Non-muscle myosin IIA and IIB differentially regulate cell shape changes during zebrafish brain morphogenesis

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During brain morphogenesis, the neuroepithelium must fold in specific regions to delineate functional units, and give rise to conserved embryonic brain shape. Individual cell shape changes are the basis for the morphogenetic events that occur during whole tissue shaping. We used the zebrafish to study the molecular mechanisms that regulate the first fold in the vertebrate brain, the highly conserved midbrain-hindbrain boundary (MHB). Since the contractile state of the neuroepithelium is tightly regulated by non-muscle myosin II (NMII) activity, we tested the role of NMIIA and NMIIB in regulating cell shape changes that occur during MHB morphogenesis. Using morpholino knockdown, we show that NMIIA and NMIIB are both required for normal MHB tissue angle. Quantification of cell shapes revealed that NMIIA is required for the shortening of cells specifically at the MHB constriction (MHBC), while NMIIB is required for the proper width of cells throughout the MHB region. NMIIA and NMIIB knockdown also correlated with abnormal distribution of actin within the cells of the MHBC. Thus, NMIIA and NMIIB perform distinct functions in regulating cell shape during MHB morphogenesis.

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Introduction

Morphogenesis is a key developmental process that shapes all organs and is required for proper organ function. Regulation of individual cell shape changes are at the core of morphogenetic events which together give rise to whole tissue shape (Heisenberg and Bellaiche, 2013; Lecuit and Lenne, 2007). During vertebrate brain development, neuroepithelial cells of the neural tube fold in specific regions giving rise to the characteristic embryonic vertebrate brain shape. The fold at the midbrain-hindbrain boundary (MHB) functions as a crucial organizing center for the developing embryo and is one of the earliest and most highly conserved folds in the vertebrate brain (Brand et al., 1996; Rhinn and Brand, 2001). Given the high degree of conservation of the MHB across vertebrate species in terms of function and form, understanding the molecular mechanisms underlying its development is critical to our understanding of brain morphogenesis.

Our previous work characterized the basic morphogenetic events that occur to form this highly conserved fold in the zebrafish (Gutzman et al., 2008). We demonstrated that cell shortening and basal constriction occur in cells at the point of deepest constriction of the MHB, the midbrain-hindbrain boundary constriction (MHBC) (Gutzman et al., 2008). While we previously determined that basal constriction is dependent on laminin, the mechanisms that regulate cell shortening at the MHBC are unknown.

Neuroepithelial cell shape and brain morphogenesis are also dependent upon the contractile state of the neuroepithelium (Gutzman and Sive, 2010). This contractile state is tightly regulated by non-muscle myosin II (NMII) activity. We demonstrated that mypt1, the myosin phosphatase regulatory subunit, is required for cell shape regulation during hindbrain morphogenesis and important for regulating the activity of NMII (Gutzman and Sive, 2010). Myosin phosphatase dephosphorylates the myosin regulatory light chain (MRLC) where MRLC, in the phosphorylated state, activates the contraction of NMII. NMII proteins are critical regulators of cell motility, cytokinesis, polarity, and adhesion. In addition, it has been established in multiple systems that cell shape is critically dependent upon NMII function (Vicente-Manzanares et al., 2009).

Depending on the tissue and cell type, NMIIA and NMIIB may have either overlapping or distinct roles during embryonic development (Wang et al., 2011). For example, NMIIA and NMIIB knockout mice have different phenotypes. NMIIA knockouts are embryonic lethal and die before gastrulation due to defects in cell-cell adhesion (Conti et al., 2004). In contrast, NMIIB knockout mice display heart defects, hydrocephalus, and abnormal neuronal migration (Ma et al., 2007; Ma et al., 2004). In migrating cells, NMIIA and NMIIB have different localization and function, which depend on the rigidity of the specific migratory substrate (Raab et al., 2012).

Given that non-muscle myosins have important roles in regulating cell and tissue shape, we hypothesized that NMIIA and...
NMIIA regulate cell shape changes that occur to form the MHBC. Here we used the zebrafish MHB as a model for determining the molecular mechanisms regulating the initial cell shape changes that occur during brain morphogenesis. We discovered distinct roles for NMIIA and NMIIB in MHB morphogenesis using live imaging to quantify changes in cell shape. We determined that NMIIA controls the length of the cells specifically at the MHB, while NMIIB regulates the width of cells throughout the MHB tissue. Thus NMIIA and NMIIB perform non-redundant functions in regulating the morphogenesis of the MHB.

Materials and methods

Zebrafish maintenance and husbandry

Standard procedures were used for zebrafish maintenance and husbandry (Kimmel et al., 1995; Westerfield, 2000). Wild-type AB zebrafish embryos were used for all experiments. Embryos were staged according to Kimmel et al., 1995. For all experiments somite number was counted to allow for consistent staging comparisons and to eliminate the possibility of phenotypes resulting from developmental delay. The following equivalent staging points were used; 18 somite stage (ss) is equal to 18 hours post fertilization (hpf); 22 ss is equal to 20 hpf; and 24 ss is equal to 22 hpf.

Embryonic gene expression analysis

For all of our studies we used the following sequence information from Zebrafish Ensembl (Flieck et al., 2013). myh9a located on the reverse strand of chromosome 6, ENSDART00000149823; myh9b located on the reverse strand of chromosome 3, ENSDART00000137705. myh10 homolog was found to be located on the forward strand of a different region of chromosome 6, ENSDART00000151114. RT-PCR was conducted on RNA isolated from wild-type embryos over time. Primers used include: myh9a forward primer (5′-AAATCTCCAGAGGTGAGGACGA-3′); myh9a reverse primer (5′-TTGGGTGTCTCCTGTTTCTCC-3′); myh9b forward primer (5′-CTGTCCCCACTCTACTACAGGGGAC-3′); myh9b reverse primer (5′-TGTGGAAGGTCTCTCTCTC-3′); myh10 forward primer (5′-CTCTGAAACACGTAGATT-3′); myh10 reverse primer (5′-TTGGTATCTCCCTAAGGAT-3′); Ef1α forward primer: (5′-GATGCACCACGAGTCTCTGA-3′); Ef1α reverse primer: (5′-TGATGACCTGACCGTTGAGG-3′).

Antisense morpholino oligonucleotide injections

Splice site-blocking morpholino antisense oligonucleotides (morpholino: MO) were used for all knockdown experiments. MO details for each gene are as follows: myh9a MO (5′-AGCAAGAGAGACCTACAAAATCAGA-3′; Gene Tools); myh9b MO (5′-ATGCTCTAAACAGGATGTGATCAGATG-3′; Gene Tools); myh10 MO (5′-CTCTCAAAATGTGCTCTAC-3′; Gene Tools); mypt1 MO (5′-ATTTTTTGTGACTTACTCAGCGATG-3′; Gene Tools). For all MO knockdown experiments, the following concentrations were used and injected into one cell stage embryos either alone or in conjunction with membrane targeting GFP (mGFP). 4 ng myh9a MO, 3 ng myh9b MO with 3 ng p53 MO, 3 ng myh10 MO, 5 ng mypt1 MO. The concentration of control MO was equal to the highest concentration of any experimental MO used in that experiment. Up to 6 ng of myh9a MO was used with no obvious brain phenotype. All confocal evaluation for myh9a MO was conducted at 4 ng.

In situ hybridization

In situ hybridization was conducted according to standard procedures. RNA probes were designed to unique UTR sequences due to the high sequence similarities in the coding regions of these genes (probe regions are shown in Fig. 1). The myh9a probe was cloned from the 3′ end into the 3′ UTR of myh9a from 24 hpf wild type embryos using the following primers. myh9a primer 3′ end forward (5′-TGAGGAGACCTGAGGGAGGAA-3′), myh9a primer 3′ UTR reverse (5′-GAACAGAGCCCCATGAACTAC-3′) resulting in a 502 bp probe. The myh9b probe in situ probe was cloned from the 5′ UTR into the 5′ end of myh9b from 24 hpf wild type embryos using the following primers. myh9b primer 5′ UTR forward (5′-TGAGGAGACCTGAGGGAGGAA-3′), myh9b primer 5′ end reverse (5′-AACACAGCCCTACCACCGTCTC-3′) resulting in a 290 bp probe. The PCR fragments above were subcloned into pGEM using the pGEM T-Easy Vector System Kit (Promega) for probe synthesis. The myh10 in situ probe was made from Image clone 8801976 from Open Biosystems and is located in the 3′ UTR of the gene and results in an 810 bp probe. Sense and antisense probes were made and used for each in situ experiment to test for specificity.

Actin staining

Embryos were fixed in 4% paraformaldehyde for 2 h at room temperature or overnight at 4 °C and washed in PBT. Embryos were incubated at 4 °C overnight in Alexa Fluor 488 phalloidin (Invitrogen A12379) 1:40 in PBT, washed 3X in PBT, mounted in glycerol, and imaged using a Nikon CS2 laser-scanning confocal microscope. Images were analyzed with Nikon Elements software and Photoshop (Adobe).

Non-muscle myosin IIA and IIB immunostaining

Embryos were fixed in Dents for 2 h at room temperature, deyolked, blocked overnight at 4 °C, and then washed in PBT. Embryos were incubated in primary antibody (anti-myosin IIA antibody raised in rabbit, Sigma-Aldrich, M8064, 1:500; and anti-myosin IIB antibody raised in mouse, Santa Cruz Biotechnology, sc-376942, 1:100) overnight at 4 °C, washed in PBT, then incubated in secondary antibody (Alexa Fluor 488 goat anti-rabbit, Life Technologies, 1:500 and/or Alexa Fluor 555 goat anti-mouse, Life Technologies, 1:500). Embryos were flat mounted in glycerol, imaged using a Nikon CS2 laser-scanning confocal microscope, and images were analyzed with Nikon Elements and Photoshop (Adobe) software.

Imaging

All live confocal imaging was conducted as previously described (Graeden and Sive, 2009) using a Nikon CS2 scanning confocal and Nikon Elements software. Briefly, embryos were co-injected at the one-cell stage with membrane GFP (mGFP) mRNA (GFP-CAXX) and the morpholinos indicated. Live embryos were then mounted in agarose wells on a slide and oriented for imaging. A z-series of images was taken for each embryo. Live confocal images presented in each figure are single slices taken from a z-series of images approximately 15–20 μm into the tissue from the dorsal surface. Brightfield and in situ hybridization imaging was conducted using an Olympus SZX12 stereomicroscope with an Olympus DP72 camera. All images were processed using Nikon Elements software or Photoshop (Adobe).

Cell shape analysis

For all cell measurements, single cells were selected based on the ability to see one single cell spanning the entire width of the neuroepithelium from apical to basal in a single z-plane in the
region of interest. Cell length was determined using the Nikon Imaging Systems (NIS) Elements software measurement tool by measuring from apical to basal of a single cell spanning the neuroepithelium. The cell width measurement was obtained from the NIS-Elements software as an average width of a cell. Single cells were manually outlined in a single z-series image to create an object. Then the NIS-Elements software determined the average width of the object (cell) by first calculating the area and the perimeter of the object (cell). The software then calculated the length of the object using the formula: Length = \( \frac{ Perimeter + \sqrt{ Perimeter^2 - 16 \times Area} }{4} \). The width of the object was in turn calculated using the formula: Width = Area/Length.

Statistical analysis

Statistical analysis for all ANOVA data presented was computed using R-3.0.1.

Results

Expression of non-muscle myosin IIA and IIB during embryonic development

Non-muscle myosins are known to be key regulators of cell shape during embryonic morphogenesis (Lecuit and Lenne, 2007; Vicente-Manzanares et al., 2009). We hypothesized that these proteins also play an integral role in shaping the cells that contribute to the MHB tissue fold. In order to test this hypothesis we first determined the zebrafish homologs of human non-muscle myosin II proteins. Zebrafish have two homologs of human MYH9 (myh9a and myh9b) encoding for NMIIA, one homolog for MYH10 (myh10) which encodes for NMIIB, and one homolog for MYH14 (myh14), which encodes for NMIIC. According to the current zebrafish genome assembly, the sequence homology for myh9a and myh9b is 77% and 79% respectively, compared to the human MYH9 (Flicek et al., 2013). Zebrafish myh10 is 90% similar to the human homolog, while myh14 is only 62% similar to the human homolog. It has been demonstrated in the mouse that all three isoforms are expressed broadly throughout the embryo; however, there are tissues that express relatively higher levels of one isoform compared to others (Golomb et al., 2004). myh14 appears to have the lowest expression level in the developing mouse brain (Golomb et al., 2004), and has the lowest sequence homology from human to zebrafish; therefore, we did not investigate the role of NMIIC in cell shape changes during MHB morphogenesis in the zebrafish.

Diagrams of the three zebrafish genes investigated in this study are shown (Fig. 1A). Using RT-PCR we confirmed that all three genes were expressed embryonically during the time of MHB development (Fig. 1B). We further analyzed gene expression using in situ hybridization to determine the localization of expression

![Gene diagram](image)
within the developing embryo (Fig. 1C). We found low levels of expression for \textit{myh9a} maternally (4 hpf) and at early stages (12 hpf). There was some localized expression of \textit{myh9a} within the forebrain, eyes, and tail between 18 and 24 hpf. Non-specific staining for \textit{myh9a} was detected within the brain ventricle space and in the yolk in both antisense and sense controls. \textit{myh9a} did not have obvious expression in the neuroepithelium of the MHB region during morphogenesis. \textit{myh9b} was found to be expressed maternally (4 hpf) and expression was detected throughout the whole embryo and brain at each time point analyzed. \textit{myh10} had a low level of maternal expression at 4 hpf; however, by 12 hpf \textit{myh10} was also found to be expressed throughout the whole embryo and brain. Together these data indicate that the zebrafish non-muscle myosin II genes \textit{myh9b} and \textit{myh10} are expressed during the time of MHB morphogenesis and are found throughout the embryo and brain. While \textit{myh9a} is also expressed, it does not appear to be as highly localized to the brain or MHB region as \textit{myh9b} or \textit{myh10} at these times. These data are consistent with the reported expression patterns for \textit{myh9} and \textit{myh10} during mouse embryonic development (Golomb et al., 2004). Next, we investigated the role for \textit{myh9a}, \textit{myh9b}, and \textit{myh10} in regulating the cell shape changes that are required for the formation of the MHB fold.

Characterization of early MHB morphogenesis and cell shape changes

In order to better characterize the initial tissue and cell shape changes that lead to the MHB tissue fold, we performed detailed analysis of wild type MHB development during early stages of MHB formation when cells are shortening (Gutzman et al., 2008). We injected wild type embryos with membrane GFP (mGFP) mRNA and imaged the developing MHB in live embryos using scanning confocal microscopy at the somite stages indicated (Fig. 2). We quantified changes in the tissue over this time frame by analyzing tissue angle, cell length, and cell width. We found that the angle of the fold changes from 140 degrees to less than 100 degrees between 18 and 24 somite stage (ss) (Fig. 2A–D). We found that cells at the MHBC shorten from 50 μm to less than 40 μm during this time frame (Fig. 2E). We also found that cells outside the MHB get slightly shorter during this time; however, there is still a large difference in cell length at the MHBC compared to cells outside (Fig. 2F). By 24 ss cells at the MHBC are approximately 75% of the length of the outside cells (Fig. 2G) which is consistent with our previous findings (Gutzman et al., 2008). Importantly, we also discovered that the width of the cells changes between 22 and 24 ss (Fig. 2H) and cells in the MHB

![Fig. 2.](image)

**Fig. 2.** Quantification of wild type tissue and cell shape changes during MHB morphogenesis between 18 and 24 ss. (A–C) Live confocal imaging of wild type embryos injected with mGFP and imaged at the stages indicated. (A’–C’) Magnifications of images in A–C with individual cells outlined at the MHBC and posterior to the MHBC towards the hindbrain. (D) Quantification of the MHB angle on the basal side of the neuroepithelium (see dotted lines in A–C). (E) Measurement of neural tube width as a representation of cell length in cells at the MHBC over time. (F) Measurement of neural tube width as a representation of cell length in cells 40 μm posterior to the MHBC over time. (G) Changes in the percentage of cell shortening for cells at the MHBC compared to cells 40 μm outside the MHBC over time. (H) Quantification of cell width measurements over time. Arrowheads indicate MHBC. Asterisks in A–C indicate cells outlined in the images below (A’–C’). One-way ANOVA with multiple t-test comparisons was performed to determine significance, asterisks indicate $p < 0.001$. Results are shown as ± s.e.m. 18 ss, $n = 9$; 22 ss, $n = 11$; 24 ss, $n = 11$. Scale bars: 25 μm.
region get narrower as the morphology of the MHB is changing and the MHBC is forming.

**myh9b and myh10 are required for MHB development**

In order to define the role for NMIIA and NMIB in embryonic MHB morphogenesis, we conducted knockdown experiments using splice-site targeting, antisense oligonucleotide morpholino (MO) knockdown of *myh9a*, *myh9b*, and *myh10*. Splice targeting morpholinos were chosen due to the essential requirement for non-muscle myosins during early development and cell division (Conti et al., 2004; Ma et al., 2007; Maciver, 1996; Urven et al., 2006). We were able to carefully titrate the concentration of each splice targeting morpholino to prevent abnormal levels of cell death while maintaining normal levels of cells division, which allowed us to determine the role for these proteins in regulating cell shape during morphogenesis. Splice blocking morpholinos were confirmed at the concentrations used for all of the experiments presented here using RT-PCR or Western blot analysis (Fig. 5I).

Embryos were injected with morpholinos at the one-cell stage and analyzed for gross embryonic phenotypes and for overall brain morphology at 24 ss using brightfield microscopy. Although we focused specifically on brain morphogenesis defects and cell shape changes at the MHB for this study, we did observe other gross phenotypes in morpholino injected embryos (Fig. S2). *myh9b* morphants demonstrated somite defects, abnormal tail curvature, pigmentation defects, heart abnormalities, slight edema, and abnormal eye and ear formation. *myh10* morphants had abnormal body axis curvature, heart abnormalities, and abnormal eye development. *myh9a* morphants injected with our splice site targeting MO did not appear to have any observable gross morphology defects with the concentrations of morpholino tested (Fig. S2). This is in contrast to the studies by Muller et al. where they demonstrated that knockdown of *myh9a* (previously called *zmyh5* and *myh9-like2*) using a MO targeting the 5'-untranslated region and translational start site, leads to abnormal development of the glomerulus and causes gross embryonic edema at five days post fertilization (Muller et al., 2011). In those studies Muller et al. did not investigate the role of the other zebrafish *myh9* gene, *myh9b* (previously called *myh9z* and *myh9-like1*). This difference in overall gross phenotype for *myh9a* knockdown between our studies and Muller et al. is likely due to the timing of phenotypic analysis, the nature of the morpholinos utilized, and their respective target sequences.

Analysis of overall MHB morphology in control MO injected embryos at 24 ss showed normal formation of the MHB, visible with brightfield microscopy. Although we focused specifically on brain morphogenesis defects and cell shape changes at the MHB for this study, we did observe other gross phenotypes in morpholino injected embryos (Fig. S2). This is in contrast to the studies by Muller et al. where they demonstrated that knockdown of *myh9a* (previously called *zmyh5* and *myh9-like2*) using a MO targeting the 5'-untranslated region and translational start site, leads to abnormal development of the glomerulus and causes gross embryonic edema at five days post fertilization (Muller et al., 2011). In those studies Muller et al. did not investigate the role of the other zebrafish *myh9* gene, *myh9b* (previously called *myh9z* and *myh9-like1*). This difference in overall gross phenotype for *myh9a* knockdown between our studies and Muller et al. is likely due to the timing of phenotypic analysis, the nature of the morpholinos utilized, and their respective target sequences.

Analysis of overall MHB morphology in control MO injected embryos at 24 ss showed normal formation of the MHB, visible with a clear and distinct fold in the tissue at the point of deepest constriction (MHBC) and normal openings in the midbrain and hindbrain ventricles (Fig. 3A). These results are consistent with our previous reports (Gutzman et al., 2008). A morpholino designed specifically to target only *myh9a* did not result in any visible abnormal MHB or brain phenotype when imaged with brightfield microscopy (Fig. 3B). In contrast, embryos injected with the *myh9b* MO or the *myh10* MO had abnormal MHB development. *myh9b* morphants did not have a sharp fold in the tissue at the point of deepest constriction, instead the fold was a curved shape (Fig. 3C). *myh10* morphants, similar to *myh9b* morphants, failed to form a sharp tissue bend at the MHB (Fig. 3D). *myh10* morphants also had decreased midbrain ventricle opening (Fig. 3D). We have previously shown that abnormal brain ventricle inflation, due to lack of cerebrospinal fluid as found in the *snakehead* mutant, did not affect cell shape at the MHB (Gutzman et al., 2008). Therefore, the *myh10* morphant ventricle defect is likely due to abnormal dorsolateral hinge-point formation which is also dependent on non-muscle myosin II (Nyholm et al., 2009). Knockdown of both *myh9a* and *myh9b* together, or *myh9a* and *myh10* together, at the same concentration of morpholino used for single knockdown, did not worsen or change the MHB phenotype observed (data not shown). However, the double knockdown of *myh9a* and *myh9b* did appear to worsen the gross whole embryo tail and eye phenotypes at 24 ss, which is consistent with the localization of *myh9a* gene expression at this time point (data not shown).

We rescued the 24 ss brain phenotypes in *myh9b* and *myh10* morphants by co-injection of human MYH9 or MYH10 mRNA (Fig. S3). Quantification and representative images are shown for normal, mild, and severe *myh9b* and *myh10* morphant phenotypes (Fig. S3). For all of the experiments presented here investigating MHB defects and cell shape analysis, only mild phenotypes were analyzed. This is consistent with the level of protein knockdown we detect using the *myh10* morpholino, where our knockdown results in approximately 40% loss of the NMIIB protein (Fig. S1D). Together these data demonstrate that knockdown of both *myh9b* and *myh10*, but not *myh9a* knockdown, results in abnormal formation of the MHB indicating a role for *myh9b* and *myh10* in brain morphogenesis. More detailed and quantitative comparisons of the knockdown MHB phenotypes are described in the following sections.

**MHB tissue angle is dependent upon non-muscle myosin IIA and IIB**

After determining that knockdown for both *myh9b* and *myh10* led to defects in MHB formation by brightfield microscopy, we wanted to determine the specific role for these non-muscle myosin proteins in regulating cell and tissue shape during morphogenesis. We performed detailed analyses on our non-muscle myosin II morphant brains and compared them to control morpholino injected embryos. We first examined the MHB tissue angle. Single-cell embryos were injected with MO and mGFP, and then imaged live using confocal microscopy. The angle at the MHBC was measured and compared (Fig. 3E–J). The average angle at 24 ss in control MO injected embryos was approximately 100 degrees, *myh9a* morphants had a normal tissue angle; however, *myh9b* MO injected embryos had a broader tissue angle of 125 degrees, and *myh10* MO injected embryos had an abnormal MHB tissue angle of 140 degrees (Fig. 3E–J). These results indicate that both *myh9b* and *myh10* contribute to the formation of the tissue angle at the MHB. We also analyzed the tissue angle in mypt1 morphants. mypt1 is the regulatory subunit of myosin phosphatase and mypt1 knockdown results in non-functional myosin phosphatase and overactive non-muscle myosin II activity (HartsHorne et al., 2004). In addition, our previous report demonstrated that mypt1 knockdown leads to abnormal tissue and cell shape in the hindbrain (Gutzman and Sive, 2010). With mypt1 knockdown, and therefore overactive NMIIA and NMIIB, we found that the mypt1 morphants also had an abnormal tissue angle with an average of 130 degrees at 24 ss (Fig. 3J–J). This result further supports the observation that regulation of non-muscle myosin activity is important for this morphogenetic process.

Since non-muscle myosin II is known to be required for normal cell proliferation, we confirmed that the brain phenotypes observed were not a result of increased or decreased cell proliferation or cell death. We analyzed cell proliferation with PH3 staining and cell death with TUNEL staining. We found that cell proliferation and cell death were normal in *myh9b* and *myh10* morphants at the concentrations of MO used for these experiments (Fig. S4). Together, these results indicate that *myh9b* and *myh10* have critical roles in determining the proper angle of tissue folding at the MHB, and that *myh9a* does not appear to be involved in this process. The mypt1 knockdown phenotype also confirms the importance of specific regulation of the contractile state of the NMII proteins during MHB morphogenesis.

**myh9b is required for cell shortening at the MHBC**

During MHB morphogenesis, the first cell shape change occurs between 17 and 22 h post fertilization (equivalent to 16 to 24 ss) where cells at the MHBC shorten to 75% of the length of cells.
Fig. 3. myh9b, myh10, and mypt1 are required for MHB tissue morphogenesis. (A–D) Brightfield dorsal view images of 24 ss embryos following injection with (A) control MO, (B) myh9a MO, (C) myh9b MO, (D) myh10 MO. Anterior is to the left in all images. Arrowheads indicate MHBC. Scale bars: 100 μm. (E–I) Live confocal images showing the MHB region of 24 ss zebrafish embryos injected with mGFP mRNA and coinjected with control MO (E), myh9a MO (F), myh9b MO (G), myh10 MO (H), or mypt1 MO (I). (J) Quantification of the MHB angle on the basal side of the neuroepithelium (see angle lines in E–I). One-way ANOVA with multiple t-test comparisons was performed to determine significance between control and test groups. Asterisks indicate *p < 0.001. Results are shown as ± s.e.m. For E–I; control MO, n = 48; myh9a MO, n = 10; myh9b MO, n = 16; myh10 MO, n = 18; mypt1 MO, n = 20.
outside of the MHBC (Fig. 2 and Gutzman et al., 2008). The mechanisms that regulate cell shortening at the MHBC are unknown. We hypothesized that NMII proteins regulate this cell shape change which is required for the formation of the normal MHB tissue angle. We tested this hypothesis using knockdown experiments and then quantifying the length of the cells at the MHBC and outside of the MHBC (Fig. 4). Embryos were injected with mGFP and specific morpholinos targeting myh9a, myh9b, myh10, or mypt1, and then live imaged at 24 ss using confocal microscopy. Cell length was quantified at the MHBC. At this stage of development neuroepithelial cells span the entire epithelium from apical to basal; therefore, we used the width of the single layer of pseudostratified epithelium as a measure of cell length. We measured cell length on one side of the neural tube at the MHBC (X) (Fig. 4A). Then we measured the length of cells 40 μm posterior to the MHBC (approximately 15 cells) outside of the MHBC region (Y) (Fig. 4B). We found that myh9b morphants had significantly longer cells at the MHBC compared to controls, while the length of the MHBC cells in myh9a and myh10 morphant cells were unchanged (Fig. 4C–G). In the surrounding region posterior to the MHBC we found that cell length in myh9b morphants was the same as control, but myh10 cells were slightly, but significantly, shorter (Fig. 4H). However, this small change in cell length outside of the MHBC from myh10 morphants would still not account for the dramatic angle change observed at the MHBC.

At the MHBC, and outside of the MHBC, mypt1 morphant cells were shorter than control cells (Fig. 4F, H). This observation continues to support the role for mypt1 in regulating myosin contraction. mypt1 morphants have overactive myosin, which causes increasing actomyosin contraction within the cells and leads to a shortening of the cells, consistent with the cell shape phenotype previously observed in the hindbrain (Gutzman and Sive, 2010).

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**Fig. 4.** myh9b is required for cell shortening at the MHBC during morphogenesis. (A–F) Live confocal images showing the MHB region of 24 ss zebrafish embryos injected with mGFP and coinjected with control MO (A–B), myh9a MO (C), myh9b MO (D), myh10 MO (E), or mypt1 MO (F). (G) Quantification of the cell length (X) at the MHBC (see lines in A, C–F). (H) Quantification of cell length 40 μm outside of the MHBC (Y) (see lines in B–F). One-way ANOVA with multiple t-test comparisons was performed to determine significance between control and test groups. Asterisks indicate *p* < 0.001. Double asterisks indicate *p* < 0.05. Results are shown as ± s.e.m. Control MO, *n* = 48; myh9a MO, *n* = 10; myh9b MO, *n* = 16; myh10 MO, *n* = 18; mypt1 MO, *n* = 20. Scale bars: 25 μm.
Together these data indicate that cell shortening at the MHBC is dependent upon the function of myh9b, and not myh10, suggesting a different role for these two non-muscle myosin proteins in regulating cell shape changes during MHB morphogenesis.

myh10 is required for regulating cell width in the MHB

In our detailed analysis of wild type embryos we discovered that cells throughout the MHB region become narrower during the formation of the fold (Fig. 2). While conducting our knockdown experiments and confocal imaging, we noticed that cells of the MHB in some morpholino-injected embryos looked wider than in control-injected embryos. Therefore, we conducted additional quantification of cell shapes by determining cell width and area in the MHB neuroepithelium in knockdown embryos. Embryos were injected with mGFP and morpholinos, and then live imaged at 24 ss using confocal microscopy. We quantified cell width and cell area in cells at the MHBC and in the posterior part of the MHB (Fig. 5). We found that cells in the myh9a and myh9b morphants had normal cell width and area (Fig. 5A–C, F, G); however, cells from embryos injected with myh10 morpholino had significantly increased cell width and area (Fig. 5D, F, G). mypt1 morphants also had significant differences in cell area and width (Fig. 5E–G) as would be predicted by over activation of non-muscle myosin and as previously observed in the hindbrain (Gutzman and Sive, 2010). These data indicate that myh10, and not myh9b, is critical for the regulation and maintenance of cell width in the MHB region of the neuroepithelium. This further supports the differential role for myh9b and myh10 in regulating cell shape at the MHB.

Abnormal distribution of actin at the MHB with NMIIA and NMIIIB knockdown

The discovery that NMIIA and NMIIIB have different roles in regulating cell shape changes at the MHB led us to ask, what are the mechanisms for this differential regulation? We have previously demonstrated that later in MHB formation (24 hpf) actin is enriched at the MHBC (Gutzman et al., 2008), and since we know that non-muscle myosin proteins are actin motors, we asked whether or not the distribution of actin in our knockdown embryos was also differentially disrupted with NMIIA or NMIIIB knockdown.

Embryos were injected with the morpholino indicated, stained with phalloidin, and imaged using confocal microscopy to show actin localization in the MHB region (Fig. 6). We quantified the relative distribution of actin within the MHB in three regions. We determined the amount of actin within the neuroepithelium at the MHBC compared to the neuroepithelium outside the MHBC.
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regulating the actin cytoskeleton and neuroepithelial cell shape

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Fig. 6A). We compared the amount of actin located apically at the midline of the neural tube compared to the amount of actin within the neuroepithelium at the apical edge of the MHBC cells (Fig. 6B), and we compared the amount of actin located on the basal side of the neural tube compared to the amount of actin within the neuroepithelium at the basal edge of the MHBC cells (Fig. 6C). These different regions of the neuroepithelium were used for comparison in B and C to more carefully address changes at the extreme apical or basal edges of the cells compared to just inside the adjacent neuroepithelium. We used the ratio of mean fluorescence intensity of actin staining in two regions within each embryo for comparison, shown by box 1 and box 2 (Fig. 6A–C). The actin mean intensity ratio for box 1 was divided by box 2 in that given region. A ratio of 1 would indicate equal distribution of actin in box 1 and box 2. In control embryos there is approximately 1.5 times more actin within the neuroepithelium at the MHBC compared to more posterior neuroepithelium. This reflects the actin distribution previously described (Gutzman et al., 2008). There is two times more actin localized to the apical region of cells compared to the adjacent apical side of the neuroepithelial cells, and there is 1.5 times more actin on the basal side of the epithelium compared to the adjacent basal side of the neuroepithelium in the region of the MHBC (Fig. 6A–C, G). When we investigated actin distribution with myh9b knockdown we found a decrease in actin localization within the MHBC neuroepithelium and apically, while basal actin was unchanged (Fig. 6D, G). With myh10 knockdown there was a significant decrease in actin localized to the MHBC neuroepithelium, apically, and basally indicating that actin in all areas of the MHBC cells was disrupted (Fig. 6E, G). mypt1 knockdown did not affect apical actin distribution, as previously reported in the hindbrain (Gutzman and Sive, 2010); however, actin within the MHBC neuroepithelium was disrupted, as was basal actin distribution (Fig. 6F–G). Together, the differences in actin distribution with myh9b knockdown compared to myh10 knockdown are consistent with myh9b and myh10 having differential effects on regulating the actin cytoskeleton and neuroepithelial cell shape changes at the MHBC. myh9b affected actin only in the neuroepithelium and apically at the midline, while myh10 affected actin within the neuroepithelium, apically, and basally.

Non-muscle myosin II A and II B protein localization

We also hypothesized that NMIIA and NMIIB would be differentially localized within the cells which could in turn explain their ability to differentially regulate cell shape. In order to test this hypothesis we used antibody staining specific for either NMIIA or NMIIB to see their localization pattern within the MHB region (Fig. 7). We did not detect any obvious differences between NMIIA and NMIIB localization in control embryos (Fig. 7A–C). The localization pattern was consistent with what has been demonstrated in the mouse neural tube (Ma et al., 2007). We did, however, see changes in both NMIIA and NMIIB localization with mypt1 knockdown where non-muscle myosin II proteins accumulated apically and basally in the neuroepithelium as seen previously for NMIIA (Fig. 7D and (Gutzman and Sive, 2010)). Interestingly, when we stained for NMIIA with knockdown of myh10 we found a change in NMIIA localization from generally cytoplasmic and apical to more diffuse and basally localized (Fig. 7E). We also found a change in NMIIB localization with myh9b knockdown, again from mostly cytoplasmic to more diffuse with increased basal localization (Fig. 7F). No primary controls are shown to indicate the specificity of the staining (Fig. 7G–H).

These data indicate that normally, the localization of NMIIA and NMIIB is overlapping within the neuroepithelium of the MHB region at this time, suggesting that the mechanism by which NMIIA and NMIIB are differentially regulating cell shape changes during brain morphogenesis is not due to differential localization, but likely due to differential regulation of activity. Furthermore, our analysis of localization of one NMII protein with knockdown of other indicates that NMIIA and NMIIB depend on each other for proper localization.

Discussion

myh9b and myh10 differentially regulate cell shape during MHB morphogenesis

In wild type embryos, the basal angle of the MHB changes over time from 140 degrees at 18 ss to a more acute angle of 95 degrees
by 24 ss (Fig. 2). During this time, cells at the MHBC are changing shape to allow this angle to form. Cells at the MHBC shorten by approximately 25%, while cells outside the MHBC also shorten slightly over this time window (Fig. 2). Concurrently, cells throughout the MHB region become narrower between 22 ss and 24 ss (Fig. 2). Together these morphogenetic changes lead to the formation of the highly conserved MHB fold (Fig. 8A).

Here, we demonstrate for the first time that non-muscle myosin II A and II B have distinct roles in regulating cell shape changes during brain morphogenesis. We discovered that NMIIA is required for the shortening of the cells specifically at the MHBC, while NMIIIB is required for the narrowing of the cells throughout the MHB region (Fig. 8B and C). In contrast to our knockdown studies, we investigated non-muscle myosin II gain of function using mypt1 knockdown. We found that cells in mypt1 morphants, where there is over activation of non-muscle myosins, were both shorter and wider cells throughout the MHB region (Fig. 8D), which is consistent with the cell shape phenotype found with mypt1 loss of function in the hindbrain (Gutzman and Sive, 2010). Together, these data demonstrate that NMIIA is required for regulating the length of cells specifically at the MHBC and not in surrounding regions, while NMIIIB is required for regulation of cell width throughout the MHB region. This uncovers a novel differential role for mechanisms by which these two proteins regulate cell shape during brain morphogenesis.

Cell length and cell width during development

Morphogenetic processes require specific changes in cell shape to cause bending of epithelial sheets, tissue invagination, and tube formation. We propose that regulating cell length and cell width may be as important in developmental processes as apical constriction or cell migration; however, investigation and quantification of these more subtle changes in cell shape has been limited. Apical constriction results in decreased surface area on the apical side of the polarized cell changing shape, and is critical in development during gastrulation and vertebrate neural tube formation (Haigo et al., 2003; Lee and Harland, 2007; Martin et al., 2009). Various mechanisms of apical constriction have been described depending on cell type and context, typically involving apical actomyosin networks linked to cell-cell apical junctions (Martin and Goldstein, 2014). Other cell shape changes including changes in cell length, width, or basal constriction have been less well defined and are likely to be regulated through both overlapping and distinct mechanisms. For example, during neural tube formation, cells of the neural plate have been described to lengthen before they apically constrict (Karfunkel, 1974), and although this cell shape change has been defined for decades, the mechanism for this cell lengthening has not been studied. Distinct mechanisms have been uncovered for regulation of basal constriction. We determined that basal constriction at the MHB, following cell shortening, is laminin dependent, and basal constriction in optic cup morphogenesis requires the novel gene ojoplano (Gutzman et al., 2008; Martinez-Morales et al., 2009; Wang et al., 2012). In contrast, follicle cells in drosophila egg chamber elongation are regulated by actomyosin contraction, as in apical constriction; however, the orientation of the filaments is different and the constraction occurs basally, not apically, to shape the tissue (He et al., 2010).

The Kupffer’s vesicle also undergoes regional cell shape changes during development. Interestingly, in the Kupffer’s vesicle the anterior cells are elongated and the posterior cells shorten and widen over time (Wang et al., 2012), cell shape changes that are similar to those described here for MHB morphogenesis. Furthermore, these cell shape changes are regulated by non-muscle myosin II activity (Wang et al., 2012); however, it was not determined if NMIIA and NMIIIB had distinct functions in the shortening or widening of cells. Future experiments to determine the mechanisms that regulate cell shape changes such as length, width, and basal constriction are essential for understanding complex morphogenetic processes.

Function of NMIIA versus NMIIIB

The DNA sequence and protein structural similarities between NMIIA and NMIIIB might suggest that these proteins are
redundant; however, it is becoming more apparent that each isoform has distinct functions. The function of these proteins has been determined using knockdown studies in many systems. NMIIA and NMIIB knockout mice display different phenotypes, where NMIIA knockouts are embryonic lethal due to cell adhesion defects (Conti et al., 2004), and NMIIB knockouts present with heart, brain, and neuronal migration defects (Ma et al., 2007; Ma et al., 2004). The ablation of NMIIB in mice resulted in structural abnormalities in the brain of mice, consistent with the role for NMIIB neuroepithelial morphogenesis (Tullio et al., 2001). Experiments to test for functional redundancy between NMIIA and NMIIB have suggested only a limited ability for the proteins to compensate for each other. For example, replacement of NMIIB with NMIIA in the mouse rescues brain abnormalities, but does not rescue cardiac defects (Bao et al., 2007). Here we have uncovered new isoform-distinct functions in regulating cell shape at the MHB during brain morphogenesis.

NMIIA and NMIIB have different enzymatic properties during ATP-hydrolysis which determine their distinct roles in regulating cell shape. Only a small fraction of the head domain of NMIIA is strongly bound to actin at any one time (Kovacs et al., 2003). In contrast, NMIIB is one of the slowest myosins with regard to the rate in which it translocates actin filaments by having a slow ATPase cycle; therefore it spends a significantly longer time strongly bound to actin (Wang et al., 2003). This longer binding may make NMIIB better suited for maintaining cellular tension. These differences in enzymatic activity may account for the role of NMIIB in regulating cell width and area throughout the MHB region, while NMIIA is working more quickly to shorten cells in a specific brain region.

Differential regulation of NMIIA and NMIIB

We determined that NMIIA and NMIIB proteins are not differentially distributed within the cells at the MHB; however, we did discover that knockdown of one can influence the localization of the other (Fig. 7). NMIIA and NMIIB are both activated through phosphorylation of the myosin regulatory light chain (MRLC) (Bresnick, 1999; Ito et al., 2004). Yet, NMIIA and NMIIB are differentially regulating cell shape in the same cell resulting in distinct changes. From our previous work, we know that the level of phosphorylated MRLC in the brain increases from 18 hpf to 21 hpf and then goes back down by 24 hpf (Gutzman and Sive, 2010), indicating the NMII activity is high in the brain during the time of morphogenesis investigated here. MRLC activation can occur through multiple signaling pathways; including through myosin light chain kinase (MLCK) and/or Rho-kinase (ROCK). In cell culture, MLCK and ROCK specifically localize to regulate MRLC phosphorylation in a spatially dependent manner. In 3T3 cells, ROCK is more active in phosphorylating MRLC at the center of the cell, while MLCK is more active in phosphorylating MRLC at the cell periphery (Totsukawa et al., 2004; Totsukawa et al., 2000). It was demonstrated in migratory cells that a given cellular microenvironment may play a role in determining the localization and function of specific NMII isoforms (Raab et al., 2012). Although we do not see a difference in NMIIA and NMIIB localization in normal tissue here, potentially the differential function of the two proteins is determined by differential localization of their upstream activators which have yet to be identified. Another possibility is that, although the proteins are localized in the same parts of the cell,
the orientation of the non-muscle myosin heavy chains may be oriented in opposing directions. For example, NMIIA fibers may run in an apical to basal direction to regulate cell length, while NMIIIB fibers may orient perpendicularly to NMIIA to regulate cell width.

Non-muscle myosin heavy chains IIA and IIB can both be phosphorylated on various sites to affect filament assembly and protein function (Vicente-Manzanares et al., 2009). This raises the question as to whether or not there is a difference in the phosphorylation state of the non-muscle myosin heavy chains themselves at the MHB. Phosphorylation of the non-muscle myosin heavy chains facilitates filament disassembly and NMIIA and NMIIIB have different sites in their tail domains making this a possible level of differential activation and regulation. It was also recently demonstrated that NMII isoforms can co-assemble in living cells, forming heterotypic filaments that can perform both isoform specific and redundant functions (Beach et al., 2014). It remains to be seen if these heterotypic filaments are present in the neuroepithelium during development.

Differential distribution of actin with non-muscle myosin knockdown

It is established that NMII protein activity, in response to extra or intracellular signals, contributes to the spatial organization of the actin network, resulting in contractility and physiological functions (Kohler et al., 2011). Both NMIIA and NMIIIB knockdown resulted in abnormal actin distribution in the MHB region during morphogenesis. Knockdown of NMIIA caused changes in actin localization within the neuroepithelium and at the apical surface of the neural tube, while knockdown of NMIIA caused abnormal actin distribution within the neuroepithelium, and at both the apical and basal surfaces of the neural tube. The location of actin affected by NMIIA knockdown may provide some additional clues as to the regulation of the NMII protein. Since mypt1 appears to regulate NMII activity apically, it is likely to be regulating both NMIIA and NMIIIB (Gutzman and Serie, 2010), which is consistent with the mypt1 knockdown cell shape phenotypes as well. Actomyosin activity is regulated on the basal surface of follicle cells by Rho, ROCK, and cell-cell adhesion during Drosophila egg chamber development to cause contraction (He et al., 2010). However, Drosophila have only one non-muscle myosin heavy chain (zipper), indicating the importance for in vivo vertebrate studies to determine how NMIIA and NMIIIB are differentially regulating cell shape. These studies will be essential to elucidate additional mechanisms of morphogenetic processes.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.10.017.

References


