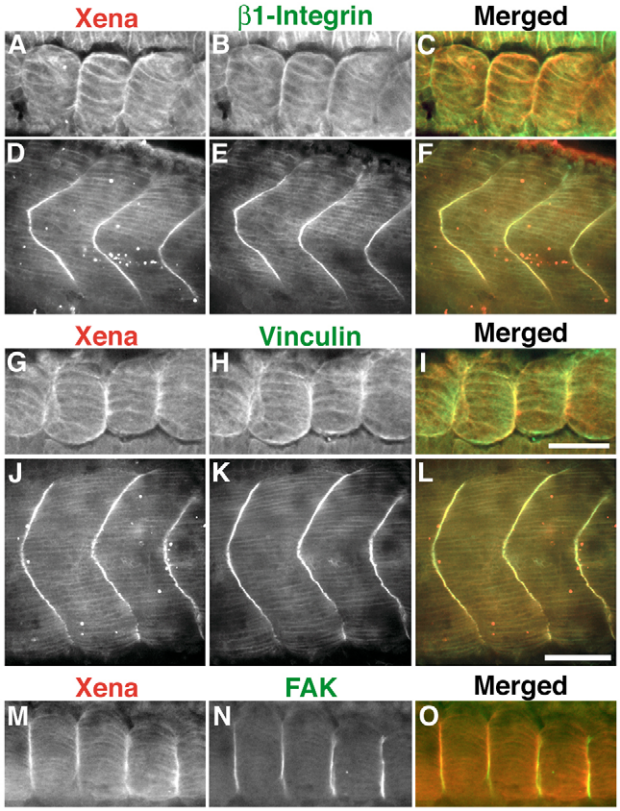

Fig. 2. Xena colocalizes with components of integrin adhesion complexes at intersomitic and intermyotomal junctions. Confocal images of stage 22 (A-C,G-I,M-O) and stage 35 (D-F,J-L) embryos stained for Xena (A,D,G,J,M; red in C,F,I,L,O), β1-integrin (B,E, green in C,F), vinculin (H,K, green in I,L) and FAK (N, green in O). Xena colocalizes with β_1-integrin, vinculin and FAK at intersomitic boundaries and with β_1-integrin and vinculin at intermyotomal junctions (co-localization appears yellow). Xena staining was performed with anti-Xena antibodies. Anterior is to the left in all images. Scale bars: 100 μm.

published reports (Bear et al., 2000; Bear et al., 2002; Goh et al., 2002), demonstrate that the FP_4-mito dominant negative provides an effective means to neutralize Ena/VASP function during development.

Ena/VASP function is not required for cell rearrangements in the PSM during gastrulation

Given the established role of Ena/VASP proteins in cell migration (Krause et al., 2003), we first analyzed whether neutralization of Ena/VASP activity impaired gastrulation movements of the PSM. Development of the PSM was assessed by immunostaining for β-tubulin, which stains cortical microtubules and facilitates visualization of cell morphology. We found that the distribution and morphology of cells in the PSM was unaffected by either AP_4-mito or FP_4-mito expression (Fig. 4A,B), suggesting that Ena/VASP function is not required for cell shape changes or movements that generate the PSM. To corroborate these findings, we used in situ hybridization to assess the expression of two markers of the PSM, *MyoD* (Hopwood et al., 1989) and *PAPC* (Kim et al., 2000). No difference in the intensity or distribution of these markers was observed in uninjected, AP_4-mito- or FP_4-mito-injected embryos (Fig. 4C-H), indicating that specification and patterning of the PSM is not dependent on Ena/VASP function.

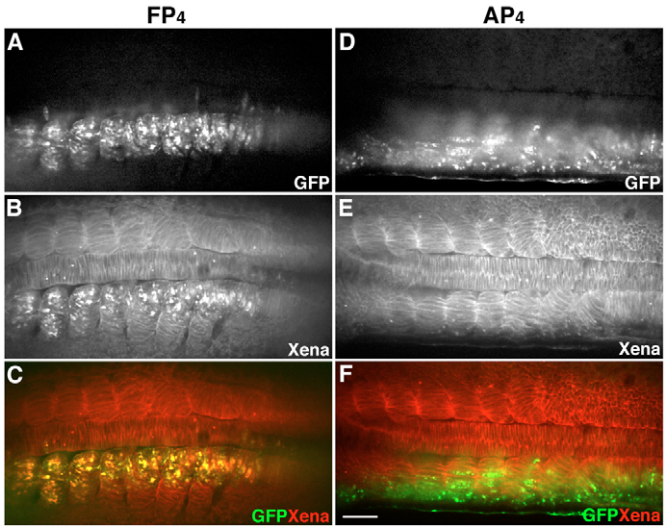

Fig. 3. FP_4-mito causes mis-localization of Xena in the PSM and somites. (**A-C**) Expression of FP_4-mito results in the mis-localization of Xena to the surface of mitochondria within the cell where it co-localizes with GFP. (**D-F**) Xena localization is unaffected in AP_4-mito-expressing cells. Embryos were double-stained for GFP to detect FP_4-mito (A, green in C) and AP_4-mito (D, green in F) and Xena (B,E, red in C,F). Anterior is towards the left. Scale bar: 100 μm.

Ena/VASP function is required for somitogenesis

To address whether Ena/VASP proteins are required for somitogenesis, FP_4-mito and AP_4-mito expressing embryos were analyzed for defects in somite formation by immunostaining for the somite/muscle marker 12/101 (Fig. 5), β1-integrin (Fig. 6) and FAK (data not shown). We found that somites of FP_4-mito injected embryos appeared disorganized with cells adopting random orientations (Fig. 5A,B,E, 93.9%, n=33; Fig. 6A-C). FP_4-mito expression also led to abnormal somite boundary formation, evidenced by the disruption of β1-integrin (Fig. 6A-C) and FAK (data not shown) staining, the presence of irregular somite borders, and the failure of cells to extend from one end of the somite to the other. Conversely, a significantly lower percentage of AP_4-mito-injected embryos showed only a mild disruption in somite morphology (Fig. 5C-E; 26.9%, n=26), which correlated with weak mis-localization of Xena at sites of high AP_4-mito protein. In addition, neither β1-integrin (Fig. 6D-F) nor FAK (data not shown) localization was disrupted by AP_4-mito. We were unable to examine potential changes in the actin cytoskeleton at high resolution because of the relative opacity of embryonic *Xenopus* cells and the requirement that embryos and explants are dehydrated and cleared prior to imaging, which precludes the use of phalloidin and many commercial antibodies for visualization of actin.

Further analysis of the defects associated with Ena/VASP inhibition revealed that the phenotype caused by Ena/VASP inhibition manifested as an expansion in somite area, as measured from digital tracings of cross-sectional images of 12/101 staining (Fig. 5F-I). Somite expansion was quantified by calculating the ratio between somite areas of injected and uninjected sides of the embryo. We found a statistically significant increase in somite area in FP_4-mito-injected embryos compared with both AP_4-mito injected and uninjected embryos (Fig. 5J). Closer examination revealed that much of this expansion was due to an increase in the number of cells that lay parallel or at oblique angles to the plane of the section, instead of the appropriate perpendicular orientation. Similar defects

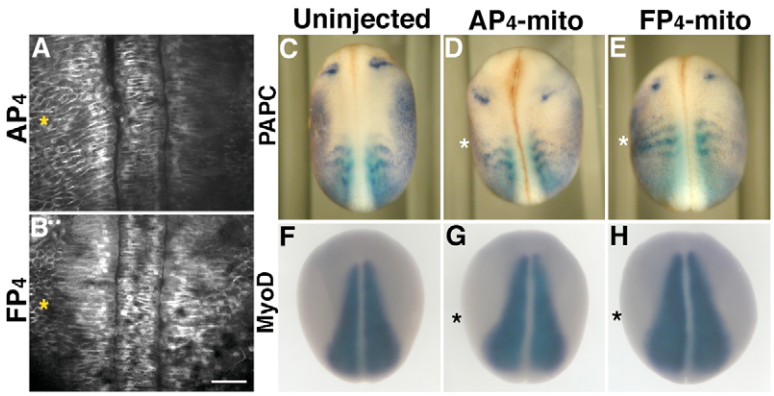

Fig. 4. Inhibition of Ena/VASP function does not perturb patterning of the PSM. (**A**,**B**) Embryos stained for β-tubulin, which outlines the periphery of cells, shows that expression of AP_4-mito (A) or FP_4-mito (B) does not alter the distribution or morphology of cells in the PSM following gastrulation. (**C-H**) Inhibition of Ena/VASP function does not affect the intensity or distribution of paraxial protocadherin (*PAPC*; C-E) or *MyoD* (F-H). Injected side is marked with an asterisk in each panel. Anterior is at the top. *PAPC*: AP_4-mito, *n*=10; FP_4-mito, *n*=17. *MyoD*: AP_4-mito, *n*=7; FP_4-mito, *n*=17. Scale bar: 100 μm.

in somitogenesis were observed in embryos expressing an EVH1-GFP dominant-negative protein (Eigenthaler et al., 2003; Vasioukhin et al., 2000), providing additional evidence that the phenotype is specific to inhibition of Ena/VASP function (data not shown).

FP_4-mito expression also resulted in disruption of myotomal junctions, as assessed by immunostaining of tenascin, β1-integrin and vinculin (data not shown). By contrast, AP_4-mito-injected embryos showed a significantly lower number of embryos displaying minor myotome dysmorphology, which correlated with weak mis-localization of Xena in cells expressing high levels of the AP_4-mito protein (data not shown). Together, these data suggest that Ena/VASP function is required for somite rotation and boundary formation, and that these defects lead to later myotomal abnormalities in tadpoles.

FN matrix assembly at somite boundaries is dependent on Ena/VASP function

Past studies show that a FN-rich matrix is assembled around each somite (Davidson et al., 2004; Koshida et al., 2005; Wedlich et al., 1989) and its deposition is required for somitogenesis in mice and zebrafish (George et al., 1993; Julich et al., 2005; Koshida et al., 2005). Thus, we examined FN localization to gain information about how Ena/VASP proteins might regulate somite formation. Immunostaining sections of stage 20 embryos for FN revealed a tendency of FP_4-mito-expressing PSM and somites to separate from the overlying dorsal epidermis and/or adjacent neural tube (Fig. 7A,B, arrows; 53.8%, *n*=26). In affected embryos, the FN matrix between somites and adjacent neural tube or notochord appeared fragmented and poorly defined (bracket in Fig. 7A), and an apparent shredding of the FN matrix was often observed between somites (arrowheads in Fig. 7A). This phenotype was rarely seen in AP_4-mito-injected embryos (Fig. 7C,D; 7.1%, *n*=14), and never observed in uninjected controls (*n*=14; data not shown). Furthermore, within sections where intersomitic junctions were discernible, we observed gaps in the FN matrix at somite borders of FP_4-mito-injected embryos (Fig. 7A, asterisks; 68.2%, *n*=22), whereas similar gaps were observed at a much lower frequency in AP_4-mito (30.8%, *n*=13) and uninjected embryos (14.3%, *n*=14).

Ena/VASP function is required for FN matrix assembly

Given the effect of Ena/VASP inhibition on the FN matrix, we used integrin α5β1-dependent FN fibril assembly on the blastocoel roof (BCR) as an assay to test whether Ena/VASP function is required for

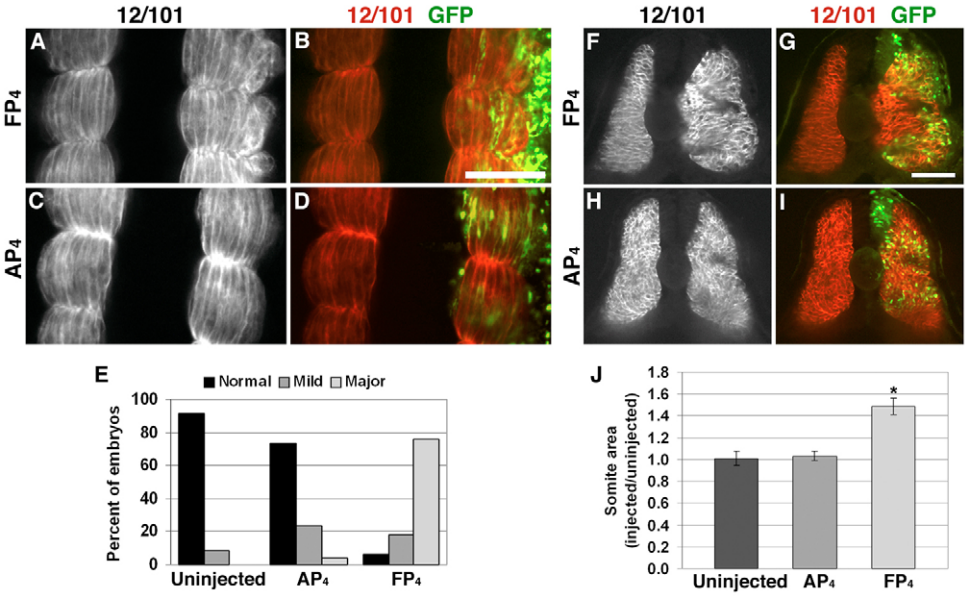

Fig. 5. Inhibition of Ena/VASP function disrupts somite rotation and leads to expanded somite area. (**A-D**) Dorsal explants of stage 22 embryos were co-stained for 12/101 (A,C; red in B,D) to visualize somite morphology and GFP (green in B,D) to visualize FP_4-mito and AP_4-mito expression. Expression of FP_4-mito (A,B) resulted in disruption of somite organization and cohesion, whereas somite morphology appeared normal in AP_4-mito (C,D) expressing embryos. Scale bar: 100 μm. (**E**) Percentage of stage 22 embryos showing normal somite morphology and mild or major disruption of somites, as assessed by 12/101 immunostaining. Results are pooled from three independent experiments; *n*=24-33 embryos/group. Anterior is at the top in A-D. (**F-I**) Somite area is increased and cells are misoriented in FP_4-mito (F,G), but not AP_4-mito (H,I) injected embryos. Scale bar: 100 μm. (**J**) Quantitative analysis of cross-sectional area of somites from uninjected, AP_4-mito or FP_4-mito injected embryos reveals that inhibition of Ena/VASP results in a significant increase in somite area (see Materials and methods). **P*<0.001 (Student's *t*-test). Error bars indicate s.e.m. Results shown in graph are from four independent experiments, *n*=10-14 embryos per treatment group.

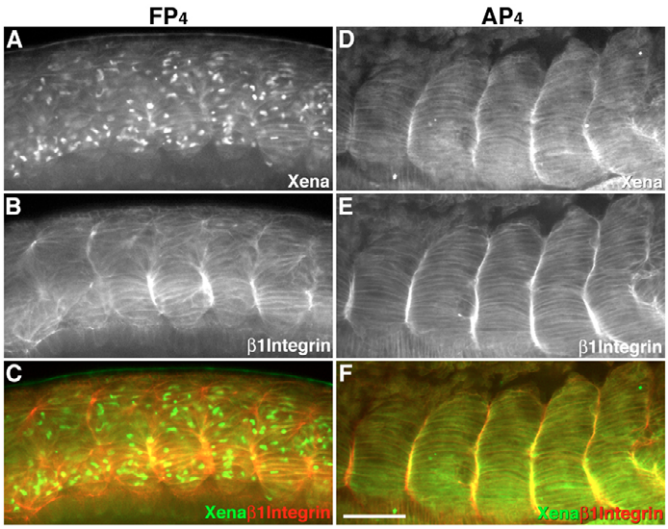

Fig. 6. Inhibition of Ena/VASP results in defects in somite rotation and boundary formation. Dorsal explants of stage 25 embryos expressing FP_4-mito (A-C) or AP_4-mito (D-F) were co-immunostained for Xena (A,D; green in C,F) and β1-integrin (B,E; red in C,F). (**A**-**C**) FP_4-mito expression leads to mis-localization of Xena that is accompanied by the presence of misoriented cells within somites and disruption of β1-integrin localization at intersomitic boundaries. (**D**-**F**) AP_4-mito expression does not alter Xena or β1-integrin localization, and is accompanied by normal somite formation. Scale bar: 100 μm.

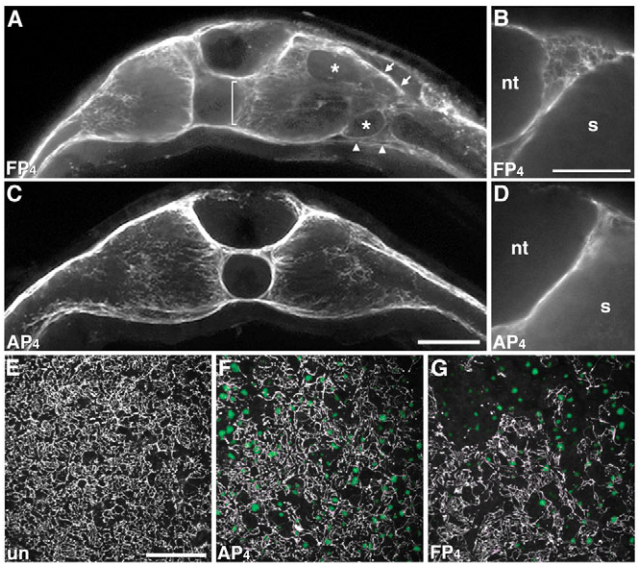

Fig. 7. Inhibition of Ena/VASP leads to disruption of the FN matrix. (**A-D**) Stacked *z*-series of FN staining reveals a fragmentation of the matrix (arrowheads), separation of somites from overlying ectoderm (arrows), gaps in the intersomitic FN matrix (asterisks) and loss of defined boundaries between the somite and neural tube or notochord in FP_4-mito (bracket) (A), but not AP_4-mito (C) injected embryos. Injected side is on the right. High magnification images of the boundary between the dorsal somite and neural tube reveals a disruption in the integrity of the FN matrix in FP_4-mito (B) but not AP_4-mito (D) injected embryos. (**E-G**) Stacked *z*-series show a dense FN matrix (white) on the interior BCR surface of an uninjected embryo (green, E) and an embryo injected with AP_4-mito (green, F). By contrast, expression of FP_4-mito (G) blocks FN matrix assembly as revealed by a thin or absent FN matrix in areas of GFP signal. s, somite; nt, neural tube. Scale bars: 100 μm.

integrin-dependent FN matrix assembly. During gastrulation, cells lining the inner surface of the BCR assemble a dense FN matrix (Winklbauer and Stoltz, 1995), the formation of which can be blocked by antibodies raised against α_5- and β_1-integrin, or FN (Davidson et al., 2002; Marsden and DeSimone, 2001; Ramos and DeSimone, 1996). Thus, this event provides a simple model with which to investigate the regulation of integrin-mediated FN fibrillogenesis. In uninjected embryos, FN covered the BCR in a dense matrix (Fig. 7E; n=10). This pattern was recapitulated in BCRs expressing AP_4-mito, although in areas of high GFP expression a mild thinning was observed (Fig. 7F, n=8). By contrast, all FP_4-mito explants displayed a marked disruption of the FN matrix that coincided with regions of FP_4-mito expression (Fig. 7G, n=17). We also found that gastrulation was delayed in FP_4-mito-expressing embryos, suggesting that disruption of the FN matrix affects mesendoderm migration across the BCR during gastrulation. Thus, Ena/VASP function is required for FN fibrillogenesis in vivo.

Ena/VASP function is required for spreading of somitic cells on FN

Next, we tested whether Ena/VASP function is required for adhesion and/or spreading of somitic cells on FN. In *Xenopus*, all early embryonic cells are able to adhere to FN, but mesodermal cells acquire the ability to spread and migrate on FN during gastrulation in a process that requires integrin activation (Ramos et al., 1996). To investigate whether Ena/VASP proteins modulate adhesion of somitic cells to FN, stage 20 dorsal explants from uninjected, AP_4-mito or FP_4-mito-injected embryos were dissociated, and triplicate samples of cells were allowed to adhere to FN coated coverslips for 30 minutes. The number of adherent cells was then counted before and after washing. This analysis revealed a slight, but non-significant, decrease in adhesion of FP_4-mito expressing cells compared with AP_4-mito and uninjected cells (data not shown).

To assess whether Ena/VASP function is required for spreading of somitic cells on FN, dissociated somitic cells expressing either FP_4-mito or AP_4-mito were incubated overnight on FN coated coverslips. The coverslips were gently washed to remove non-adherent cells, fixed and stained with 12/101 and anti-GFP antibodies to identify cells expressing the FP_4-mito and AP_4-mito proteins. Spreading was then quantified by counting the numbers of cells that adhered and flattened on the substrate (cells with two or more discernible points of adhesion) versus those that remain rounded. We found that 49% (n=350) of 12/101/FP_4-mito-positive

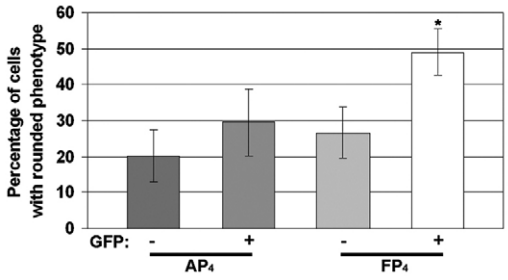

Fig. 8. Inhibition of Ena/VASP function impairs spreading of somitic cells on FN. Percentage of cells with a rounded phenotype from uninjected embryos and embryos injected with AP_4-mito or FP_4-mito with (+) or without (–) detectable GFP signal show a significant increase in the occurrence of a rounded phenotype in cells expressing FP_4-mito. *P<0.05 (Student's t-test). Error bars indicate s.e.m. Results are from four independent experiments, n=864-888 cells per treatment group.

cells failed to spread and displayed a rounded phenotype, whereas 29.6% (n=295) 12/101/AP_4-mito-positive cells displayed a rounded phenotype (Fig. 8). Similar numbers of rounded cells were also seen for control 12/101-positive/GFP-negative cells derived from AP_4-mito- or FP_4-mito-injected embryos (20.1%, n=569, and 26.7%, n=538, respectively), and for cells derived from uninjected embryos (data not shown).

Ena/VASP function is required for FAK activation

The requirement for Ena/VASP function in cell spreading and FN matrix assembly suggests that Ena/VASP proteins might participate in modulating integrin activation and/or signaling during somitogenesis. Integrin binding to FN leads to phosphorylation and activation of FAK, which is enriched at intersomitic boundaries (Crawford et al., 2003; Henry et al., 2001; Hens and DeSimone, 1995) and is required for FN fibrillogenesis in cultured mammalian cells (Ilic et al., 2004). Given these results, we tested whether FAK activation, as assessed by levels of autophosphorylation at tyrosine 397, is blocked by inhibition of Ena/VASP activity. Protein lysates prepared from uninjected stage 20 dorsal explants or explants expressing either AP_4-mito or FP_4-mito were blotted and probed with anti-FAK pY397 antibodies. We found that expression of FP_4-mito caused a significant decrease in the levels of phospho-FAK, when compared with AP_4-mito or uninjected control explants (Fig. 9).

FAK is required for somite formation and FN matrix deposition

The colocalization of Xena and FAK in somites and the correlation between Ena/VASP activity and FAK autophosphorylation, suggests that Ena/VASP and FAK proteins may function in a common pathway to regulate somite formation. To investigate this idea, we performed FAK loss-of-function studies to determine the requirement for FAK in somite formation in *Xenopus*. FAK activity was blocked by injection of mRNA encoding FAK-related non-kinase (FRNK), a naturally occurring dominant-negative form of FAK that can inhibit autophosphorylation, phosphorylation of downstream substrates and cell migration (Gilmore and Romer, 1996; Ilic et al., 1998; Richardson et al., 1997; Richardson and Parsons, 1996; Sieg et al., 1999; Slack et al., 2001; Taylor et al., 2001). The relative expression levels and activity of FRNK were first examined by western blot analysis, which demonstrated that injection of 500 pg of FRNK mRNA resulted in expression of FRNK at levels similar to that of endogenous FAK (Fig. 10A). We also found that FRNK expression resulted in a significant decrease in autophosphorylation of FAK (Fig. 10B), indicating that FRNK is an effective inhibitor of FAK activity in vivo.

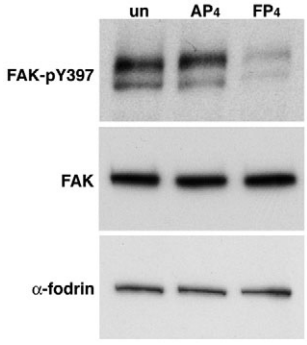

Fig. 9. Inhibition of Ena/VASP function leads to a decrease in FAK-Y397 phosphorylation. Protein lysates prepared from stage 20 dorsal explants were probed with anti-FAK-pY397, anti-FAK, or anti-α-fodrin antibodies. Expression of FP_4-mito caused a significant decrease in the levels of FAK-pY397.

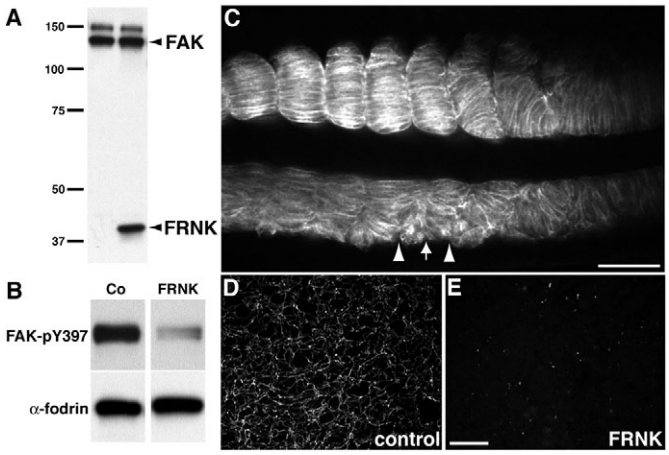

Fig. 10. FAK is required for somitogenesis and FN matrix deposition. (**A**) Immunoblot showing levels of FAK and FRNK in control (left lane) and FRNK-injected (right lane) embryos. (**B**) FRNK expression inhibits autophosphorylation at Y397; α-fodrin serves as a loading control. (**C**) Inhibition of FAK results in somite defects including misorientation of cells (arrow) and impaired somite boundary formation (arrowheads). FRNK-injected side is at the bottom and anterior is to the left. (**D**,**E**) Immunostaining of FN matrix in control (D) and FRNK-injected (E) BCRs show that FAK is required for FN matrix deposition. Scale bars: 100 μm.

To test the requirement for FAK in somitogenesis, 500 pg of FRNK mRNA was injected unilaterally at the four-cell stage into regions fated to become somites. FRNK expression led to defects in somite rotation, evidenced by the presence of misoriented cells and disruption of intersomitic boundaries (Fig. 10C; 83%, n=18). Next, we tested whether FAK is required for FN matrix assembly in the BCR. We found that in contrast to uninjected or GFP-injected BCRs (Fig. 10D; n=9), FRNK expression blocked FN matrix assembly in the BCR (Fig. 10E; n=12). Thus, FAK is required for somite formation and FN matrix deposition in *Xenopus*. In addition, the similarity of the phenotypes observed in FRNK and FP_4-mito-injected embryos supports the idea that FAK and Ena/VASP function in a common pathway to regulate FN matrix assembly and somitogenesis in *Xenopus*.

FAK modulates Xena localization

To further explore the relationship between Ena/VASP and FAK, we examined whether FAK regulates the subcellular distribution of Xena. In these experiments, 500 pg of FRNK mRNA was injected into the animal pole region of two-cell stage embryos, animal caps were harvested at stage 10 and Xena localization was determined by confocal microscopy. In GFP-injected animal caps, Xena is enriched and tightly localized to the cell cortex (Fig. 11A; n=5), whereas in FRNK-injected caps Xena displays a more diffuse cortical staining pattern (Fig. 11B; n=7). Comparison of the ratios of pixel intensities at membrane versus juxtamembrane regions of representative cells from control and FRNK-injected animal caps demonstrate that inhibition of FAK results in a significant decrease in membrane-associated Xena staining (Fig. 11C-E; GFP=1.26±0.08, FRNK=1.11±0.05, n=50 cells per treatment). Overall levels of Xena

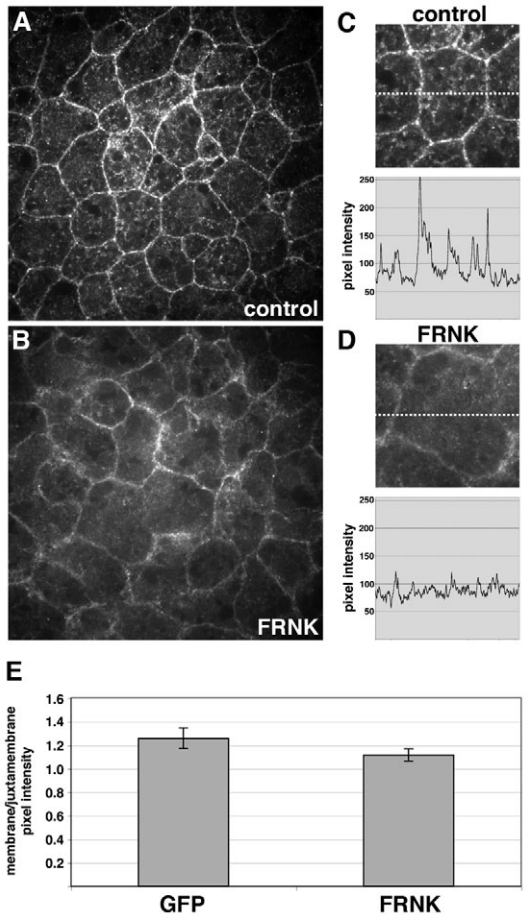

Fig. 11. Inhibition of FAK alters Xena localization. (**A**) Xena is enriched at the cortex in control animal caps. (**B**) Inhibition of FAK results in decrease in the levels of Xena at the cortex. (**C**) Plot of pixel intensity in control animal caps showing enrichment of Xena at the cortex. (**D**) Plot of pixel intensity in FRNK-injected animal caps reveals that Xena is de-localized from the cortex and displays a more uniform distribution. (**E**) Quantitative analysis of Xena staining in control (GFP) and FRNK injected BCRs. For 10 randomly selected cells, pixel intensity was measured along a line extending across the region of cell-cell contact. Average pixel intensity at the membrane (two peak intensities 0.3 μm apart) was compared with the average pixel intensity of the juxtamembrane region (3.3 μm adjacent to membrane). The ratio of the average membrane intensity to average juxtamembrane intensity was significantly lower for FRNK-expressing embryos. $P<0.001$ (Student's *t*-test). Error bars indicate s.d. $n=50$ cells per treatment.

were not affected by FRNK expression, indicating that the observed redistribution is not due to altered levels of Xena (data not shown). These data suggest that FAK regulates Xena localization and provides further evidence that Ena/VASP proteins and FAK functionally interact in vivo.

DISCUSSION

One of the major events in vertebrate development is the subdivision of the paraxial mesoderm into serially repeated units, called somites. Somitogenesis in *Xenopus* is characterized by the 90° rotation of blocks of cells in the rostral region of the presomitic mesoderm to form somites, which primarily differentiate into myotome. The molecular regulation of cell rearrangements during somitogenesis involves changes in the adhesive and migratory properties of cells. Cells must break adhesive interactions at the somite/notochord boundary, acquire polarized motile behavior and dynamically regulate adhesions during rotation to allow for cell rearrangements while maintaining tissue integrity. Following rotation, cells re-establish stable cell-matrix adhesions at the nascent intersomitic boundary. Here, we have used targeted expression of dominant-negative proteins that neutralize Ena/VASP and FAK activity to investigate the mechanisms that control morphological segmentation of the somitic mesoderm. We found that inhibition of Ena/VASP or FAK leads to abnormal somite rotation and failure of intersomitic boundary formation. With respect to the mechanism by which Ena/VASP and FAK control somitogenesis, we found that inhibition of Ena/VASP disrupts integrin-dependent processes, including cell spreading on FN, assembly of the FN matrix and activation of FAK. Neutralization of FAK also blocks FN matrix assembly and disrupts localization of Xena to the cell cortex. Together, these data support a model in which Ena/VASP proteins and FAK regulate somite formation by modulating integrin activity and integrin/FN interactions to govern the migratory and adhesive properties of cells during somitogenesis.

How might Ena/VASP proteins and FAK regulate integrin-dependent adhesion and migration during somitogenesis? The most obvious role for Ena/VASP proteins is as a molecular link between cell-surface integrins and the actin cytoskeleton. Previous studies have shown that Ena/VASP proteins localize to focal adhesions and bind F-actin, as well as several components of integrin adhesion complexes, including vinculin and zyxin (Brindle et al., 1996; Drees et al., 1999). Thus, Ena/VASP proteins are appropriately positioned to act as a key regulatory link between integrins and the actin cytoskeleton to orchestrate changes in adhesive strength and cell motility during somitogenesis. By modulating the link between integrins and actin, Ena/VASP proteins could modulate integrin clustering and formation of juxtamembrane adhesion complexes. Such a role for Ena/VASP may also explain how Ena/VASP proteins regulate FAK autophosphorylation, as FAK activity is dependent on integrin clustering and targeting of FAK to focal adhesions (Hagel et al., 2002; Shen and Schaller, 1999).

It is widely accepted that FAK transmits signals to a variety of targets to govern focal adhesion remodeling associated with changes in cell adhesion and movement (Schlaepfer et al., 2004). Thus, the phenotypes caused by inhibiting FAK function support the idea that integration of integrin-dependent events by FAK is required for somitogenesis in *Xenopus*. Specifically, FAK might facilitate transduction of integrin signals into local changes in cell motility, adhesive strength and FN matrix assembly/patterning. Interestingly, we found that FAK activity correlates with localization of Xena to the cortex of cells in the BCR, although it is not known whether Xena is a direct or indirect target of FAK. These data support the idea that Xena and FAK functionally interact during somitogenesis and predict that Xena would be enriched at sites where FAK activity is high. In agreement with this notion, Xena and FAK co-localize at somite boundaries and phosphorylated (active) FAK is enriched at intersomitic boundaries (Crawford et al., 2003; Henry et al., 2001).

A second potential mechanism by which Ena/VASP proteins and FAK might regulate cell behaviors during somitogenesis is through inside-out activation of integrin adhesion and FN matrix assembly. Inside-out regulation of integrin activity is an important mechanism underlying changes in cell adhesion and movements that drive morphogenesis (Coppolino and Dedhar, 2000; Miranti and Brugge, 2002). Studies in *Xenopus* have shown that developmentally regulated changes in integrin activity govern a variety of morphogenetic behaviors, including initiation of gastrulation

movements, spreading of mesodermal cells on FN and FN matrix assembly (Marsden and DeSimone, 2003; Na et al., 2003; Ramos and DeSimone, 1996; Ramos et al., 1996). Here, we show that Ena/VASP and FAK are required for FN matrix assembly in the BCR and inhibition of Ena/VASP function leads to disruption of the FN matrix surrounding somites and blocks spreading of somitic cells on FN. Our interpretation of these results is that Ena/VASP proteins and FAK mediate inside-out regulation of integrin activity during somitogenesis.

A number of studies have shown that inside-out activation of integrins and integrin-mediated FN fibrillogenesis is dependent on an intact actin cytoskeleton (Pankov et al., 2000; Wu et al., 1995; Zaidel-Bar et al., 2003). Thus, it seems likely that the underlying cause of defective FN matrix assembly in Ena/VASP and FAK inhibited embryos is disruption of cytoskeletal organization and linkages between the actin cytoskeleton and cell-surface integrins. Consistent with this idea, $FAK^{-/-}$ cells display defects in actin stress fiber organization and integrin-mediated FN matrix assembly and patterning (Ilic et al., 2004). Likewise, loss of Ena/VASP function would be predicted to disrupt actin dynamics leading to dysregulation of integrin activity. In support of this idea, roles for Ena/VASP proteins in the regulation of integrin-mediated adhesion have been reported, although these studies reveal that the function of Ena-VASP proteins in cell adhesion may be cell-type dependent. In osteoclasts, VASP function was found to correlate with αvβ3-integrin adhesion and redistribution of VASP was linked to increased cell motility (Yaroslavskiy et al., 2005). Ena/VASP activity also correlates with T-cell receptor-mediated actin remodeling and integrin activation in lymphocytes (Griffiths and Penninger, 2002; Krause et al., 2000). Moreover, *Dictyostelium* cells lacking VASP show defects in cell migration that are attributed to the inability of VASP-null cells to properly adhere to the substratum (Han et al., 2002). However, knockout studies in mice have shown that VASP negatively regulates αIIbβ3-integrin activity adhesion in platelets (Aszodi et al., 1999; Hauser et al., 1999). Thus, a clear connection exists between Ena/VASP, FAK and integrins, although further studies are required to elucidate the precise mechanisms by which Ena/VASP and FAK regulate integrin activity during somitogenesis.

If Ena/VASP proteins and FAK work through integrins to control somite formation, then one would expect that loss of integrin function would be associated with defects in migration and adhesion during somitogenesis. Consistent with this idea, studies in several systems have demonstrated a requirement for integrins in somite formation or maintenance (Drake et al., 1992; Goh et al., 1997; Julich et al., 2005; Koshida et al., 2005; Krotoski and Bronner-Fraser, 1990; Yang et al., 1993; Zagris et al., 2004). Furthermore, studies in *Xenopus* have shown that expression of a dominant-negative form of β1-integrin blocks FN matrix assembly and results in marked defects in somite formation (Marsden and DeSimone, 2003). In addition, inhibition of α5-integrin with a function-blocking antibody results in abnormal segmentation and the loss of intersomitic boundaries (B. Hoffstrom and D. DeSimone, personal communication). Loss of α5-integrin function, however, does not appear to affect somite rotation, as most cells appear to orient themselves properly with their long axis parallel to the anteroposterior axis. The observation that initial somite morphogenesis appears to occur normally following loss of α5-integrin function is consistent with genetic studies in zebrafish showing that itga5 is not required for somite formation, but is required for maintenance of somite boundaries (Julich et al., 2005; Koshida et al., 2005). These data contrast phenotypes associated with inhibition of Ena/VASP, FAK and β1-integrin (Marsden and DeSimone, 2003) where somite formation is impaired. One potential explanation for these differences would be that additional α-integrin subunits, such as α3- or α6-integrin, might play essential roles in somite formation.

Additional mechanisms by which Ena/VASP proteins could govern somitogenesis that are consistent with our data include regulation of polarized protrusive activity and cell-cell adhesion. During somite formation in *Xenopus*, cells display polarized protrusive behavior which is thought to help drive rotation (Wilson et al., 1989). Ena/VASP proteins are known to bind the barbed-ends of actin filaments to promote actin polymerization and filopodia formation at the leading edge, activities that could contribute to the protrusive behavior of cells during somite formation. In addition, the localization of Xena to cell-cell contacts in the PSM and somitic mesoderm suggests a potential role for Ena/VASP proteins in cell-cell adhesion during somitogenesis. Evidence from several model systems underscores the importance of cadherin-based adhesion in somite formation (Giacomello et al., 2002; Horikawa et al., 1999; Kim et al., 2000; Linask et al., 1998) and of Ena/VASP proteins in modulating cadherin function (Grevengoed et al., 2003; Grevengoed et al., 2001; Vasioukhin et al., 2000). In *Xenopus*, inhibition of cadherin function results in misorientation of cells and overall disorganization of the myotome (Giacomello et al., 2002), defects similar to those caused by neutralization of Ena/VASP function. These observations leave open the possibility that Ena/VASP proteins are required for modulating cell-cell adhesion during somite formation. Interestingly, Marsden and DeSimone (Marsden and DeSimone, 2003) have shown that integrins regulate cadherin adhesion during gastrulation, raising the possibility that Ena/VASP proteins may indirectly regulate cadherin function during somitogenesis by influencing integrin activity. Addressing potential roles for Ena/VASP proteins in regulating protrusive activity and cell-cell adhesion during somitogenesis will be one of our next challenges.

The results presented in this paper indicate that Ena/VASP proteins and FAK are key components of the molecular machinery that drives somite formation in *Xenopus*. Moreover, our data indicates an important role for Ena/VASP proteins and FAK in the modulation of integrin activity during somitogenesis. Despite differences in the cellular behaviors that accompany somitogenesis among vertebrates, the molecular pathways that control morphological segmentation appear to be conserved (Holley and Nusslein-Volhard, 2000; Keller, 2000; Pourquie, 2000; Pourquie, 2001; Stickney et al., 2000). In particular, the dynamic regulation of integrin-mediated adhesion and migration appears to play crucial roles in coordinating cell behaviors during somitogenesis (Drake et al., 1992; Goh et al., 1997; Julich et al., 2005; Koshida et al., 2005; Krotoski and Bronner-Fraser, 1990; Yang et al., 1993; Zagris et al., 2004). Thus, our studies help set the stage for future experiments that will be needed to determine the precise molecular mechanisms regulating somite formation.

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