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Basal constriction during midbrain–hindbrain boundary morphogenesis is mediated by Wnt5b and focal adhesion kinase

Jennifer H. Gutzman1,*†, Ellie Graened1,2,**, Isabel Brachmann1, Sayumi Yamazoe3,§, James K. Chen3,4 and Hazel Sive1,2,**

ABSTRACT
Basal constriction occurs at the zebrafish midbrain–hindbrain boundary constriction (MHBC) and is likely a widespread morphogenetic mechanism. 3D reconstruction demonstrates that MHBC cells are wedge-shaped, and initially constrict basally, with subsequent apical expansion. wnt5b is expressed in the MHB and is required for basal constriction. Consistent with a requirement for this pathway, expression of dominant negative Gsk3β overcomes wnt5b knockdown. Immunostaining identifies focal adhesion kinase (Fak) as active in the MHB region, and knockdown demonstrates Fak is a regulator of basal constriction. Tissue specific knockdown further indicates that Fak functions cell autonomously within the MHBC. Fak acts downstream of wnt5b, suggesting that Wnt5b signals locally as an early step in basal constriction and acts together with more widespread Fak activation. This study delineates signaling pathways that regulate basal constriction during brain morphogenesis.

KEY WORDS: Basal constriction, Cell shape, Morphogenesis, Zebrafish, Wnt5b, Focal adhesion kinase, Midbrain–hindbrain boundary

INTRODUCTION
Basal constriction is a cell shape change associated with zebrafish midbrain–hindbrain boundary constriction (MHBC) (Gutzman et al., 2008). This process contrasts with the widely studied mechanism of apical constriction (Martin and Goldstein, 2014). Following our initial identification of the process, basal constriction has been described in several other systems and developmental processes. It is associated with zebrafish and medaka optic cup morphogenesis (Bogdanović et al., 2012; Martinez-Morales et al., 2009; Nicolás-Pérez et al., 2016; Sidhaye and Norden, 2017), notochord cell elongation in Ciona, (Dong et al., 2011) and egg chamber elongation in Drosophila (He et al., 2010). Together these findings suggest that basal constriction is a conserved and fundamental morphogenetic process.

We previously demonstrated that basal constriction in the MHBC cells of the zebrafish neuroepithelium requires an intact basement membrane and is laminin-dependent (Gutzman et al., 2008). During optic cup morphogenesis, basal constriction has been demonstrated to require actomyosin contraction and is also dependent on laminin (Nicolás-Pérez et al., 2016; Sidhaye and Norden, 2017). However, the upstream signaling pathways that promote basal constriction have not been identified.

Since basal constriction at the zebrafish MHBC occurs within a small group of cells, one hypothesis is that a localized signaling process is involved. Wnt-PCP signaling is one candidate regulatory pathway, since this is crucial for many morphogenetic events, including gastrulation, convergent extension, cell migration, and cell adhesion (Ciani and Salinas, 2005) and has been studied during the development of the midbrain–hindbrain boundary (Buckles et al., 2004; Gibbs et al., 2017). Wnt5b is a PCP ligand and regulator of cell shape and movement. It is required during gastrulation (Jopling and den Hertog, 2005; Kilian et al., 2003; Lin et al., 2010), mesenchymal cell migration and adhesion (Bradley and Drissi, 2011), Xenopus bottle cell apical constriction (Choi and Sokol, 2009) and tail morphogenesis (Marlow et al., 2004). In this communication, we demonstrate expression of wnt5b at the zebrafish MHBC and find a connection between Wnt5b, Gsk3β and focal adhesion kinase (Fak), providing the first delineation of a signaling pathway required for basal constriction.

RESULTS AND DISCUSSION
Basally constricted cells are wedge-shaped
To delineate steps in basal constriction, we examined cell shape during midbrain–hindbrain boundary constriction (MHBC) by injecting wild-type embryos with membrane targeted GFP (mGFP) and imaging live embryos using confocal microscopy (Fig. 1A–D). MHBC morphogenesis takes place beginning at approximately the 18 somite stage (ss) and extending to the prim-6 stage. At the start, the neural tube is composed of a pseudostratified epithelium with established apical-basal polarity (Fig. 1A). We identified three sequential morphogenetic changes during MHBC formation. First, by 21 hpf MHBC cells become approximately 25% shorter than surrounding cells. Second, at 24 hpf, 3–4 cells within a single imaging plane at the point of deepest indentation of the MHBC each show basal constriction. Third, by 24 hpf MHBC cells expand apically by 60% relative to that of surrounding cells (Fig. 1A–H) (Gutzman et al., 2008, 2015). Three dimensional (3D) reconstruction of MHBC cells revealed that as the cells basally constrict and apically expand they become wedge-shaped (Fig. 1C–D,G–H). The average basal anteroposterior width of the MHBC cells decreases from 2.1 μm to less than 0.5 μm between 14 ss and prim-6 (Fig. 1I). The progression of MHBC cell shape change is summarized in Fig. 1J–M.
The ability to temporally separate morphogenetic steps supports the hypothesis that discrete molecular and mechanical processes underlie each step in MHBC formation. Our additional data are consistent with this hypothesis. For example, MHBC cell basal constriction can occur in the absence of apical expansion. Thus, in *snakehead* mutants that have reduced function in the *atp1a1* gene encoding a Na⁺K⁺ ATPase, MHBC cells constrict basally, but fail to expand apically (Gutzman et al., 2008). In *sleepy* mutants, mapping to the *lamc1* gene encoding for the gamma chain of Laminin 1-1-1, cell shortening occurs in the MHBC region, but both basal constriction and apical expansion fail to occur, indicating that cell shortening is not sufficient to drive subsequent steps and that apical expansion may depend on prior basal constriction (Gutzman et al., 2008). Additional studies support that independent molecular mechanisms govern various parts of MHBC morphogenesis. Prior to 24 ss, non-muscle myosin IIA and calcium signals are required to specifically shorten MHBC cells, but these molecules do not appear to have a direct role in mediating cell width (Gutzman et al., 2015; Sahu et al., 2017). In turn, non-muscle myosin IIB is not required for cell shortening, but is required to decrease cell width to fold the MHBC (Gutzman et al., 2015; Sahu et al., 2017). Together, these data suggest that each step of MHBC morphogenesis is regulated by separable molecular mechanisms.

**wnt5b regulates basal constriction, possibly through Gsk3β.**

We hypothesized that genes required for basal constriction would be expressed prior to the start of MHBC formation and that expression would be restricted to cells undergoing basal constriction or adjacent cells. In assessing the literature, we identified *wnt5b* expression as correlating with MHBC morphogenesis both temporally and spatially (Montero-Balaguer et al., 2006; Thisse and Thisse, 2005). Consistently, we demonstrated that *wnt5b* RNA was enriched at the MHBC using in situ hybridization (Fig. 2A–D). There is a low level of *wnt5b* expression throughout the embryo; however, expression increases at the MHBC shortly before morphogenesis begins and persists in this region throughout basal constriction (Fig. 2A–D). To determine the functional significance of Wnt5b in MHBC basal constriction, we used the established *wnt5b* antisense-morpholino modified oligonucleotide (MO) to inhibit function (Lele et al., 2001; Robu et al., 2007; Young et al., 2014). One-cell stage embryos were co-injected with control MO or *wnt5b* splice-site targeting MO with mGFP and live confocal imaging was employed to examine cell shape. Knockdown of *wnt5b* was associated with failure of basal constriction at the MHBC (Fig. 2E–F'). MHBC defects with Wnt5b knockdown could be a result of anomalous patterning in the MHB, which occurs earlier during development. However, expression of the patterning genes *fgf8* and *pax2*, that are required for MHB formation, was unchanged in *wnt5b* morphants relative to control (Fig. S1). We also confirmed that *wnt5b* knockdown did not disrupt neuroepithelial cell apical-basal polarity (Fig. S1). These data suggest that requirement for *wnt5b* in basal constriction is not due to loss of early tissue patterning or polarity but is a later effect, perhaps directly impacting morphogenesis. At the tissue level, a slight constriction of the MHB...
was observable at prim-6 stage even after Wnt5b knockdown (Fig. 2F), suggesting additional forces or signals are involved in MHB morphogenesis. One possibility is that fluid secretion during embryonic brain ventricle inflation plays a role in promoting folding of the MHB tissue (Gutzman et al., 2008). The basement membrane, and specifically laminin, localized to the basal side of the cells, may also provide mechanical signals to influence tissue shape, which could be Wnt5b independent (Gutzman et al., 2008).

Wnt5b is a ligand that can activate non-canonical Wnt signaling through Rho and JNK, and can also act through inactivation of Gsk3β (De Rienzo et al., 2011; Niehrs and Acerbon, 2010; Terrand et al., 2009; Torii et al., 2008). In zebrafish, Wnt5b functions as a negative regulator of Wnt/β-catenin activity (Westfall et al., 2003) and studies in Hydra suggest that, during evagination, Wnt5b may also be involved with crosstalk between the canonical and non-canonical Wnt signaling pathways (Philipp et al., 2009). We asked whether inhibition of Gsk3β is required for basal constriction, using a kinase-dead Gsk3β (dnGsk3β) that is an established dominant negative construct (De Rienzo et al., 2011; Yost et al., 1996). Supporting a connection between Wnt5b and inhibition of Gsk3β, we observed that co-injection of dnGsk3β with wnt5b MO prevented defects in basal constriction seen after injection of wnt5b MO alone (Fig. 2G–H). Consistent with this, abnormalities in the gross morphology of the MHB after injection of wnt5b MOs was prevented by expression of the dnGsk3β (Fig. S2). These data are consistent with a pathway in which Wnt5b regulates basal constriction through inhibition of Gsk3β.

**Fak is required at the MHBC for basal constriction**
Basal constriction at the MHBC and in the optic cup both require laminin (Gutzman et al., 2008; Nicolás-Pérez et al., 2016), a component of the underlying basement membrane, which interacts with integrins to regulate epithelial cell adhesion, migration and differentiation (Yamada and Sekiguchi, 2015; Yurchenco, 2015). Fak, a non-receptor tyrosine kinase, is a regulator of adhesion and cell migration that is activated through intracellular interactions with integrins (Parsons et al., 1994; Schaller, 2010). We therefore hypothesized that Fak plays a role in MHB basal constriction. Since a primary mechanism for Fak activation is via autophosphorylation at Tyr397 (Schaller, 2010), we specifically hypothesized that autophosphorylated FakY397 would be localized to the MBHC. An antibody specific to Fak autophosphorylation site Y397 stained both the apical and basal surfaces in the neural tube at 18 ss, 24 ss, and prim-6 (Fig. 3A–D).

We tested a role for Fak in basal constriction using knockdown with antisense-morpholino modified oligonucleotide injection. One-cell stage embryos were injected with control MO or a splice-site morpholino targeting the fak gene encoding one of the two fak genes in zebrafish. fak MO efficacy was confirmed by RT-PCR and western blot analysis (Fig. S3). At the concentration used here, fak MO injections did not disrupt MHB tissue patterning or apical polarity markers (Fig. S1). fak morphants demonstrated disruption in MHB formation and abnormal basal constriction at the MBHC (Fig. 3E–F’). We tested activity of FakY397 in regulation of basal constriction using a phosphomimetic mutation of Tyr397 to Glu397 (FakY397E). Consistent with activation of Fak during basal constriction, co-injection of FakY397E with fak MO was able to prevent abnormal basal constriction (Fig. 3G,G’).

We further tested the spatial and temporal requirement of Fak in the MHB to mediate basal constriction using an injection of a photoactivatable cyclic fak MO. With UV activation, the cyclic fak MO becomes linear and binds to its target site (Yamazoe et al., 2012). We injected wild-type embryos with cyclic fak MO, Kaede mRNA and mGFP mRNA. UV activation was performed at 14–16 ss, just before MHBC morphogenesis begins, in the MHB region as delineated by the change of Kaede from green to red. Basal constriction was disrupted after photoactivation of the cyclic fak MO, with no effect without activation or after UV treatment of the

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**Fig. 2. Wnt5b regulates basal constriction possibly through Gsk3β.** (A–D) In situ hybridization of wnt5b expression during MHB development at 18 ss (A), 22 ss (B), and prim-6 (C). (D) prim-6 sense probe control. (E–G′) Live confocal images of the MHB region in prim-6 stage embryos. Single-cell wild-type embryos were injected with mGFP to label cell membranes and co-injected with control MO (E,E′), wnt5b MO (F,F′), or wnt5b MO and dnGsk3β mRNA (G,G′). (H) Quantification of basal cell width in control MO, wnt5b MO, dnGsk3β mRNA (image not shown), and wnt5b MO+dnGsk3β mRNA injected embryos. (H) For each treatment group, n=3 embryos. For each embryo, 6 cells located at the MHBC were measured, 3 cells on each side. Arrowheads indicate MHBC. M, midbrain; H, hindbrain. Scale bars: 26 µm.
Fig. 3. See next page for legend.
control MO-injected animals. These data show that Fak is required in the MHB region to mediate basal constriction (Fig. 3G–K). We further determined that Fak functions cell autonomously using MHB targeted cell transplantation (Fig. S4). Together these data indicate that Fak activity is required for basal constriction, and that Fak functions in the cells of the region that is undergoing basal constriction, beginning just prior to the start of the process.

**Wnt5b signals through Fak to mediate MHBc basal constriction**

Since both Wnt5b and Fak are required for basal constriction, we asked whether Wnt5b functions upstream of Fak. To address this, we tested whether human FAK mRNA encoding the activated FAK*Y397E* was able to prevent the basal constriction defect seen after Wnt5b inhibition. Indeed, we found that this mRNA was able to rescue basal constriction in *wnt5b* morphants (Fig. 4A–G). This effect was not general, as FAK*Y397E* did not rescue basal constriction defects found in laminin mutants ([Gutzman et al., 2008] and Fig. S5), which is consistent with a structural role for laminin in the constriction process. These data indicate that Fak acts downstream of Wnt5b in activation of basal constriction at the MHB.

Together, our results uncover key signaling factors contributing to basal constriction during MHBC morphogenesis. Our data point to a model in which Wnt5b signals locally at the MHBC as an early step in basal constriction, and acts together with more widespread Fak activation (Fig. 4H). We do not know whether or not Wnt5b and Fak function at the same time during this process, nor whether their activity is also necessary for the earlier cell shortening or the subsequent apical expansion. Future experiments will uncover the molecular details of this signaling interaction and the role in other steps of MHBC formation.

**MATERIALS AND METHODS**

**Zebrafish husbandry**

Zebrafish lines were maintained and embryo stages were determined as previously described ([Kimmel et al., 1995]; Westerfield, 2000). Zebrafish strains used include wild-type AB and *slx10ab* (Schier et al., 1996). Due to the stages of development analyzed, we are unable to determine the sexes of the embryos. However, a large number of embryos were utilized for these experiments and each sex should be represented. All experimental procedures on live animals and embryos were reviewed and approved by the Institutional Animal Care and Use Committee of the Massachusetts Institute of Technology and were carried out in accordance with the recommendations in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

**mRNA injections**

All mRNA was *in vitro* transcribed with the mMessage mMachine kit (Thermo Fisher Scientific). Membrane-bound GFP (mGFP) mRNA was injected at 100–200 pg/embryo (kindly provided by J. B. Green, Dana-Farber Cancer Institute Boston, USA). Membrane-bound Cherry (mCherry) mRNA was injected at 50 pg/embryo (kindly provided by Dr Roger Tsien, University of California San Diego, USA). Photoconvertible Kaede mRNA was injected at 100 pg/embryo. pCS2+-Kaede was kindly provided by Atsushi Miyawaki (RIKEN) (Ando et al., 2002). Human focal adhesion kinase (FAK) (accession number BC035404) was purchased from Open Biosystems (EHS1001-5481173) and constructs were generated by subcloning into the pCS2+ expression plasmid. mRNA was *in vitro* transcribed and injected at 200–250 pg/embryo. pCS2+-FAK was used as the backbone to generate the FAK*Y397E* phosphomimetic using QuickChange Site-Directed Mutagenesis (Agilent). For each mRNA injection and rescue experiment, all embryos were injected with equal amounts of total mRNA. This included total mGFP when needed for imaging by scanning confocal microscopy. All microinjection experiments were performed at least three times.

**Live imaging and cell shape analysis**

Live imaging of whole embryos was conducted using brightfield and fluorescent microscopy (SteREO Discovery.V8, Zeiss). Live scanning confocal imaging was conducted as previously described (Graeden and Sive, 2009). Briefly, embryos were mounted in 0.7% agarose and imaged using a 40× water immersion lens. Imaging was conducted using a Leica DMI6000B microscope. Live confocal images were analyzed using Imaris (Bitplane). Individual cells at the MHBC were manually outlined in each z-section and rendered in 3D. A minimum of six embryos were imaged by scanning confocal microscopy and analyzed for basal constriction for each condition. Quantification of cell width was conducted using Imaris (Bitplane). The width of six cells at the MHBC from each of three embryos was measured at 30× zoom. Measurements were averaged and error bars reflect standard deviation for each condition.

**Morphinol injections**

Splice site-blocking morpholin (MO) antisense oligonucleotides were injected into embryos at the one-cell stage. Morpholinos and concentrations used are as follows: 3 ng/embryo of *wnt5b* MO targeting the exon5/6 splice donorn 5'-GTGTTATTTCTCACCACCTTTCCGG-3' ([Kim et al., 2005]; Robu et al., 2007); 0.75 ng/embryo of *fak* MO (pkh2.1) targeting the exon 12/13 splice donor 5'-GTTGTTGTTGTTTGTCTACCCCTTGC-3'; non-specific sequence control standard control MO 5'-CTCTTACCTCAGTCTTATATAAT-3' at the concentration equal to the test condition; and p53 MO 5'-GCACCTTCTTTGCAGAGAGTTG-3' was co-injected at a concentration equal to 1.5 times the concentration of the test condition. Morpholinos were purchased from Gene Tools, LLC. Embryo phenotypes were classified as normal, mild, or severe. Normal embryos appeared as wild type. Mild embryos exhibited a consistent tail morphology defect, but basal constriction occurred normally. Severe phenotype embryos exhibited extensive tail defects and a basal constriction defect. Severe phenotype embryos were used for confocal imaging.

**Region specific knockdown by morpholin photoconversion**

For photoactivatable morpholin experiments, we injected one-cell stage embryos with 1 ng/embryo of splice site-targeting cyclic fak MO (pkh2.1) 5'-GGGGGTCTAATGCTCGTCTCATATT-3'. The fak MO was cyclized with a photoactivatable linker as previously described ([Yamazoe et al., 2012]) and remains inactive until ‘uncaging’ by UV light. Linker photolysis reverts the MO to a linear oligonucleotide that can target the fak splice site.
Embryos were co-injected with mGFP mRNA and Kaede mRNA together with either cyclic fak MO or control MO at the one-cell stage. Region and time specific UV activation was conducted at the 10–16 somite stage on cells located in the prospective MHB using a Zeiss Axioplan2 fluorescent microscope with a UV filter and adjustable iris. The tissue region that was activated by UV light is visible with the Kaede color change from green to red. Only cells that were photoconverted at the MHBC were analyzed for basal constriction as described.

In situ hybridization
Antisense and sense RNA probes containing digoxigenin-11-UTP were synthesized from linearized plasmid DNA for wnt5b was obtained from Addgene #21282 (Stoick-Cooper et al., 2007). Standard methods for hybridization and for single color labeling were used as described (Sagerstrom et al., 1996). After staining, embryos were de-yolked, flat-mounted in glycerol and imaged with a Nikon compound microscope or a Zeiss Discovery V8.
Embryos were fixed in 4% paraformaldehyde; blocked in 2% normal goat serum, 1% BSA, and 0.1% Triton-X100 in PBT; incubated overnight at 4°C in primary antibody (anti-phosphoY397-FAK, 44-624 BioSource, Life Technologies), 1:200; then incubated in secondary antibody (goat anti-rabbit IgG conjugated with Alexa Fluor 488, Invitrogen, 1:500). Embryos were de-yolked and mounted in glycerol. Images were collected using scanning confocal microscopy (Zeiss LSM510 or 710) and analyzed using Photoshop (Adobe).

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**SUPPLEMENTARY MATERIAL**

**Fig. S1.** Tissue patterning and polarity are retained in wnt5b and fak morphants. (A-F) in situ hybridization patterns for fgf8 and pax2a expression in control MO (A,D), wnt5b MO (B,E), and fak MO (C,F) injected embryos at 18 ss. Expression patterns are normal in both wnt5b and fak morphants. RNA probes containing digoxigenin-11-UTP were synthesized from linearized plasmid DNA for pax2.1 (Krauss et al., 1991), and fgf8 (Reifers et al., 1998) as previously described (Harland, 1991). Standard methods for hybridization and for single color labeling were used as described (Sagerstrom et al., 1996). After staining, embryos were de-yolked, flat-mounted in glycerol and imaged with a Nikon compound microscope. (G-L) Immunohistochemistry staining for the apical junction marker ZO-1 (G-I) and the cell polarity maker aPKC (J-L) in control MO (G,J), wnt5b MO (H,K), and fak MO (I,L) injected embryos at prim-6. Apical localization of ZO-1 appears normal in each condition and indicates establishment of apical junctions. aPKC is also normal in each condition indicating that the cells have established cell polarity. For immunostaining experiments, embryos were fixed in 4% paraformaldehyde or Dent’s (70% methanol: 30% DMSO) for ZO-1. Embryos were blocked in 2% normal goat serum, 1% BSA, and 0.1% Triton-X100 in PBT; incubated overnight at 4°C in primary antibody (anti-aPKC (C-20), SC-216, Santa Cruz Biotechnology, 1:1000; anti-ZO1, 33-9100, Invitrogen, 1:200); then incubated in secondary antibody (goat anti-rabbit or anti-mouse IgG conjugated with Alexa Fluor 488, Invitrogen, 1:500). Arrowheads indicate MHBC. Scale bars: G-I, 20 μm; J-L, 35 μm.
**Fig. S2.** Gross morphology images of dnGsk3β and wild-type Gsk3β overexpression phenotypes and dnGsk3β rescue of wnt5b morphants. (A-D) Brightfield dorsal and lateral images of control MO (A,B) and wnt5b MO (C,D) injected embryos, co-injected with mGFP (A,C), or dnGsk3β (B,D) mRNA. (A) Control morphants co-injected with mGFP mRNA demonstrating a normal MHBC basal constriction phenotype. (B) Control morphants co-injected with dnGsk3β mRNA exhibit an eyeless phenotype, but undergo basal constriction normally with this concentration of dnGsk3β (n=9). (C) Wnt5b morphants co-injected with control mRNA exhibit abnormal MHBC morphogenesis, tail defects, and fail to undergo basal constriction (n=6). (D) Wnt5b morphants co-injected with dnGsk3β mRNA exhibit a loss of eyes and tail defects, but the gross morphology of basal constriction is rescued and occurs normally (n=6). Arrowheads indicate the MHBC. Scale bars: 100μm.
Fig. S3. fak MO efficacy and specificity. (A) RT-PCR for control MO and fak MO injected embryos. Whole tissue lysate was analyzed. fak MO injection resulted in 2 abnormal mRNA products (arrows 1 and 2). EF1α was used as an RT-PCR control. Primers used for RT-PCR: fak exon 11 forward 5'-CACCTTGCAACTTCACTCA-3'; fak exon 22 reverse 5'-GTGAATCGTGGGCGTTTACT-3; EF1α forward, 5'-GATGCACCACGAGTCTCTGA-3; and EF1α reverse, 5'-TGATGACCTGAGCGTTGAAG-3. fak RT-PCR products were cloned into pGEM using the pGEM T-Easy Vector System Kit (Promega) and sequenced. (B) Sequence analysis of the two RT-PCR product variants from (A) resulted in the detection of two truncated mRNA amplicons caused by partial or complete exon deletion, each resulting in early stop codons as indicated in the diagram. Both truncations eliminate the autophosphorylation site Y397. (C) Western Blot analysis of fak morphant lysate demonstrating downregulation of full length Fak (asterisks ** at 125 kDa). Injected embryos were manually dechorionated and deyolked. Proteins were analyzed on 8% SDS-PAGE gels. Blots were blocked in 4% non-fat milk or 5% BSA and probed with antibody in 3% BSA. FAK C-20 (1:1000 dilution; sc-558) and FAK (1:200 dilution; Invitrogen AHO0502). GAPDH was used as a loading control (1:25,000 dilution, ab22555). Blots were visualized using Enhanced Chemiluminescence. (D) Representative brightfield images and quantification of fak morphant embryo rescue experiments. Control MO or fak MO was co-injected with mGFP, wild-type human FAK, or human FAK<sup>Y397E</sup> mRNA. Each condition had equal total amounts of mRNA injected. Embryos were analyzed at prim-6 for MHB defects. Each embryo was scored as having a normal, mild, or severe phenotype. Mild phenotypes are represented in D. Control MO + mGFP (n=38), control MO + wt hFAK (n=42), fak MO + mGFP (n=55), fak MO + hFAK (n=63), control MO + hFAK<sup>Y397E</sup> (n=51), fak MO + hFAK<sup>Y397E</sup> (n=54). Anterior is to the left in all images. Arrowheads indicate MHB.
**Fig. S4. Fak is required at the MHBC for basal constriction.** Schematic for transplant procedure. 1. One-cell stage wild-type embryos were co-injected with mGFP (donor) or mCherry (host) and control or fak MO. 2. Cells from donors (sphere stage) were transplanted into hosts (shield stage). Transplanted cells were targeted to the presumptive MHB region (Woo and Fraser, 1995). 3. Embryos were incubated until prim-6 then imaged with live confocal microscopy. (E-F') Donor cells are outlined in yellow and host cells in blue. (B,B') Control donor and control host cells basally constricted normally when transplanted to the MHBC, (n=6). (C,C') Cells from fak morphant donors transplanted into control hosts failed to undergo basal constriction at the MHBC, (n=4). Basal constriction occurred normally in control host cells, even when immediately adjacent to fak morphant donor cells.
**Fig. S5.** Expression of human FAK does not rescue the laminin mutant basal constriction phenotype. (A-F') Live confocal images showing the MHB region of prim-6 embryos injected with mGFP (A,D), mGFP + wt FAK mRNA (B,E), mGFP + FAK-Y397E (C,F). (A'-C') *sleepy* (*sly*m86) (Schier et al., 1996) heterozygous sibling or wild-type sibling embryos showing normal basal constriction at the MHBC. (D-F') *sleepy* mutants showing defects in basal constriction both without and with the co-injection of human wild-type FAK and human phosphomimetic FAK-Y397E mRNA. Representative images from 3 independent experiments with n>3 for each condition. (A'-F') Magnifications of the neuroepithelium shown in A-F with individual cells outlined at the MHBC. Arrowheads indicate MHBC. M, midbrain. H, hindbrain. Scale bars: 35 μm.