Epithelial relaxation mediated by the myosin phosphatase regulator Mypt1 is required for brain ventricle lumen expansion and hindbrain morphogenesis

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SUMMARY
We demonstrate that in the zebrafish hindbrain, cell shape, rhombomere morphogenesis and, unexpectedly, brain ventricle lumen expansion depend on the contractile state of the neuroepithelium. The hindbrain neural tube opens in a specific sequence, with initial separation along the midline at rhombomere boundaries, subsequent openings within rhombomeres and eventual coalescence of openings into the hindbrain ventricle lumen. A mutation in the myosin phosphatase regulator mypt1 results in a small ventricle due to impaired stretching of the surrounding neuroepithelium. Although initial hindbrain opening remains normal, mypt1 mutant rhombomeres do not undergo normal morphological progression. Three-dimensional reconstruction demonstrates cell shapes within rhombomeres and at rhombomere boundaries are abnormal in mypt1 mutants. Wild-type cell shape requires that surrounding cells are also wild type, whereas mutant cell shape is autonomously regulated. Supporting the requirement for regulation of myosin function during hindbrain morphogenesis, wild-type embryos show dynamic levels of phosphorylated myosin regulatory light chain (pMRLC). By contrast, mutants show continuously high pMRLC levels, with concentration of pMRLC and myosin II at the apical side of the epithelium, and myosin II and actin concentration at rhombomere boundaries. Brain ventricle lumen expansion, rhombomere morphology and cell shape are rescued by inhibition of myosin II function, indicating that each defect is a consequence of overactive myosin. We suggest that the epithelium must ‘relax’, via activity of myosin phosphatase, to allow for normal hindbrain morphogenesis and expansion of the brain ventricular lumen. Epithelial relaxation might be a widespread strategy to facilitate tube inflation in many organs.

KEY WORDS: Myosin, mypt1 (ppp1r12a), Neuroepithelium, Rhombomere, Brain ventricle, Lumen, Epithelial relaxation, Morphogenesis, Hindbrain, Cell shape, Zebrafish

INTRODUCTION
During vertebrate brain development, the lumen of the neural tube fills with embryonic cerebrospinal fluid (eCSF) to form the brain ventricles, a conserved system of interconnected cavities that is essential for neurogenesis and normal brain function (Gato and Desmond, 2009; Lowery and Sive, 2009). Brain ventricle formation is intimately connected to morphogenesis of the neuroepithelium. In the hindbrain, rhombomeres are segments of neuroectoderm that differentiate into specific cranial nerves. Rhombomeres are morphologically apparent during neural tube and initial brain ventricle formation, but concomitant with ventricle lumen expansion, rhombomeres lose morphological distinctness, although they retain different molecular identities (Hanneman et al., 1988; Heyman et al., 1993; Moens and Prince, 2002).

Zebrafish mutants lacking eCSF fail to form a brain ventricular lumen, confirming the role of fluid secretion in inflating the ventricular lumen (Lowery and Sive, 2005). However, several zebrafish mutants with abnormal ventricle shape do not appear to have defects in eCSF secretion (Lowery and Sive, 2009). One such mutant corresponds to Mypt1 (Ppp1r12a – Zebrafish Information Network), a regulatory inhibitor of myosin phosphatase. Myosin phosphatase regulates the phosphorylation state of myosin regulatory light chain (MRLC), which, in turn, regulates contractility of non-muscle myosin II. In order for myosin phosphatase to be functional, Mypt1 must be non-phosphorylated and bound to PP1 (the catalytic subunit of myosin phosphatase). Thus, phosphorylated Mypt1 inhibits myosin phosphatase activity (Hartshorne et al., 2004), and mutations in Mypt1 that prevent binding to PP1 result in non-functional myosin phosphatase (Huang et al., 2008).

Cell shape and movement are intimately correlated with myosin function (Lecuit and Lenne, 2007; Quintin et al., 2008). In Drosophila, Myosin II is required for morphogenetic furrow formation during eye development and for regulation of cell shape and arrangement within the eye imaginal disc (Escudero et al., 2007). Myosin II is required for proper sheet movement and cell shape during Drosophila dorsal closure (Mizuno et al., 2002; Tan et al., 2003). In zebrafish, Mypt1 is important for kidney formation and for convergent extension during gastrulation (Huang et al., 2008; Weiser et al., 2009).

The role of myosin during vertebrate hindbrain formation is not well understood. We demonstrate that regulation of myosin function through Mypt1 is required for normal hindbrain cell shape, rhombomere morphogenesis and, unexpectedly, for brain ventricle lumen expansion, through a process that we have termed ‘epithelial relaxation’. This process might play a role during tube lumen expansion in multiple organs.

MATERIALS AND METHODS
Fish lines and husbandry
Zebrafish were maintained and staged using standard procedures (Kimmel et al., 1995; Westerfield, 1995). Zebrafish lines included wild-type AB and mypt1 hi2653 kindly provided by Nancy Hopkins (Amsterdam et al., 2004).
Primers to identify mypt1 mutants by genomic PCR: 2653c1, 5'-GAT-AGGCTCGGAGACGGACC-3' (480 bp 5' of viral insertion); 2653-3, 5'-CACAAAACGTAGCTAATAGGTGGC-3' (220 bp 3' of viral insertion); and IPl3, 5'-GTCCTTTGGGAGGCTCCTC-3' (within viral insertion, 55 bp from the 3' end of the insertion). The PCR product for the wild-type allele is 700 bp, for the mutant allele 275 bp.

**Brightfield brain imaging, ventricle injections and forced neuroepithelial expansion**

Brain ventricle injections were carried out as described (Gutzman and Sive, 2009). Forced expansion was conducted as above with increased injection pressure (30 psi) for extended time and captured with video. The ventricle opening was measured at the ear before injection and at the point of maximal expansion during injection.

**Antisense morpholino oligonucleotide injections**

A splice site-blocking morpholino antisense oligonucleotide (morpholino:MO) (5'-ATTTTTTTGTGACTTACTACGCGATG-3'; Gene Tools) that targets exon 2 intron 2 of zebrafish mypt1 was injected into one-cell stage embryos. Wild-type embryos were injected with 5 ng of splice site MO or control MO. The head/brain portion of each embryo was removed posterior to the ear and anterior to the first somite and collected in buffer comprising control MO. The head/brain portion of each embryo was removed posterior to the ear and anterior to the first somite and collected in buffer comprising 25 mM Tris (pH 8.0), 2 mM EDTA (pH 8.0), 10% glycerol, 1% Triton X-100 and protease inhibitor cocktail (Roche, 0469312401). Anti-pMRLC (Cell Signaling, #3671, 1:1000) and β-actin (Sigma, A5441, 1:1000) were used. Films were scanned and quantified using Photoshop.

**Western blot analysis**

Wild-type embryos were injected with 5 ng of mypt1 splice site MO or control MO. The head/brain portion of each embryo was removed posterior to the ear and anterior to the first somite and collected in buffer comprising 25 mM Tris (pH 8.0), 2 mM EDTA (pH 8.0), 10% glycerol, 1% Triton X-100 and protease inhibitor cocktail (Roche, 0469312401). Anti-pMRLC (Cell Signaling, #3671, 1:1000) and β-actin (Sigma, A5441, 1:1000) were used. Films were scanned and quantified using Photoshop.

**Immunohistochemistry**

Embryos were fixed in Dents (myosin II) or in 4% paraformaldehyde (pMRLC), blocked in 5% normal goat serum in PBT, incubated in primary antibody (anti-myosin IIA, M8064, Sigma, 1:500; or anti-pMRLC, #3671, Cell Signaling, 1:20) then in secondary antibody (goat anti-rabbit or anti-mouse IgG conjugated with Alexa Fluor 488, Invitrogen, 1:500), some in combination with propidium iodide (PI) (P3566, Invitrogen, 1:1000). Actin staining was as previously described (Gutzman et al., 2008). Images were analyzed using Imaris, LSM software (Zeiss) and Photoshop. For vibratome sections, fixed embryos were embedded in 5% low-melting-point agarose. Sections (50 μm) were stained as described above.

**Mosaic analysis**

Wild-type embryos were co-injected with 5 ng control MO or 5 ng mypt1 MO and either memCherry (kindly provided by Dr Roger Tsien, University of California San Diego) or memGFP mRNA at the one-cell stage. Cells were transplanted from the memGFP-injected donors at sphere stage into memCherry-injected hosts at shield stage targeted to the presumptive neuroepithelium through a combination with propidium iodide (PI) (P3566, Invitrogen, 1:1000). Actin staining was as previously described (Gutzman et al., 2008). Images were analyzed using Imaris, LSM software (Zeiss) and Photoshop. For vibratome sections, fixed embryos were embedded in 5% low-melting-point agarose. Sections (50 μm) were stained as described above.

**Three-dimensional (3D) cell reconstruction**

Confocal images were imported into 3D Doctor (Able Software). Single cells in r4 and at the r4/r5 boundary were chosen for 3D reconstruction, based on the ability to see an entire cell from the apical to the basal side of the neuroepithelium through a z-series. Individual cells were manually outlined in each z-section and rendered in 3D. For mosaic analysis, transplanted cells were reconstructed using automated surface rendering of the green channel (transplanted cells) in Imaris.

**Blebbistatin treatment**

Wild-type and mypt1 mutant embryos were treated with 50 μM blebbistatin (B0560, Sigma) or 0.1% DMSO as control at 22 hpf. After treatment, embryos were imaged by brain ventricle injection (Gutzman and Sive, 2009). Alternatively, an mypt1 mutant clutch of embryos was treated with 50 μM blebbistatin or 0.1% DMSO at 17 hpf. Embryos were imaged at 19 hpf and then collected and analyzed for genotype following imaging.

**RESULTS**

**A specific sequence of hindbrain ventricle opening**

As the brain ventricles form, the closed zebrafish neural tube must separate at the midline and undergo lumen expansion as it fills with eCSF (Lowery and Sive, 2005). In order to determine how these separation and expansion processes occur, we analyzed the temporal sequence of neural tube opening during formation of the hindbrain ventricle. In all vertebrates, the hindbrain neuroepithelium is divided into segmented rhombomeres, which are transient anteroposterior bulges (Lumsden, 2004; Vaage, 1969) with distinct gene expression and fates. Our focus was on a 7-hour period during zebrafish brain development, 17-24 hours post-fertilization (hpf), beginning after completion of neurulation and encompassing the time of initial neural tube opening, neuroepithelial morphogenesis and ventricle expansion. Emphasis was on the hindbrain as this forms the largest ventricle. The sequence of hindbrain opening can be seen in live embryos imaged by spinning disk confocal microscopy (Fig. 1 and see Movie 1 in the supplementary material). At 17 hpf the neural tube has formed a clear midline, and outlines of rhombomeres are apparent as shallow indentations on the outside of the neural tube (Fig. 1A). An initial hindbrain opening was present at ~18 hpf, between the boundary of rhombomere 0 (r0) and r1 (Fig. 1B); therefore, we hypothesized that ventricle inflation would occur in an anterior-to-posterior direction. However, by 19 hpf, when rhombomeres were clearly visible, additional openings appeared at the r3/r4 boundary and the r4/r5 boundary, in the middle of the hindbrain (Fig. 1C). The openings surrounding r4 were consistent with reported r4 determination, as assessed by gene expression and by the sequence of visible rhombomere boundary morphology in zebrafish (Maves et al., 2002; Moens and Prince, 2002). By 20 hpf, the lumen of the neural tube had begun to open anteriorly and posteriorly from r1, while a new opening was seen at the r2/r3 boundary (Fig. 1D). By 21 hpf, the lumen was open within r2 (Fig. 1E) and, by 22 hpf, lumen opening was seen within r4, leaving the only visible rhombomere morphology at r3 and r5, the two largest rhombomeres (Fig. 1F). Between 23 and 24 hpf, separation of r3 and r5 occurred and the ventricle lumen expanded (Fig. 1G,H). Thus, by 24 hpf, the neural tube was open, with a large ventricular space. The more posterior rhombomeres, r6 and r7, opened in a sequence that was consistent with rhombomere determination by gene expression (data not shown); however, owing to the curvature of the embryo, we could not image the entire length of the hindbrain in any one experiment, and focused on more anterior rhombomeres.

These data show that, contrary to our hypothesis, the hindbrain ventricle does not open in an anterior-to-posterior sequence. Rather, opening occurs in a stereotypical sequence along the length of the hindbrain, with initial openings between rhombomeres, and later coalescence of these openings to form the hindbrain ventricle lumen.
**mypt1 mutants have abnormal hindbrain ventricle expansion and retain early rhombomere morphology**

In order to define the mechanisms underlying hindbrain ventricle opening and expansion, we focused on the mypt1^hi2653 insertional mutant, which has been identified as having small and abnormally shaped brain ventricles (Amsterdam et al., 2004) (L. A. Lowery and H.S., unpublished). The retroviral insertion is located in the mypt1 gene (also called ppp1r12a), which encodes myosin phosphatase subunit 1 (Mypt1), a regulator of the myosin phosphatase holoenzyme (Ito et al., 2004; Matsumura and Hartshorne, 2008) (see Fig. S1A,B in the supplementary material). Gene assignment for mypt1^hi2653 was confirmed by rescue of mutants with wild-type mypt1 mRNA, by RT-PCR for mypt1 mRNA in mutants (see Fig. S1C,D in the supplementary material), by injection of a mypt1 splice site morpholino antisense oligonucleotide, which phenocopies mypt1 mutants, and by rescue of the MO-injected phenotype with mypt1 mRNA (see Fig. S1E-G in the supplementary material). mypt1 is expressed maternally and zygotically throughout the embryo (see Fig. S2 in the supplementary material). The events we describe occur prior to the onset of heartbeat, indicating that circulation is not involved.

In brain ventricles injected with Texas Red dextran (Gutzman and Sive, 2009) at the early stages of hindbrain opening (18-21 hpf), mypt1 mutants appeared the same as the wild type (see Fig. S3 in the supplementary material). However, by 24 hpf, mypt1 mutants had defects in brain structure (Fig. 2A-D) and dorsal images showed that specific regions of the hindbrain remained apposed, without ventricle expansion (Fig. 2A,B). Strikingly, lateral images showed that whereas rhombomere morphology was not visible in wild-type embryos, distinct rhombomeres were visible in mypt1 mutants (Fig. 2C-F).

The mypt1 mutant phenotype was examined more closely by high-resolution confocal imaging at 18, 21 and 24 hpf. Wild type and mypt1 mutants appeared grossly similar at 18 hpf, although the neural tube was narrower in the mutant (Fig. 2G,H), and at 21 hpf hindbrain openings at rhombomere boundaries (r3/r4 and r4/r5) were present in both mutant and wild type, indicating that steps in initial hindbrain opening occurred normally (Fig. 2I,J). However, at 24 hpf, although the neural tube had opened in mypt1 mutants, the hindbrain ventricle was poorly expanded relative to that in the wild type (Fig. 2K,L’). Consistent with brightfield imaging, by 24 hpf, wild-type embryos had lost distinct rhombomere morphology, whereas in the mutants rhombomere morphology persisted (compare Fig. 2K,K’ with 2L,L’).

We excluded the possibility that mypt1 phenotypes were consequences of changes in cell death or cell proliferation using TUNEL and phosphorylated histone H3 (PH3) staining (see Fig. S4 in the supplementary material). In addition, rhombomeric gene expression patterns progressed normally, as did expression of the post-mitotic neuronal marker HuC (Elavl3 – Zebrafish Information Network), in mypt1 mutants, indicating that the phenotypes are specific to morphogenesis (see Fig. S5 in the supplementary material).

The mypt1 mutant ventricle could have expanded poorly because too little eCSF was produced, or because the neuroepithelium was incapable of expanding fully. We asked how much the mutant and wild-type hindbrain ventricles could be forced to open by high-pressure injection (30 psi) of fluid into the ventricle (Fig. 2M). In wild-type embryos, hindbrain ventricle width could be increased 1.5-fold relative to the uninjected ventricle (Fig. 2N and see Fig. S6 in the supplementary material). However, in mutants, hindbrain ventricle width could be maximally increased only 1.3-fold relative to its original size. Thus, the mypt1 mutant neuroepithelium was less able to expand than that of the wild type.

These data show that in the mypt1 mutant, a clear hindbrain phenotype is apparent by 24 hpf, after the hindbrain ventricle has opened. Rhombomere morphology does not progress normally and the hindbrain ventricle is poorly expanded. The data demonstrate that the mutant neuroepithelium is unable to stretch as much as that of the wild type, explaining the small hindbrain ventricle lumen in mypt1 mutants.

**mypt1 mutants have abnormally shaped neuroepithelial cells**

The persistence of rhombomere morphology in mypt1 mutants led us to ask whether mutant hindbrain cell shape was abnormal. Laser-scanning confocal imaging at cellular resolution was performed in wild-type and mutant embryos at 19 hpf, when the neuroepithelial cells are aligned so that the entire length of the cell can be imaged. At later stages, neuroepithelial cells are tilted and the full length of a cell is difficult to capture. r4, r5 and the r4/r5 boundary were imaged, and 3D cell shapes were reconstructed (see Materials and methods).
In wild-type embryos, cells within the rhombomeres were spindle shaped, i.e. narrow apically and basally, whereas boundary cells between rhombomeres were club shaped, i.e. wide at either the apical or basal end and long and narrow at the other (Fig. 3A-C and see Fig. S7 in the supplementary material). By contrast, mypt1 mutants within rhombomeres were shorter and wider, and lacked the narrow ends (Fig. 3D-F and see Fig. S7 in the supplementary material). Cells at the r4/r5 boundary were also shorter and wider, without the characteristic wild-type club shape (Fig. 3D-F and see Fig. S7 in the supplementary material).

Since mypt1 is broadly expressed, we asked whether the mypt1 mutant phenotype was a result of activity within the neuroepithelium or whether surrounding tissues directed the mutant phenotype. In addition, we asked whether the phenotype within the neuroepithelium was cell autonomous. These questions were addressed using transplants of late blastula cells from mypt1 or control MO-injected embryos into the presumptive hindbrain region of gastrula stage wild-type or mypt1 mutants (H,J,L,L') after memGFP mRNA injection. (G,H) Wild type (G) and mypt1 mutants (H) at 18 hpf. (I,J) Wild type (I) and mypt1 mutants (J) at 21 hpf showing similar morphology and hindbrain openings (arrows). (K-L') Wild type (K) and mypt1 mutants (L) at 24 hpf. (K',L') The same images as in K and L but with neuroepithelial tissue outlined in yellow. Dashed lines denote rhombomere morphology (L'). n>10 for all panels. mypt1 mutants have a normal neural tube, suggesting maternal gene expression is sufficient for brain development until the end of neurulation. Consistent with this, inhibition of Mypt1 expression using a start site MO led to a disorganized neural tube (data not shown), whereas a splice site MO (see Fig. S1 in the supplementary material) showed a phenotype only after the neural tube had formed. (M) Schematic of the hindbrain before and at maximum forced ventricle expansion by high-pressure injection of fluid; a and b indicate the regions that were measured. (N) Quantitation of the expandability of the hindbrain neuroepithelium in wild type and mypt1 mutants. Two independent experiments were averaged; wild type, n=12; mypt1, n=15. Data are represented as an expansion ratio ± s.d. Results are significantly different (P<0.001, Mann-Whitney U-test). Asterisks denote the ear. Anterior is to the left. H, hindbrain ventricle. Scale bars: 100 μm in A-F; 50 μm in G-L'.

Phosphorylation of myosin regulatory light chain is abnormally elevated and apically localized in the developing brain of mypt1 mutants

Active myosin phosphatase dephosphorylates myosin regulatory light chain (MRLC) (Ito et al., 2004). Dephosphorylation of MRLC inactivates non-muscle myosin II and limits contraction (Fig. 4A). Based on this scheme, and knowing that the mutation in mypt1 would disrupt the binding of Mypt1 and PP1 and render myosin phosphatase inactive, we hypothesized that phosphorylated MRLC (pMRLC) levels would be higher in mypt1 mutant brains than in wild-type brains (Fig. 4B). The level of pMRLC was examined in brain tissue microdissected from 18, 21 and 24 hpf embryos (Fig. 4C), with or without Mypt1 function. In order to obtain enough brain tissue for analysis by western blot, we used MO-injected embryos for these experiments.
At all time points examined, pMRLC levels were 3- to 5-fold higher in embryos injected with the mypt1 MO than in control embryos (Fig. 4C,D). Further, the level of pMRLC in control embryos changed with time, such that pMRLC levels peaked at 21 hpf and were 60% lowered both 18 and 24 hpf (Fig. 4E). These data show that pMRLC levels change during normal brain development, and that in the mypt1 mutant, very high levels of pMRLC are present throughout hindbrain ventricle opening.

These changes in pMRLC levels prompted us to ask where pMRLC is localized. Immunocytochemistry was performed on wild-type siblings and mypt1 mutants at 19, 21 and 24 hpf (Fig. 4F-Q). In wild-type embryos at 19 hpf, pMRLC was localized on the basal side of the neural tube, with some apical localization and diffuse expression throughout the cells (Fig. 4F,G). At 21 hpf, pMRLC expression appeared stronger apically, consistent with western blot data (Fig. 4H,J), but by 24 hpf the apical staining had disappeared (Fig. 4J,K). In mypt1 mutants, apical pMRLC staining was very strong at all time points assayed (Fig. 4L-Q). Similar to wild-type embryos, mypt1 mutants showed basal pMRLC immunostaining (Fig. 4L-Q).

These data show that in wild-type embryos, phosphorylation of MRLC is dynamic, peaking during neural tube opening and then falling as the hindbrain ventricle expands. By contrast, mypt1 mutants show strongly elevated, apically localized pMRLC expression, indicating that Mypt1 regulates both pMRLC levels and its localization in the developing brain.

**Non-muscle myosin concentrates apically and both non-muscle myosin II and actin are concentrated at rhombomere boundaries in mypt1 mutants**

Myosin regulatory light chain is bound at the head region of myosin to form the non-muscle myosin II functional unit (Landsverk and Epstein, 2005); therefore, increased apical pMRLC in mypt1 mutants might lead to apical concentration of non-muscle myosin II (myosin heavy chain). To test this, we performed immunostaining for myosin II on wild type and mypt1 mutants (Fig. 5). At all stages, myosin II was present basally and within cells. At 19 hpf, wild-type and mypt1 mutant embryos showed a similar pattern of myosin II localization in the brain, whereas at 21 hpf mypt1 mutants showed slightly increased staining of myosin II at the apical surface of the neuroepithelium compared with wild type (see Fig. S8 in the supplementary material). However, at 24 hpf, mypt1 mutants showed much stronger apical myosin II staining than the wild type (Fig. 5A-C,G-I).

In order to examine the apical surface of the rhombomeres more closely, we removed the roof plate and flat-mounted the neural tube (see Fig. 5F). Strikingly, in mypt1 mutants, but not in wild type, myosin II staining was present at the rhombomere boundaries, in a line along the apical surfaces of boundary cells (Fig. 5D, arrows in Fig. 5E). This staining was consistent with the persistence of rhombomere morphology in mypt1 mutants.

We also analyzed the localization of actin, another crucial component of the cytoskeleton, which interacts with myosin (Lecuit and Lenne, 2007). In wild-type embryos, actin surrounded cells and was concentrated at the apical cell surfaces lining the brain ventricle, consistent with actin localization at adherens junctions (Fig. 5J,M, Fig. 5L,O). The levels and apical localization of actin did not appear altered in mypt1 mutants (Fig. 5N). However, dorsal 3D projections of the mypt1 mutant embryo hindbrain showed strong accumulation of actin at rhombomere boundaries (Fig. 5K, arrows), consistent with myosin II localization.
These data indicate that in *mypt1* mutants, non-muscle myosin II, along with pMRLC, is concentrated apically in the hindbrain neuroepithelium. Non-muscle myosin II and actin are abnormally localized at rhombomere boundaries, suggesting that this might be responsible for the failure of rhombomere morphology progression.

**The abnormal ventricle morphogenesis in *mypt1* mutants is myosin dependent**

We used an independent approach to ask whether overactive myosin was responsible for the *mypt1* mutant phenotype by ascertaining whether the myosin II inhibitor, blebbistatin (Kovacs et al., 2004), would rescue the hindbrain ventricle phenotype (Fig. 6A,B). At 22 hpf, mutant or wild-type embryos were treated with DMSO as a control or with 50 µM blebbistatin. After 3 hours of treatment, embryos were ventricle injected and then imaged (Fig. 6C-J). Blebbistatin slightly reduced the size of the wild-type hindbrain ventricle (Fig. 6E,F), but did not alter rhombomere morphology. However, blebbistatin treatment fully rescued the *mypt1* mutant phenotype (Fig. 6L,J), with the hindbrain ventricle fully open and rhombomere morphology no longer apparent. Other phenotypes of the mutant embryo were also rescued, including the abnormal notochord and somites (Fig. 6G,H). Additionally, the inability of *mypt1* mutant neuroepithelium to fully expand when forced open with high-pressure injection (Fig. 2N) was also rescued by blebbistatin treatment (see Fig. S9 in the supplementary material). These data confirm that the *mypt1* phenotype is a result of overactive myosin contraction, and that myosin II inactivation is required for hindbrain morphogenesis and full ventricle expansion.

**mypt1** mutant neuroepithelial cells are abnormally shaped due to myosin contraction

We extended this analysis (Fig. 3) to ask whether blebbistatin would correct mutant cell shape. A *mypt1* mutant clutch of embryos was injected with memGFP mRNA and treated with 50 µM blebbistatin at 17 hpf. At 19 hpf, embryos were imaged and 3D cell shapes reconstructed. Since the mutant phenotype is not grossly visible at this time, embryos were genotyped by PCR (Fig. 7A). *mypt1* mutants treated from 17 to 19 hpf with blebbistatin showed normal cell shapes within and between rhombomeres (compare Fig. 7Bc,g with 7Bd,h).

As a corollary to cell shape changes, we quantified the width of the neuroepithelium in all treatment groups, by measuring the distance from the apical side of the epithelium to the basal side at r4 and at the r4/r5 boundary. In *mypt1* mutants, tube width was significantly narrower than in wild-type embryos at r4 (13%) and at the r4/r5 boundary (10%) (Fig. 7C and see Fig. S10 in the
supplementary material). In mypt1 mutants treated with blebbistatin, neural tube width was restored to the wild-type width (Fig. 7C and see Fig. S10 in the supplementary material). These data demonstrate that hindbrain cell shape and neural tube width are regulated by myosin activity.

DISCUSSION

Cell shape, rhombomere morphogenesis and ventricle expansion are regulated by myosin contractility

We demonstrate for the first time that modulation of myosin activity by myosin phosphatase affects three aspects of normal hindbrain development: neuroepithelial cell shape, morphogenesis of the rhombomeres and expansion of the hindbrain ventricle (Fig. 8). Of particular significance is our finding that unless myosin contractility is reduced, the lumen of the neural tube cannot expand to form the normal hindbrain ventricle.

Regulation of neuroepithelial cell shape via apical modulation of myosin function

The earliest hindbrain phenotype we observed in mypt1 mutants was abnormal cell shape. Spindle-shaped rhombomeric cells and club-shaped boundary cells lost their characteristic shapes with inhibition of mypt1 and overactive myosin. It is not clear what normally drives formation of these disparate shapes; however, cells within rhombomeres are more adhesive than those between (Cooke et al., 2005), perhaps contributing to cell shape differences. Mosaic analysis showed that mypt1 mutant cells always assumed a mutant shape, regardless of the genotype of the surrounding cells, indicating cell-autonomous Mypt1 function. However, wild-type cell shape required that surrounding cells are also wild type,
suggesting a non-autonomous function. One possible explanation for these results is that wild-type cells cannot push or pull enough against the stiff mypt1 neuroepithelium to realize their normal shapes. Another possibility is that wild-type cells must be fully connected through junctions in the epithelial sheet in order to undergo correct shape change. Interestingly, a similar duality of cell-autonomous and non-autonomous phenotype has also been observed for mypt1 function during convergent extension (Weiser et al., 2009).

Loss of asymmetric shape in cells with overactive myosin raises the question of whether Mypt1 acts through the apical or basal side of the cell. In embryos lacking mypt1 function, pMRLC was concentrated at the apical surface of the neuroepithelium. Since the strong expression of pMRLC and myosin II basally appeared unaltered after mypt1 loss of function, the effects of Mypt1 are likely to be mediated through the apical side of the epithelium.

Consistent with regulation of cell shape via apical myosin activity, Kinoshita et al. demonstrated Rho localization at the apical surface during chick neurulation, and connected some of its effects with pMRLC (Kinoshita et al., 2008). In the developing Drosophila eye, Myosin II regulates changes in both cell shape and cell clustering, and in spaghetti squash (MRLC mutant) and zipper (myosin heavy chain mutant) the apical surface does not constrict (Escudero et al., 2007; Hildebrand, 2005; Lee and Treisman, 2004).

Sequence of hindbrain opening and rhombomere progression

A later phenotype in the mypt1 mutant was a failure of rhombomeres to progress through their normal sequence of morphogenesis, whereby rhombomere morphology is normally lost over the 6-hour time period that we analyzed. This progression is dependent on inhibition of myosin activity and is coupled to the normal dynamic changes in pMRLC levels, which peak as the neural tube midline separates and decrease as rhombomere morphology progresses. In the mypt1 mutant, during the time period when rhombomere morphology would normally have been lost, myosin II and actin were strongly concentrated at rhombomere boundaries, whereas they were not visible at wild-type boundaries. Thus, an overactive actinomyosin network at the rhombomere boundaries in the mypt1 mutant might hold the rhombomeres in their morphologically segmented state.

Failure of morphological rhombomere progression in mypt1 mutants was independent of the changes in gene expression characteristic of differentiating rhombomeres, indicating that morphology, and not cell type determination, is under actinomyosin regulation. This failure of rhombomere progression is also independent of fluid pressure within the ventricle space, as snakehead mutants lacking eCSF do not have arrested rhombomere morphology (Lowery and Sive, 2005). Further, the mypt1 phenotype did not involve control of cell number, as neither cell proliferation nor cell death was affected, further pointing to myosin contractility as the major player in this morphogenetic change.

Epithelial relaxation and the role of myosin in ventricle expansion

Opening of the hindbrain ventricle along the neural tube midline begins normally in mypt1 mutants, but later the ventricle does not expand completely as a result of a ‘stiff’ mutant neuroepithelium.
In many organs (e.g. McCray et al., 1992), tubes initially form without a lumen, or with only a small lumen, which must expand to allow the tube to function. Part of this expansion is through fluid secretion, and, in the embryonic brain, the ventricular lumen expands as it fills with eCSF (Lowery and Sive, 2009). However, this study shows that ventricular lumen expansion also requires a stretchy neuroepithelium that can expand appropriately with eCSF. We have coined the term ‘epithelial relaxation’ to describe the acquisition of stretchiness, which depends upon inhibition of myosin contractility, and suggest that this might be a widespread mechanism underlying the expansion of epithelial tubes in other organs.

How would myosin activity regulate cell stretching or elasticity? The elasticity of a cell is determined by the cytoskeleton, primarily through the cortical actinomysin network. Cortical tension is created when myosin II contracts, pulling actin filaments together (Clark et al., 2007). In mypt1 mutants, cortical actin localization does not change, except at rhombomere boundaries, but the excessive myosin contraction would increase cytoskeletal tension in all cells, resulting in a more rigid, less ‘stretchy’ cell, and in a less elastic neuroepithelium. Our data complement the observation that in all cells, resulting in a more rigid, less ‘stretchy’ cell, and in a less elastic neuroepithelium. This might be a widespread mechanism underlying the expansion of epithelial tubes in other organs.

Another consideration is that because cells are connected by junctions, the neuroepithelium may act as a unit during morphogenesis and ventricle formation (Lowery and Sive, 2009). In tissue culture cells, Mypt1 interacts with ERM proteins in the apical junction complex (Eto et al., 2005; Kawano et al., 1999), suggesting that apical junctions might be abnormal in the mypt1 mutant. However, immunostaining with several apical junction markers showed no abnormalities in the mutants described here (Fig. 5 and data not shown).

Epithelial relaxation has not previously been described during development, but might be similar to the changes in the contraction and relaxation of tubes that are surrounded by smooth muscle. Like non-muscle myosin, smooth muscle is also regulated by Mypt1 and myosin phosphatase (Pfizer, 2001), and processes akin to epithelial relaxation may exist in vascular smooth muscle (Hirano, 2007), smooth muscle of the urinary bladder (Ding et al., 2009; Foley et al., 2008), gastrointestinal smooth muscle (Huang et al., 2005; Ihara et al., 2007) and in secretion of bile by the gall bladder (Camello-Almaraz et al., 2009).

**Is there a connection between all mypt1 mutant hindbrain phenotypes?**

Downstream of myosin function, it is not clear whether each of the events described is under the same molecular control, and whether one of these events affects another. For example, cell shape abnormalities are the earliest phenotypes that we observe in the mypt1 mutant. Do these abnormally shaped cells contribute to a failure of rhombomere morphology progression, or to a failure of the hindbrain ventricle to expand? Since myosin II and actin accumulated at rhombomere boundaries, excess contraction along a line of boundary cells, in effect functioning as a unit, might prevent progression of rhombomere morphogenesis. Thus, rhombomere progression may correlate with abnormally shaped cells, but might not be caused by the abnormal cell shape per se. With regard to ventricle opening, we believe that it is most relevant to view this process as a result of epithelial sheet activity, rather than as dependent on individual cell shape. However, mypt1 mutant cells might be more rigid than wild-type cells and, as a group, might contribute to epithelial ‘tightness’ and failure of hindbrain ventricle expansion. Are rhombomere progression and ventricle opening linked? In wild-type embryos, rhombomere progression and ventricle expansion both occur as pMRLC levels decline, but it is unclear whether these events are interdependent. Future studies will focus on additional mechanisms underlying epithelial relaxation, the connection between the events described here, and the regulation of mypt1 activity in the neuroepithelium.

**Acknowledgements**

We thank our colleagues for useful criticism, particularly Amanda Dickinson, Alena Shikumata and Ellie Graedel; Olivier Pougois for expert fish husbandry; Nancy Hopkins and Adam Amsterdam for the kind gift of the mytp1<sup>−/−</sup> fish line; the Zebrafish International Resource Center (ZIRC) for emr cDNA; David Wilkinson (MRC National Institute for Medical Research, London) for radical fringe cDNA; and Paul Mastusudaira and James Evans for advice and tuition in imaging. This work was conducted using the Whitehead-MIT Bioimaging Center and the W. M. Keck Foundation Biological Imaging Facility at the Whitehead Institute. H.S. is supported by the NIH (grant number MH077253). J.H.G. is supported by an MIT CSBi/Merck postdoctoral fellowship. Deposited in PMC for release after 12 months.

**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.042705/-/DC1

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