Development 136, 2375-2384 (2009) doi:10.1242/dev.034892

Rho-regulated Myosin phosphatase establishes the level of protrusive activity required for cell movements during zebrafish gastrulation

Douglas C. Weiser*, Richard H. Row and David Kimelman†

Rho-dependent amoeboid cell movement is a crucial mechanism in both tumor cell invasion and morphogenetic cell movements during fish gastrulation. Amoeboid movement is characterized by relatively non-polarized cells displaying a high level of bleb-like protrusions. During gastrulation, zebrafish mesodermal cells undergo a series of conversions from amoeboid cell behaviors to more mesenchymal and finally highly polarized and intercalative cell behaviors. We demonstrate that Myosin phosphatase, a complex of Protein phosphatase 1 and the scaffolding protein Mypt1, functions to maintain the precise balance between amoeboid and mesenchymal cell behaviors required for cells to undergo convergence and extension. Importantly, Mypt1 has different cell-autonomous and non-cell-autonomous roles. Loss of Mypt1 throughout the embryo causes severe convergence defects, demonstrating that Mypt1 is required for the cell-cell interactions involved in dorsal convergence. By contrast, mesodermal Mypt1 morphant cells transplanted into wild-type hosts undergo dorsally directed cell migration, but they fail to shut down their protrusive behavior and undergo the normal intercalation required for extension. We further show that Mypt1 activity is regulated in embryos by Rho-mediated inhibitory phosphorylation, which is promoted by non-canonical Wnt signaling. We propose that Myosin phosphatase is a crucial and tightly controlled regulator of cell behaviors during gastrulation and that understanding its role in early development also provides insight into the mechanism of cancer cell invasion.

KEY WORDS: Bleb, Blebbing, Myosin phosphatase, Mypt1 (Ppp1r12a), Zebrafish, Gastrulation, Convergent extension, Convergence, Amoeboid, RhoA, Rock, Metastasis

INTRODUCTION

Gastrulation is a tightly regulated series of cell rearrangements that establishes the vertebrate body plan (Keller, 2002). A major driving force in vertebrate gastrulation is convergent extension (CE), a mechanism by which cells move towards the future dorsal side of the embryo and then intercalate between neighboring cells, resulting in an overall dorsoventral narrowing (convergence) and anteroposterior lengthening (extension) of the embryo (Hammerschmidt and Wedlich, 2008; Keller, 2002; Solnica-Krezel, 2006). Zebrafish lateral mesodermal cells undergo a series of characteristic cell behavior changes during this process. Early in gastrulation, lateral mesodermal cells are highly motile and extend numerous short-lived and randomly oriented lamellipodia and bleb-like protrusions (Concha and Adams, 1998; Sepich et al., 2005; Solnica-Krezel, 2006). As gastrulation proceeds, the cells become more highly polarized and stabilize their protrusions with a dorsal orientation (Concha and Adams, 1998). When the cells approach the dorsal midline they reduce their migratory and protrusive behaviors (Concha and Adams, 1998), pack tightly together and undergo intercalation (Yin et al., 2008). Interestingly, several studies have shown that convergence and extension are genetically separable in zebrafish, indicating that some aspects of these processes are unique (Bakkers et al., 2004; Formstone and Mason, 2005; Glickman et al., 2003; Myers et al., 2002; Weiser et al., 2007; Yin et al., 2008).

One of the major regulators of CE movements is the non-canonical (β-catenin-independent) Wnt pathway, the vertebrate version of the *Drosophila* planar cell polarity (PCP) pathway (Mlodzik, 2002). Interestingly, both gain- and loss-of-function of regulators of PCP pathway members results in severe gastrulation defects (Carreira-Barbosa et al., 2003; Carreira-Barbosa et al., 2009; Heisenberg et al., 2000; Jessen et al., 2002; Wallingford et al., 2000), demonstrating that proper cell movements during gastrulation require precise and tightly balanced signaling. The non-canonical Wnts activate several downstream PCP effectors, including the small GTPase Rho, which mediates many of the effects of the PCP pathway during gastrulation (Habas et al., 2003; Marlow et al., 2002) by affecting cell polarity, cell shape changes and movement (Etienne-Manneville and Hall, 2002; Hall, 1998). Although most work on small G-proteins during gastrulation has focused on the role of non-canonical Wnts in controlling their activity (Habas et al., 2003), several other regulators of Rho are required for proper gastrulation, including the scaffolding protein Gravin (Akap12), which is essential for mesodermal cells to convert from amoeboid cell movement to intercalative cell behaviors at the end of gastrulation in order for extension to occur (Weiser et al., 2007; Weiser et al., 2008). RhoA activates the Formins, which control actin polymerization, and Rho-dependent kinase (Rock), which phosphorylates the regulatory light chain of non-muscle myosin (Mlc) (Etienne-Manneville and Hall, 2002). Both Formin and Rock signaling are required for normal CE movements (Marlow et al., 2002; Sato et al., 2006; Zhu et al., 2006).

In this work, we investigated the role of the Myosin phosphatase complex in CE movements, exploiting the advantages of zebrafish to study its role in cell behavior in vivo. The Myosin phosphatase complex is composed of a catalytic subunit of Protein phosphatase 1 (Ppp1cb) and a targeting subunit called Mypt1 (Ppp1r12a) (Ito et al., 2004; Matsumura and Hartshorne, 2008). Several sites on Mypt1

Department of Biochemistry, Box 357350, University of Washington, Seattle, WA 98195-7350, USA.

*Present address: Department of Biological Sciences, University of the Pacific, 3601 Pacific Avenue, Stockton, CA 95211, USA
†Author for correspondence (e-mail: kimelman@u.washington.edu)

Accepted 14 May 2009

are regulated by phosphorylation (Ito et al., 2004; Matsumura and Hartshorne, 2008; Muranyi et al., 2002; Wooldridge et al., 2004), including Rock-mediated inhibitory phosphorylation (Feng et al., 1999; Kimura et al., 1996). In addition, a family of inhibitory proteins including Cpi-17 (Ppp1r14a) and Phi-1 (Ppp1r14b) (Eto et al., 1999; Eto et al., 2000) interact with Mypt1 and block Mlc dephosphorylation (Eto et al., 2004; Eto et al., 2000).

Mypt1 is an important regulator of cell migration, adhesion and retraction. Knockdown of Mypt1 in mammalian cells causes excessive cell contractility (Eto et al., 2005). Mypt1 (Mbs) loss in *Drosophila* results in disorganized epithelial movements, cell shape defects and failure of dorsal closure (Mizuno et al., 2002; Tan et al., 2003), whereas loss of Mypt1 (MEL-11) function in *C. elegans* disrupts cell movements during gastrulation (Piekny et al., 2003; Wissmann et al., 1999). In contrast to these systems, the role of Mypt1 in early vertebrate development is unclear. The *Mypt1* mouse knockout is embryonic lethal, but the phenotype remains uncharacterized (Okamoto et al., 2005). Zebrafish *mypt1* mutants develop fairly normally although they fail to form the liver owing to incorrect placement of Bmp-expressing cells (Huang et al., 2008). We reasoned that the lack of earlier defects in zebrafish might be due to maternal *mypt1* mRNA, and we show here that Mypt1 is essential for CE movements. Mypt1 acts cell-autonomously to control protrusive activity, which is essential for intercalation and extension, whereas it acts non-autonomously to regulate convergence movements. Importantly, inhibitory phosphorylation mediated by Rock downstream of non-canonical Wnt signaling tightly regulates Mypt1 activity, establishing Mypt1 as a key downstream mediator of Wnt/Rho signaling. With the similarity between amoeboid cell movements in gastrulation and in metastatic cells, our work suggests that Mypt1 might function as a tumor suppressor regulating Rho-dependent amoeboid cell behavior in metastasis.

MATERIALS AND METHODS

Zebrafish

Wild-type WIK/AB zebrafish (*Danio rerio*) embryos were obtained through natural spawning and were maintained, injected and staged according to established procedures (Westerfield, 1993).

Plasmids, mRNA and morpholinos

Human *MYPT1*, zebrafish *cpi-17* and zebrafish *phi-1* clones were obtained from Open Biosystems, and constitutively active *mlck* (*mylk*) was a kind gift of Dr Erez Raz (Blaser et al., 2006). Each clone was inserted into pCS2 for the production of mRNA (details of plasmid construction are available upon request). Embryo injection, mRNA synthesis and in situ hybridization were performed as described (Weiser et al., 2007).

Mammalian tissue culture

HEK 293T cells, L-cells and Wnt5a/L-cells (obtained from the ATCC) were maintained in DMEM containing 10% FBS and cultured in 5% CO_2. Cells were transfected as described (Weiser et al., 2008). Conditioned media were obtained by culturing L-cells or Wnt5a/L-cells in DMEM containing 10% FBS without selective antibiotics for 5 days. The medium was then removed and placed on the HEK 293T cells for 4 hours. The following antibodies were used: anti-Mypt1 (Cell Signaling, 1:1000), anti-phospho-T696 Mypt1 (Upstate, 1:500) and mouse anti-Actin (Chemicon, 1:1000).

Uncaging experiment

Embryos were injected and imaged at bud stage for analysis of convergence and/or extension as described (Weiser et al., 2007).

Time-lapse microscopy

Time-lapse recordings were performed with a Zeiss Axiovert 200M microscope using AxioVision 4 software, DIC optics and a 40× objective at 28°C as described (Weiser et al., 2007). Cell tracking experiments were performed using the ImageJ manual-tracking program to record tracks of cell movement and calculate dorsal migration. Net dorsal migration was calculated by determining the distance between the location of each cell during the first and last frame of a 15-minute time-lapse recording. Total movement was calculated as the sum of the distances between 15 1-minute time points. Persistence is defined as the net migration divided by the total migration. The number of bleb-like protrusions per cell was scored by observing individual mesodermal cells (at least 25 in total from three separate embryos) two to three cell layers away from the notochord through a 15-minute time-lapse movie, and reported as blebs per cell per hour.

Inhibition of Rho-Myosin signaling

Embryos were treated with the Myosin inhibitor blebbistatin (50 μM), with Rho kinase inhibitor III (200 μM) or with DMSO as a control as described (Weiser et al., 2007).

Immunoprecipitation

Myc-tagged *mypt1* mRNA (100 pg) was injected into one-cell-stage embryos. At the beginning of gastrulation, 300 embryos of each condition were collected, dechorionated, deyolked and lysed. The myc-tagged Mypt1 was immunoprecipitated using anti-myc antibody (Covance). Fifty embryos equivalents were loaded on an acrylamide gel and the level of phosphorylation determined by western blot.

Transplants

One-cell-stage embryos to be used as donors were injected with 1% fluorescein dextran or with 1 ng *mypt1* MO and 1% rhodamine dextran. Fifteen to twenty-five cells of each control and morphant cells were removed from sphere-stage embryos and injected into shield-stage host embryos near the margin, ~90 degrees from the shield.

Data analysis

Statistical calculations were performed using Microsoft Excel. Error bars represent s.d. and *P*-values were determined using Student's *t*-test, comparing each sample with the uninjected control at the same stage.

RESULTS

Myosin phosphatase is required for body axis elongation and morphogenesis

To inhibit maternal Myosin phosphatase we designed a translation-blocking morpholino to *mypt1* (*mypt1* MO) and also tested ectopic expression of the specific Mypt1 inhibitors Cpi-17 and Phi-1 (Eto et al., 2005; Eto et al., 2000; Totsukawa et al., 2000). *mypt1* MO (1 ng) reduced endogenous Mypt1 protein levels (Fig. 1I). After 48 hours, *mypt1* morphant embryos had a dramatically shorter body axis, wavy notochord, broad somites and serious defects in heart morphology (Fig. 1B) as compared with control embryos (Fig. 1A). This phenotype differs considerably from that of embryos lacking only zygotic *mypt1*, which show no defect in body axis elongation (Huang et al., 2008). Similar morphogenetic defects were seen in embryos injected with *cpi-17* mRNA (Fig. 1C) or *phi-1* mRNA (not shown). Injection of constitutively active *myosin light chain kinase* mRNA (ca-*mlck*), which we predicted would cause the same biochemical effect as a loss of Myosin phosphatase, produced the same phenotype (Fig. 1D). Injection of *cpi-17* or ca-*mlck* resulted in complete cyclopia (Fig. 1C,G), whereas injection of *phi-1* or the *mypt1* MO resulted in partial cyclopia and small eyes (Fig. 1B and data not shown). A truncated body axis is a common phenotype in embryos with defects in CE movements (Jessen et al., 2002; Solnica-Krezel et al., 1996; Topczewski et al., 2001), whereas cyclopia is often seen in embryos with defects in prechordal plate migration (Heisenberg et al., 2000; Marlow et al., 1998). Interestingly, injection of 100 pg of human *MYPT1* mRNA has no effect on zebrafish development (Fig. 1E), as reported previously (Huang et al., 2008). However, injection of 100 pg of human *MYPT1* mRNA rescued the phenotype of *mypt1* morphant embryos (Fig. 1F,J); human MYPT1

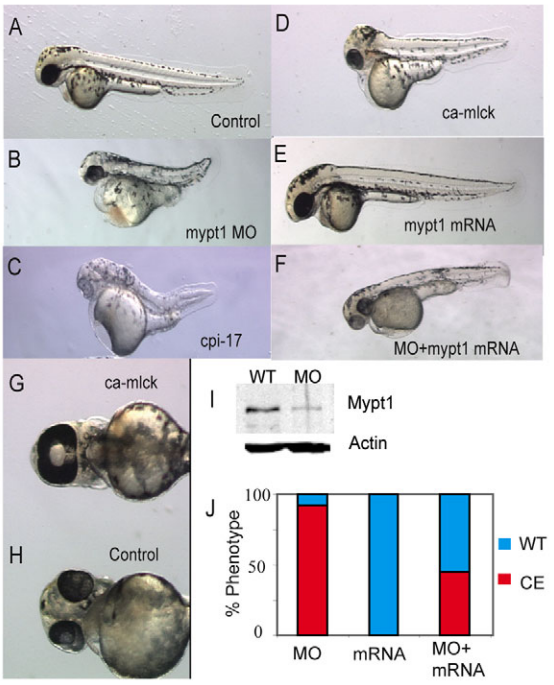

Fig. 1. Myosin phosphatase is required for body axis elongation in zebrafish. (**A-F**) Dorsal views of 48 hpf zebrafish embryos injected with (A) control, (B) 1 ng *mypt1* MO, (C) 200 pg *cpi-17* mRNA, (D) 100 pg ca-*mlck* mRNA, (E) 100 pg of human *MYPT1* mRNA. (F) A partially rescued embryo injected with 100 pg human *MYPT1* mRNA and 1 ng *mypt1* MO. (**G**,**H**) Ventral views of the head of 48 hpf zebrafish embryos showing a ca-*mlck*-injected embryo displaying cyclopia (G) and a control embryo (H). (**I**) Western blot showing endogenous Mypt1 levels in the presence and absence (WT) of 1 ng *mypt1* MO. (**J**) Quantification of the truncated body axis phenotype in morphant and mRNA-injected embryos. The *y*-axis displays the percentage of embryos exhibiting a severe axis extension defect (red) or normal axis extension (blue). The *x*-axis displays the injected reagent.

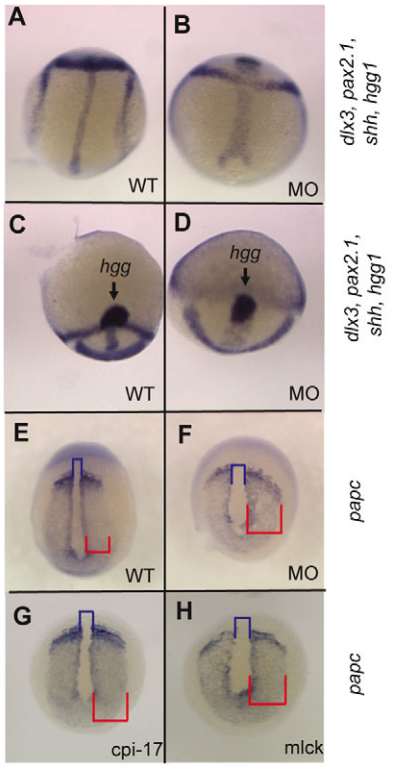

Fig. 2. Mypt is required for morphogenetic cell movements during gastrulation. Zebrafish embryos at bud stage stained for (**A-D**) *hgg1* (to mark the prechordal plate), *shh* (midline), *pax2.1* (*pax2a*; midbrain-hindbrain boundary) and *dlx3* (neural plate) or (**E-H**) *papc* (*pcdh8*; presomitic mesoderm). The blue bracket indicates the width of the notochord and the red bracket indicates the width of the presomitic mesoderm.

is 75% identical and 85% similar to zebrafish Mypt1 and the mRNA matches the MO at only 9 of 25 positions. In addition, co-injection of *mypt1* MO with either ca-*mlck* or *cpi-17*, each at a dose that does not cause a CE defect when injected alone, resulted in a clear synergistic CE phenotype (65 of 71 embryos for *cpi-17* plus *mypt1* MO; 52 of 61 embryos for ca-*mlck* plus *mypt1* MO). To confirm that the short body axis phenotype observed at 48 hours was caused by a gastrulation defect, embryos were scored for elongation defects at the end of gastrulation and again at 48 hours. The penetrance of the phenotype was the same at both time points (see Fig. S1 in the supplementary material). The results of all these experiments demonstrate that decreasing Myosin phosphatase activity and increasing Myosin contractility results in specific defects in body axis formation.

To determine the underlying morphogenetic defects that cause the morphant phenotype, in situ hybridization was performed on *mypt1* morphants, *cpi-17*, ca-*mlck* and control embryos. Morphants (Fig. 2B,D,F; 44 of 52 embryos) and *cpi-17* (Fig. 2G; 38 of 49 embryos) and ca-*mlck* (Fig. 2H; 32 of 51 embryos) embryos displayed short, broad notochords and broadened presomitic domains as compared with the wild type (WT) (Fig. 2A,C,E). Anterior migration of the prechordal plate was also defective, as indicated by the mislocalization of *hgg1* (*ctsl1b*) staining (39 of 52 embryos). This phenotype indicates that CE is defective during gastrulation in both axial and paraxial tissues. Interestingly, other morphogenetic movements appeared normal, with no apparent defects in epiboly in morphant or *cpi-17* embryos (data not shown). This pattern of mislocalized prechordal plate and shortened broadened notochord and presomitic mesoderm is a hallmark of defects in convergence and extension during zebrafish gastrulation (Heisenberg et al., 2000; Marlow et al., 2002; Topczewski et al., 2001). Given the similarity in phenotype between the morphant and mRNA-injected embryos, the *mypt1* MO was used for the rest of this work.

Myosin phosphatase is required for convergence and extension

We set out to directly assay the ability of lateral cells to both converge dorsally and extend in the anterior-posterior direction using photo-uncaging (Sepich and Solnica-Krezel, 2005). Groups of cells were uncaged at shield stage, ~90 degrees from dorsal (Fig. 3A,D). Four hours later (bud stage), the embryos were analyzed to determine the extent of cell migration. Lateral cells displayed severe defects in both convergence and extension (Fig. 3E,F) compared with controls (Fig. 3B,C). The cells were unable to migrate dorsally to the extent seen in controls, and they also failed to undergo full extension (Fig. 3G,H). Interestingly, morphant cells displayed a

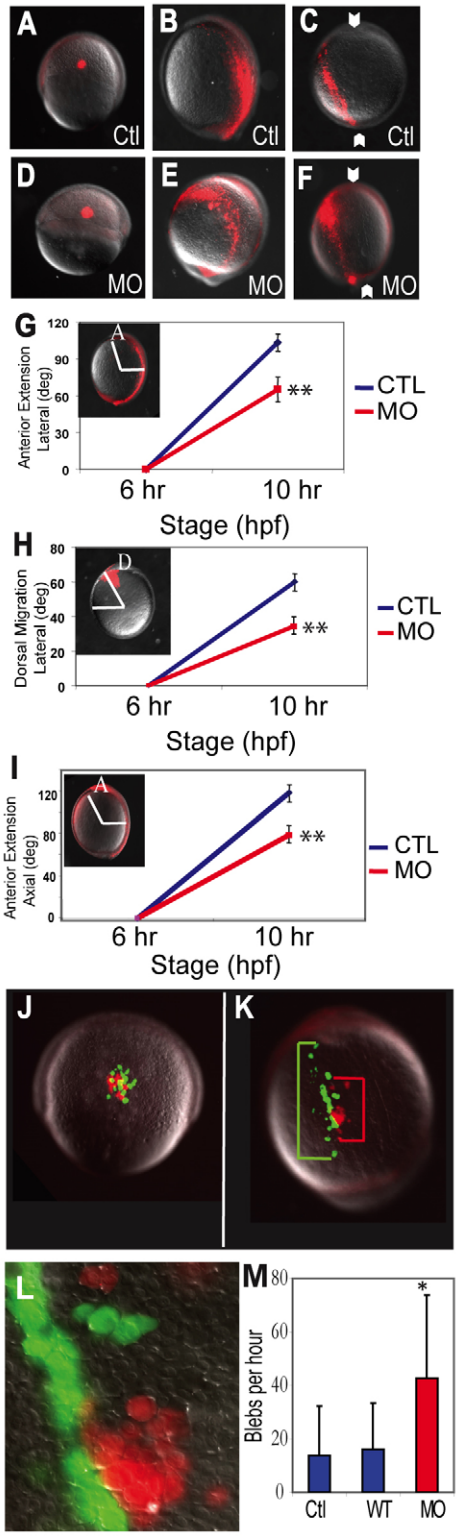

Fig. 3. Mypt1 regulates convergence and extension.
(**A-F**) Distribution of labeled lateral cells at shield and bud stages. The fluorescent dye was uncaged at shield stage (A,D), and examined at bud stage in lateral (B,E) and dorsal (C,F) views of control (Ctl) and *mypt1* morphant (MO) zebrafish embryos. Arrowheads indicate the notochord. (**G**,**H**) Quantification of the dorsal convergence and anterior extension of lateral cells in control and morphant embryos. (G) Anterior extension is defined as the angle between the anterior-most labeled cell and the dorsal side (lateral view, anterior at top). (H) Dorsal migration is defined as the angle between the site of activation at shield stage and the site of labeled cells at bud stage (animal pole view, dorsal at top, lateral to the left). (**I**) Migration of the axial mesoderm is calculated by measuring the angle between the dorsal side and the head mesoderm in the laterally labeled embryos (A-F). A, anterior; D, dorsal. (**J-L**) WT (green) and *mypt1* MO (red) cells were transplanted into WT shield-stage hosts (J, lateral view) and the host embryos were allowed to develop until bud stage (K, dorsal view 50×; L, dorsal view 400×), when the extent of migration of both WT and MO cells was determined. Green bracket indicates the total extension of WT cells and the red bracket indicates the extension of morphant cells.
(**M**) Quantification of blebs per hour of host control cells, transplanted WT cells and transplanted MO cells. *, $P<0.05$; **, $P<0.01$.

highly disorganized distribution pattern (Fig. 3F) as compared with the tightly packed and organized cells in the controls (Fig. 3C). This disorganization has been observed in embryos that lack the proper intercalative cell behaviors of presomitic mesoderm late in gastrulation (Weiser et al., 2007). Anterior migration of the axial mesoderm was also affected (Fig. 3I), consistent with the observed head defects.

We next used cell transplantation to determine whether the CE defects in *mypt1* morphants are cell-autonomous or non-autonomous. WT and morphant cells, separately labeled, were transplanted together into WT embryos at shield stage (Fig. 3J). The embryos were then allowed to complete gastrulation and were assayed for convergence and extension. Transplanted control cells underwent both convergence and extension and formed a narrow extended belt of intercalated cells on the dorsal side of the embryo (green in Fig. 3K). Surprisingly, *mypt1* morphant cells were able to migrate dorsally to the same extent as WT cells; however, these cells failed to undergo extension properly (red in Fig. 3K; 13 of 15 transplants). Morphant cells remained primarily in a clump with other morphant cells and failed to undergo intercalation (Fig. 3L). Taken together with the whole-embryo results, these observations demonstrate that Mypt1 has a cell-autonomous role in extension, and a non-autonomous role in convergence. Moreover, our studies further support the emerging view that convergence and extension are mechanistically separable (Bakkers et al., 2004; Glickman et al., 2003; Weiser et al., 2007).

Myosin phosphatase regulates amoeboid cell behaviors of mesodermal cells

We next focused on the underlying cell behaviors of *mypt1* morphants to determine which aspect of gastrulation is defective. We analyzed the polarity of mesodermal cells by calculating the length-width ratios of mesodermal cells at bud stage in control embryos compared with embryos injected with the *mypt1* MO (Fig. 4A). Control mesodermal cells are highly polarized and elongate at bud stage in both presomitic and axial mesoderm (Fig. 4B,C). Both lateral and axial mesodermal cells were significantly less polarized in morphant embryos (Fig. 4B,C). This clearly indicates that *mypt1* morphant cells are less polarized than control cells.

To determine the effect of Mypt1 loss-of-function on cell motility, we analyzed presomitic mesodermal cells just prior to bud stage using time-lapse and DIC optics. Migration speeds and paths for individual cells were calculated and recorded (from 25 cells in total

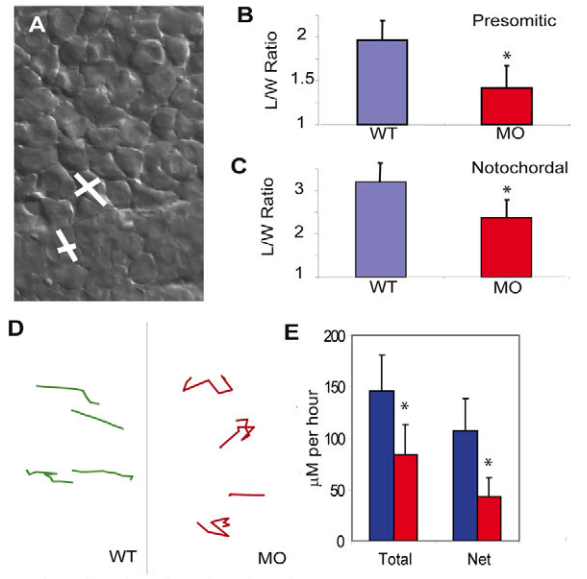

Fig. 4. Mesodermal cell polarity and migration are controlled by Mypt1. (**A-C**) The cell polarity of presomitic (B) and notochordal (C) mesodermal cells was determined by calculating the length-to-width (L/W) ratio (*y*-axis). (A) WT cells showing the long axis length and the short axis width. Note that *mypt1* morphant cells are more rounded than the WT in both the notochord and presomitic mesoderm. (**D**,**E**) The migration ability of mesodermal cells was measured by tracking individual presomitic mesoderm cells at bud stage. Representative tracks of WT and morphant cells (D) are quantified (E). The *y*-axis displays dorsal movement in µm per hour; both total migration and net dorsal migration are shown. Note that morphant cells are more erratic in their movement and migrate less overall. *, $P<0.05$.

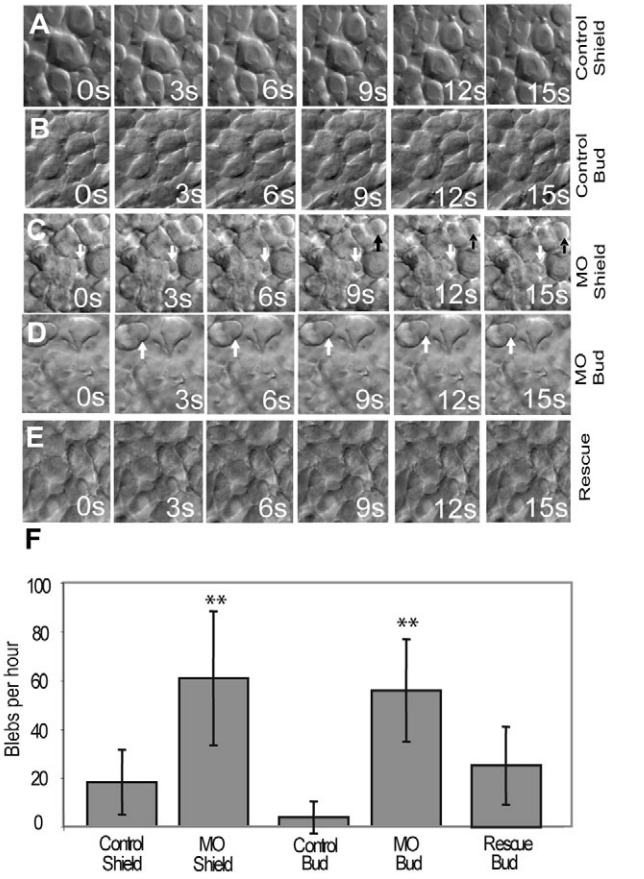

Fig. 5. Mypt1 inhibits bleb-like protrusive activity. (**A-E**) Membrane protrusive activity of mesodermal cells at shield and bud stages was determined by time-lapse microscopy. Shown is the behavior of mesodermal cells in control zebrafish embryos at shield (lateral cells, A) and bud (dorsal cells, B) stages, *mypt1* morphants at shield (C) and bud (D) stages, and *mypt1* mRNA and MO co-injected embryos (E). White and black arrows indicate bleb-like protrusions. s, seconds. (**F**) Quantification of the blebbing behavior shown in A-E. The *y*-axis shows the number of blebs per cell per hour. Note the large increase in protrusive activity in *mypt1* morphants relative to the control at both time points. **, $P<0.01$.

from two embryos of each condition; Fig. 4D,E). Morphant cells moved more slowly than control cells, with a ~50% reduction in net dorsal migration. Control cells at this stage migrated in a highly polarized manner, whereas morphant cells not only moved more slowly (total migration reduced), but they also changed direction more often [persistence (the ratio of total to net migration) increased from 1.36 to 1.95], further reducing convergence movements (Fig. 4E). These observations indicate that Mypt1 is required for the proper dorsal migration of mesodermal cells, without which convergence is impaired.

Since *mypt1* morphants are defective in extension as well as in dorsal migration of paraxial mesoderm (Fig. 3), we hypothesized that *mypt1* is required for mediolateral intercalation. To test this, we utilized time-lapse analysis of mesodermal cell shape throughout gastrulation in control and morphant embryos. Control cells during early gastrulation are highly active and extend many bleb and lamellipodial protrusions and migrate in an amoeboid fashion (Fig. 5A and see Movie 1 in the supplementary material). By the end of gastrulation, control cells were densely packed, exhibited few protrusions and were mesenchymal in cell behavior (Fig. 5B and see Movie 2 in the supplementary material). The few protrusions that were observed at this stage were primarily either bleb-like protrusions along the notochordal-paraxial boundary or lamellipodial protrusions that extended between cells. By contrast, *mypt1* morphant mesodermal cells extended increased numbers of bleb-like protrusions at both early and late gastrula stages and exhibited amoeboid behaviors throughout gastrulation (Fig. 5C,D and see Movies 3 and 4 in the supplementary material). Control cells averaged less than 20 blebs per hour at early stages and less than four at late gastrula stages, whereas morphant embryos displayed over 50 blebs per hour throughout gastrulation (Fig. 5F). This increased blebbing correlates with defects in dorsal migration, as shown in the dose-response curve (see Fig. S2 in the supplementary material). In contrast to lamellipodia, which display a strong dorsal bias in orientation (Solnica-Krezel, 2006), blebs in both WT and morphant cells appeared to be randomly oriented (data not shown). Interestingly, co-injection of *mypt1* mRNA not only rescued axis elongation of *mypt1* morphant embryos (Fig. 1), but also rescued blebbing (Fig. 5E,F).

Transplanted morphant cells, which converge but fail to extend (Fig. 3), were also less polarized than transplanted and host WT cells and they extended numerous bleb-like protrusions (Fig. 3M). These results demonstrate that these highly blebbing amoeboid cells are unable to undergo extension, but are fully capable of dorsal

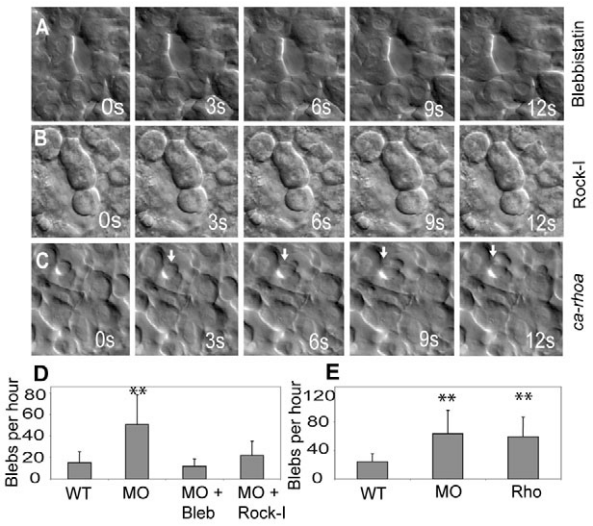

Fig. 6. Mypt1 inhibits the Rho-Rock-Myosin pathway. (**A**,**B**) *mypt1* morphant zebrafish embryos were treated with blebbistatin (A) or Rho kinase inhibitor III (B, Rock-I) and assayed for blebbing activity by time-lapse microscopy at bud stage. s, seconds. (**C**) ca-*rhoA* mRNA results in excessive blebbing of lateral mesoderm at shield stage. (**D**,**E**) Quantification of data in A and B (D) and C (E). The *y*-axis shows the number of blebs per cell per hour. **, $P<0.01$.

convergence. This property is very reminiscent of *gravin* morphant cells, which, uniquely among the genes involved in CE, are highly blebbing and fail to extend (Weiser et al., 2007). However, whereas *gravin* morphant cells exhibit normal blebbing activity at the start of gastrulation and fail to shut down blebbing after migration to the dorsal side, *mypt1* morphant cells show increased blebbing activity from the start of gastrulation (Fig. 5C). We therefore conclude that while the failure to shut down blebbing prevents intercalation and extension in a cell-autonomous manner, increased blebbing does not prevent convergence as long as the surrounding cells have normal Mypt1 activity.

Mypt1 regulates the Rho-Myosin II pathway

We sought to determine whether the *mypt1* MO phenotype was mediated by effects on acto-myosin contractility and Rho signaling. Rho, acting through Rock, phosphorylates and activates non-muscle myosin (Etienne-Manneville and Hall, 2002). We used two potent pharmacological inhibitors of Rho-Myosin signaling, blebbistatin, which inhibits non-muscle myosin, and Rho kinase inhibitor III, a cell-permeable Rock inhibitor, both of which are effective in zebrafish embryos (Berndt et al., 2008; Weiser et al., 2007). Treatment with blebbistatin completely abrogated blebbing in *mypt1* morphants (Fig. 6A,D), providing direct evidence that Mypt1 is upstream of Myosin II. Interestingly, blebbing was reduced but not completely eliminated in *mypt1* morphants when treated with the Rock inhibitor (Fig. 6B,D and see Movie 5 in the supplementary material). This contrasts with *gravin* morphants, in which both blebbistatin and the Rock inhibitor eliminate ectopic blebbing (Weiser et al., 2007). Other Mlc kinases are present during zebrafish gastrulation, including Mlck and Dap (Blaser et al., 2006). We suggest that in the absence of opposing Myosin phosphatase activity, these other kinases can elicit sufficient phosphorylation of Mlc to cause some membrane blebbing, even if Rock is inhibited.

Myosin phosphatase activity is regulated by inhibitory phosphorylation

Mypt1 is not a constitutively active phosphatase but instead is regulated by many inputs, including binding proteins and phosphorylation at various sites. Since the Rho-Rock pathway plays an essential role in zebrafish gastrulation (Habas et al., 2001; Marlow et al., 2002), we asked whether Rock not only activates Mlc to regulate cell movements, but also targets Mypt1 in embryos. Cell culture experiments have shown that inhibitory phosphorylation of Mypt1 at threonine 696 is induced by Rock, either directly (Feng et al., 1999; Kimura et al., 1996) or by activating Dap kinase (also called Zip kinase), which also phosphorylates this site (Hagerty et al., 2007). Phosphorylation at this site reduces the affinity of Mypt1 for Myosin and thus inhibits the function of the Myosin phosphatase complex (Kimura et al., 1996).

We hypothesized that if Rock regulates Mypt1 then loss of this inhibitory phosphorylation site would render Mypt1 hyperactive during gastrulation. To test this, we mutated T696 to either alanine or aspartic acid to eliminate phosphorylation and create a phosphomimetic, respectively. Injection of 100 pg of *mypt1* mRNA (Fig. 7B), or as much as 400 pg (data not shown), had no effect on early development. By contrast, injection of 100 pg of *mypt1* T696A mRNA caused a severe CE defect (Fig. 7A,B), as well as a partially cyclopic head. Examination of cells expressing T696A *mypt1* revealed inhibition of cell movement and protrusive activity (Fig. 7C). T696A cells displayed a rounded cell morphology, with reduced polarity and migratory ability, resembling cells with reduced acto-myosin contractility. The phosphomimetic mutant T696D, like WT *mypt1*, failed to elicit a CE defect (Fig. 7B). Importantly, whereas 100 pg of WT *mypt1* mRNA was required to rescue the CE defect in *mypt1* morphants, just 20 pg of the T696A mRNA produced the same extent of rescue (Fig. 7B). By contrast, *mypt1* T696D completely failed to rescue the phenotype (Fig. 7B). These results indicate that mutation of threonine 696 to an alanine renders Mypt1 constitutively active in vivo, as has been observed in vitro (Feng et al., 1999), whereas mutation to aspartic acid renders Mypt1 constitutively inactive. Since WT Mypt1 is much less active than Mypt1 T696A, these results demonstrate that Mypt1 activity is regulated in vivo through residue 696.

Mypt1 phosphorylation is regulated by Wnt and Rho

The crucial role of non-canonical Wnts in controlling RhoA signaling during gastrulation led us to hypothesize that non-canonical Wnts, working through Rho and Rock, might promote inhibitory phosphorylation of Mypt1 at position 696. To test this, we first turned to an in vitro system. WNT5A- and control-conditioned media were added to HEK 293T cells transfected with myc-tagged Mypt1, and the level of Mypt1 phosphorylation was detected using an anti-phospho-T696 antibody. T696 exhibits basal levels of phosphorylation in HEK 293 cells; however, upon treatment with Rock inhibitor, the level of phosphorylation was considerably reduced, indicating that much of this phosphorylation is Rock dependent (Fig. 7E). Treatment with human WNT5A-conditioned medium resulted in a greater than 2-fold increase in Mypt1 phosphorylation. Expression of constitutively active RhoA, or incubation with the phosphatase inhibitor okadaic acid, increased Mypt1 phosphorylation to a similar extent (Fig. 7E). Importantly, inhibition of Rock blocked the increase in Mypt1 phosphorylation caused by WNT5A, and reduced phosphorylation to below control levels.

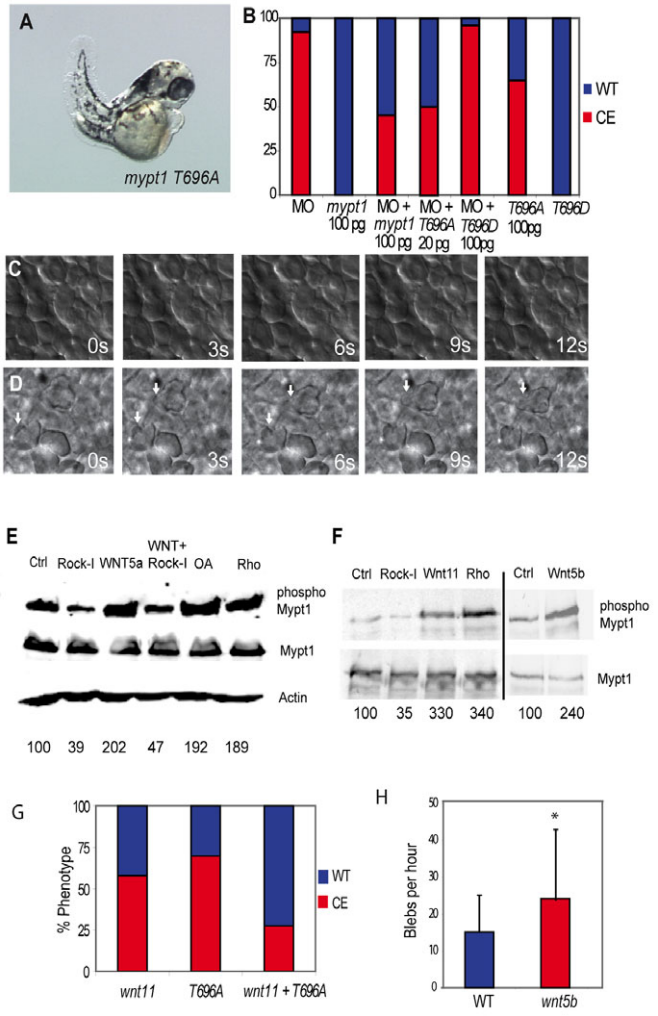

Fig. 7. Rock and non-canonical Wnts mediate the inhibitory phosphorylation of Mypt1. (**A**) *mypt1* T696A mRNA (100 pg) expression elicits gastrulation defects in zebrafish embryos. (**B**) Quantification of convergent extension (CE) defects in *mypt1* T696A- and T696D-injected embryos and rescue of the MO phenotype. Red indicates a CE defect; blue indicates a normal body axis length. (**C**) Lack of protrusive activity of dorsal mesodermal cells in *mypt1* T696A-injected embryos at bud stage. s, seconds. (**D**) Increased protrusive activity of dorsal mesodermal cells in *wnt5b* (100 pg)-injected embryos at bud stage. Arrows indicate bleb-like protrusions. (**E**) Western blot of HEK 293 lysates expressing myc-tagged Mypt1 treated with WNT5A-conditioned medium, or control-conditioned medium. Cells were also either co-transfected with ca-*rhoA* or treated with Rock inhibitor (Rock-I) or the phosphatase inhibitor okadaic acid (OA) and blotted for Mypt1, phospho-Mypt1, Mypt1 and Actin. (**F**) Myc-tagged Mypt1 was immunoprecipitated from zebrafish embryos and blotted for Mypt1 and phospho-Mypt1. Embryos were also injected with 10 pg ca-*rhoA*, 100 pg *wnt11*, 100 pg *wnt5b* mRNA or treated with Rock inhibitor. Numbers beneath the lanes in E,F indicate the percentage of phospho-Mypt1 relative to the control. (**G**) Quantification of the rescue of the *wnt11* (100 pg) overexpression phenotype by co-expression of *mypt1* T696A (100 pg) mRNA. (**H**) Induction of blebbing of dorsal mesodermal cells at bud stage in *wnt5b* (100 pg)-injected embryos. *, $P<0.05$.

We next determined whether Mypt1 phosphorylation is regulated in zebrafish. We injected 100 pg of myc-tagged *mypt1* mRNA into embryos, a level that causes no phenotypic effects on embryo development, and determined the level of phosphorylation by western blot. In zebrafish, basal Mypt1 phosphorylation is Rock-dependent and was stimulated by expressing constitutively active RhoA, or the non-canonical Wnts Wnt11 or Wnt5b (Pipetail) (Fig. 7F), providing clear in vivo evidence that Mypt1 phosphorylation is regulated by non-canonical Wnt signaling in zebrafish embryos. In addition, co-injection of *mypt1* T696A with *wnt11* mRNA rescued the CE phenotype in *wnt11* embryos (Fig. 7G). Together, these experiments provide strong evidence that stimulation of the non-canonical Wnt pathway can promote Rock-dependent inhibitory phosphorylation of Mypt1, both in mammalian cells and in zebrafish embryos.

Since our loss-of-function data demonstrated that Mypt1 acts to limit the extent of blebbing, and because we found that Rho causes phosphorylation of an inhibitory site in Mypt1, we hypothesized that hyperactivation of the RhoA pathway would phenocopy the ectopic blebbing seen in *mypt1* morphants. To test this, we injected 10 pg of constitutively active *rhoA* mRNA (ca-*rhoA*) and assayed protrusive activity early in gastrulation. ca-*rhoA*-expressing cells displayed a high level of protrusive activity and reduced movement (Fig. 6C and see Movie 6 in the supplementary material). These cells extended a large number of membrane blebs and the tissue organization was severely perturbed. Mesodermal cells overexpressing Wnt5b also displayed rounded cell morphology and increased cell blebbing (Fig. 7D,H). These experiments strongly support the hypothesis that Rho-dependent inhibition of Mypt1 is essential for regulating protrusive activity during gastrulation.

DISCUSSION

Mypt1 is a highly conserved regulator of acto-myosin contractility (Matsumura and Hartshorne, 2008). In this work, we demonstrate that Myosin phosphatase is a tightly controlled regulator of essential cellular behaviors during vertebrate gastrulation. Two key functions of Mypt1 are required for gastrulation. Mypt1 cell-autonomously controls cell shape and protrusive activity. Cells lacking Mypt1 fail to convert from amoeboid to mesenchymal behavior and do not undergo intercalative movements required for extension. These cells are, however, able to migrate dorsally without delay, indicating that the amoeboid-to-mesenchymal transition is not required cell-autonomously for convergence. If the entire tissue is lacking Mypt1, however, the cells remain amoeboid and fail to undergo net dorsal migration, resulting in severe convergence defects. Finally, we demonstrate that Mypt1 is tightly regulated by inhibitory phosphorylation downstream of the non-canonical Wnt pathway.

Mypt1 is required cell-autonomously for extension

During gastrulation, paraxial mesodermal cells undergo a characteristic series of changes beginning with cells that produce blebs, lamellipodia and filopodia and move in an amoeboid manner, followed by more dorsal oriented, mesenchymal type movements as the cells approach the dorsal midline (Concha and Adams, 1998; Sepich et al., 2005; Weiser et al., 2007). At the midline, cells shut down protrusive activity, pack tightly together and intercalate to extend the axis (Fig. 8A). In our previous work we found that paraxial mesodermal cells lacking Gravin showed normal protrusive activities at the start of gastrulation and converged dorsally, but the cells specifically failed to shut down blebbing when they reached the dorsal side and were unable to undergo normal intercalative behavior, resulting in extension but not convergence defects (Weiser et al., 2007). *mypt1* morphant cells transplanted into WT hosts act similarly to *gravin* morphant cells, converging normally but failing

to intercalate and extend. Unlike *gravin* morphants, however, the *mypt1* morphant cells have hyper-blebbing activity throughout gastrulation, demonstrating that this type of protrusive activity is fully consistent with dorsal migration of the paraxial mesodermal cells, but, as in the case of *gravin* morphants, it is inconsistent with packing, intercalation and extension (Fig. 8A). Interestingly, metastatic tumor cells are able to move at very similar speeds when using either lamellipodial or blebbing-type protrusive activities (Sanz-Moreno et al., 2008; Wolf and Friedl, 2006; Wolf et al., 2003), and zebrafish germ cells move rapidly using only blebbing activity (Blaser et al., 2006). Thus, hyper-blebbing activity is consistent with rapid movement, but as we suggest below, there are other reasons why mesodermal cells do not only adopt this type of protrusive activity.

Mypt1 is required non-cell-autonomously for convergence

In contrast to the cell-autonomous role of Mypt1 in extension, Mypt1 has a surprising, non-cell-autonomous essential role in convergence. When all the cells in the embryo are depleted of Mypt1 (or are overexpressing either of two Myosin phosphatase inhibitors or a constitutively active Myosin light chain) the embryos have severe convergence defects. During convergence, the paraxial mesoderm cells migrate up an adhesive gradient that is under the control of Bmp signaling, with the lowest adhesion ventrally and the highest adhesion dorsally (Hammerschmidt and Wedlich, 2008; Myers et al., 2002; Solnica-Krezel, 2006; von der Hardt et al., 2007). Since the transplanted *mypt1* morphant cells are able to migrate dorsally in WT hosts, they are able to follow the adhesive gradient. However, when all the cells lack Mypt1, the mesodermal cells are much less dorsally directed in their migration, which explains the overall reduction in convergence (Fig. 8A).

Interestingly, acto-myosin contractility is required for the maintenance of tension in gastrula-stage cells in zebrafish and in *Xenopus*, and in zebrafish for the separation of germ layers and for mechano-transduction in other tissues (Clark et al., 2007; Krieg et al., 2008; Skoglund et al., 2008). Our results suggest that in zebrafish, in which mesodermal cells migrate dorsally as individual cells, they make productive adhesive interactions (von der Hardt et al., 2007) and pull against each other to gain traction. Thus, we suggest that if cells have too much acto-myosin contractility, owing to a reduction in Mypt1 function, they are unable to productively move towards the dorsal side, explaining the observed non-cell-autonomous defects in *mypt1* morphants. Similarly, too little acto-myosin contractility, caused by overexpression of a constitutively active *mypt1*, also results in severe CE defects. We propose that the mesodermal cells very carefully regulate their level of acto-myosin contractility in order to establish optimal conditions for convergence (Fig. 8B). An essential role for Mypt1, therefore, is to modulate the level of Myosin phosphorylation in order to establish this balance.

One of the conundrums in the study of CE is that for many factors, both inhibition and overexpression cause the same general phenotype. The same is true in our studies, in which the *mypt1* MO and overexpression of the constitutively active *mypt1* produce embryos with severe CE defects. However, examination of the mesodermal cell behaviors reveals very different effects. Whereas the morphant cells have hyper-blebbing activity throughout gastrulation, the cells overexpressing the constitutively active *mypt1* have much reduced protrusive activity. A similar effect was seen with the hyaluronan synthetase Has2, which acts via Rac1 activation (Bakkers et al., 2004). Both gain- and loss-of-function of Has2 caused CE defects. However, gain of Has2 function produced supernumerary lamellipodia, whereas loss of Has2 caused an absence of lamellipodia. Taken together, these results suggest that several features of CE are finely balanced such that tipping either way causes different cell behaviors, with the net effect being a defect in convergence and extension.

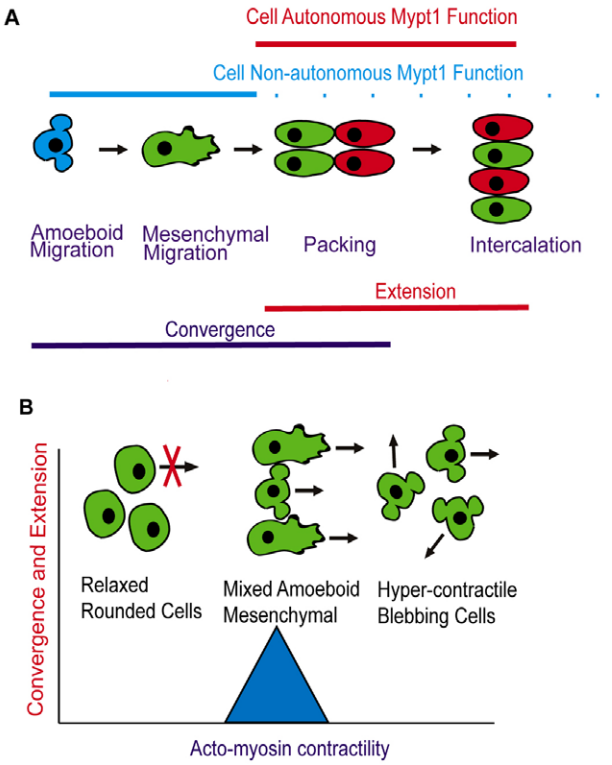

Fig. 8. A model of the cell-autonomous and non-cell-autonomous roles of Mypt1 in mesodermal cells undergoing convergent extension. (**A**) Mesodermal cells convert from amoeboid to mesenchymal morphology during gastrulation. Both behaviors are highly migratory and contribute to convergence. Late in gastrulation, cells pack tightly against one another and inhibit protrusive activity by contact inhibition. Cells intercalate, driving extension. Mypt1 is required cell-autonomously for inhibition of protrusive activity and intercalation, but not dorsal migration. Mypt1 is required non-autonomously for convergence. (**B**) In order for mesodermal cells to undergo both convergence and extension, a precise balance of acto-myosin contractility must be maintained. Cells with excessive contractility, such as *mypt1* morphants, display a hyper-blebbing phenotype that interferes with tissue cohesion and CE. Cells with insufficient acto-myosin contractility, such as *mypt1* T696A mRNA-injected embryos, display rounded cell morphologies and a greatly reduced migratory speed. Only cells with the precise balance of signaling can undergo all the cellular behaviors required for convergent extension.

Regulation of Myosin phosphatase by inhibitory phosphorylation

A large body of work has established the importance of the non-canonical Wnt signaling pathway as an essential regulator of CE movements in vertebrate gastrulation (Veeman et al., 2003). Among the essential targets is Rho, which activates Rock (Habas et al., 2001). One major target of Rock is Myosin light chain, which induces acto-myosin contractility (Clark et al., 2007). Here, we provide the first in vivo evidence that non-canonical Wnt signaling, acting through Rock, also regulates Mypt1 phosphorylation. We

show in both cultured cells and zebrafish embryos that two non-canonical Wnts and activated Rho enhance T696 phosphorylation, whereas the Rock inhibitor suppresses phosphorylation at this site. Importantly, conversion of T696 to a phosphomimetic aspartic acid inactivates Mypt1, whereas conversion to an alanine produces a constitutively active Mypt1 that causes severe CE defects when expressed in zebrafish embryos, as well as rescuing the morphant phenotype at a 5-fold lower dose than is effective for WT Mypt1. Therefore, we propose that one of the crucial functions of the non-canonical Wnt pathway during gastrulation is not only to promote phosphorylation of Mlc through Rho/Rock, but also to partially inhibit Mypt1, thus creating the balance in blebbing behavior and contractility needed to maintain migration and intercalation (Fig. 8B). This precise molecular control would allow the Wnts to fine-tune acto-myosin contractility and precisely control the conversion from amoeboid to mesenchymal to intercalation of mesodermal cells as gastrulation continues.

Cell behaviors controlled by Mypt1: implications for tumor cell invasion

The most striking phenotype of cells lacking Mypt1 is their excessive bleb-like protrusive activity. Blebs differ from other cellular protrusions in that they are driven by acto-myosin contraction and hydrostatic pressure on the membrane, and not by actin polymerization (Charras et al., 2005). Blebbing is an important form of locomotion (Charras and Paluch, 2008) observed not only in zebrafish gastrula-stage mesodermal cells (Weiser et al., 2007), zebrafish germ cells (Blaser et al., 2006) and delaminating zebrafish neural crest cells as they undergo an epithelial-to-mesenchymal transition (Berndt et al., 2008), but also in *Fundulus* blastula- and gastrula-stage deep cells (Trinkaus, 1973). Perhaps the most studied form of amoeboid motion is in tumor cell invasion (Friedl and Wolf, 2003). Highly metastatic cells can utilize an amoeboid form of movement (Friedl, 2004; Wolf and Friedl, 2006) characterized by hyperactivation of the Rho-Rock pathway leading to increased acto-myosin contractility and blebbing (Sanz-Moreno et al., 2008; Wolf et al., 2003).

In our current and previous studies of cell movement in zebrafish, we observed that mesodermal cells undergo a conversion from amoeboid to mesenchymal movements during gastrulation and that these cellular behaviors contribute differently to gastrulation. Loss of the metastasis inhibitor Gravin in zebrafish embryos results in a failure to shut down blebbing, which led us to propose that Gravin has an important role as a tumor suppressor that inhibits amoeboid movement (Weiser et al., 2007). As we show here, loss of Mypt1 results in mesodermal cells that not only fail to shut down blebbing but that also exhibit a hyper-blebbing phenotype even at the start of gastrulation. This raises the intriguing possibility that Mypt1 could also function as a metastasis inhibitor. Several recent lines of research have indicated that Rock is a key regulator of invasive cell behaviors in vivo (Sanz-Moreno et al., 2008; Wilkinson et al., 2005; Wolf and Friedl, 2006; Wyckoff et al., 2006). Our work indicates that a key target of Rock in promoting amoeboid behavior is Mypt1. This raises the interesting possibility that loss of Mypt1 function, or constitutive inhibition of Mypt1 by Cpi-17 or Phi-1, could provide an alternative route toward amoeboid behavior in tumor cells, resulting in enhanced metastatic behavior. Thus, it would be interesting to carefully examine Mypt1 regulation and function in controlling tumor cell invasion in vivo.

In conclusion, we have identified Mypt1 as a crucial regulator of several cell behaviors required for gastrulation. Mypt1 regulates cell shape and protrusive activity and is required for intercalation cell-autonomously. Mypt1 also has a non-autonomous role in dorsal convergence, most likely by maintaining the cohesive tissue tension required for mesodermal cells to pull against each other. Finally, we demonstrated that Mypt1 is under the strict control of the non-canonical Wnt pathway and RhoA signaling. Thus, Mypt1 is a crucial regulator of morphogenesis during gastrulation, and is a likely candidate to be involved in mediating invasive cell behavior in metastasis.

Acknowledgements

We thank Steve Hauschka for the use of his microscope and tissue culture facility, Ben Martin for critical reading of the manuscript, and Dianne Slusarski for the Wnt5b/Pipetail plasmid. This work was supported by NIH grants R01HD27262 to D.K., F32HD053189 to D.C.W. and T32HD007183-26A1 to R.H.R. Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/14/2375/DC1

References

Bakkers, J., Kramer, C., Pothof, J., Quaedvlieg, N. E., Spaink, H. P. and Hammerschmidt, M. (2004). Has2 is required upstream of Rac1 to govern dorsal migration of lateral cells during zebrafish gastrulation. *Development* **131**, 525-537.

Berndt, J. D., Clay, M. R., Langenberg, T. and Halloran, M. C. (2008). Rho-kinase and myosin II affect dynamic neural crest cell behaviors during epithelial to mesenchymal transition in vivo. *Dev. Biol.* **324**, 236-244.

Blaser, H., Reichman-Fried, M., Castanon, I., Dumstrei, K., Marlow, F. L., Kawakami, K., Solnica-Krezel, L., Heisenberg, C. P. and Raz, E. (2006). Migration of zebrafish primordial germ cells: a role for Myosin contraction and cytoplasmic flow. *Dev. Cell* **11**, 613-627.

Carreira-Barbosa, F., Concha, M. L., Takeuchi, M., Ueno, N., Wilson, S. W. and Tada, M. (2003). Prickle 1 regulates cell movements during gastrulation and neuronal migration in zebrafish. *Development* **130**, 4037-4046.

Carreira-Barbosa, F., Kajita, M., Morel, V., Wada, H., Okamoto, H., Martinez Arias, A., Fujita, Y., Wilson, S. W. and Tada, M. (2009). Flamingo regulates epiboly and convergence/extension movements through cell cohesive and signalling functions during zebrafish gastrulation. *Development* **136**, 383-392.

Charras, G. and Paluch, E. (2008). Blebs lead the way: how to migrate without lamellipodia. *Nat. Rev. Mol. Cell Biol.* **9**, 730-736.

Charras, G. T., Yarrow, J. C., Horton, M. A., Mahadevan, L. and Mitchison, T. J. (2005). Non-equilibration of hydrostatic pressure in blebbing cells. *Nature* **435**, 365-369.

Clark, K., Langeslag, M., Figdor, C. G. and van Leeuwen, F. N. (2007). Myosin II and mechanotransduction: a balancing act. *Trends Cell Biol.* **17**, 178-186.

Concha, M. L. and Adams, R. J. (1998). Oriented cell divisions and cellular morphogenesis in the zebrafish gastrula and neurula: a time-lapse analysis. *Development* **125**, 983-994.

Etienne-Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. *Nature* **420**, 629-635.

Eto, M., Karginov, A. and Brautigan, D. L. (1999). A novel phosphoprotein inhibitor of protein type-1 phosphatase holoenzymes. *Biochemistry* **38**, 16952-16957.

Eto, M., Wong, L., Yazawa, M. and Brautigan, D. L. (2000). Inhibition of myosin/moesin phosphatase by expression of the phosphoinhibitor protein CPI-17 alters microfilament organization and retards cell spreading. *Cell Motil. Cytoskeleton* **46**, 222-234.

Eto, M., Kitazawa, T. and Brautigan, D. L. (2004). Phosphoprotein inhibitor CPI-17 specificity depends on allosteric regulation of protein phosphatase-1 by regulatory subunits. *Proc. Natl. Acad. Sci. USA* **101**, 8888-8893.

Eto, M., Kirkbride, J. A. and Brautigan, D. L. (2005). Assembly of MYPT1 with protein phosphatase-1 in fibroblasts redirects localization and reorganizes the actin cytoskeleton. *Cell Motil. Cytoskeleton* **62**, 100-109.

Feng, J., Ito, M., Ichikawa, K., Isaka, N., Nishikawa, M., Hartshorne, D. J. and Nakano, T. (1999). Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. *J. Biol. Chem.* **274**, 37385-37390.

Formstone, C. J. and Mason, I. (2005). Combinatorial activity of Flamingo proteins directs convergence and extension within the early zebrafish embryo via the planar cell polarity pathway. *Dev. Biol.* **282**, 320-335.

Friedl, P. (2004). Prespecification and plasticity: shifting mechanisms of cell migration. *Curr. Opin. Cell Biol.* **16**, 14-23.

Friedl, P. and Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat. Rev. Cancer* **3**, 362-374.

Glickman, N. S., Kimmel, C. B., Jones, M. A. and Adams, R. J. (2003). Shaping the zebrafish notochord. *Development* **130**, 873-887.

Habas, R., Kato, Y. and He, X. (2001). Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel Formin homology protein Daam1. *Cell* **107**, 843-854.
Habas, R., Dawid, I. B. and He, X. (2003). Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation. *Genes Dev.* **17**, 295-309.
Hagerty, L., Weitzel, D. H., Chambers, J., Fortner, C. N., Brush, M. H., Loiselle, D., Hosoya, H. and Haystead, T. A. (2007). ROCK1 phosphorylates and activates zipper-interacting protein kinase. *J. Biol. Chem.* **282**, 4884-4893.
Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-514.
Hammerschmidt, M. and Wedlich, D. (2008). Regulated adhesion as a driving force of gastrulation movements. *Development* **135**, 3625-3641.
Heisenberg, C. P., Tada, M., Rauch, G. J., Saude, L., Concha, M. L., Geisler, R., Stemple, D. L., Smith, J. C. and Wilson, S. W. (2000). Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. *Nature* **405**, 76-81.
Huang, H., Ruan, H., Aw, M. Y., Hussain, A., Guo, L., Gao, C., Qian, F., Leung, T., Song, H., Kimelman, D. et al. (2008). Mypt1-mediated spatial positioning of Bmp2-producing cells is essential for liver organogenesis. *Development* **135**, 3209-3218.
Ito, M., Nakano, T., Erdodi, F. and Hartshorne, D. J. (2004). Myosin phosphatase: structure, regulation and function. *Mol. Cell. Biochem.* **259**, 197-209.
Jessen, J. R., Topczewski, J., Bingham, S., Sepich, D. S., Marlow, F., Chandrasekhar, A. and Solnica-Krezel, L. (2002). Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. *Nat. Cell Biol.* **4**, 610-615.
Keller, R. (2002). Shaping the vertebrate body plan by polarized embryonic cell movements. *Science* **298**, 1950-1954.
Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K. et al. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* **273**, 245-248.
Krieg, M., Arboleda-Estudillo, Y., Puech, P. H., Kafer, J., Graner, F., Muller, D. J. and Heisenberg, C. P. (2008). Tensile forces govern germ-layer organization in zebrafish. *Nat. Cell Biol.* **10**, 429-436.
Marlow, F., Zwartkruis, F., Malicki, J., Neuhauss, S. C., Abbas, L., Weaver, M., Driever, W. and Solnica-Krezel, L. (1998). Functional interactions of genes mediating convergent extension, knypek and trilobite, during the partitioning of the eye primordium in zebrafish. *Dev. Biol.* **203**, 382-399.
Marlow, F., Topczewski, J., Sepich, D. and Solnica-Krezel, L. (2002). Zebrafish Rho kinase 2 acts downstream of Wnt11 to mediate cell polarity and effective convergence and extension movements. *Curr. Biol.* **12**, 876-884.
Matsumura, F. and Hartshorne, D. J. (2008). Myosin phosphatase target subunit: Many roles in cell function. *Biochem. Biophys. Res. Commun.* **369**, 149-156.
Mizuno, T., Tsutsui, K. and Nishida, Y. (2002). Drosophila myosin phosphatase and its role in dorsal closure. *Development* **129**, 1215-1223.
Mlodzik, M. (2002). Planar cell polarization: do the same mechanisms regulate Drosophila tissue polarity and vertebrate gastrulation? *Trends Genet.* **18**, 564-571.
Muranyi, A., MacDonald, J. A., Deng, J. T., Wilson, D. P., Haystead, T. A., Walsh, M. P., Erdodi, F., Kiss, E., Wu, Y. and Hartshorne, D. J. (2002). Phosphorylation of the myosin phosphatase target subunit by integrin-linked kinase. *Biochem. J.* **366**, 211-216.
Myers, D. C., Sepich, D. S. and Solnica-Krezel, L. (2002). Bmp activity gradient regulates convergent extension during zebrafish gastrulation. *Dev. Biol.* **243**, 81-98.
Okamoto, R., Ito, M., Suzuki, N., Kongo, M., Moriki, N., Saito, H., Tsumura, H., Imanaka-Yoshida, K., Kimura, K., Mizoguchi, A. et al. (2005). The targeted disruption of the MYPT1 gene results in embryonic lethality. *Transgenic Res.* **14**, 337-340.
Piekny, A. J., Johnson, J. L., Cham, G. D. and Mains, P. E. (2003). The Caenorhabditis elegans nonmuscle myosin genes nmy-1 and nmy-2 function as redundant components of the let-502/Rho-binding kinase and mel-11/myosin phosphatase pathway during embryonic morphogenesis. *Development* **130**, 5695-5704.
Sanz-Moreno, V., Gadea, G., Ahn, J., Paterson, H., Marra, P., Pinner, S., Sahai, E. and Marshall, C. J. (2008). Rac activation and inactivation control plasticity of tumor cell movement. *Cell* **135**, 510-523.
Sato, A., Khadka, D. K., Liu, W., Bharti, R., Runnels, L. W., Dawid, I. B. and Habas, R. (2006). Profilin is an effector for Daam1 in non-canonical Wnt signaling and is required for vertebrate gastrulation. *Development* **133**, 4219-4231.
Sepich, D. S. and Solnica-Krezel, L. (2005). Analysis of cell movements in zebrafish embryos: morphometrics and measuring movement of labeled cell populations in vivo. *Methods Mol. Biol.* **294**, 211-233.
Sepich, D. S., Calmelet, C., Kiskowski, M. and Solnica-Krezel, L. (2005). Initiation of convergence and extension movements of lateral mesoderm during zebrafish gastrulation. *Dev. Dyn.* **234**, 279-292.
Skoglund, P., Rolo, A., Chen, X., Gumbiner, B. M. and Keller, R. (2008). Convergence and extension at gastrulation require a myosin IIB-dependent cortical actin network. *Development* **135**, 2435-2444.
Solnica-Krezel, L. (2006). Gastrulation in zebrafish – all just about adhesion? *Curr. Opin. Genet. Dev.* **16**, 433-441.
Solnica-Krezel, L., Stemple, D. L., Mountcastle-Shah, E., Rangini, Z., Neuhauss, S. C., Malicki, J., Schier, A. F., Stainier, D. Y., Zwartkruis, F., Abdelilah, S. et al. (1996). Mutations affecting cell fates and cellular rearrangements during gastrulation in zebrafish. *Development* **123**, 67-80.
Tan, C., Stronach, B. and Perrimon, N. (2003). Roles of myosin phosphatase during Drosophila development. *Development* **130**, 671-681.
Topczewski, J., Sepich, D. S., Myers, D. C., Walker, C., Amores, A., Lele, Z., Hammerschmidt, M., Postlethwait, J. and Solnica-Krezel, L. (2001). The zebrafish glypican knypek controls cell polarity during gastrulation movements of convergent extension. *Dev. Cell* **1**, 251-264.
Totsukawa, G., Yamakita, Y., Yamashiro, S., Hartshorne, D. J., Sasaki, Y. and Matsumura, F. (2000). Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J. Cell Biol.* **150**, 797-806.
Trinkaus, J. P. (1973). Surface activity and locomotion of Fundulus deep cells during blastula and gastrula stages. *Dev. Biol.* **30**, 69-103.
Veeman, M. T., Axelrod, J. D. and Moon, R. T. (2003). A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev. Cell* **5**, 367-377.
von der Hardt, S., Bakkers, J., Inbal, A., Carvalho, L., Solnica-Krezel, L., Heisenberg, C. P. and Hammerschmidt, M. (2007). The Bmp gradient of the zebrafish gastrula guides migrating lateral cells by regulating cell-cell adhesion. *Curr. Biol.* **17**, 475-487.
Wallingford, J. B., Rowning, B. A., Vogeli, K. M., Rothbacher, U., Fraser, S. E. and Harland, R. M. (2000). Dishevelled controls cell polarity during Xenopus gastrulation. *Nature* **405**, 81-85.
Weiser, D. C., Pyati, U. J. and Kimelman, D. (2007). Gravin regulates mesodermal cell behavior changes required for axis elongation during zebrafish gastrulation. *Genes Dev.* **21**, 1559-1571.
Weiser, D. C., Julien, K. R., Lang, J. S. and Kimelman, D. (2008). Cell shape regulation by Gravin requires N-terminal membrane effector domains. *Biochem. Biophys. Res. Commun.* **375**, 512-516.
Westerfield, M. (1993). *The Zebrafish Book: a Guide for the Laboratory Use of Zebrafish (Brachydanio rerio)*. Eugene, OR: University of Oregon Press.
Wilkinson, S., Paterson, H. F. and Marshall, C. J. (2005). Cdc42-MRCK and Rho-ROCK signalling cooperate in myosin phosphorylation and cell invasion. *Nat. Cell Biol.* **7**, 255-261.
Wissmann, A., Ingles, J. and Mains, P. E. (1999). The Caenorhabditis elegans mel-11 myosin phosphatase regulatory subunit affects tissue contraction in the somatic gonad and the embryonic epidermis and genetically interacts with the Rac signaling pathway. *Dev. Biol.* **209**, 111-127.
Wolf, K. and Friedl, P. (2006). Molecular mechanisms of cancer cell invasion and plasticity. *Br. J. Dermatol.* **154 Suppl. 1**, 11-15.
Wolf, K., Mazo, I., Leung, H., Engelke, K., von Andrian, U. H., Deryugina, E. I., Strongin, A. Y., Brocker, E. B. and Friedl, P. (2003). Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J. Cell Biol.* **160**, 267-277.
Wooldridge, A. A., MacDonald, J. A., Erdodi, F., Ma, C., Borman, M. A., Hartshorne, D. J. and Haystead, T. A. (2004). Smooth muscle phosphatase is regulated in vivo by exclusion of phosphorylation of threonine 696 of MYPT1 by phosphorylation of Serine 695 in response to cyclic nucleotides. *J. Biol. Chem.* **279**, 34496-34504.
Wyckoff, J. B., Pinner, S. E., Gschmeissner, S., Condeelis, J. S. and Sahai, E. (2006). ROCK- and myosin-dependent matrix deformation enables protease-independent tumor-cell invasion in vivo. *Curr. Biol.* **16**, 1515-1523.
Yin, C., Kiskowski, M., Pouille, P. A., Farge, E. and Solnica-Krezel, L. (2008). Cooperation of polarized cell intercalations drives convergence and extension of presomitic mesoderm during zebrafish gastrulation. *J. Cell Biol.* **180**, 221-232.
Zhu, S., Liu, L., Korzh, V., Gong, Z. and Low, B. C. (2006). RhoA acts downstream of Wnt5 and Wnt11 to regulate convergence and extension movements by involving effectors Rho kinase and Diaphanous: use of zebrafish as an in vivo model for GTPase signaling. *Cell Signal.* **18**, 359-372.