Has2 is required upstream of Rac1 to govern dorsal migration of lateral cells during zebrafish gastrulation

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Summary

The large extracellular polysaccharide Hyaluronan (HA) and its synthesizing enzymes (Has) have been implicated in regulating the migratory potential of metastatic cancer cells. Here, we analyze the roles of zebrafish Has2 in normal development. Antisense morpholino oligonucleotide (MO)-mediated knockdown of zebrafish Has2 leads to the loss of HA, and severe migratory defects during gastrulation, somite morphogenesis and primordial germ cell migration. During gastrulation, ventrolateral cells of *has2* morphant embryos fail to develop lamellipodia and to migrate dorsally, resulting in a blockage of dorsal convergence, whereas extension of the dorsal axis is normal. The effect is cell autonomous, suggesting that HA acts as an autocrine signal to stimulate the migration of HA-generating cells. Upon ectopic expression in axial cells, *has2* causes the formation of supernumerary lamellipodia and a blockage of axis extension. Epistasis analyses with constitutively active and dominant-negative versions of the small GTPase Rac1 suggest that HA acts by Rac1 activation, rather than as an essential structural component of the extracellular matrix. Together, our data provide evidence that convergence and extension are separate morphogenetic movements of gastrulation. In addition, they suggest that the same HA pathways are active to auto-stimulate cell migration during tumor invasion and vertebrate embryogenesis.

Key words: Hyaluronan, Has, Dg42, Rac1, Cell migration, Convergence extension, Adaxial cells, Slow muscle, Sclerotome, Germ cells, Metastasis, Zebrafish

Introduction

Hyaluronan (HA) is a linear polysaccharide of high molecular weight, consisting of glucuronic acid (GlcA) and *N*-acetyl-glucosamine (GlcNAc) disaccharide repeats, linked by alternating β1-3 and β1-4 glycosidic bonds. In contrast to most other polysaccharides, HA is not covalently linked to a core protein. HA is synthesized at the inner face of the plasma membrane by HA synthases (Has), and is directly extruded to the extracellular space. However, HA can also re-enter the cell, and can even translocate to the nucleus (for reviews, see DeAngelis, 1999; Lee and Spicer, 2000). In vertebrates, three HA synthases (Has1, Has2 and Has3) encoded by three distinct genes have been identified by complementing HA-deficient cell lines.

Numerous functions have been associated with HA in cell proliferation, cell adhesion and cell migration (for reviews, see Lee and Spicer, 2000; Toole, 2001). Some of these can be attributed to the ability of HA to create and fill space by organizing and modifying the ECM. Other roles are related to its ability to interact and signal through cell surface or cytoplasmic receptors, such as CD44 or RHAMM. Different cellular responses can be induced via various signal transduction pathways, involving the GTPases Ras, RhoA and Rac (reviewed by Lee and Spicer, 2000; Toole, 2001; Turley et al., 2002). In the case of Rac1, which can be activated by CD44 via the nucleotide exchange factor Tiam1 (Bourguignon et al., 2000), these responses include the local reorganization of the cytoskeleton and lamellipodia formation (Oliferenko et al., 2000).

Most of the experiments addressing the functions of HA have been carried out in vitro. Comparably few in vivo analyses have been reported, most of which deal with the role of HA in cancer. It was shown that most malignant solid tumors contain elevated HA levels (reviewed by Toole, 2001). In addition, Has overexpression promotes fibrosarcoma and mammary carcinoma growth (Kosaki et al., 1999), whereas perturbation of endogenous HA interactions inhibits mammary carcinoma growth (Peterson et al., 2000). Furthermore, HA-induced clustering of CD44 (Yu and Stamenkovic, 2000), and overexpression of RHAMM (Hall et al., 1995), lead to enhanced invasiveness of tumor cells and metastasis. Together, these data indicate an important role of HA in promoting cell proliferation and migration in malignant tumor cells.

To study the role of HA during normal vertebrate development, *Has2* has been knocked out in mouse. *Has2* deficient embryos die during midgestation (E9.5-E10), exhibiting reduced body size and severe cardiac and vascular abnormalities (Camenisch et al., 2000). In the developing heart, *Has2* and HA are required for an epithelial-to-mesenchymal transformation, and subsequent migration of

endothelial cells at the atrioventricular boundary during early steps of heart valve formation. However, it remains unclear whether, being also involved in other morphogenetic and migratory processes that take place in vertebrate embryos, HA and Has proteins might have more widespread roles.

In this paper we focus on the essential role of zebrafish *has2* for cell migrations during zebrafish gastrulation. Based on cell tracing analyses, three different morphogenetic movements of gastrulation have been distinguished, epiboly, involution, and convergent extension (CE) (Warga and Kimmel, 1990). During CE, cells from lateral regions of the gastrula embryo move towards the dorsal side (convergence), which extends accordingly (extension). Initially, convergence and extension were supposed to be closely linked, driven by mediolateral cell-cell intercalations (Warga and Kimmel, 1990). Indeed, several mutants were isolated in which both convergence and extension are affected, such as *knypek*/*glypican6* (Topczewski et al., 2001) and *trilobite*/*strabismus* (Jessen et al., 2002; Park and Moon, 2001). However, a recent study of cell behavior within the notochord of *no tail* (*ntl*) mutant embryos shows that extension can be driven by mechanisms other than convergence (Glickmann et al., 2003). According to this notion, mediolateral intercalation is largely restricted to dorsal regions, driving extension and narrowing of the axis, whereas convergence of lateral cells entails migration of individual cells and small groups of cells without any cell rearrangements (reviewed by Wallingford et al., 2002; Myers et al., 2002b; Solnica-Krezel and Cooper, 2002).

Mutant analysis has revealed some of the signaling pathways that are involved in instructing cellular CE behavior. Although the non-canonical Wnt signals Wnt11 and Wnt5, mutated in *silberblick*/*wnt11* and *pipetail*/*wnt5* mutant embryos, respectively (Heisenberg et al., 2000; Rauch et al., 1997; Wallingford et al., 2002), are required for both convergence and extension movements, Bone morphogenetic proteins (Bmps) appear to be essential for convergence only. In *bmp* mutant embryos, convergence is blocked, whereas extension is normal or even elevated, resulting in the characteristic cylinder shape of the mutant embryo (Myers et al., 2002a). However, in addition to cell movements, Bmps also determine differential cell fates along the dorsoventral axis of gastrula embryos, and it is currently unclear how these two roles are interconnected (for a review, see Hammerschmidt and Mullins, 2002; Myers et al., 2002b).

Here we show that Has2 is specifically required for the migration of lateral cells driving dorsal convergence. By contrast, extension within the axial and paraxial mesoderm is normal in *has2* morphant embryos, but blocked upon *has2* overexpression. The loss of dorsal convergence in *has2* morphants results in a cylinder shape of the embryos, similar to that of *bmp* mutants. However, in contrast to *bmp* mutants, *has2* morphants do not display a dorsalization of cell fates. Thus, *has2* allows us to genetically dissect convergence and extension as two separate morphogenetic movements of gastrulation, specifically regulating active cell migration of converging lateral cells independent of dorsoventral cell fate specification. In addition, we show that the effect of Has2 on dorsal convergence is mediated by the small GTPase Rac1, promoting lamellipodia outgrowth. This indicates that HA regulates migrating cells during embryogenesis and tumor invasion by activating the same signal transduction pathway.

Materials and methods

Isolation of zebrafish *has1*, *has2* and *has3*

A cDNA library derived from zebrafish embryos from dome to 80% epiboly stages was screened under high stringency, using a previously described partial zebrafish *dg42* cDNA as a probe (Semino et al., 1996). A 2.4 kb and a 3 kb cDNA clone were isolated (GenBank Accession Number AF190742), both of which contain the same open reading frame, encoding a protein of 552 amino acids. Comparison of the predicted amino acid sequence of the full-length protein with Has proteins from other species revealed that it is zebrafish Has2, rather than Has1 [*Xenopus* Dg42 is Has1 (Rosa et al., 1988)]. In order to search for other zebrafish Has genes, a genomic library was screened with low stringency, using the *has2* gene as a probe. Three clones were isolated, two of which contained exons of *has2*, whereas an 18 kb clone contained all exons, encoding a Has protein most similar to mammalian Has3 (GenBank Accession Number AF190743). A third Has gene was identified by searching the Ensemble06 zebrafish genomic sequence from The Sanger Center using the Fugu *has1* sequence. Two exons of a putative Has gene were found. The corresponding cDNA was amplified from 5-day-old larvae and sequenced (GenBank Accession Number AY437407). The predicted protein has 53% homology with zebrafish Has2 and Has3, and 60% homology with human and mouse Has1, indicating that this is most likely to be zebrafish Has1.

Generation of constructs, mRNA synthesis and microinjection

has2 was amplified with primers containing *Eco*RI and *Xho*I restriction sites from full-length cDNA using Cloned *Pfu* DNA polymerase (Stratagene), and cloned into pCS2+ to create a pCS2-*has2* expression construct. Human caRac1 G12V was obtained from the Guthrie cDNA Resource Center (Sayre, PA) (Lennon et al., 1996), and was re-cloned by *Bam*HI and *Xho*I digest into pCS2+ to create pCS2-caRac1G12V. Capped RNA was prepared with the Message Machine kit (Ambion). RNA was dissolved in water, and 1 nl per embryo was injected at indicated concentrations as described (Hammerschmidt et al., 1999).

RT-PCR analyses

RT-PCR was carried out using the Titan One Tube RT-PCR kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. RNA was isolated from dechorionated embryos using the Ultraspec™ RNA isolation system (Biotecx, Houston, USA), according to the manufacturer's instructions. The sequence of the primers used were:

zf ef1α (GenBank Accession Number X77689), 5′-GGCCACGTCGACTCCGGAAAGTCC-3′ and 5′-CTCAAAACGAGCCTGGCTGTAAGG-3′;

has1, 5′-AGTCGGTCCGGGCTCTTCGTAT-3′ and 5′-GGACTGGCAGGACCTCTCAATGTT-3′;

has2, 5′-ACCATGCTGGACCCGGCCTCC-3′ and 5′-GAGCCAGCGCAGGTAGGTAAT-3′; and

has3, 5′-GACAAATGCGAGGCCCCTCCAGAC-3′ and 5′-CCGCACCACTCTGGCCACTCGTGC-3′.

RT-PCR reaction products after 20, 25, 30 and 35 cycles, were separated on a 2% agarose gel and blotted on Hybond N+ membrane (Amersham Pharmacia Biotech, UK). *has1*, *has2* and *ef1α* DNA probes were labeled with alkaline phosphatase by using AlkPhosDirect (Amersham), and hybridization and detection were carried out according to the manufacturer's instructions. *has3* DNA probe was labeled with ^{32}P by random primed DNA synthesis.

Morpholino oligonucleotides

Morpholino oligonucleotides (MOs; Gene Tools) were dissolved in water to a concentration of 4 mM. For injection (1 nl per embryo), MOs were diluted in 1×Danieu's buffer (Nasevicius and Ekker, 2000).

Sequences of MOs used were: *has2* MO1 AGCAGCTCTTTGGAGATGTCCCGTT; control *has2* 4mm-MO AGCACCTCTATGGAGTTGTCGCGTT; *has2* MO2 CGTTAGTTGAACAGGGATGCTGTCC. All MOs were injected at 0.25 mM.

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization and antibody counterstaining were carried out as previously described (Hammerschmidt et al., 1996). For *has2* in situ probe synthesis, plasmid pBS-zfhas2 was linearized with *Xba*I and transcribed with T7 RNA polymerase. In addition, riboprobes of the following cDNAs were used: *hgg1* (Thisse et al., 1994), *krox20* (Oxtoby and Jowett, 1993), *pax2.1* (Krauss et al., 1992), *gata1* (Detrich et al., 1995), *myoD* (Weinberg et al., 1996), *smbpc* (Xu et al., 2000), *sox17* (Alexander and Stainier, 1999), *twist* (Morin-Kensicki and Eisen, 1997) and *vasa* (Yoon et al., 1997). Whole-mount immunostaining with anti-Fibronectin (Sigma, F3648, 1:200), and anti-Laminin and anti-pan Cadherin antibodies (Crawford et al., 2003), was carried out using reported concentrations and protocols as described (Hammerschmidt et al., 1996). 15 μm sections of stained embryos were cut, using glass knifes, after mounting in Technovit 8100 (Haraeus-Kulzer), according to the supplier's instructions.

HA staining

Staining was performed essentially as described by Köprunner et al. (Köprunner et al., 2000), with the following modifications. After sectioning, the partially (30%) rehydrated specimens were refixed in 4% paraformaldehyde. The sections were incubated for 3 hours at room temperature with a 1/10 dilution of serum free medium of HEK293 cells expressing a neurocan-alkaline phosphatase fusion protein (generous gift from Uwe Rauch, University of Lund) in 10 mM sodium phosphate buffer (pH 7.0), 1.5 M NaCl, 0.08% BSA.

Uncaging experiments

Embryos were injected at the 1-cell stage with 0.25% caged fluorescein dextran (10,000 MW; Molecular probes, Eugene, USA). Uncaging was carried out at shield stage using a Zeiss Axiophot Microscope equipped with a UV light source, adjustable pinhole and a 40× objective. Pictures were taken at 80% epiboly and tailbud stage, and the angle for dorsal convergence or the length of extension was measured using NIH image software.

Cell transplantations

To visualize cell shape and cellular processes in wild-type, *has2* mRNA, *has2* MO, *caRac* mRNA, *dnRac1* mRNA or double-injected embryos, donor embryos were injected with mRNA encoding membrane-localized GFP (Moriyoshi et al., 1996). At the shield stage, 5-10 lateral or dorsal marginal donor cells were transplanted into the same region of the same type of recipient embryo (lateral wild-type→lateral wild-type; lateral *has2* MO→lateral *has2* MO; dorsal *has2* mRNA→dorsal *has2* mRNA, and so on). To show that *has2* and *Rac1* act in a cell autonomous manner, heterologous transplantation of labeled wild-type cells into *has2* MO or *dnRac1* mRNA-injected recipients, or transplantation of labeled *has2* MO or *dnRac1* mRNA-injected cells into wild-type recipients were performed. Chimeric embryos were mounted in 1% methylcellulose between bridged coverslips, and photos were taken immediately after the transplantation (shield stage) and at the 80-90% epiboly stage, using a Hamamatsu digital camera (C4742-95) and Openlab software (Improvision).

Results

The zebrafish *has1*, *has2* and *has3* genes

We have isolated three members of the Has gene family from zebrafish, *has1*, *has2* and *has3*, which are closely related to the corresponding orthologs from human and mouse (Fig. 1A). The coding region of *has2*, 1656 nucleotides in length, contains a short previously cloned cDNA fragment originally named *dg42* (Semino et al., 1996), according to the first member of the gene family from *Xenopus* (Rosa et al., 1988), which in the meantime has been identified as *XHas1*. Hydrophobicity plots of the zebrafish Has proteins show six putative transmembrane domains, classifying them as membrane-integrated glycosyltransferases. The catalytic domains contain all conserved amino acid residues involved

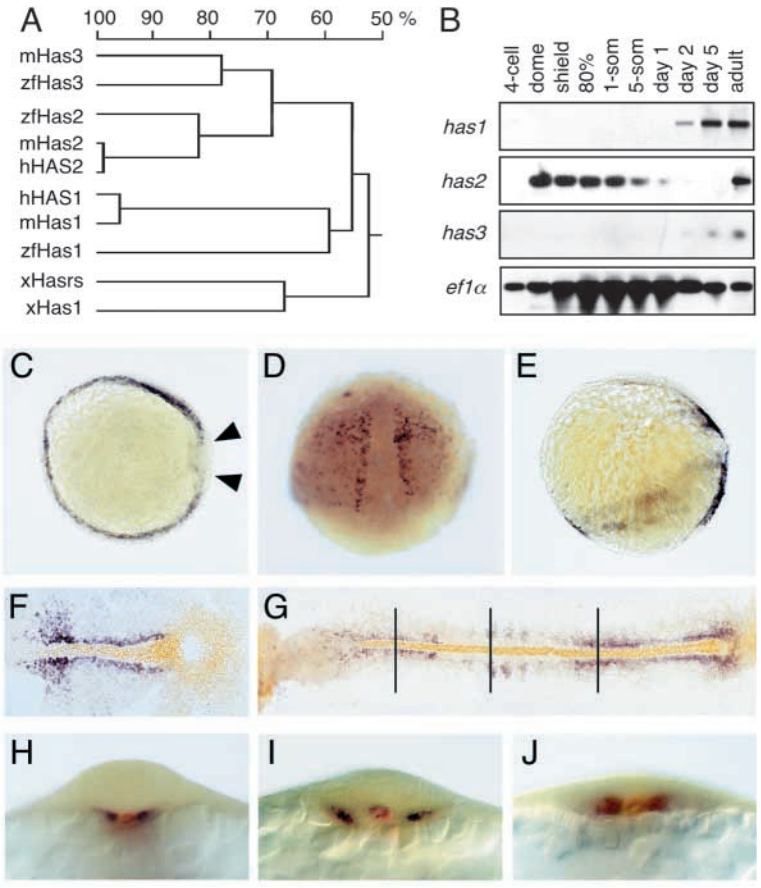


Fig. 1. Zebrafish Has genes and their expression during embryogenesis. (A) Homology tree of Has1, Has2, and Has3 family members based on amino acid sequences. The GenBank accession numbers for the sequences listed above are: *Xenopus laevis* xHas1 (X52958), *Xenopus laevis* xHasrs (AF015780), mouse mHas3 (MMU86408), human hHAS2 (HSU54804), mouse mHas2 (MMU52524), human hHAS1 (HSU59269), mouse mHas1 (D82964). (B) RT-PCR analysis of zebrafish *has1, has2 and has3* expression during different stages of development. (C-J) Wild-type embryos stained for *has2* mRNA (blue), and No tail (Ntl) protein (brown; F-J). (C) Early gastrulation (shield stage), animal pole view. Arrowheads indicate the dorsal shield devoid of staining. (D) Midgastrulation (80% epiboly stage), dorsal view. (E) 90% epiboly stage, lateral view, dorsal to the right. (F) End of gastrulation (100% epiboly stage), dorsal view on flat-mounted embryo; anterior to the left. (G-J) 8-somite stage: (G) dorsal view on flat-mounted embryo, anterior to the left; (H-J) optical cross sections at levels indicated in G.

in both GlcNAc and GlcA transferase activity (Yoshida et al., 2000).

The temporal expression profiles of *has1*, *has2* and *has3* during zebrafish development were determined through semi-quantitative RT-PCR analysis. Of the three, only *has2* shows expression during embryogenesis. No *has2* mRNA was detected in fertilized eggs (4-cell embryo), pointing to the absence of maternal contribution, whereas zygotic expression is already high at the dome stage, shortly after midblastula transition (MBT). *has2* continues to be strongly expressed during gastrulation, although transcript levels decrease during segmentation stages, and are very low at 24 hours post fertilization (hpf). In addition to in embryos, zebrafish *has2* is also expressed in adult animals, similar to the human and mouse members of the class 2 genes (Spicer and McDonald, 1998).

In contrast to zebrafish *has2*, no *has1* or *has3* transcripts could be detected during early developmental stages (Fig. 1B). Expression of both genes is initiated in 2-day-old larvae, and continues in adult tissues, corresponding with the expression profile reported for the human and mouse class 3 genes (Spicer and McDonald, 1998).

The spatial expression pattern of *has2* was determined using whole-mount in situ hybridization. At blastula stages, *has2* transcripts are ubiquitously distributed throughout the zebrafish embryo (data not shown). Starting with the onset of gastrulation, a restricted expression pattern can be observed. At the shield stage, *has2* transcripts are confined to the presumptive mesendoderm in the entire marginal zone of the embryo, with the exception of the dorsal shield (Fig. 1C). During gastrulation *has2* is expressed in cells of the ventrolateral mesoderm and endoderm, whereas cells of the axial mesendoderm lack *has2* transcripts (Fig. 1D,E). Towards the end of gastrulation, *has2* expression becomes progressively stronger in paraxial regions of the mesoderm (Fig. 1F), whereas during segmentation stages it is predominantly expressed in adaxial cells of the head (Fig. 1G,H), the sclerotome of formed somites (Fig. 1G,I) and adaxial cells of the presomitic mesoderm (Fig. 1G,J). The trunk and tail adaxial cells will give rise to slow muscle cells (see below).

The *has2* expression pattern anticipates the distribution of HA in the embryo

Has2 catalyses the synthesis of HA, which is supposed to be secreted into the extracellular space. To determine the distribution of HA in zebrafish embryos, sections of late gastrula and early segmentation embryos were stained with the Hyaluronan-binding protein Neurocan, coupled to alkaline phosphatase (Rauch et al., 1992; Köprunner et al., 2000). Although at late gastrula stages, HA levels were too low to be detected (data not shown), the HA distribution in 10-somite stage embryos perfectly reflects the expression pattern of *has2*, with high HA levels in the somites and the presomitic mesoderm, whereas neural tube and notochord are devoid of HA (Fig. 2A).

Has2 is required for dorsoventral morphogenesis, but not for dorsoventral patterning

To assess the function of Has2 during embryogenesis, we blocked translation of *has2* by injecting antisense morpholino oligonucleotides (MOs) (Nasevicius and Ekker, 2000) targeting the 5′ UTR of *has2*. Injection of 0.25 pmol of two different *has2* MOs (see Materials and methods) led to a reduction of HA to non-detectable levels at early segmentation stages (Fig. 2B). By contrast, other extracellular components appeared unaffected, as revealed by immunostaining against Fibronectin (Fig. 2D,E), Laminin and Cadherins (data not shown). During gastrula stages, *has2* morphants develop striking morphogenetic defects. At the end of gastrulation they display a characteristic cylinder-like shape (Fig. 3B), thus far only described for dorsalized embryos mutated in components of the Bmp signaling

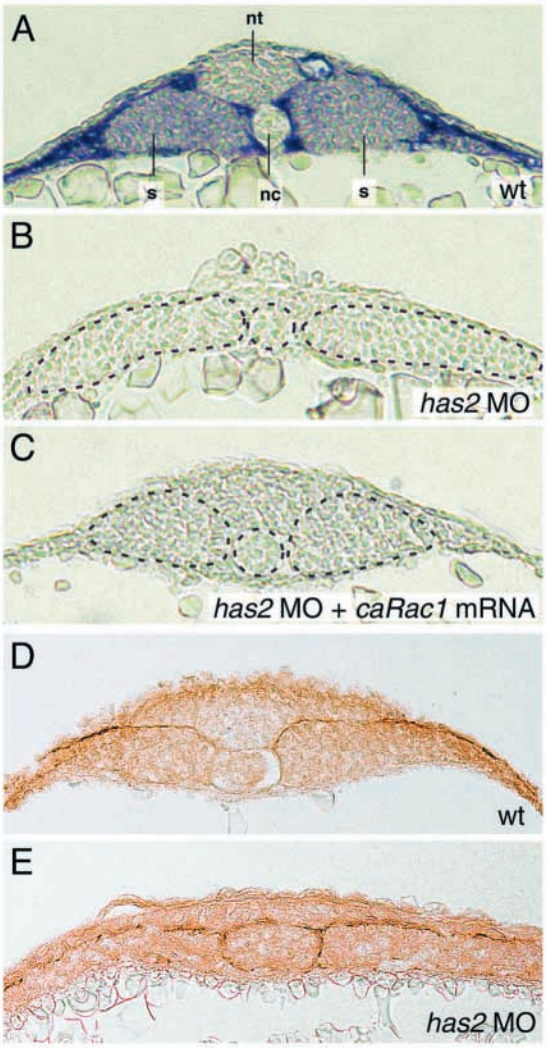


Fig. 2. Presence of HA depends on Has2 activity and cannot be restored by constitutively active Rac1, whereas Fibronectin is unaffected in *has2* morphants. (A-C) HA staining on transverse sections through the posterior region of 10-somite stage embryos. (A) Wild type; (B) embryo injected with *has2* MO; (C) embryo co-injected with *has2* MO and mRNA encoding constitutively active Rac1 (*caRac1*). At tailbud stage, the embryo in B was elongated, indicating convergence defects, the embryo in C was round like the wild-type control, indicating that convergence was rescued (compare with Fig. 4). Notochord and somites are outlined in B,C. (D,E) Anti-Fibronectin immunostaining. Section through posterior region of 3-somite stage embryos. (D) Wild type; (E) embryo injected with *has2* MO. nc, notochord; nt, neural tube; s, somite.

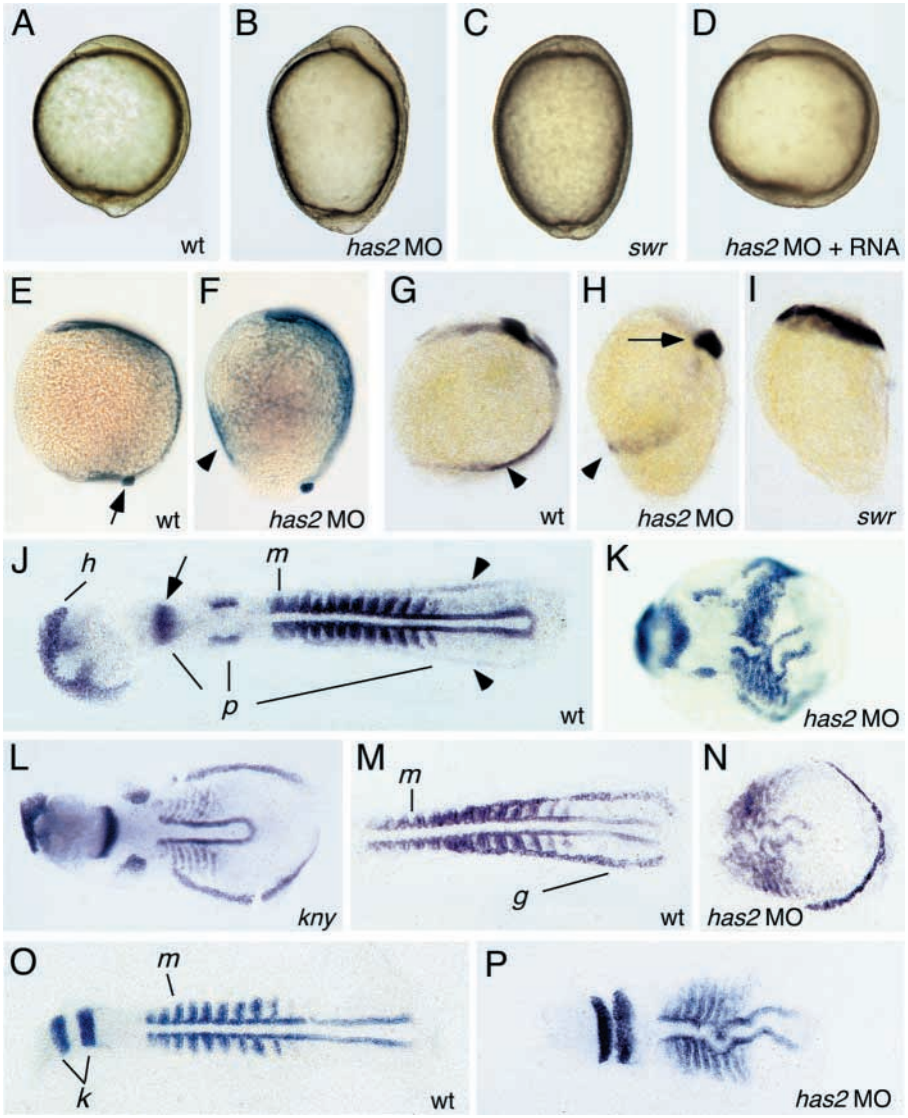


Fig. 3. *has2* morphants are elongated, similar to Bmp mutants, but are not dorsalized. (A-D) Live embryos at tailbud stage, lateral view, dorsal to the right. (A) Wild type; (B) *has2* morphant; (C) *bmp2b/swr* mutant; (D) embryo co-injected with *has2* MO and *has2* mRNA. (E,F) *sox17* expression at tailbud stage, lateral views, dorsal to the right. (E) Wild type; (F) *has2* morphant. Arrow (E) indicates forerunner cells; arrowhead (F) endodermal cells that have remained on the ventral side. (G-I) *pax2.1* expression at the 4-somite stage, lateral view. Arrow (H) indicates midbrain-hindbrain boundary; arrowheads (G,H) indicate presumptive pronephric duct cells. (G) Wild type; (H) *has2* morphant; (I) *swr* mutant. (J-L) *pax2.1* (marked as 'p'), *myoD* ('m'), *hgg1* ('h') expression at the 10-somite stage; dorsal views on regular (K) or flat-mounted (J,L) embryos. (J) Wild type; (K) *has2* morphant; (L) *kny/glypican6* mutant. In J, midbrain-hindbrain boundary is marked by an arrow, pronephric ducts by arrowheads. (M,N) *gata1* ('g') and *myoD* ('m') expression at the 10-somite stage; dorsal view on flat-mounted embryos. (M) Wild type; (N) *has2* morphant. (O,P) *krox20* ('k') and *myoD* ('m') expression at the 10-somite stage; dorsal view on flat-mounted embryos. (O) Wild type; (P) *has2* morphant.

pathway, such as *swr*/*bmp2b* (Fig. 3C). This effect of the *has2* MOs is very specific. It can be perfectly rescued by co-injection of low amounts (25 ng/μl) of *has2* mRNA lacking the 5′ sequence targeted by the MOs (Fig. 3D). Furthermore, injection of a control MO with four nucleotide exchanges (4mm-MO) had no effect (data not shown).

Despite this striking similarity in embryonic morphology, Bmp mutants and *has2* morphants display crucial differences in early embryonic patterning, as revealed by whole-mount in situ hybridization. Bmp mutants are dorsalized, characterized by a ventral expansion of the expression domain of dorsal marker genes, such as *pax2.1*, a marker for the midbrain-hindbrain boundary region (Fig. 3I; compare with wild-type control in 3G), and *myoD*, a marker for presumptive muscle cells in the somitic mesoderm (data not shown) (Mullins et al., 1996). In parallel, Bmp mutants display a loss of ventral-specific marker gene expression, such as *pax2.1*, in presumptive pronephric cells (Fig. 3I), and *gata1*, a marker for blood precursors (data not shown) (Mullins et al., 1996). No such shifts in dorsoventral marker gene expression are observed in *has2* morphants, which show the normal number of dorsally and ventrally specified cell types (Fig. 3H,K,N; compare with wild-type controls in 3G,J,M). However, the spatial organization of the different cell types is severely altered in *has2* morphant embryos. Most strikingly, at the 4-somite stage, presumptive pronephric cells are located in ventral-most regions of the morphant embryo (Fig. 3H), rather than in paraxial regions as in control siblings (Fig. 3G). According to cell lineage analyses, pronephric cells derive from such ventral regions of the late blastula embryo (Kimmel et al., 1990). However, during normal development, they move dorsally during gastrulation (Warga and Kimmel, 1990) to end up in paraxial regions at early segmentation stages (Fig. 3G). In this respect, the ventral localization of pronephric cells in *has2* morphants could be interpreted as a result of an impaired dorsal movement of its precursors during gastrulation. The same appears to be true for endodermal cells, as reflected by the expression pattern of *sox17*. Whereas in wild-type embryos, endodermal cells are shifted dorsally during gastrulation (Fig. 3E), in *has2* morphants, the earlier dorsoventral *sox17* pattern is maintained, with positive cells present ventrally and laterally, but absent from the posterior dorsal axis itself (Fig. 3F). At the 10-somite stage, *has2* morphants display a generally shorter and broader body axis, as revealed by the broadened somites (Fig. 3K,N,P; compare with 3J,M,O for wild-type control), and the increased distance of pronephric and blood precursors from the dorsal midline (Fig. 3K,N; compare with 3J,M). The phenotype is very similar

to that of *knypek/glypican6* (Fig. 3L) (Solnica-Krezel et al., 1996; Topczewski et al., 2001) and *trilobite/strabismus* (Hammerschmidt et al., 1996; Solnica-Krezel et al., 1996; Jessen et al., 2002; Park and Moon, 2001) mutants, both of which have been previously shown to display specific defects in convergent extension movements of gastrulation. However, in contrast to such mutants, *has2* morphants display a severe undulation of the notochord, reflected by the *myoD* expression pattern in adaxial cells (Fig. 3K,N,P). Thus, it appears that in contrast to Glypican6 and Strabismus, Has2 is not required for the extension of axial tissue. Interestingly, *has2* morphants also display a broadening of the neuroectoderm, as reflected by the expression domains of *pax2.1* in the midbrain-hindbrain boundary region (Fig. 3K), and of *krox20* in hindbrain rhombomeres 3 and 5 at the 10-somite stage (Fig. 3P). As *has2* is not expressed in the ectoderm (see above; Fig. 1), and because it acts in a cell autonomous fashion (see below; Fig. 5), this broadening of the neuroectoderm appears to be a secondary consequence of the defects in the mesendoderm, indicating that dorsal convergence of the neural plate depends on proper convergence of the underlying mesendodermal cells.

In summary, the in situ analyses indicate that dorsoventral-specific cell fate specification processes are normal in *has2* morphants, whereas morphogenetic movements of gastrulation are affected. In particular, dorsal convergence of ventrolateral cells appears to be severely impaired, while extension of axial tissues seems largely unaffected.

Has2 is required for dorsal convergence, but not for extension

In order to directly study the effect of Has2 on convergent extension movements, lateral marginal (mesoderm and endoderm) cells located 90° from the dorsal shield were labeled by un-caging a fluorescent dye at the onset of gastrulation (6 hpf), and movement of labeled cells was followed until gastrulation was completed (10.5 hpf), as previously described (Sepich et al., 2000; Topczewski et al., 2001). *has2* morphant embryos display a severely reduced movement of labeled lateral cells towards the dorsal axis (Fig. 4D-F; see Fig. 4J for graph; distance from dorsal axis at 10.5 hpf: wild type 20±9°, *has2* MO 63±9°; $P=1\times10^{-10}$). Co-injection of *has2* MO together with low amounts (25 ng/μl) of *has2* mRNA, which does not contain the sequence targeted by the MOs, results in a complete rescue of dorsal convergence and morphology of lateral cells (Fig. 4J; distance from dorsal axis at 10.5 hpf: 23±7°, $P=0.5$; and data not shown), indicating that the effects are specific.

Most of the thus far described zebrafish mutants with defects in dorsal convergence also display impaired extension movements (see above for *knypek*). Therefore, we measured extension movements during gastrulation in *has2* morphants. Despite their failed convergence, lateral marginal cells of *has* morphants display rather normal extension movements (Fig. 4D-F; see Fig. 4K for graph; length of labeled clones along anteroposterior axis at 10.5 hpf: wild type 532±43 μm, *has2* MO 507±28 μm; $P=0.3$). When extension of dorsal cell populations was measured by labeling cells within the shield, *has2* morphants only displayed a slight reduction compared with wild-type control embryos (Fig. 6A,B,I,J; Fig. 6Q for graph; lengths of labeled clones along anteroposterior axis at 10.5 hpf: wild type 780±36 μm, *has2* MO 690±76 μm; $P=0.04$). By contrast, an approximately 2.5-fold increase in extension was observed for ventral cells of *has2* morphants compared with wild-type embryos (wild type 86±25 μm, *has2* MO 230±54 μm; $P=6.6\times10^{-4}$; data not shown). Together, these results indicate that Has2 is primarily required for dorsal migration of lateral cells to allow convergence movements, whereas the shifts in extension are most likely secondary consequences of the impaired convergence (see Discussion).

Has2 and Rac1 function are required for lamellipodia formation

To investigate the cellular basis of failed dorsal convergence in *has2* morphant embryos, we visualized cell membranes and

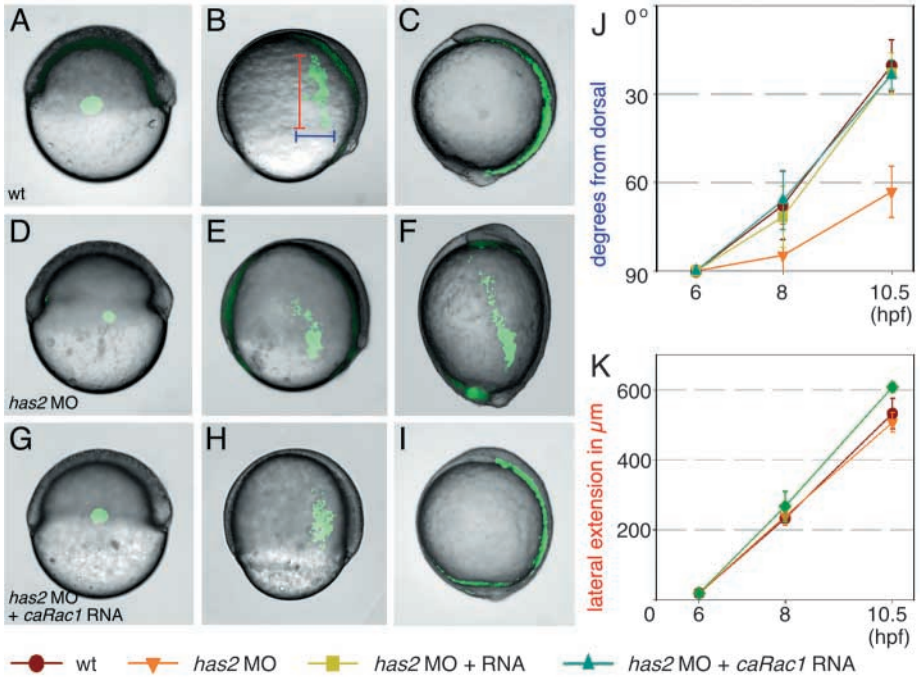


Fig. 4. Loss of Has2 function leads to blockage of dorsal convergence, but not of extension, and can be compensated by constitutively active Rac1.
(A-I) Distribution of labeled cell clusters at onset of gastrulation, mid-gastrulation and end of gastrulation; lateral views, dorsal to the right. (A-C) Wild type; (D-F) *has2* morphant; (G-I) embryo co-injected with *has2* MO and *caRac1* RNA. (A,D,G) Shield (6 hpf), directly after labeling of cell clusters by fluorochrome uncaging; (B,E,H) 80% epiboly (8 hpf); (C,F,I) tailbud (10.5 hpf). (J) Graph showing angle between labeled cells and dorsal axis at different time points, measuring dorsal convergence (indicated in B by blue bar). All clones start at 90° from the shield. (K) Graph showing the anteroposterior extension of labeled lateral cell populations (indicated in B by red bar) at different time points. In J and K, 10 embryos per treatment were evaluated, and standard deviations are indicated.

cellular protrusions of individual lateral cells with a membrane-localized GFP. At midgastrulation stages (8 hpf), lateral cells in wild-type embryos are flattened and polarized, with multiple lamellipodia (n=4.3±0.95; from 20 cells in 4 embryos) (Fig. 5A), broad cellular protrusions acting as leading edges during directed cell migration (for a review, see Webb et al., 2002). By contrast, labeled cells of *has2* morphants lack lamellipodia (n=0.6±0.98; from 31 cells in 5 embryos), and are roundish, without any apparent polarity (Fig. 5B).

A role to induce lamellipodia formation in metastatic tumor cells has also been described for the small GTPase Rac1 (reviewed by Hall, 1998), a component of the HA signal transduction pathway downstream of the HA receptor CD44 (Oliferenko et al., 2000). In order to investigate whether Rac1 might also be involved in driving cell migrations of converging lateral cells during zebrafish gastrulation, we injected embryos with mRNA encoding a dominant-negative version of human RAC1. Indeed, in injected embryos labeled lateral cells are roundish, lack lamellipodia (n=0.3±0.63; from 29 cells in 3 embryos) (Fig. 5C), and fail to converge dorsally as in *has2* morphant embryos (data not shown).

Has2 and Rac1 act in a cell autonomous fashion

In metastatic cancer cells, migration-stimulating signaling by HA and secreted proteoglycans is supposed to occur in an autocrine, cell autonomous fashion (Kosaki et al., 1999; Rauvala et al., 2000). To study whether the effect of Has2 on lamellipodia formation in lateral cells during zebrafish gastrulation is cell autonomous or non-cell autonomous, *has2* morphant cells labeled with membrane-localized GFP were transplanted into the lateral margin of wild-type recipients. Despite the wild-type environment, such *has2* morphant cells failed to form lamellipodia (n=0.5±0.62; from 32 cells in 3 embryos) (Fig. 5F). By contrast, labeled wild-type cells transplanted into the lateral margin of *has2* morphant recipients formed lamellipodia in normal numbers (n=4.3±0.82; from 24 cells in 3 embryos) (Fig. 5E). The same cell-autonomous effect was also observed for Rac1. *dnRac1*-expressing cells transplanted into a wild-type environment lack lamellipodia (n=0.3±0.57; from 32 cells in 3 embryos) (Fig. 5H), whereas wild-type cells transplanted into a host expressing *dnRac1* display normal lamellipodia formation (n=4.0±1.0; from 18 cells in 2 embryos) (Fig. 5G).

Together, the data show that both Has2 and Rac1 affect lamellipodia formation in lateral cells in a cell autonomous fashion. This suggests that HA, the likely product of Has2 activity, does not have to be secreted far to govern cell migration. Rather, it appears to be involved in some kind of autocrine self-stimulation of migrating cells (see also Discussion).

Ectopic dorsal Has2 and constitutively active Rac1 induce supernumerary lamellipodia, blocking extension movements within the axis

In contrast to dorsal convergence, extension of the embryonic axis is largely driven by other cell movements, such as mediolateral cell-cell intercalation, rather than by directed cell migration (Myers et al., 2002b; Wallingford et al., 2002). As the axial mesoderm is devoid of *has2* transcripts (see Fig. 1D), we injected *has2* mRNA into zebrafish eggs to investigate the effect of ectopic *has2* expression on the dorsal side of gastrulating embryos. Embryos injected with threefold higher amounts of *has2* mRNA (75 ng/μl) than used for the rescue experiments (see above; Fig. 3) form a shortened body axis and display partial cyclopia (data not shown), similar to the phenotype of *wnt11/silberblick* mutants, which are characterized by impaired extension of axial tissue (Heisenberg et al., 2000).

Labeling cells in the shield of *has2* mRNA-injected embryos and following their behavior during gastrulation revealed a strongly reduced extension of the axial mesoderm (Fig. 6E,F), compared with wild-type siblings (Fig. 6A,B; see Fig. 6Q for graph; dorsal extension: wild type 780±36 μm, *has2* RNA 321±74 μm; P=10^{-8}). Whereas wild-type cells acquire an elongated bipolar shape, forming single lamellipodia along the mediolateral axis (Fig. 6C,D), axial cells of *has2* mRNA-injected embryos display no apparent mediolateral polarization (Fig. 6G). They form many lamellipodia that can point in any direction (wild type: n=0.9±0.9, from 14 cells in 2 embryos; *has2* RNA: n=4.0±1.13, from 27 cells in 6 embryos; P=0.0005; Fig. 6H). Within one cell, the lamellipodia occupy a large portion of the cell surface, similar to the arrangement observed in migrating lateral cells (compare Fig. 5A with Fig. 6H). Thus, *has2*-expressing axial cells display the characteristics of cells undergoing active migration, rather than mediolateral cell-cell intercalation.

In agreement with our observation that loss of Has2 and loss of Rac1 function cause similar migratory defects in lateral cells, we also observed very similar effects of *has2* mRNA and

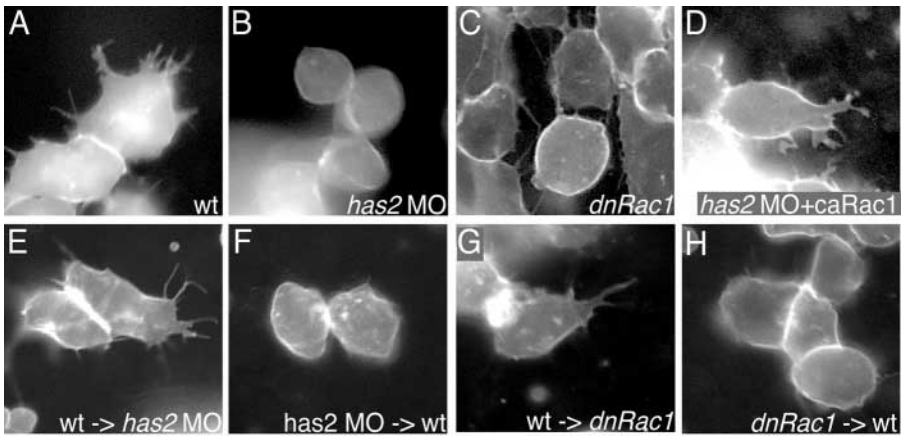
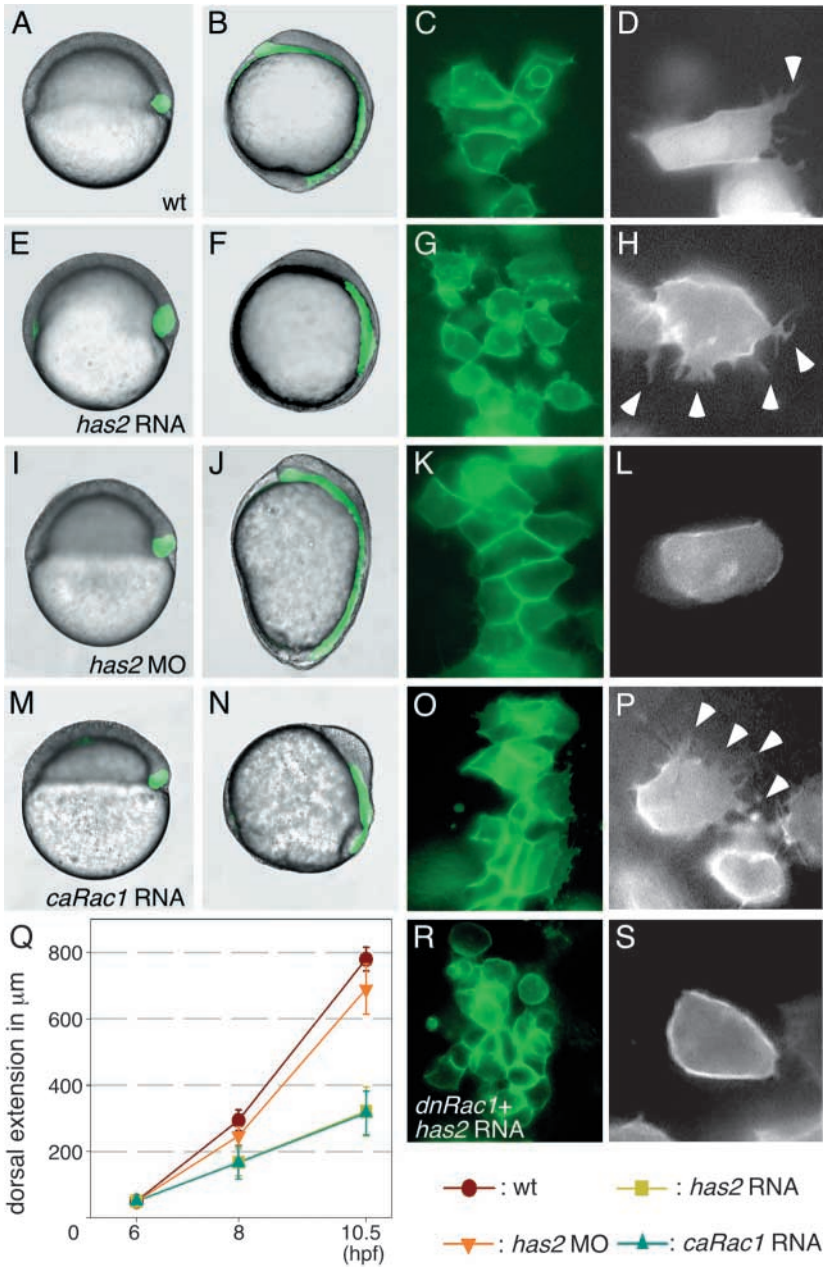


Fig. 5. Has2 and Rac1 act in a cell autonomous fashion, promoting lamellipodia formation. All panels show individual lateral mesodermal cells at the 80% epiboly stage, visualized with membrane-localized GFP. Dorsal is to the right, anterior to the top. (A) Wild type; (B) *has2* morphant; (C) wild type injected with mRNA encoding dominant-negative Rac1 (dnRac1); (D) *has2* morphant injected with mRNA encoding constitutively active Rac1 (caRac1); (E) wild-type cells transplanted into *has2* morphant host; (F) *has2* morphant cells transplanted into wild-type host; (G) wild-type cells transplanted into host expressing dnRac1; (H) dnRac1-expressing cells transplanted into wild-type host.

mRNA encoding a constitutively active version of human RAC1 (*caRac1*) in axial cells. Like *has2* mRNA-injected embryos, embryos injected with *caRac1* mRNA (10 ng/μl) display the formation of supernumerary lamellipodia in axial cells (n=5.1±0.86, from 12 cells in 2 embryos; Fig. 6P), and show a severe reduction in axis extension (Fig. 6M,N; see Fig. 6Q for graph; dorsal extension: 316±67 μm, $P=10^{-4}$). In summary, the data suggest that Has2 and Rac1 promote dorsal convergence in lateral regions of the embryo, while they can block extension of the axis.

Has2 function can be substituted by constitutively active, and blocked by dominant-negative Rac1

To test whether Rac1 acts downstream of Has2 in a linear HA signal transduction pathway, we carried out epistasis analyses, co-injecting either *has2* MOs with mRNA encoding constitutively active Rac1 (*caRac1*), or *has2* mRNA with mRNA encoding dominant-negative Rac1 (*dnRac1*). Indeed, we found that small amounts (2 ng/μl) of co-injected *caRac1* mRNA or larger amounts (25 ng/μl) of wild-type *Rac1* mRNA lead to a perfect rescue of *has2* morphant embryos, which show normal lamellipodia formation in lateral cells (*has2* MO + *caRac1* RNA n=4.7±1.4, from 15 cells in 4 embryos; P=0.2; Fig. 5D), and normal convergence and extension movements (Fig. 4G-J). Such rescued embryos lack HA like regular *has2* morphants do (Fig. 2C), suggesting that HA is dispensable for dorsal convergence when Rac1 is active. Furthermore, dnRac1 blocked the formation of supernumerary lamellipodia caused by ectopic *has2* mRNA (*has2* RNA + *dnRac1* RNA: n=0.04±0.21, from 22 cells in 2 embryos) (Fig. 6R,S). Together, these data strongly suggest that Has2 promotes lamellipodia formation and cell migration by activating the small GTPase Rac1. Biochemical studies and analyses of potential HA receptors will be necessary to investigate whether this effect of HA is direct, or mediated through other components of the ECM (see Discussion).



Has2 is required for proper migration of presumptive slow muscle cells, sclerotomal cells and primordial germ cells

To study the general relevance of cell migration regulation by HA, we investigated whether Has2 is also required during other developmental processes involving cell migration. After gastrulation, *has2* expression is maintained and/or upregulated in adaxial and sclerotomal cells of the somites (see Fig. 1). The adaxial cells display a very striking migratory behavior. After somite regionalization into a dorsal and a ventral half, they leave their adaxial locations and migrate to the lateral walls of the somites, where they become slow muscle cells

Fig. 6. Forced dorsal expression of *has2* and constitutively active Rac1 lead to supernumerary lamellipodia, blocking axis extension. Extension movement of labeled populations of dorsal cells. (A-D) Wild-type control embryos; (E-H) embryos injected with *has2* mRNA; (I-L) *has2* morphant embryos; (M-P) embryos injected with *caRac1* RNA; (R,S) embryos co-injected with *has2* mRNA and *dnRac1* mRNA. The first two columns show uncaging experiments. Lateral view of embryos at shield stage (6 hpf), directly after uncaging (A,E,I,M), or at tailbud stage (10.5 hpf; B,F,J,N). Columns 3 and 4 show morphology of clusters (C,G,K,O,R) or individual (D,H,L,P,S) dorsal cells labeled with membrane-localized GFP. Arrowhead (D) indicates single lamellipodium sometimes visible on narrow dorsal side of highly polarized axial cells of wild-type embryos. Arrowheads (H,P) indicate multiple lamellipodia in axial cells of *has2* RNA- or caRac1 mRNA-injected embryos. (Q) Graph showing the amount of extension in the axis of wild-type, *has2* MO-, *has2* mRNA- or *caRac1* mRNA-injected embryos. Per treatment, ten different embryos were evaluated, and standard deviations are indicated.

(Devoto et al., 1996; Stickney et al., 2000). During this migration, different routes can be taken, either along the horizontal myoseptum, or through the presumptive fast muscle tissue, the major component of the somites. A similar directional migration has been reported for sclerotomal cells. Initially located in ventromedial positions of the somites, they migrate dorsally into adaxial regions (Morin-Kensicki and Eisen, 1997; Stickney et al., 2000), eventually surrounding the entire neural tube to form the vertebral column (Morin-Kensicki et al., 2002).

To study whether Has2 is involved in these migratory processes, weakly affected *has2* morphant embryos that had survived early segmentation stages were stained for *smbpc* mRNA, a marker for specified postmigratory slow muscle cells (Xu et al., 2000), or for *twist* mRNA, a marker for premigratory and migrating sclerotomal cells (Morin-Kensicki and Eisen, 1997; Stickney et al., 2000). Whereas in control embryos (Fig. 7A,C), *smbpc*-positive cells are located in the lateral walls of the somites, *smbpc*-positive cells of *has2* morphants are largely confined to adaxial regions and the horizontal myoseptum, with a few isolated cells stuck within the presumptive fast muscle tissue (Fig. 7B,D). Similarly, sclerotomal cells of *has2* morphant embryos fail to migrate dorsally as in control embryos (Fig. 7E), but remain in ventrolateral positions of the somites (Fig. 7F). Together, these data suggest that expression of *has2* in adaxial and sclerotomal cells is essential for their migration during somite reorganization. However, *has2* does not appear to be required for the specification of cells. Thus, despite their failed migration, adaxial cells acquire slow muscle characteristics in ectopic locations.

Another cell type that depends heavily on cell migration to reach the proper location within the embryo are primordial germ cells (PGCs) (reviewed by Raz and Hopkins, 2002). PGCs stem from rather random positions within the embryo, from where they actively migrate to the developing genital ridges, taking common routes along the blastoderm margin and through the lateral mesoderm, the sites of *has2* expression during late gastrulation and early segmentation stages. In situ hybridization with *vasa*, an early marker for PGCs (Yoon et al., 1997), shows that in *has2* morphant embryos, the PGCs are present in normal numbers. However, in contrast to the situation in wild-type embryos (Fig. 7G), most of them fail to reach their proper location in the genital ridges, but remain randomly distributed over the yolk (Fig. 7H).

Together, these data show that in addition to dorsal convergence during gastrulation, *has2* is required for proper migration of several mesodermal cell types during later steps of zebrafish development.

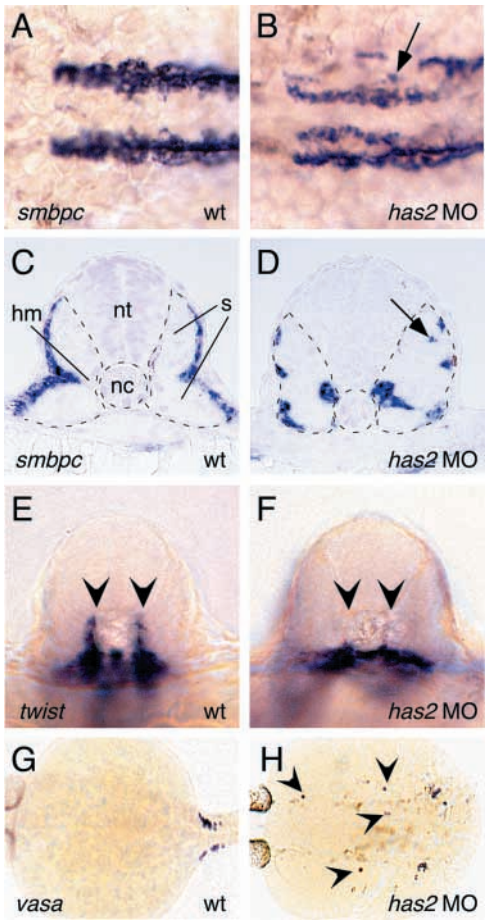


Fig. 7. Has2 is required for migration of presumptive slow muscle and sclerotomal cells during morphogenesis of somites, and for the migration of primordial germ cells. (A-D) 30 hpf; *smbpc* in situ hybridization to mark slow muscle cells of trunk somites: (A,C) wild type; (B,D) *has2* morphant. (A,B) Dorsal view, anterior left; (C,D) transverse sections. Arrows (B,D) mark *smbpc*-positive cells located within the presumptive fast muscle tissue. The lower side of the embryo in B is less affected, which might have been the reason for its survival. The effects are restricted to the first 5-8 somites, consistent with previous findings indicating different properties between such anterior and more posterior somites (see van Eeden et al., 1996). (E,F) *twist* in situ hybridization, at 24 hpf, to mark sclerotomal cells of trunk somites. Arrowheads indicate dorsally migrated sclerotomal cells in wild type (E), which remain ventral in *has2* morphant embryos (F). (G,H) Distribution of *vasa*-positive primordial germ cells, at 24 hpf, in wild type (G) and *has2* morphant (H). Arrowheads (H) point to ectopic PGCs outside the genital ridges. hm, horizontal myoseptum; nc, notochord; nt, neural tube; s, somite.

Discussion

Compared with the amount of data concerning the roles of HA, its synthesizing enzymes, Has, and its putative receptors and signal transduction pathways in metastatic cancer cells, little has been reported about their roles during normal vertebrate development. Here, we show that zebrafish *has2* is required for the migration of multiple cell types during multiple developmental processes, including the dorsal convergence of ventrolateral mesodermal cells during gastrulation, the migration of adaxial and sclerotomal cells during somite reorganization, and the migration of primordial germ cells to the genital ridges. We further show data indicating that during gastrulation, the synthesis product of Has2 – most likely HA – acts in a cell autonomous or autocrine fashion to activate the small GTPase Rac1, thereby promoting the formation of lamellipodia in *has2*-positive cells themselves. This appears to result in some kind of auto-stimulation of migrating cells, whereas *has2*-negative cells of the environment remain stationary.

Has2 helps to dissect the cellular events driving convergence and extension independent of cell fate determination

According to current understanding, convergent extension (CE) movements of gastrulating zebrafish embryos involve different cellular events, depending on the position of cells along the dorsoventral axis (Myers et al., 2002b; Wallingford et al., 2002). Convergence of lateral cells towards the dorsal shield is regarded as an active migration of individual or small groups of cells along a chemoattractant gradient. This view is largely based on microscopic observations, revealing that ventral and lateral mesodermal cells at the onset of gastrulation meander without much directionality, whereas their movement becomes faster and more directed when they get closer to the shield during gastrulation (Myers et al., 2002a; Jessen et al., 2002; Trinkaus, 1998). Dorsally, cells not only pile up on each other, leading to a thickening of the axis, but also undergo mediolateral intercalations, leading to axis extension. Dorsal migration of lateral cells and mediolateral intercalation of dorsal cells might involve both shared and distinct cellular events. Thus, dorsal convergence appears to require migration of cells on the extracellular matrix, while mediolateral intercalation might involve a tight control of cell-cell adhesion, and a migration of cells on the surface of neighboring cells as substrate. Another specific driving force of extension appears to be the active anteriorwards migration of cells at the anterior edge of the mesendoderm, also called the polster or prechordal plate (Winklbauer and Nagel, 1991; Winklbauer and Selchow, 1992; Yamashita et al., 2002).

Genetic evidence for such different cellular mechanisms underlying dorsal convergence and axis extension had been rather limited thus far. Impaired convergence with normal or even enhanced extension had only been described for mutants lacking Bmp signaling (Myers et al., 2002a) or the T-box transcription factor Spadetail (Griffin et al., 1998). However, such mutants, in addition to CE morphogenesis, also have dramatic defects in differential dorsoventral cell fate specification (Hammerschmidt and Mullins, 2002; Solnica-Krezel and Cooper, 2002). This is not the case for *has2*, which is required for CE morphogenesis only, but not for dorsoventral patterning.

has2 morphants also differ from all previously described mutants and morphants with defective CE, but normal dorsoventral patterning. Such embryos show either defects in extension only (Glickman et al., 2003), or a combined reduction of both convergence and extension, making it difficult to dissect the two processes. The combination of convergence and extension defects could have different reasons. In morphant embryos deficient in Stat3, a transcription factor mediating cytokine signaling, convergence defects seem to be a secondary consequence of the impaired anterior migration of the anterior dorsal mesendoderm (Yamashita et al., 2002), whereas in *silberblick*, *knypek* and *trilobite* mutants, and in *dnrok2*-injected embryos lacking the non-canonical Wnt signal Wnt11 or its interacting partners Glypican6, Strabismus, or RhoA-associated kinase, respectively, cellular processes shared by dorsal migration and extension seem to be affected, such as the general establishment of cell polarity (Heisenberg et al., 2000; Jessen et al., 2002; Topczewski et al., 2001; Marlow et al., 2002). In contrast to such genes, *has2* appears to be required for dorsal convergence only, while it has a negative effect on cellular processes specifically involved in axis extension. Thus, forced *has2* expression in axial cells appears to transform them from intercalating to migratory cells similar to converging lateral cells. They form multiple lamellipodia and move. However, in contrast to lateral cells, their movement appears random, rather than directed (J.B. and M.H., unpublished), suggesting that guiding cues are only present laterally, and are missing in the axis.

In summary, our data provide evidence that during gastrulation, directed dorsal migration of cells in lateral regions and axis extension on the dorsal side are rather independent of each other. *has2* seems to be selectively required for cellular events underlying dorsal convergence, such as for the aforementioned migration of cells on the ECM, whereas axis extension appears to be achieved by different means, independently of *has2* (see above).

Despite the normal or even elevated extension during gastrula stages, *has2* morphants later become significantly shorter than their siblings. This, however, does not necessarily mean that later extension processes require Has2. Clearly, the dorsal mesoderm itself extends normally, as indicated by the undulation of the notochord. Thus, the reduced length of the embryos during segmentation stages is more likely to be a secondary consequence of the block of convergence, indicating that later anteroposterior growth of the dorsal axis heavily depends on the immigration of cells from lateral regions. In this respect, convergence and extension are linked in the sense that convergence supplies the dorsal side with cells required to build up the embryo.

Mechanism of HA function during zebrafish gastrulation

Different modes of HA function have been suggested. In all cases, HA is supposed to be extruded into the extracellular space directly after its Has-catalyzed synthesis at the cytoplasmic side of the plasma membrane. Thus, even intracellular HA binding proteins (IHABPs), such as cytoplasmic versions of the receptor RHAMM, are supposed to bind HA after it has re-entered the cells by endocytosis (reviewed by Lee and Spicer, 2000). After its secretion into the extracellular space, HA could act to govern cell migrations in several ways. First, HA might function as a space filling substrate, on which cells are able to move. In addition, HA might influence the arrangement of other components of the ECM, such as fibronectins, ligands of integrins that have been reported to regulate Cadherin-dependent cell adhesion and convergent extension movements during *Xenopus* gastrulation (Mardsen and DeSimone, 2003). In this case, our observed effect of *has2* during zebrafish development would be very indirect. Alternatively, HA could act by direct signaling to migrating cells, mediated by specific HA receptors such as CD44 and RHAMM. Such signaling can activate different signal transduction pathways, involving transcription factors such as Myc, Erk1 and NFκb, and various Rho GTPases, which have distinct effects on the cytoskeleton (reviewed by Lee and Spicer, 2000; Ridley, 2001; Turley et al., 2002). Although RhoA is supposed to promote the formation of cytoplasmic fibers, influencing overall cell polarity and inducing cell body contractions, Rac1 has been reported to have more local effects, promoting the formation and outgrowth of lamellipodia (reviewed by Hall et al., 1998; Ridley, 2001).

The data obtained in our *Has2* loss- and gain-of-function studies strongly suggest that the migration of converging lateral cells during zebrafish gastrulation is governed by a Has2-HA-Rac1 pathway. As all of our experiments were carried out with Has2, rather than with HA, we cannot rule out the possibility that the observed effects are due to an HA-independent, parallel function of Has2. However, no additional roles of Has proteins have been reported, except their ability to catalyze the synthesis of chitins, HA-related oligosaccharides consisting of *N*-acetyl-glucosamine units only. To specify which activity of Has2 is required during zebrafish gastrulation, we carried out experiments with a mutant version of Has2, carrying a single amino acid residue exchange in the glucuronic acid binding pocket. This mutation has been shown to specifically block the HA-synthesizing, but not the chitin-synthesizing activity of Has proteins (Yoshida et al., 2000). The corresponding mutant version of zebrafish Has2 failed to rescue the *has2*-morphant phenotype (J.B. and M.H., unpublished), indicating that it is indeed the HA-synthesizing activity of Has2 that is required to drive dorsal convergence in zebrafish embryos. Consistent with this notion, we found that the loss of *has2* function leads to highly reduced HA levels in morphant embryos, according to the reported 96% reduction in HA content in *Has2* mutant mice (Camenisch et al., 2000). By contrast, content and distribution of other ECM components, such as Fibronectin and Cadherins, were unaltered. In addition, we have evidence that Has2 acts by activating the Rho GTPase Rac1, which is in agreement with data obtained for HA in cell culture systems, showing that local application of HA induces strong activation of Rac1, local lamellipodia formation and cell migrations (Oliferenko et al., 2000; Bourguignon et al., 2000). In zebrafish embryos, loss and gain of function of Rac1 has the same effects on lamellopodia formation as loss and gain of function of Has2. Co-injection experiments combining Has2 loss of function with Rac1 gain of function, and vice versa, further show that Rac1 is epistatic to Has2, suggesting that Rac1 acts downstream of, rather than in parallel to, Has2. Together with the aforementioned data obtained in tissue culture, our results strongly suggest that dorsal migration of converging lateral cells in zebrafish embryos is regulated by HA through activation of Rac1.

We do not know as yet how the activation of Rac1 by HA is achieved. Clearly, *Rac1* can be activated by many different transmembrane receptors with different ligand specificities (reviewed by Ridley, 2001). However, as we failed to detect any changes in the composition of the ECM of *has2* morphants, except the loss of HA, we favor a model according to which the effect of HA on Rac1 activation and cell migration is quite direct, mediated by binding to HA transmembrane receptors such as CD44.

HA appears to act as an autocrine signal

In all studied systems, no activation of Rac1 independent of extracellular signaling has been reported (reviewed by Ridley, 2001). This is important for the interpretation of our finding that Has2 – like Rac1 – acts in a cell autonomous fashion. Given the epistatic relationship of Has2 and Rac1 (see above), we conclude that HA acts as an autocrine or extracellular signal with an extremely short-range effect, rather than as a cytoplasmic trigger. Such an autocrine effect would allow a differential cellular regulation of migratory behavior within tissues, because *has2*-positive cells would only activate themselves, not neighboring cells. This appears particularly important when certain cell types invade or pass tissues of stationary cells. One example are the *has2*-positive presumptive slow muscle cells, which on their way to the lateral walls of the somites migrate through the *has2*-negative tissue of presumptive fast muscle (Fig. 7).

The cell autonomous fashion of Has2 action also indicates that the broadly discussed role of HA to fill the extracellular space is not relevant for cell migrations during zebrafish gastrulation. This notion is further supported by our finding that Rac1 can rescue the migratory defects of *has2* morphant embryos in the absence of detectable levels of HA. In summary, the data suggest that HA has not a structural but an instructive function. Rather than preparing the extracellular space as a substrate for migrating cells, it appears to induce cells to migrate.

Parallels between HA function in embryogenesis and tumor invasion

Most of the previous studies concerning the roles of HA and HA signaling pathways had been carried out with tumor cells lines. There are many lines of in vitro and in vivo evidence that HA signaling plays a crucial role in governing the metastatic potential of malignant tumor cells, transmitted via the HA receptors CD44 and RHAMM, and with Rac1 as one of the intracellular effectors (Lee and Spicer, 2000; Toole, 2001; Turley et al., 2002).

Our findings that both Has2 and Rac1 are required in a linear pathway to govern lamellipodia formation and active cell migration during zebrafish gastrulation suggests that the same HA signaling pathway might be used to drive cell migrations during both normal development and tumor invasion. In support of this notion, HA-regulated embryonic and tumor cells are similar in several other respects. As reported here for migrating lateral cells in the zebrafish gastrula, Has2 protein appears to promote metastasis of tumor cells in a cell autonomous fashion. Thus, in vivo tumorigenicity of cells is enhanced after cell transfection with Has2 (Kosaki et al., 1999), indicating that Has2 and HA are required as an autostimulus in migrating cells themselves. Similar autocrine effects promoting tumor invasion have also been described for other secreted proteoglycans (see Rauvala et al., 2000). In addition, Has2-dependent migrating cells in zebrafish embryos share morphological similarities with metastatic tumor cells. In contrast to Has2-independent migrating embryonic cell types, such as cells at the leading edge of the hypoblast during involution, all Has2-dependent cells migrate as small groups of cells or individual cells. In particular the Has2-dependent migration of adaxial cells through the somites (Devoto et al., 1996; Stickney et al., 2000) is very reminiscent of tissue invasion by metastatic cancer cells. If the mechanisms of the HA-dependent migration of Has2-expressing cells during zebrafish embryogenesis can indeed be compared with tumor invasion, a systematic search for additional genes involved in these processes in zebrafish might be helpful to shed further light onto the mechanisms of metastasis.

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