RESEARCH ARTICLE

Eph-Ephrin signaling and focal adhesion kinase regulate actomyosin-dependent apical constriction of ciliary band cells

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ABSTRACT
Apical constriction typically accompanies inward folding of an epithelial sheet. In recent years there has been progress in understanding mechanisms of apical constriction and their contribution to morphogenetic processes. Sea urchin embryos form a specialized region of ectoderm, the ciliary band, which is a strip of epithelium, three to five cells wide, encircling the oral ectoderm and functioning in larval swimming and feeding. Ciliary band cells exhibit distinctive apical-basal elongation, have narrow apices bearing a cilium, and are planar polarized, so that cilia beat away from the mouth. Here, we show that filamentous actin and phosphorylated myosin light chain are uniquely distributed in ciliary band cells. Inhibition of myosin phosphorylation or actin polymerization perturbs this distribution and blocks apical constriction. During ciliary band formation, Sp-Ephrin and Sp-Eph expression overlap in the presumptive ciliary band. Knockdown of Sp-Eph or Sp-Ephrin, or treatment with an Eph kinase inhibitor interferes with actomyosin networks, accumulation of phosphorylated FAK (pY397FAK), and apical constriction. The cytoplasmic domain of Sp-Eph, fused to GST and containing a single amino acid substitution reported as kinase dead, will pull down pY397FAK from embryo lysates. As well, pY397FAK colocalizes with Sp-Eph in a JNK-dependent, planar polarized manner on latitudinal apical junctions of the ciliary band and this polarization is dissociable from apical constriction. We propose that Sp-Eph and pY397FAK function together in an apical complex that is necessary for remodeling actomyosin to produce centripetal forces causing apical constriction. Morphogenesis of ciliary band cells is a unique example of apical constriction in which receptor-mediated cell shape change produces a strip of specialized tissue without an accompanying folding of epithelium.

KEY WORDS: Morphogenesis, Apical constriction, Eph-ephrin signaling, Focal adhesion kinase, Planar cell polarity, Ciliary band, Sea urchin

INTRODUCTION
Apical constriction is a cellular shape change that reduces apical surface area, producing bottle-shaped cells. This process is fundamental to morphogenetic movements of cells and cellular sheets that are integral to embryogenesis. It occurs coordinately in specific cell groups and is crucial to events such as gastrulation (Sweeton et al., 1991), neurulation (Nagele and Lee, 1987) and formation of specialized epithelia (Pilot and Lecuit, 2005; Sawyer et al., 2010). The mechanisms for achieving apical constriction appear to vary between organisms and even cell types within an organism (Sawyer et al., 2010), therefore characterizing these complex regulatory mechanisms is crucial in developing a generalized understanding of morphogenesis.

It is widely accepted that actin filaments interact with myosin II motor units to provide mechanical force for apical constriction, and live imaging reveals concomitant organizational changes of actomyosin networks in apically constricting cells (Hildebrand, 2005; Lee and Harland, 2007). One of the most elaborate models for apical constriction occurs during Drosophila ventral furrow formation where the process is coordinated by a subcellular, ratcheting mechanism that produces a series of local, pulsed contractions in the supracellular, actomyosin meshwork; incrementally reducing apical surface area (Martin et al., 2009). Although constriction of the apical surface occurs in pulses, contraction and cortical tension within the actomyosin network occur prior to, and in the absence of, apical surface area reduction. This implies a regulatory mechanism controlling transient linkage of tensioned actomyosin networks to contact zones on the membrane surface (Roh-Johnson et al., 2012).

Echinoderm embryos have a distinctive ectodermal structure, the ciliary band. This region is a continuous, three- to five-cell wide, band of cells encircling the oral field and forming a boundary between oral and aboral ectoderm. The ciliary band, like most ectoderm, is specified in late cleavage as a consequence of TGFβ signaling (Angerer et al., 2000; Duboc et al., 2004). Nodal is expressed ventrally and establishes oral ectoderm whereas BMP2/4, also expressed ventrally, acts with BMP5/8 to specify aboral ectoderm (Lapraz et al., 2009; Ben-Tabou de Leon et al., 2013). A band of cells between the major ectoderm domains is protected from this signaling and adopts the default state of becoming ciliary band ectoderm (Saudemont et al., 2010; Bradham et al., 2009; Yaguchi et al., 2010). The presumptive ciliary band cells are distinguished by expression of Hnf6, a putative core element of the gene regulatory network that specifies ciliary band cells (Otton et al., 2004; Poustka et al., 2004). Each ciliary band cell bears a cilium and their coordinated action provides feeding and locomotory functions for the larva (Strathmann, 1971; Strathmann et al., 1972). The cilia normally beat with their power stroke directed away from the oral field (Strathmann, 2007) and are functionally polarized in the plane of the epithelium. In Strongylocentrotus purpuratus, the ciliary band is first apparent at ~60 hours of development (Strathmann, 1971) when cells apparently reduce their apical surface area and become bottle shaped (Burke, 1978). The mechanism of this shape change has not been explored.

The Eph receptor tyrosine kinases and their Ephrin ligands constitute the largest class of receptor tyrosine kinases in vertebrates. They are cell-surface molecules with roles in diverse biological processes, although they typically function at the interface of patterning and morphogenesis. Eph and Ephrin function in adhesion and regulate cytoskeletal organization by influencing regulatory protein complexes (Wilkinson, 2000; Klein, 2012). These include
interaction with focal adhesion kinase (FAK) (Carter et al., 2002), a non-receptor tyrosine kinase that regulates many cellular functions and has long been identified as a cytoskeletal regulator (Arold, 2011). Recent data identify FAK as an effector of Eph-Ephrin signaling, remodeling the cytoskeleton through recruitment and activation of Src-family kinases (Thomas et al., 1998; Parri et al., 2007; Shi et al., 2009; Darie et al., 2011).

Here, we describe ciliary band morphogenesis in the developing sea urchin embryo. The ciliary band forms in the embryonic region where ectodermal expression domains of Sp-Eph and Sp-Ephrin overlap. We show that apical constriction is independent of cell division, and loss-of-function experiments indicate that actin, myosin, Eph-Ephrin signaling and FAK are necessary for apical constriction. We propose that Eph-Ephrin signaling in the ciliary band provides a proximate cue initiating formation of a planar polarized, FAK-containing complex that regulates apical constriction in ciliary band cells. Apical constriction of ciliary band cells is a distinctive model in which there is no inward folding of epithelium.

RESULTS
Apical surface area of ciliary band cells

The shape of ciliary band cells suggests apical constriction may be a feature of their development and we investigated whether apical surface area of ciliary band cells decreases during ciliary band formation. Between 48 and 96 hours of development, the ectoderm is transformed from a uniform sheet of cells into clearly defined regions of oral, aboral and ciliary band (Fig. 1). At 48 hours there is no measurable difference in surface area between ciliary band cells and non-ciliary band cells (Fig. 1A,B,E). At 55 hours, Hnf6-positive cells have a noticeable reduction in their surface area (not shown). Over the next 17 hours the surface area of ciliary band cells is reduced by roughly one half; the majority of this occurring between 60 and 72 hours (Fig. 1A,B,E). Reduction in surface area is largely completed after 96 hours (Fig. 1D'). When viewed in cross section, ciliary band cells change their shape from having almost equal width and depth (Fig. 1F) to bottle-shaped (Fig. 1F'). This shape change appears to be an important event in ciliary band formation and we focused on identifying the underlying mechanism.

Change in surface area in the absence of cell division

To assess whether reduction in apical surface area of ciliary band cells is due to localized cytokinesis, we inhibited DNA polymerase at 60 hours using aphidicolin and cultured embryos in the presence of 5-ethynyl-2'-deoxyuridine (EdU) to label newly synthesized DNA and confirm inhibition of cytokinesis as an indirect effect. After 72 hours development, ciliary band cells in control embryos reduce their apical surface area (Fig. 2A) and incorporate EdU into their nuclei (Fig. 2A), indicating DNA synthesis and subsequent cell division. Although aphidicolin completely blocks DNA synthesis (Fig. 2B) and indirectly prevents cytokinesis, ciliary band cells in treated embryos also appear to apically constrict (Fig. 2B'). Apical surface area measurements of ciliary band cells in embryos in which cell division is blocked and control embryos are not significantly different (P=0.062; Fig. 2C), indicating that reduction of apical surface area occurs independently of cytokinesis.
Actin cytoskeleton of the ciliary band

At 35 hours, actin and phosphorylated myosin light chain (pS19MLC) are distributed uniformly around the apical margin of ciliary band cells and colocalize with apical junction components (Fig. 3A-A″). Beginning at about 40 hours, actin and pS19MLC are not associated solely with cell junctions (Fig. 3B-B″) but are distributed throughout the apical cortex of ciliary band cells. At 72 hours, pS19MLC is in discontinuous patches at the cell periphery and actin is dispersed in strands and patches in the apical cortex of ciliary band cells (Fig. 3C-C″). This rearrangement suggests a role during apical surface area reduction in ciliary band cells.

We hypothesized that the actomyosin network provides some of the mechanical force that contributes to apical constriction and that interfering with actomyosin contractility would lead to a loss of apical constriction in ciliary band cells. We tested this using cytochalasin D (Miyoshi et al., 2006) to disrupt actin filaments (Fig. 4A-A″), or ML 7 (Uehara et al., 2008) to inhibit myosin light chain kinase (Fig. 4B-B″). Cytochalasin D-treated embryos are rounded with loosely packed cells expressing Hnf6 (Fig. 4A) and distribution of actin and pS19MLC are perturbed (Fig. 4A′,A″). Specifically, pS19MLC occurs in circular structures in the ciliary band (Fig. 4A″, arrows and inset). Similarly, actin networks in the ciliary band are discontinuous and appear as hollow circles (Fig. 4A″, arrows and inset). Embryos treated with ML 7 are rounded and Hnf6-expressing cells are loosely packed (Fig. 4B). Latitudinal distribution of pS19Myo is irregular (Fig. 4B′, arrowheads and inset). Similarly, actin networks are irregular and
discontinuous in these embryos (Fig. 4B″) and apical cortices often lack actin accumulation. As can be seen from Fig. 4C ciliary band cells in embryos treated with ML 7 (dotted line) or cytochalasin D (dotted line) are less apically constricted than those of control embryos (solid line). This indicates polymerization of actin filaments and phosphorylation of myosin light chain are necessary for apical constriction of ciliary band cells.

**Ectodermal expression of Sp-Eph and Sp-Ephrin and interaction with FAK**

Preliminary data indicated that Sp-Eph and Sp-Ephrin were expressed in ectoderm beginning at gastrulation. Further analysis using antibodies against pY397FAK and Sp-Eph indicated that Sp-Eph is expressed throughout the oral and ciliary band ectoderm (Fig. 5E,F) and becomes phosphorylated in the ciliary band during apical constriction (Fig. 5A-B″; ciliary band boundary to oral ectoderm marked by arrowheads, boundary to aboral ectoderm marked by arrows). In the ciliary band, beginning at 48 hours, Sp-Eph is polarized to latitudinal membranes in some areas of the ciliary band (Fig. 5E,F, arrows). This contrasts with the expression in the oral ectoderm, where Sp-Eph is expressed uniformly around the periphery of cells (Fig. 5E, asterisks). Antibodies against Sp-Ephrin indicate that the protein is expressed on aboral ectoderm and the ciliary band (Fig. 5A,A′;B,B′). Expression of Sp-Ephrin could not be detected in the oral ectoderm during ciliary band formation (Fig. 5B,B′). The overlapping expression of Sp-Eph and Sp-Ephrin in the region in which the ciliary band is forming and the detection of the phosphorylated form of the Sp-Eph receptor suggests that Sp-Eph is signaling in presumptive ciliary band cells. A ciliary band expression pattern similar to that of Sp-Eph is observed using pY397FAK antibody (Fig. 5C,E,F″), and these colocalize during apical constriction (Fig. 5E,F″) suggesting an interaction.

Following these observations, we investigated interactions between Sp-Eph and pY397FAK in vitro using a GST-tagged, cytoplasmic domain (322-762) of native Sp-Eph (Fig. 5D). This protein failed to reliably pull down pY397FAK in epithelial cell lysate from 72-hour embryos (Fig. 5D, lower panel). Because kinase domains often bind substrates transiently, releasing them upon phosphorylation, we created a kinase-dead form of this construct as a substrate trap (Roos et al., 2005) and we found it pulls down pY397FAK (Fig. 5D, top panel). This indicates a potential physical interaction between Sp-Eph and pY397FAK in ciliary band cells.

**Eph-Ephrin signaling is necessary for apical constriction**

To assess the role of Eph-Ephrin signaling during apical constriction, we blocked translation of Sp-Eph or Sp-Ephrin in the embryo by morpholino-substituted antisense oligonucleotide (MASO) injection (Fig. 6). For each protein, we injected two, non-overlapping oligonucleotides to knock down expression with 42.4% (±16.4% s.e.m.) mean knockdown based on fluorescence intensity. When we knocked down Sp-Eph or Sp-Ephrin, a distinct ciliary band failed to form after 72 hours and embryos were ovoid in shape (Fig. 6A-B″). At 72 hours, actin and pS19MLC in Sp-Eph knockdown embryos appear in circular or crescent-shaped patches throughout the ciliary band (Fig. 6C-C″). These patches appear restricted to individual cells for pS19MLC (Fig. 6C,F, arrow and inset) and for actin (Fig. 6C″, arrow and inset) and do not form an interconnected, supacellular network as seen in untreated embryos (Fig. 3), indicating Sp-Eph is necessary for actomyosin reorganization during ciliary band formation. Furthermore, ciliary band cells in MASO-injected embryos appear larger than in control embryos (Fig. 6D-F) and when we quantified apical surface area, we found apical constriction is significantly reduced in knockdown embryos (P<0.0001; Fig. 6G), indicating Sp-Eph and Sp-Ephrin are necessary for apical constriction of ciliary band cells.

**Ciliary band-specific accumulation of pY397FAK is regulated by Eph-Ephrin signaling**

We investigated whether ciliary-band-specific FAK phosphorylation is dependent on Eph-Ephrin signaling (Fig. 7). Prior to ciliary band
Distinct latitudinal polarization of Sp-Eph and pY397FAK during apical constriction of ciliary band cells. (A) Sp-Ephrin is primarily on aboral and ciliary band ectoderm. (A') Prior to apical constriction, pY379Eph is not detected. (B-B') Ciliary band-specific colocalization of pY379Eph and Sp-Ephrin during apical constriction. (B') Ephrin expression is restricted to aboral and ciliary band ectoderm. (B') Ephrin expression indicates ciliary band cells are forming at the ciliary band boundary. Arrows mark aboral ectoderm-ciliary band boundary. (C) During apical constriction, pY379Eph is polarized to latitudinal membrane junctions of ciliary band cells. (D) Sp-Eph cytoplasmic domain and pY397FAK interact physically. (Top panel) A GST fusion protein of kinase-dead Sp-Eph (EphKD) cytoplasmic domain acts as a substrate trap, showing a specific interaction with pY397FAK in an ectodermal cell lysate from S. purpuratus embryos at 72 hours. (Bottom panel) A native form of Eph cytoplasmic domain does not reliably pull down detectable quantities of pY397FAK from an ectodermal lysate. (E-E') Prior to apical constriction, Sp-Eph and pY397FAK are apparent in the presumptive ciliary band. (E) Sp-Eph is evident on junctions within oral and ciliary band ectoderm, and latitudinal polarization is evident on junctions bordering the aboral ectoderm (arrows). (E') pY397FAK becomes abundant on apical junctions in the ciliary band where it colocalizes with Eph (F'). Distinct latitudinal polarization of Sp-Eph and pY397FAK during apical constriction of ciliary band cells. (F) Sp-Eph distribution is polarized in the ciliary band and does not extend into aboral ectoderm (arrows) but is uniformly distributed on junctions of oral ectoderm (asterisks). (F') Polarized expression of pY397FAK is restricted to the ciliary band where it colocalizes with Eph (F'). Puncta in E and F appear to represent recognition of material within the basal body of each cell in the embryo. Scale bars: 10 μm.

**Fig. 5.** During apical constriction, Eph and Ephrin expression overlap in the ciliary band and Eph becomes phosphorylated along latitudinal membranes where it colocalizes with pY397FAK. Antigens and developmental stages are indicated. Arrowheads mark oral ectoderm-ciliary band boundary; arrows mark aboral ectoderm-ciliary band boundary. (A-A') Region-specific expression of Sp-Ephrin prior to apical constriction. (A) Sp-Ephrin is primarily on aboral and ciliary band ectoderm. (A') Prior to apical constriction, pY379Eph is not detected. (B-B') Ciliary band-specific colocalization of pY379Eph and Sp-Ephrin during apical constriction. (B') Ephrin expression is restricted to aboral and ciliary band ectoderm. (B') Ephrin expression indicates ciliary band cells are forming at the ciliary band boundary. (C) During apical constriction, pY379Eph is polarized to latitudinal membrane junctions of ciliary band cells. (D) Sp-Eph cytoplasmic domain and pY397FAK interact physically. (Top panel) A GST fusion protein of kinase-dead Sp-Eph (EphKD) cytoplasmic domain acts as a substrate trap, showing a specific interaction with pY397FAK in an ectodermal cell lysate from S. purpuratus embryos at 72 hours. (Bottom panel) A native form of Eph cytoplasmic domain does not reliably pull down detectable quantities of pY397FAK from an ectodermal lysate. (E-E') Prior to apical constriction, Sp-Eph and pY397FAK are apparent in the presumptive ciliary band. (E) Sp-Eph is evident on junctions within oral and ciliary band ectoderm, and latitudinal polarization is evident on junctions bordering the aboral ectoderm (arrows). (E') pY397FAK becomes abundant on apical junctions in the ciliary band where it colocalizes with Eph (F'). Distinct latitudinal polarization of Sp-Eph and pY397FAK during apical constriction of ciliary band cells. (F) Sp-Eph distribution is polarized in the ciliary band and does not extend into aboral ectoderm (arrows) but is uniformly distributed on junctions of oral ectoderm (asterisks). (F') Polarized expression of pY397FAK is restricted to the ciliary band where it colocalizes with Eph (F'). Puncta in E and F appear to represent recognition of material within the basal body of each cell in the embryo. Scale bars: 10 μm.

Screening for a role of pY397FAK, we used PF573 to block phosphorylation of Y397 and we measured the effect in ciliary band cells. Apical constriction is blocked by PF573 (10 μM; Fig. 8C,D), indicating that FAK phosphorylation on Y397 is crucial. These data support a model in which forward Eph-Ephrin signaling regulates apical constriction of the ciliary band cells through pY397FAK.

**Apical constriction requires Eph-Ephrin signaling and phosphorylation of FAK**

By adding specific inhibitors and measuring apical surface area, we further assessed the roles of Eph-Ephrin signaling and FAK catalytic activity on apical constriction of ciliary band cells (Fig. 8). In the presence of NVP (1.75 μM), an Eph kinase inhibitor, ciliary band formation and accumulation of pY397FAK are disrupted (Fig. 8B), and apical surface area of ciliary band cells does not change (Fig. 8D), indicating a lack of apical constriction. To determine the role of pY397FAK, we used PF573 to block phosphorylation of Y397 and we measured the effect in ciliary band cells. Apical constriction is blocked by PF573 (10 μM; Fig. 8C,D), indicating that FAK phosphorylation on Y397 is crucial. These data support a model in which forward Eph-Ephrin signaling regulates apical constriction of the ciliary band cells through pY397FAK.

**Planar cell polarity in ciliary band cells**

Using an inhibitor specific to the non-canonical Wnt pathway, we investigated the role of planar cell polarity in the ciliary band (Fig. 9). We treated embryos with a cell permeant, e-Jun N-terminal peptide (Range et al., 2013) and examined polarization of pY397FAK or pY397Eph in ciliary band cells (Fig. 9A-B'). In control embryos, the abundance of pY397FAK on latitudinal junctions of the ciliary band is 12.21 times greater than on longitudinal junctions when measured by normalized fluorescence intensity (Fig. 9A). When treated with JNK inhibitor, the abundance of pY397FAK is equal on latitudinal junctions is significantly reduced (P<0.001) to 1.02 times (nearly equal) the amount on longitudinal junctions (Fig 9A'). Similarly, polarization of Y397Eph to latitudinal membranes is significantly reduced (3.28 times greater in controls versus 2.27 times greater in treated embryos; P<0.001) when JNK is inhibited (Fig. 9B,B'), indicating polarization of these signaling molecules in the ciliary band is dependent on the non-canonical Wnt pathway. Interestingly, when we quantify apical surface area of ciliary band cells in these JNK-treated embryos and compare them with control cells, there is no significant difference (P=0.303; Fig. 9C), implying that the mechanisms driving apical constriction and polarization of pY397Eph and pY397FAK are independent (Fig. 9D).

**DISCUSSION**

Our data support a model in which Eph-Ephrin forward signaling initiates formation of a planar-polarized, pY397FAK-containing, signaling complex that regulates actomyosin-mediated...
its catalytic activity is necessary for apical constriction, pY397FAK appears to be a role for FAK during ciliary band morphogenesis; Moeller et al., 2006; Carter et al., 2002; Ohashi et al., 2000. There cytoskeletal reorganization and cell shape (Shi et al., 2009; 1080 autophosphorylation on Y397 with downstream effects on FAK following Ephrin stimulation of Eph. These include increased and Sp-Ephrin expression appears to activate Sp-Eph within the presumptive ciliary band. A number of studies describe effects on contractility, producing apical constriction. The overlap of Sp-Eph and Sp-Ephrin expression appears to activate Sp-Eph within the presumptive ciliary band. A number of studies describe effects on cytoskeletal reorganization and cell shape (Shi et al., 2009; Moeller et al., 2006; Carter et al., 2002; Ohashi et al., 2000). There appears to be a role for FAK during ciliary band morphogenesis; its catalytic activity is necessary for apical constriction, pY397FAK accumulates specifically on latitudinal, ciliary band membranes and Eph-Ephrin signaling is necessary for this accumulation. Furthermore, colocalization of pY397FAK and Sp-Eph on latitudinal membranes of the ciliary band and the putative molecular interaction emphasize a close functional relationship. We propose Sp-Eph and FAK act in an apical complex; regulating assembly of cytoskeletal networks necessary for transducing centripetal forces that reduce apical size during ciliary band morphogenesis (Fig. 9E). There are a number of potential mechanisms by which FAK activation can function in the regulation of actin-mediated contractility. These include transduction through Src family kinases, guanine nucleotide exchange factors, GTPase-activating proteins and Rho-family GTPases (Thomas et al., 1998; Tilghman and Parsons, 2008; Burridge and Wennerberg, 2004). This provides a potential mechanism whereby extracellular Sp-Ephrin elicits a receptor-mediated (Sp-Eph) response to change cell shape.

Loss-of-function experiments show filamentous actin and myosin light chain kinase are necessary for apical constriction, and rearrangements in their distribution occur immediately prior to shape change. The observed distribution is not consistent with a purse string model in which a circumferential band of actin in each cell is predicted. Rather, our observations are consistent with a model of apical constriction, where centripetal force is produced by contractions of the actomyosin network at the medial apical cortex (Martin et al., 2009). Furthermore, when we block apical constriction by perturbing Eph-Ephrin signaling, we also disrupt supracellular cytoskeletal networks in the ciliary band, illustrating that Eph-Ephrin signaling is involved in remodeling actomyosin networks during apical constriction of ciliary band cells (Fig. 9E).

Ciliary band cells are polarized within the epithelial plane and this is apparent functionally; cilia have their power stroke oriented away from the oral field (Strathmann, 2007). Sp-Eph and pY397FAK exhibit a polarized distribution and Eph-Ephrin signaling and FAK catalytic activity do not appear to initiate polarization, as loss of function interferes with the extent of immunoreactivity of polarized components without noticeably altering their distribution. By contrast, inhibition of JNK leads to a loss in polarization of pY397FAK and pY397Eph with no apparent effects on the level of immunoreactivity.
Eggs and sperm were collected from that drive morphogenesis. Complex mechanisms linking patterning to the mechanical forces coupling of apical constricting and adjacent tissues remains a key adjacent tissues in morphogenetic folding. The nature of the ciliary band cells is informative in that it indicates participation of large-scale morphogenetic movements. The apical constriction of that apical constriction does not function independently to produce adjacent ectoderm; rather cells change shape producing a tightly Ciliary band formation is a unique example of morphogenesis have not been resolved (Sawyer et al., 2010; Nishimura et al., 2012). have been implicated in the folding process, individual contributions although additional morphogenetic forces and planar cell polarity have been dissociable phenomena that are regulated independently (Fig. 9D,E).

In widely studied models of apical constriction, reduction of apical surface area is followed by inward folding of epithelium and although additional morphogenetic forces and planar cell polarity have been implicated in the folding process, individual contributions have not been resolved (Sawyer et al., 2010; Nishimura et al., 2012). Ciliary band formation is a unique example of morphogenesis because there is no accompanying, large-scale, inward flexion of adjacent ectoderm; rather cells change shape producing a tightly packed array of cilia with modest outward flexion. This indicates that apical constriction does not function independently to produce large-scale morphogenetic movements. The apical constriction of ciliary band cells is informative in that it indicates participation of adjacent tissues in morphogenetic folding. The nature of the coupling of apical constricting and adjacent tissues remains a key feature in the development of models that seek to describe the complex mechanisms linking patterning to the mechanical forces that drive morphogenesis.

MATERIALS AND METHODS
Embryo culture and injection
Eggs and sperm were collected from S. purpuratus adults induced to spawn with 0.55 M KCl or by shaking or gentle prodding. Sperm was diluted 1:1000 in filtered seawater prior to fertilization and embryos were grown at 14°C. Unless otherwise mentioned, inhibitors were added 48 hours postfertilization. Eggs were prepared for microinjection as described previously (Krupke et al., 2013). Injection solutions containing 22.5% glycerol, either RhodamineB-dextran (Sigma, R9379) or Fluorescein isothiocyanate-dextran (Sigma, 46945) and RNA were microfiltered at 5000 g for 1 minute using 0.22 μm Ultrafree centrifugal filters (Millipore). Morpholino antisense oligonucleotides were obtained from GeneTools and injected as previously described (Krupke et al., 2013).

Plasmids and reagents
Oligonucleotide DNA primers were obtained from Operon. Sequences encoding full-length Sp-Ephrin and Sp-Eph were obtained by PCR from cDNA isolated from 72 hour S. purpuratus embryos and cloned by using standard protocols. Morpholino antisense oligonucleotides were obtained from GeneTools, EphrinMO1: 5′-AAATTGCTCTCCTGGATGAGAC-3′. EphrinMO: 5′-CTCCAGGGTTCAAGGTCGATTTAT-3′. EphMO1: 5′-ATTGGAAGAGTAAATCCGAGATG-3′. EphMO2: 5′-AAATAGTCATTCTCTCCITCCTCGT-3′. ControlMO: 5′-GAATGAACTGTCTTTATCCATCA-3′. Inhibitors (Tocris Biosciences) were used as follows: 50 μM c-jun peptide (cat. no. 1989), 20 μM cytochalasin D (cat. no. 1233), 5 μM ML 7 (cat. no. 4310), 1.75 μM NVP BHG 712 (NVP, cat. no. 4405), 10 μM PF 573228 (PF573, cat. no. 3239).

Antibody production
Proteins were produced using pET28a vector (Novagen) for expression of 6×His-tagged proteins. The C-terminal half of Sp-Eph [amino acids (aa) 447-746] and the N-terminus of Sp-Ephrin (aa 28-169) were amplified by PCR, cloned into pET28a and protein expression was induced in E. coli using standard protocols. Bacterial lysate was prepared using BugBuster (Novagen) and protein was solubilized in binding buffer (6 M guanidine HCl, 0.5 M NaCl, 100 mM Na2HPO4, 100 mM NaH2PO4, 10 mM imidazole, 10 mM Tris, 1 mM 2-mercaptoethanol, pH 8.0) prior to affinity purification by immobilized metal ion affinity chromatography (IMAC) using Chelex 100 Resin (Bio-Rad). Proteins were purified by size exclusion on a Hi Load 16/60 Superdex 75 prep grade column (GE Healthcare) using the AKTAprime plus system (GE Healthcare). Purified protein was concentrated and dialyzed in PBS and mixed with Freund’s complete adjuvant (Sigma) for initial immunization or with Freund’s incomplete

Fig. 7. Ephrin signaling through Eph is necessary for phosphorylation of focal adhesion kinase during ciliary band formation. (A) pY397FAK is uniformly distributed on cell-cell junctions at 24 hours. (B) At the onset of apical constriction, pY397FAK accumulates on latitudinal junctions in the ciliary band. (C,D) pY397FAK intensity on latitudinal junctions in the ciliary band increases as apical constriction continues (C) and the fully formed ciliary band (D) shows abundant pY397FAK. (E,F) Knocking down Sp-Ephrin (E; EphrinMO – 200 μM solution) or Sp-Eph (F; EphMO – 250 μM solution) expression disrupts pY397FAK accumulation in the ciliary band. (G) Blocking Eph-Ephrin signaling or FAK catalytic activity significantly reduces relative abundance of pY397FAK (denotes P<0.001). In control treatments relative pY397FAK abundance in the ciliary band is >3.2 (left bar). Injection of Eph or Ephrin morpholino (as above) or addition of Eph kinase inhibitor (NVP) at 1.75 μM results in a significant loss in pY397FAK accumulation. A similar response is achieved by adding 10 μM of PF573, an inhibitor specific to pY397FAK (right bar). Relative abundance is measured by comparing fluorescence intensity of pixels within the ciliary band to those within aboral ectoderm at 60 hours. A relative abundance value of 1 is equivalent to the abundance of pY397FAK on aboral ectoderm. All pixels in the aboral and/or ciliary band ectoderm were quantified for each embryo. Untreated, n=10 embryos; EphMO, n=12 embryos; EphrinMO, n=11 embryos; Eph inhibitor, n=10 embryos; pFAK inhibitor, n=14 embryos. Mean knockdown efficiency for all MASO injections based on fluorescence intensity was 42.4%. Scale bars: 10 μm.
within the ciliary band. (B) Blocking Eph kinase (NVP at 1.75 μM) disrupts ciliary band formation. (C) Blocking FAK phosphorylation (PF573 at 10 μM) reduces FAK abundance and disrupts ciliary band formation. (D) Blocking Eph kinase or FAK phosphorylation (as above) significantly reduces the extent of apical constriction in ciliary band cells. Embryos were exposed to inhibitors at 48 hours and apical surface area was quantified at 72 hours. Control embryos (DMSO) form discrete ciliary band regions with apically constricted cells. Addition of NVP (center bar) or PF573 (right bar) results in inhibition of apical constriction along the structure of interest.

**Fig. 8. Phosphorylation of focal adhesion kinase and forward signaling through Eph are necessary for apical constriction of ciliary band cells.**

Antigens and treatments are indicated. All images are of 72 hour embryos. (A) Control embryos accumulate pY397FAK at apical membrane junctions within the ciliary band. (B) Blocking Eph kinase (NVP at 1.75 μM) reduces pY397FAK abundance and disrupts ciliary band formation. (C) Blocking FAK phosphorylation (PF573 at 10 μM) reduces pY397FAK abundance and disrupts ciliary band formation. (D) Blocking Eph kinase or FAK phosphorylation (as above) significantly reduces the extent of apical constriction in ciliary band cells. Embryos were exposed to inhibitors at 48 hours and apical surface area was quantified at 72 hours. Control embryos (DMSO) form discrete ciliary band regions with apically constricted cells. Addition of NVP (center bar) or PF573 (right bar) results in inhibition of apical constriction along the structure of interest.

**Adjuvant (Sigma) for booster immunizations at a 1:1 ratio. Animals were immunized and housed at the University of Victoria Animal Care Facility. Immunization by subcutaneous injection included 100 μg antigen in 250 μl total volume at 0 days, 21 days and 42 days. A terminal bleed by cardiac puncture was performed at 52-56 days. Blood samples were incubated for 45 minutes at 37°C and placed at 4°C overnight. Clots were centrifuged at 1000 g, serum collected and stored at -80°C (Sigma) to a final concentration of 0.02% w/v. Antibody specificity was confirmed by western blotting against the antigen and subsequently against an embryonic lysate.

**Immunofluorescence microscopy**

*Strongylocentrotus purpuratus* embryos were fixed for 5 minutes in PEM buffer (Vielkind and Swierenga, 1989) or ice-cold methanol. Embryos were washed with PBS and probed with primary antibody diluted in SuperBlock (Thermo). Primary antibodies: atypical protein kinase C ζ (aPKC, 1:300, Santa Cruz), Sp-Ephrin (1:2, 4D2 supernatant), Sp-Eph (1:500, serum), Sp-Hnf6 (Yaguchi et al., 2010) (1:700, serum), pY397FAK (1:2000, Invitrogen), pY379Eph (1:3000, Abcam), Phalloidin-Alexa Fluor 633 (1:300, Invitrogen), pS19myosin light chain II (pS19MLC, 1:200, Cell Signaling Technology). Embryos were washed three times with PBS and visualized with Alexa Fluor secondary antibodies (Invitrogen) on a Zeiss 700 LSM (Carl Zeiss) confocal microscope.

The contribution of cell division to apical constriction in ciliary band ectoderm was assessed by adding DMSO or 0.6 μM aphidicolin (Sigma, A0781) with 1 μM EdU to sea water at 60 hours. Following fixation, EdU incorporation was detected using a Click-IT detection kit (Life Technologies, C10340) according to manufacturer’s direction.

All immunofluorescence images are maximum intensity projections of whole mounted, sea urchin larvae oriented with oral ectoderm towards the top and aboral ectoderm towards the bottom. Cells shown in the insets were chosen randomly in an area of ciliary band ectoderm or chosen to highlight typical defects observed with treatments. Images used for data analyses were maximum intensity projections that extended from the cell surface to a 3–4 μm depth for cell shape (anti-aPKC) and 7 μm for all cytoskeleton (pSer19myosin and Phalloidin). Optimal gain, pinhole diameter and laser intensity settings were established for each antibody/fluorophore combination and settings were re-used consistently. Imaging and analysis was conducted using ZEN software (Carl Zeiss). Adobe Photoshop was used for cropping and assembling figures and to adjust image contrast and brightness.

**Cell surface area calculations**

For surface area measurements, anti-aPKC was used to visualize apical cell membranes, and ciliary band cells were identified using the ciliary-band-specific marker, anti-Hnf6. Apical membranes were outlined and cell surface area was calculated using ZEN software. Surface area values were exported to Microsoft Excel for further analysis.

**Relative intensity measurements**

Images were quantified using ZEN software and areas of interest selected by encircling them (when comparing intensity in ciliary band and aboral ectoderm) or by drawing a 5 pixel width line along the structure of interest (when comparing latitudinal and longitudinal expression). Dark pixels and saturated pixels were excluded in relative intensity measurements. Data sets were collected as intensity values per pixel within or along the region of interest and exported into Microsoft Excel for further analysis.

**Statistical analyses**

Grouped data were analyzed using a one-way ANOVA and the *P*-value for each comparison is reported. A *P*-value below 0.05 was considered statistically significant.

**Protein affinity**

Ectodermal cells were isolated from embryos at 72 hours (McCay and Marchase, 1979) for preparation of ectodermal cell lysate. Lysate was clarified by ultracentrifugation (4°C, 10 minutes, 100,000 g) and protein was quantified by Bradford analysis. Full-length Sp-Eph and the cytoplasmic portion (aa 322-762) of a kinase-dead Sp-Eph, Sp-EphKD[K445R], were cloned into pGEX4T-1 to create GST fusions and transformed into E. coli BL21 for expression using standard protocols. Cells were harvested by centrifugation (4°C, 15 minutes, 12,000 g), resuspended in ice-cold lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% v/v Triton X-100, 5% w/v glycerol, 0.03% w/v SDS, 1 mM diithiothreitol (DTT), 1 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 8 μM leupeptin, 1.5 μM pepstatin, 0.3 μM aprotinin) and lysed by sonication at 4°C. Lysate was clarified by ultracentrifugation (4°C, 10 minutes, 100,000 g) and protein was quantified by Bradford analysis. Fusion protein lysate was incubated for 30 minutes at 4°C with glutathione-agarose at a rate of 35 mg/ml wet resin. Resin was washed with three volumes lysis buffer and ectodermal lysate was added at 35 mg/ml wet weight.
beads and incubated at 4°C for 1 hour with rocking. Beads were washed with three volumes of lysis buffer and proteins eluted with two 0.5 volume additions of elution buffer (5 mM reduced glutathione, 50 mM Tris-HCl, pH 9.0).

SDS-PAGE used 12% polyacrylamide resolving gels with 5% stacking gel in Mini-protein TetraCell (Bio-Rad) for 50 minutes at 200 V in standard running buffer. Proteins were blotted onto polyvinylidene fluoride (PVDF) membrane in standard transfer buffer for 1 hour using Genie Blotter (Idea Scientific). Blots were probed with anti-GST (1:5000, Cedarlane) and anti-pFAK[pY397] (1:1000, Invitrogen). Infrared detection of secondary antibodies (Rockland) was done on an Odyssey Imager (Li-Cor Biosciences) and images were adjusted for brightness and contrast using Photoshop.

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Competing interests
The authors declare no competing financial interests.

Author contributions
O.A.K. developed materials, concepts and approaches, performed experiments, analyzed data and prepared the manuscript. R.D.B. developed concepts and approaches, performed experiments, analyzed data and edited the manuscript.

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