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Snail1a and Snail1b cooperate in the anterior migration of the axial mesendoderm in the zebrafish embryo

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The Snail genes are implicated in processes that involve cell movement, both during embryonic development and tumour progression. In teleosts, the vertebrate *Snail1* gene is represented by two distinct genes, *snail1a* and *snail1b* (previously *snail1* and *snail2*). These genes are expressed in complementary mesodermal domains and their combined expression matches that of their mammalian counterpart. By analysing their loss and gain of function, we found that the most-anterior axial mesendodermal cells, the precursors of the polster, move in a cohesive manner directed by the activity of *snail1a*- and *snail1b*-expressing cells surrounding these precursors. The cell-autonomous function of Snail1 proteins regulates cell motility and influences the behaviour of Snail-negative neighbouring cells. Snail1a is required by the prechordal plate for it to reach its normal position, whereas Snail1b controls the acquisition of its normal shape. These non-redundant functions of Snail1a and Snail1b in controlling axial mesendoderm migration comply with the duplication-degeneration-complementation model, and indicate that Snail genes not only act as inducers of epithelial-to-mesenchymal transition, but also as more general regulators of cell adhesion and movement.

KEY WORDS: Extension, Prechordal plate, Axial mesendoderm, E-Cadherin, Epithelial-mesenchymal transition, Cell adhesion, Cell migration, DDC model

INTRODUCTION

The Snail family of zinc-finger transcription factors fulfils important roles in vertebrate development. Indeed, these genes are involved in a variety of processes that require cell movement and shape changes within the organism. Most significantly, they are required to induce the epithelial to mesenchymal transition (EMT) through which epithelial cells loose their cell-cell adhesion contacts, becoming migratory and invasive. EMT is fundamental for the formation of many tissues during vertebrate embryonic development, implicating Snail family members in these processes (Nieto, 2002). Moreover, as EMT also occurs during pathological processes, Snail genes have been implicated in tumour progression and renal fibrosis (Nieto, 2002; Thiery, 2002; Boutet et al., 2006).

In amniotes, EMT is fundamental for the formation of tissues as important as the mesoderm and the neural crest. However, no large-scale EMT has been observed during gastrulation in anamniote embryos where a complex interplay of different morphogenetic movements gives rise to a mass sheet-like migration and the cells maintain their contacts while moving. These morphogenetic movements can be described as: (1) epiboly towards the vegetal pole over the yolk cell; (2) internalisation of the mesendoderm at the margin; and (3) dorsal convergence and extension along the anteroposterior axis (C&E) (Keller et al., 2000; Solnica-Krezel, 2006). In contrast to the *Xenopus* gastrula, C&E movements in zebrafish seem to be independent of both epiboly and mesendoderm internalisation (Myers et al., 2002), and convergence can even be separated from extension (Myers et al., 2002; Glickman et al., 2003). Indeed, the interdependence of these two processes in *Xenopus* may in part be due to the strength of the adhesive forces that maintain cells together during involution and convergent-extension. However, during internalisation in fish, cells can be found in groups or even as individual cells (Carmany-Rampey and Schier, 2001; Montero et al., 2005).

Although Snail genes have been implicated in the formation of the neural crest and the mesoderm in different vertebrate species (reviewed by Nieto, 2002), functional analyses in zebrafish have only been carried out on *snail1a*. Four Snail genes have been described in teleosts: *snail1*, *snail2*, *snail3* and *slug* (Thisse et al., 1993; Hammerschmidt and Nüsslein-Volhard, 1993; Thisse et al., 1995; Locascio et al., 2002; Manzanares et al., 2004). Phylogenetic analyses have shown that *snail1* and *snail2* are duplicates of the *Snail1* gene that arose after gene duplication in the teleost lineage (Postlethwait et al., 1998; Manzanares et al., 2001). *snail1* and *snail2* have been renamed *snail1a* and *1b*, respectively. Similarly, *slug* has been renamed *snail2*, and the most recently described family member is *snail3* (Barrallo-Gimeno and Nieto, 2005).

The patterns of *snail1a* and *1b* expression in the neural crest suggest that as in other species, Snail genes are fundamental for the development of this tissue (Thisse et al., 1993; Hammerschmidt and Nüsslein-Volhard, 1993; Thisse et al., 1995). However, in the absence of massive EMT at gastrulation these genes were considered to be less important in convergence and extension (Locascio and Nieto, 2001). Nevertheless, *snail1a* has since been implicated in the anterior movement of the axial mesendoderm in the fish embryo (Yamashita et al., 2004) where elegant time-lapse studies demonstrated that individual cells delaminate from the embryonic shield (Montero et al., 2005). Here we show that *snail1b* is important for the movements that take place during extension, with *snail1a* and *1b* fulfilling non-redundant functions in the movements that direct the migration of the axial mesendoderm.

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MATERIALS AND METHODS

Fish maintenance

Zebrafish were raised and kept at 28°C under standard conditions, and they were staged according to Kimmel et al. (Kimmel et al., 1995). AB and Tup-Lof wild-type strains were used in all experiments.

Morpholino oligonucleotide and mRNA injections

All morpholino antisense oligonucleotides were obtained from Gene Tools LLC and used as described by Nasevicius and Ekker (Nasevicius and Ekker, 2000). The antisense morpholinos (MOs) blocking *snail1b* translation hybridise to bases –28 to –4 (MO1) and –2 to +23 (MO2) with respect to the start codon, and the *snail1a* MOs are as previously described (Yamashita et al., 2004). Likewise, the *cdh1* MOs were as described by Babb and Marrs (Babb and Marrs, 2004), and the standard control morpholino was used for control injections (Gene Tools, LLC). All oligonucleotides were injected into the yolk of one- or two-cell embryos (3-20 ng/embryo) using a pressure injector (PicoSpritzer).

Rescue and overexpression analyses were performed with *snail1b* mRNA that lacked the 5′UTR region to which MO1 binds, and that were synthesised and capped using the mMessage mMachine Kit (Ambion). Rescue was achieved by co-injecting 3 ng of morpholino plus 250 pg mRNA per embryo, and 150 pg of mRNA per embryo was injected for overexpression studies. Phenotypic analyses involved at least 50 embryos for each specific marker studied under each experimental condition.

In situ hybridisation

In situ hybridisation of whole-mount zebrafish embryos was performed as described by Nieto et al. (Nieto et al., 1996), with minor modifications, and fluorescent whole-mount in situ hybridisation was carried out as described by Denkers et al. (Denkers et al., 2004). In these experiments, the following probes were used: *snail1a* (previously *snail1*) and *snail1b* [previously *snail2* (Thisse et al., 1993; Thisse et al., 1995)]; *cdh1* [*cadherin 1*; also known as *E-cadherin* (Babb and Marrs, 2004)]; *hgg1* (Vogel and Gerster, 1997) [also known as *ctsl1b* (*cathepsin L*) – ZFIN]; *spt* (Griffin et al., 1998) [also known as *tbx16* (*T-box gene 16*) – ZFIN]; *myoD* (Weinberg et al., 1996); *papc* (Yamamoto et al., 1998) [also known as *pcdh8* (*protocadherin 8*) – ZFIN]; *dlx3* (Akimenko et al., 1994); and *ntl* [*no tail* (Schulte-Merker et al., 1992)]. The zebrafish anti-Cdh1 antibody (Babb and Marrs, 2004) was used at a dilution of 1/500. Embryos were embedded in gelatin to obtain vibratome sections (50 μm).

Cell transplantations

Donor embryos were injected at the one- to two-cell stage with fluorescein-dextran (10,000 kDa; Molecular Probes), alone or together with the appropriate dose of the *snail1b* morpholino. At the shield stage, 10-20 cells were transplanted into the shield of an unlabelled sibling host. Alternatively, donor embryos were also injected with mRNA encoding a membrane-tagged RFP. After an incubation of 1 hour and at approximately 70% epiboly, grafted embryos were mounted dorsal side up in 0.8% low-melting point agarose in Danieau's solution, and images were obtained on a Leica SP2

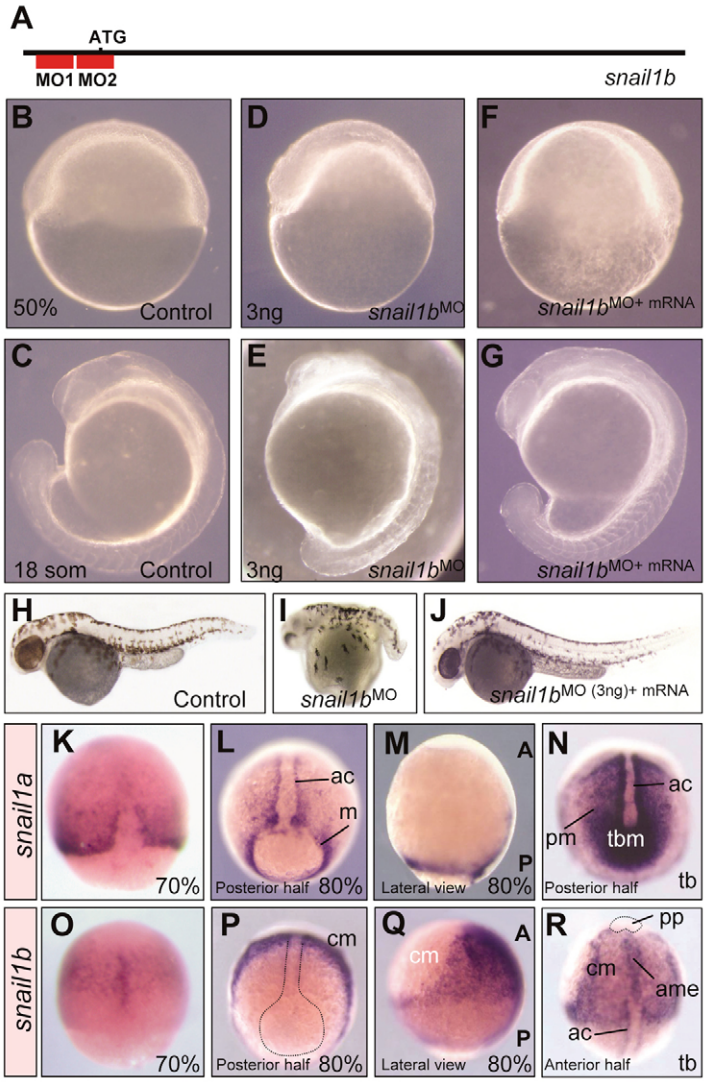

Fig. 1. *snail1b* knockdown induced morphological defects at gastrulation and segmentation stages. (**A**) Two different morpholino antisense oligonucleotides were designed against adjacent sequences in the 5′ region of the *snail1b* cDNA. (**B**,**D**) Embryos at mid-gastrulation and (**C**,**E**) at the 18-somite stage. Morpholino injection (3 ng/embryo) produced defects in extension movements at early embryonic stages that led to the shortening of the body axis at 30 hpf (compare **H** with **I**). (**F**,**G**,**J**) This phenotype was rescued by co-injection of *snail1b* mRNA. (**K**,**L**,**N**,**O**,**P**,**R**) *snail1a* but not *snail1b* was prominently expressed in the involuting and posterior mesoderm. (**M**,**Q**) Lateral views highlight the expression of *snail1a* in the posterior half of the embryo and *snail1b* in the anterior. The dotted lines indicate the position of the margin and the adaxial cells (P) and that of the prechordal plate (R). ac, adaxial cells; ame, anterior axial mesendoderm; cm, condensing mesenchyme; pm, paraxial mesoderm; tbm, tail bud mesenchyme.

confocal microscope using a 40× water immersion objective. Embryos were allowed to develop to the tail-bud stage, when they were photographed on a Leica FLIII dissecting microscope. The distances that three individual cells moved in each experiment (wt→wt, *n*=7, mo→wt, *n*=5) were assessed using the Manual Tracking plug-in for ImageJ 1.37v software.

Live imaging

For time-lapse, multiple focal plane (4D) microscopy of zebrafish embryos, the embryos were co-injected with *snail1b* morpholino and 100 ng/μl of membrane-tagged-GFP mRNA [with myristoylation and palmitoylation sequences from the Lyn tyrosine kinase (Teruel et al., 1999), kindly provided by Reinhard Köster]. The embryos were then embedded at the appropriate stage in low gelling 1% agarose in Danieau buffer. Imaging was performed on a Leica SP2 confocal system mounted on an inverted microscope using a 10× objective, and *z*-stacks were obtained every 5 minutes and processed for maximum intensity projection using ImageJ software.

RESULTS

To analyse the function of *snail1* genes during fish development, we have adopted an approach that involves the use of morpholino antisense oligonucleotides. We initially decided to study *snail1b* because of its prominent expression in the premigratory neural crest (Thisse et al., 1995). To disrupt *snail1b* activity, an oligonucleotide against the 5′UTR region (MO1) and another that overlapped the start codon of the *snail1b* cDNA (MO2; Fig. 1A) were designed. The vast majority of embryos that were injected with 1-1.5 ng of the morpholino survived, although at higher doses (3 ng) only 80% of the embryos reached the tail bud stage and only 60% survived for 24 hours after the injection. Unexpectedly, we found that injecting *snail1b* MOs induced an early phenotype at gastrulation stages and the shortening of the body axis at later stages (Fig. 1B-E,H,I). The phenotype we observed was somewhat surprising given the pattern of *snail1b* expression in the mesoderm, where *snail1a* rather than s*nail1b* is more prominently expressed (Fig. 1K-R). Indeed, the main sites of *snail1a* expression in the mesoderm at gastrulation stages are the margin, the adaxial cells (Fig. 1K,L) and the paraxial mesoderm (Fig. 1N) (see Thisse et al., 1993). By contrast, the main mesodermal sites for *snail1b* are the converging cephalic mesoderm (Fig. 1Q), the adaxial cells and a population of the axial mesendodermal cells (Fig. 1O,R) (Thisse et al., 1995).

In all cases, the phenotypes were highly penetrant with more than 90% of the living embryos developing a similar phenotype at each concentration of morpholino. No defects were observed in uninjected embryos, in embryos injected with buffer alone (Danieau), or in those embryos injected with a negative control MO.

The injection of either morpholino alone (MO1 or MO2) induced similar defects and with a similar dose response. Since MO1 was used in all our control experiments (see also Fig. S1 in the supplementary material), it was used in most of the experiments shown at a dose of 3 ng per embryo unless otherwise indicated. To confirm the specificity of the phenotype, we co-injected MO1 along with the coding region of *snail1b* mRNA. Significantly, both the phenotypic and lethality effects observed after MO1 injection could be rescued in up to 80% of embryos (*n*>100; Fig. 1F,G,J), indicating that the shortening of the body axis was induced specifically by the *snail1b* MOs. In order to assess whether we were dealing with morphogenetic defects or with a failure to specify the mesoderm, we assessed the expression of axial [*no tail* (Schulte-Merker et al., 1992)] and paraxial [*paraxial protocaherin* (Yamamoto et al., 1998)] mesoderm markers at gastrulation stages. Both these markers were detected in the embryos, indicating that fate specification was not significantly affected. In fact, somites still formed in the axial mesoderm, although they had an abnormal shape, and evidence of the compression was detected by *myoD* expression at segmentation stages (Fig. 2). Together, these data indicated that *snail1b* MOs induced a severe impairment in anteroposterior extension without affecting mesodermal fate.

Interfering with *snail1b* induces defects in extension movements by augmenting cell adhesion in the axial mesendoderm

Since the anterior migration of the axial mesendoderm is an important component of the extension movements and *snail1b* is expressed in this territory, we analysed the alterations in the *snail1b* MO-injected embryos using the prechordal plate markers, *hatching gland gene 1* [*hgg1* (Vogel and Gerster, 1997)] and *spadetail* [*spt* (Griffin et al., 1998)]. Injection of *snail1b* MO expanded the expression domains of both these markers, extending *spt* expression more than *hgg1* (Fig. 3A-C,F-H). By contrast, ectopic overexpression of *snail1b* mRNA induced the anterior compression of the *hgg1*-domain and altered the distribution of the *spt*-positive cells but not the number of expressing cells (Fig. 3K-M and data not shown). These two complementary phenotypes are compatible with *snail1b* being involved in the regulation of cell-cell adhesion during anterior migration. Snail is a strong repressor of *cdh1* (*E-cadherin*) (Cano et al., 2000; Batlle et al., 2000) and as expected, injection of the *snail1b* MO enlarged the domain of *cdh1* expression in the axial mesendoderm (Fig. 3J), as well as the *hgg1* expression domain. Furthermore, less *cdh1* was detected throughout the embryos than overexpressed *snail1b* (Fig. 3N,O). Snail1b did indeed appear to be

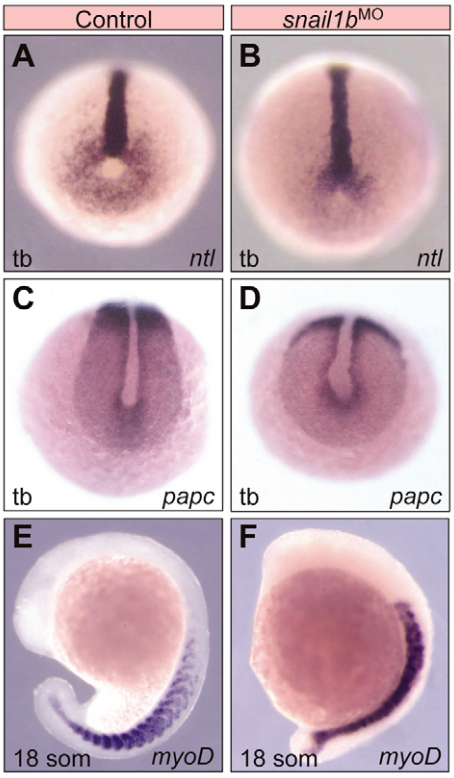

Fig. 2. *snail1b* knockdown does not affect mesodermal fate. (**A-D**) The axial and paraxial mesoderm form normally in *snail1b* morphants at tail bud stages (tb), as observed by the expression of *ntl* and *paraxial protocadherin* (*papc*), respectively. (**E**,**F**) *MyoD* expression at the 18-somite stage shows that knockdown of *snail1b* does not prevent the formation of somites.

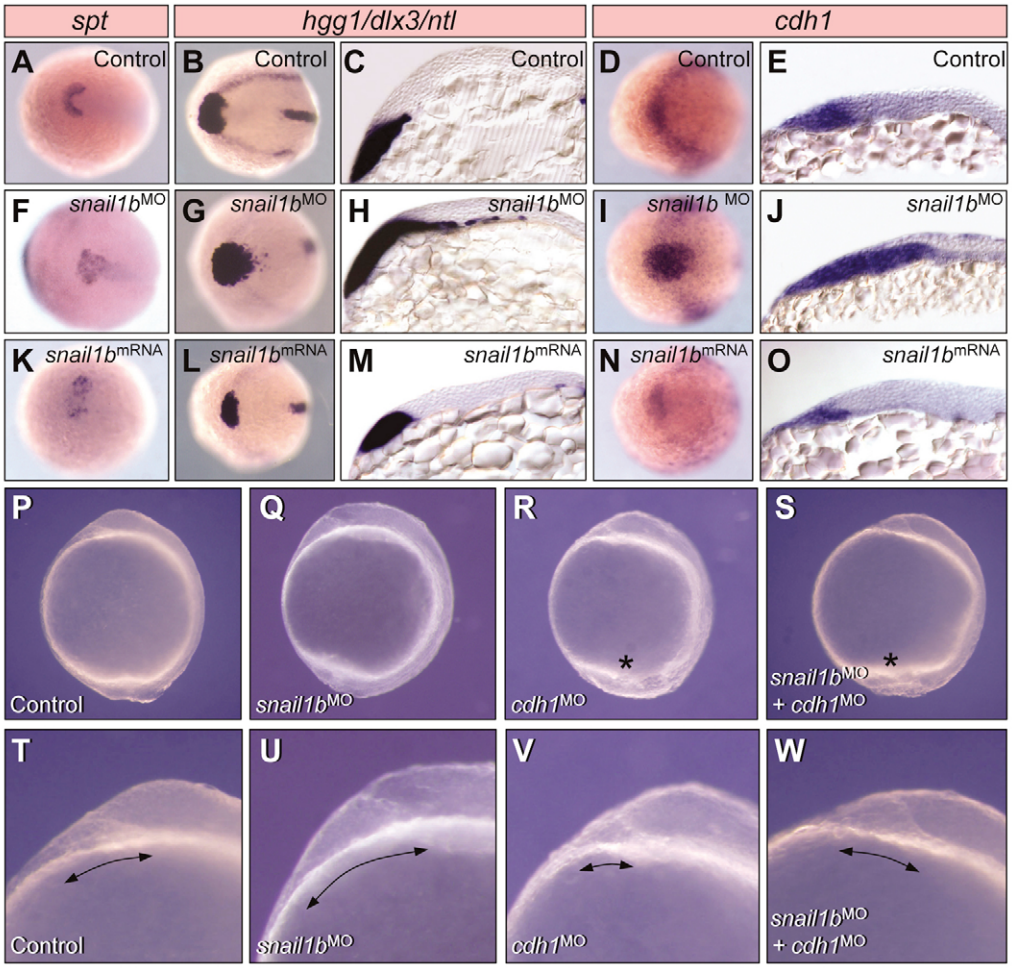

Fig. 3. Snail1b modulates extension movements during gastrulation. (**A**,**F**,**K**) Knockdown of *snail1b* caused the posterior expansion of the *spt* expression domain, whereas overexpression of *snail1b* mRNA produced anterior compression. Similar effects were observed on the expression of *hgg1* (**B**,**G**,**L**). (**D**,**I**,**N**) The expression of *cdh1* follows that of *hgg1* in the prechordal plate of control, as well as in *snail1b* morphants or overexpressing embryos. (**C**,**H**,**M**) Parasagittal sections obtained from *hgg1*-hybridised embryos reveal the position of the presumptive prechordal plate cells. Sections obtained from *cdh1*-hybridised embryos (**E**,**J**,**O**) confirm the similarity between *hgg1* and *cdh1* expression. (**P-S**) Increased cellular adhesion seems to account for the defects in extension movements in *snail1b* morphants, since the phenotype can be ameliorated by impairing cadherin activity with the injection of *cdh1* MOs. The morphology of the polster is altered in the *snail1b* morphants (Q), although this effect can be reverted by the injection of *cdh1* MOs (S). Asterisks indicate the defective posterior region where cells detach from the embryo both in the *cdh1* (R) and in the double morphants (S). (**T-W**) Higher magnification of the images shown in P-S at the level of the prechordal plate to allow a better visualisation of the polster. The arrows indicate the territory it occupies along the anteroposterior axis. All embryos in this figure are at tail bud stages.

acting as a repressor of cadherin in these embryos, as the morphant phenotype could be ameliorated by reducing cadherin function with the injection of *cdh1* MOs (Fig. 3P-S). For these rescue experiments, embryos were injected with the usual dose of *snail1b* MO and a low dose of *cdh1* MO (500 pg) to avoid the severe phenotype of strong *cdh1* knockdown (Babb and Marrs, 2004), and thus, generating a partial loss of Cdh1 function. The prechordal plate adopts a normal shape in embryos injected with both *snail1b* and *cdh1* MOs (3 ng and 500 pg, respectively), and its shape in cadherin morphants is similar to that observed in embryos with Snail1b gain of function (compare Fig. 3V with O). Cells still disaggregate in the posterior region of double morphants as observed in the *cdh1* MO-injected embryos (asterisks in Fig. 3R,S, and not shown). It therefore appears that increased cell adhesion most probably accounts for the defects in extension movements in *snail1b* morphants.

Adhesion appeared to be the main defect underlying the aberrant movement of the prechordal plate when snail1b function is compromised. However, we were puzzled by the fact that *snail1b* is not expressed in the prechordal plate but rather, in a population of axial mesendoderm that seems to lie just posterior to it (Thisse et al., 1995) (Fig. 1R). Moreover, as a transcription factor, *snail1b* should act in a cell-autonomous manner. To better define the territory of *snail1b* expression, we contrasted the distribution of *hgg1* and confirmed that these two markers identified independent cell populations that partially intermingle at their interface as they migrate during gastrulation (Fig. 4A-C). By the tailbud stage, cells that express *hgg1* and *snail1b* were completely separated such that a sharp boundary had formed (Fig. 4D). We also compared the distribution of the posterior axial mesodermal marker *no tail* (*ntl*) with the *snail1b*-expressing population and we were unable to

identify double-labelled cells. As such, the posterior limit of *snail1b* expression abuts with the anterior limit of *ntl* expression (see Fig. S2 in the supplementary material).

Our data indicate that the expression of *snail1b* in the axial mesendoderm is restricted to a segment that lies posterior to the *hgg1*-expressing cells and anterior to those expressing *ntl*. Accordingly, these cells occupy a gap between the *hgg1* and *ntl* expression domains that can be clearly observed at the tailbud stages. This expression data indicated that *snail1b* is excluded from the precursor cells of the hatching gland where it seems to induce a cell non-autonomous phenotype, as *hgg1*-positive cells lag behind in *snail1b* morphant embryos (Fig. 3H). Conversely, the *hgg1*-expressing cells accumulate significant levels of *Cdh1* (Fig. 4E-G), further evidence that they lack *snail1b*. To analyse whether *snail1b* MOs induce an additional cell autonomous effect, we grafted 10-20 wild-type or *snail1b* morphant shield cells to the shield of wild-type hosts that were left to develop to the tailbud stage. The wild-type grafted cells were found in the prechordal plate, whereas the *snail1b* morphant cells remained at much more posterior locations (Fig. 5B). Indeed, when we analysed the behaviour of the individual cells, the abundant lamellipodia that projected anteriorly in wild-type grafted cells were not observed in the *snail1b* morphant cells, which often migrated in compact groups (Fig. 5C,D and Movies 1 and 2 in the supplementary material) and more slowly (Fig. 5E). Thus, as might be expected for a transcription factor, Snail1b also fulfils a cell autonomous function favouring cell migration.

Snail1a and 1b cooperate to regulate the directed migration of the axial mesendoderm

Snail1a has been implicated in anterior movement of the axial mesendoderm in the zebrafish embryo, since the prechordal plate in morphant embryos was located at a more posterior position (Yamashita et al., 2004). However, *snail1a* is not expressed in the prechordal plate (Thisse et al., 1993), again suggesting that, like *snail1b*, this transcription factor seems to act in a cell non-autonomous manner to direct the migration of the prechordal plate cells. Accordingly, we carefully analysed *snail1a* expression sites in the gastrulating fish, identifying a previously neglected domain of extraembryonic *snail1a* expression in the yolk syncytial layer YSL located underneath and anterior to the prechordal plate cells at 70% epiboly (compare Fig. 6A with B, in territories anterior to the dotted line encircling the *hgg1*-positive region, and Fig. 6D). This expression domain can be better assessed in sections (Fig. 6D). Higher levels of *snail1a* expression surrounding the *hgg1*-expressing cells are already evident at 60% epiboly, where the previously described high levels of expression in the margin are also observed (Fig. 6E,F).

When the expression patterns of the two *snail1* genes were compared with that of *hgg1* (Fig. 6), their distribution was mutually exclusive, as they are with respect to *cdh1* (Figs 4 and 6). Indeed, as shown above, *hgg1*-expressing cells also express *cdh1* (Fig. 4E), and both gene transcripts are excluded from *snail1a* or *snail1b* expressing cells (Fig. 4 and Fig. 6G,H). Thus, a dynamic and complementary pattern of *snail1* and Cdh1 expression divides the embryo into territories with different intercellular adhesion properties (summarised in Fig. 6I with data from Figs 2, 4 and 6).

Since *snail1a* and *1b* are expressed in independent populations and they generate a distinct phenotype when disturbed individually, we wondered whether they cooperate to promote extension movements during fish gastrulation. We co-injected *snail1a* and *snail1b* MOs and found that the defects in the axial mesendoderm were compounded (Fig. 7D; $n>20$). Hence, at the end of gastrulation the double morphants show the defects produced in both single morphants (Fig. 7B-D). The anterior limit to axial mesendoderm migration in these morphant embryos was more posterior than in control embryos [as in *snail1a* morphants in Yamashita et al. (Yamashita et al., 2004) and Fig. 7B,J; $n>20$], and they developed an abnormally elongated prechordal plate, similar to the *snail1b* morphants (Fig. 3 and Fig. 7C,K; $n>100$). Significantly, the cells expressing *snail1b* in the *snail1a* morphants migrate normally and often continue migrating on top of the *hgg1*-expressing cells (Fig. 7F).

The cells that express *snail1a* located anterior to the prechordal plate are unaffected when the function of *snail1b* is compromised, explaining how the anterior limit of the prechordal plate is established correctly (Fig. 7C). However, the polster is formed before epiboly has been completed (see Fig. S3 in the supplementary material) and when the morphant embryo reaches the tailbud stage, the prechordal plate has already adopted an abnormal shape (Fig. 2H and Fig. 7C). Since the *snail1b* MO used does not induce mRNA degradation, we can follow the cells in which Snail1b function has been knockdown since they still express its mRNA (Fig. 7C,G). In these embryos, time-lapse studies of the movements of the cells containing *snail1b* transcripts (the translation of which is impaired by the morpholinos) indicated that their anterior movement was much slower than in control embryos (not shown). When we

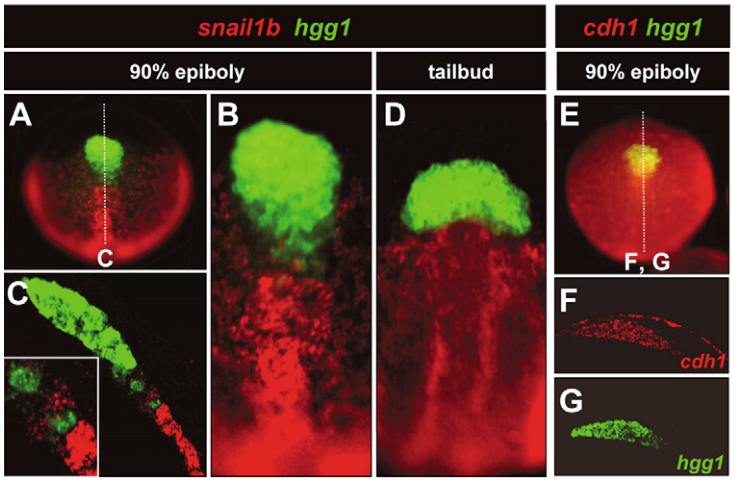

Fig. 4. *snail1b* expression is excluded from the *hgg1* expression domain within the axial mesendoderm. (**A**). Dual fluorescent in situ hybridisation demonstrates that *snail1b* expression is excluded from the *hgg1*-positive prechordal plate cells. The domains occupied by the two populations have ill-defined borders (**B**). (**C**) Sagittal sections of the embryo shown in A (dotted line) reveal the intermingling of the adjacent cell populations. Inset: border region at higher magnification. (**D**) At the tailbud stage, the cells have sorted out and a sharp boundary is defined. (**E-G**) The *hgg1*-positive prechordal plate cells also express *cdh1* transcripts.

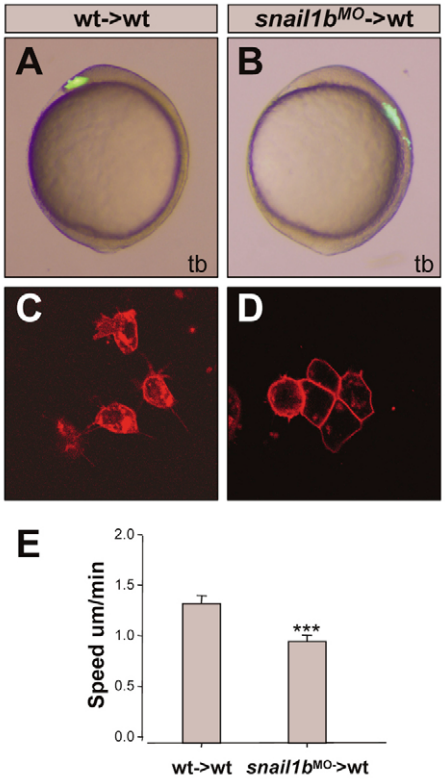

Fig. 5. Migratory behaviour of *snail1b* morphant cells in wild-type hosts. (**A**,**B**) Lateral views of wild-type live embryos after transplantation at the shield stage of 10-20 axial mesendodermal cells from wild-type (A) or *snail1b* morphant (B) fluorescein dextran-labelled embryos. (**C**,**D**) Membrane-tagged RFP-labelled grafts from wild-type (C) or *snail1b* morphant (D) embryos on wild-type hosts at 70% epiboly stage allows the visualisation of differences in individual cell morphology: wild-type cells show protrusions whereas *snail1b* morphant cells form cohesive clusters and show an epithelial-like morphology. (**E**) Speed of wild-type and *snail1b* morphant axial mesendodermal cells in wild-type hosts ($P<0.001$). In each embryo, the distances of three individual cells were measured (wt→wt, $n=7$; mo→wt, $n=5$)

observed the movements of the prechordal plate cells, we found that the anterior cells move forward normally while those at its posterior edge appeared to struggle to advance and lagged behind (for an example, see Movies 3 and 4 in the supplementary material). At the tail bud stages the lagging *hgg1* cells were still intermingled with cells that contained *snail1b* transcripts (Fig. 7G), explaining the aberrant elongated shape of the prechordal plate (Fig. 2H and Fig. 7C,K). We did not find any double-labelled cells, indicating that *snail1b* knockdown is not accompanied by a change in cell fate towards the *hgg1*-expressing prechordal plate cells. Nevertheless, the enlargement of the area occupied by the prechordal plate (*hgg1*-expressing cells) in embryos injected with *snail1b* MOs could be due to increased proliferation. This does not seem to be the case because we were unable to detect a significant change in the total number of *hgg1*-positive cells in wild-type, *snail1a*, *snail1b* and *snail1* double morphants ($n=3$ for each condition) when their nuclei were counted (Fig. 7M-P). In addition, even in the most compact areas, the number of nuclei per field in the *hgg1*-positive territory in *snail1b* or in *snail1a* plus *1b* morphants was around 50% of that observed in wt or *snail1a* morphants. (Fig. 7M-P). These data indicate that the same number of cells occupied a much larger area, explaining the elongated aberrant shape of the polster region observed in these embryos (Fig. 3 and Fig. 7C,D).

In summary, our data reveal the importance of the cooperation between the two populations of *snail1*-expressing cells and that of prechordal plate expressing *hgg1* and *cdh1* in the formation of the prechordal plate.

DISCUSSION

In this work, we describe the cooperation of *snail1* genes in the anterior migration of the axial mesendoderm. The *snail1a* and *1b* genes were generated by the duplication of the vertebrate *Snail1* gene in the teleost lineage (Manzanares et al., 2001), and their preservation may reflect the appearance of new functions associated to either gene or their subfunctionalisation. According to the duplication-degeneration-complementation model (DDC) (Lynch and Force, 2000), their subfunctionalisation would imply the complementary loss of expression between the two duplicates. Our functional analysis in the axial mesendoderm highlights the translation of this complementarity into functional cooperation.

Mutually exclusive expression of the two *snail1* genes and Cdh1 provides a framework for dynamic adhesion in the axial mesendoderm

The mutually exclusive expression of *snail1* and Cdh1 generates an interesting framework of transient and dynamic adhesion that somehow reconciles different models of the regulation of cell adhesion during the directed migration of prechordal mesoderm cells (see Solnica-Krezel, 2006). At the beginning of gastrulation, the internalisation of the first axial mesendodermal progenitors occurs through the delamination of individual cells (Montero et al., 2005). This process is reminiscent of the EMT that occurs during mesodermal delamination in amniotes as they do not express cadherin. In agreement with this view, in the shield, these individual cells express *goosecoid* and *snail1a* (Thisse et al., 1993). Similarly, the first axial mesodermal cells to ingress during gastrulation in *Xenopus* embryos delaminate as individual cells that transiently express *Snail1*, as discussed previously (see Morales and Nieto, 2004). Upon delamination, cells very quickly re-express Cdh1 and migrate as a cohesive anterior group. Cdh1 re-expression can be seen as a rapid mesenchymal to epithelium transition (MET, the reverse of EMT), which occurs concomitant with the loss of *snail1a* and appears to be necessary for the proper migration of the mesendodermal cells (Montero et al., 2005). As such, injection of *cdh1* MO (Montero et al., 2005) impairs anterior migration, as occurs in different *cdh1* hypomorphic mutants (Kane et al., 2005; Shimizu et al., 2005). Mesendodermal progenitors involute normally in these embryos (Montero et al., 2005), as expected considering that wild-type involuting cells lack Cdh1 because of the expression of *snail1a*. Thus, cadherin is required for mesendodermal extension in cells that lack *snail1a* and *1b*, in the prechordal plate and in the posterior regions but it is excluded from the delaminating cells, which undergo a very transient EMT, and in those located posterior to the *hgg1*-positive domain.

The migration of the prechordal plate cells is impaired in *snail1a* and *snail1b* morphants due to the upregulation of Cdh1 in the corresponding populations. Hence, the prechordal plate forms at an anomalous posterior position in embryos deficient in *snail1a* function (Yamashita et al., 2004). We found similar phenotypes when 20 ng of *snail1a* MO was injected, an unusually high dose when compared to that used for the *snail1b* MO (1.5-3ng).

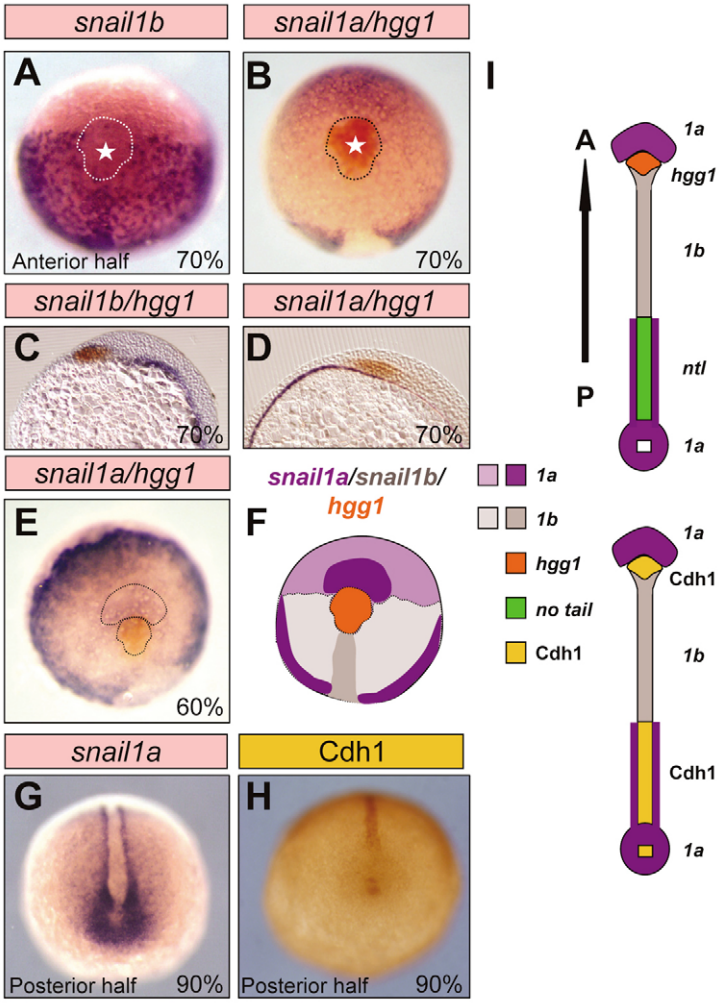

Fig. 6. The axial mesendoderm is subdivided into different territories with complementary *snail1* and E-cadherin (Cdh1) expression. (**A-H**) Expression of *snail1* genes and that of E-cadherin protein. (A,B) At 70% epiboly, *snail1a*-expressing cells surround the anterior limit of the *hgg1*-expressing domain and *snail1b* transcripts are absent from the *hgg1*-expressing prechordal plate region. (C,D) These expression domains are better observed in sections. (E) The domain of strong *snail1a* expression surrounding the *hgg1*-expressing cells is already present at 60% epiboly. (F) Diagram depicting the domains of *snail1a*, *1b* and *hgg1* expression in the anterior half of the embryo. (G,H) *snail1b* and E-cadherin (Cdh1) expression in the posterior half of an embryo at 90% epiboly. Note the complementary domains of *snail1b* and cadherin expression (compare E with F). (**I**) Diagram showing the relationship between the expression of *snail1* genes and those of *hgg1* and *ntl*. Also note the inverse correlation between *snail1* and Cdh1 expression.

Nevertheless, the expression of *hgg1* showed that the prechordal plate maintained its identity, again indicating that Snail1a is not a fate determinant but rather an inducer of movement (Barrallo-Gimeno and Nieto, 2005) and in agreement with the fact that the *hgg1* domain does not express any of the *snail1* genes.

Interestingly, although it impairs the movements of the posterior prechordal plate cells, the knockdown of *snail1b* does not prevent the correct migration of those located in its anterior domain since the pioneers seem to reach the appropriate anterior position. Again, this behaviour reinforces the crucial role of *snail1a* in driving the anterior migration of the prechordal plate (Yamashita et al., 2004). The correct movement of the prechordal plate cells depends on the expression and motility of the *snail1a* cell population located underneath and in front of them. In addition, the intermingling of the more posterior *hgg1*-expressing prechordal plate cells and those expressing *snail1b* at gastrulation stages in the wild-type embryo may explain why the polster shows an abnormally elongated shape in embryos where snail1b function is compromised. The increased cell to cell adhesion among the axial mesendodermal cells that have aberrantly lost s*nail1b* expression disturbs the motility of the prechordal plate *snail1*-negative and *hgg1*-positive cells that are forced to lag behind, and that sometimes even detach from their pioneers. This effect would only be observed when a significant proportion of axial mesendodermal cells have lost Snail1b function, as occurs in *snail1b* morphants. As such, small grafts of *snail1b* morphant cells into wild-type hosts only show a cell-autonomous effect, being unable to perturb the behaviour of surrounding normal axial mesendodermal cells. This suggests that a sort of 'community effect', i.e. certain number of abnormal cells, is required.

Our data indicate that the cell-autonomous function of *snail1b* is necessary for the proper migration of the axial mesendoderm as observed by the defects in migration of snail1b morphant cells transplanted into WT embryos. In addition, the influence of *snail1a* and *1b* loss of function on *snail1*-negative, *hgg1*-positive prechordal plate cells can be summarised as follows: the posterior cells push and the YSL is likely to provide an active substrate for prechordal plate migration (D'Amico and Cooper, 2001; Rohde and Heisenberg, 2007). The latter explains the abnormal posterior position of the prechordal plate in *snail1a* morphants (Yamashita et al., 2004) (this work). In summary, the precursors of the polster require the activity of Snail1a, which exerts a cell-non-autonomous effect on them, to reach their correct position, and they also require Snail1b function to acquire a normal shape. Selective adhesion allows *hgg1*-expressing prechordal plate cells to compact and separate from the more posterior cells while migrating. Indeed, a clear morphological boundary between the polster and the more posterior cells is evident in gastrulating fish embryos. Hence, for the axial mesendoderm to migrate properly, cells located adjacent to the *hgg1*-expression domain must migrate actively. Although this does not exclude the need for prechordal plate cells to actively migrate (Heisenberg and Tada,

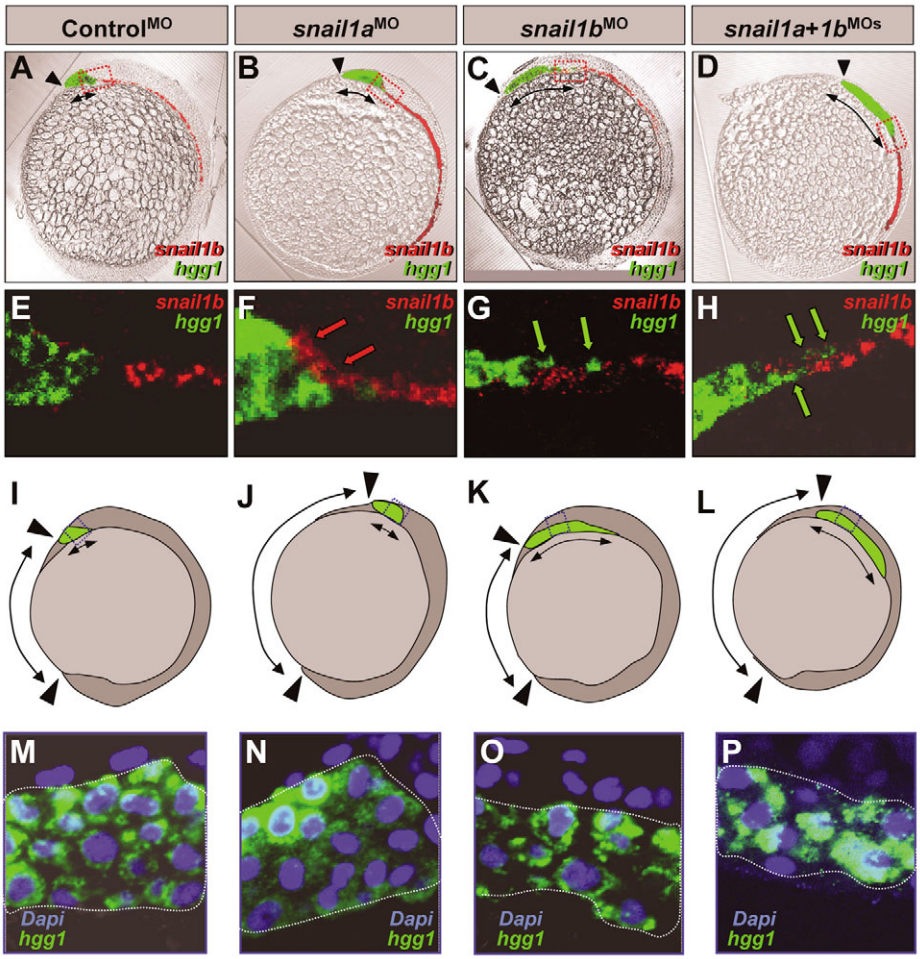

Fig. 7. Snail1a and Snail1b cooperate in the directed anterior migration of the axial mesendoderm. (**A-D**) Morphological appearance of control embryos (A), and of embryos injected with *snail1a* MOs (B), with *snail1b* MOs (C) or with both (D). The expression of *hgg1* (green) and *snail1b* (red) is superimposed. Note the abnormal position and/or shape of the prechordal plate. The arrows mark the territory occupied by the polster. (**E-H**) High-magnification confocal microscopy images of the same embryos at the regions indicated with a red square in A-D. Red arrows indicate the aberrant position of groups of *snail1b*-expressing cells, and green arrows that of *hgg1*-expressing cells. (**I-L**) Diagrams summarising the phenotypes observed in the *snail1* morphants. (J) As previously described, the injection of *snail1a* MOs altered the posterior position of the prechordal plate (shown in green, with the extent indicated by a double-headed arrow). (K) *snail1b* morphants developed an elongated prechordal plate, which reached the normal anterior position. (L) Co-injection of *snail1b* and *snail1a* MOs produced a compound phenotype in the prechordal plate, which adopted an elongated shape and was situated at an aberrant posterior position. Black arrowheads indicate the anterior and posterior limits of the embryo. The double-headed arrows on the outside of the embryos mark the distance between the embryo limits. (**M-P**) *hgg1* in situ hybridisation and DAPI (nuclei) staining show the cell density in the regions indicated with a dotted blue square in I-L. All embryos in this figure are at tail bud stages.

2002), it highlights the importance of a force driven by Snail1a in the YSL and of the influence of actively migratory *snail1b*-expressing cells located posteriorly.

We have unveiled an important function for *snail1* genes in the migration of the axial mesendoderm. The products of both genes repress cadherin expression and by downregulating adhesion, they facilitate the migration. The combination of the cell autonomous action of these two transcription factors in two independent populations influences the behaviour of the snail-free prechordal plate region destined to become the hatching gland. In addition, these results extend and confirm that Snail genes regulate cell adhesion and cell movements in different tissues, and that their function as adhesion regulators may or may not be associated with the induction of a full EMT depending on the cell context (i.e. neural crest cells or delaminating mesendodermal precursors versus mesendodermal precursors located posterior to the *hgg1*-positive cells). As such, the EMT is much more than just cell adhesion or cadherin downregulation, highlighting the need to analyse the different Snail targets and partners during the induction of cell movements in the developing embryo.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/22/4073/DC1

References

Akimenko, M. A., Ekker, M., Wegner, J., Lin, W. and Westerfield, M. (1994). Combinatorial expression of three zebrafish genes related to distal-less: part of a homeobox gene code for the head. *J. Neurosci.* **14**, 3475-3486.

Babb, S. G. and Marrs, J. A. (2004). E-cadherin regulates cell movements and tissue formation in early zebrafish embryos. *Dev. Dyn.* **230**, 263-277.

Barrallo-Gimeno, A. and Nieto, M. A. (2005). The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* **132**, 3151-3161.

Batlle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J. and Garcia de Herreros, A. (2000). The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat. Cell Biol.* **2**, 84-89.

Boutet, A., De Frutos, C. A., Maxwell. P. H., Mayol, M. J., Romero, J. and Nieto, M. A. (2006). Snail activation disrupts tissue homeostasis and induces fibrosis in the adult kidney. *EMBO J.* **25**, 5603-5613.

Cano, A., Pérez-Moreno, M. A., Rodrigo, I., Locascio, A., Blanco, M. J., del Barrio, M. G., Portillo, F. and Nieto, M. A. (2000). The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol.* **2**, 76-83.

Carmany-Rampey, A. and Schier, A. F. (2001). Single-cell internalization during zebrafish gastrulation. *Curr. Biol.* **11**, 1261-1265.

D'Amico, L. A. and Cooper, M. S. (2001). Morphogenetic domains in the yolk syncytial layer of axiating zebrafish embryos. *Dev. Dyn.* **222**, 611-624.

Denkers, N., Garcia-Villalba, P., Rodesch, C. K., Nielson, K. R. and Match, T. J. (2004). FISHing for chick genes: triple-label whole-mount fluorescence in situ

hybridization detects simultaneous and overlapping gene expression in avian embryos. *Dev. Dyn.* **229**, 651-657.
Glickman, N. S., Kimmel, C. B., Jones, M. A. and Adams, R. J. (2003). Shaping the zebrafish notochord. *Development* **130**, 873-887.
Griffin, K. J. P., Amacher, S. L., Kimmel, C. B. and Kimmelman, D. (1998). Molecular identification of spadetail: regulation of zebrafish trunk and tail mesoderm formation by T-box genes. *Development* **125**, 3379-3388.
Hammerschmidt, M. and Nüsslein-Volhard, C. (1993). The expression of a zebrafish gene homologous to Drosophila snail suggests a conserved function in invertebrate and vertebrate gastrulation. *Development* **119**, 1107-1118.
Heisenberg, C.-P. and Tada, M. (2002). Zebrafish gastrulation movements: bridging cell and developmental biology. *Semin. Cell Dev. Biol.* **13**, 471-479.
Kane, D. A., McFarland, K. N. and Warga, R. M. (2005). Mutations in half baked/E-cadherin block cell behaviors that are necessary for teleost epiboly. *Development* **132**, 1105-1116.
Keller, R., Davidson, L., Edlund, A., Elul, T., Ezin, M., Shook, D. and Skoglund, P. (2000). Mechanisms of convergence and extension by cell intercalation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355**, 897-922.
Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.
Locascio, A. and Nieto, M. A. (2001). Cell movements during vertebrate development: integrated tissue behaviour versus individual cell migration. *Curr. Opin. Genet. Dev.* **11**, 464-469.
Locascio, A., Manzanares, M., Blanco, M. J. and Nieto, M. A. (2002). Modularity and reshuffling of Snail and Slug expression during vertebrate evolution. *Proc. Natl. Acad. Sci. USA* **99**, 16841-16846.
Lynch, M. and Force, A. (2000). The probability of duplicate gene preservation by subfunctionalization. *Genetics* **154**, 459-473.
Manzanares, M., Locascio, A. and Nieto, M. A. (2001). The increasing complexity of the Snail gene superfamily in metazoan evolution. *Trends Genet.* **17**, 178-181.
Manzanares, M., Blanco, M. J. and Nieto, M. A. (2004). Snail3 orthologues in vertebrates: divergent members of the Snail zinc-finger gene family. *Dev. Genes Evol.* **214**, 47-53.
Montero, J.-A., Carvalho, L., Wilsch-Brauninger, M., Kilian, B., Mustafa, C. and Heisenberg, C.-P. (2005). Shield formation at the onset of zebrafish gastrulation. *Development* **132**, 1187-1198.
Morales, A. V. and Nieto, M. A. (2004). The Snail family in gastrulation. In *Gastrulation* (ed. C. D. Stern), pp. 631-641. New York: Cold Spring Harbor Laboratory Press.
Myers, D. C., Sepich, D. S. and Solnica-Krezel, L. (2002). Convergence and extension in vertebrate gastrulae: cell movements according to or in search of identity? *Trends Genet.* **18**, 447-455.
Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216-220.
Nieto, M. A. (2002). The Snail superfamily of zinc-finger transcription factors. *Nat. Rev. Mol. Cell Biol.* **3**, 155-166.
Nieto, M. A., Patel, K. and Wilkinson, D. G. (1996). In situ hybridization analysis of chick embryos in whole mount and tissue sections. *Methods Cell Biol.* **51**, 219-235.
Postlethwait, J. H., Yan, Y.-L., Gates, M. A., Horne, S., Amores, A., Brownlie, A., Donovan, A., Egan, E. S., Force, A., Gong, Z. et al. (1998). Vertebrate genome evolution and the zebrafish gene map. *Nat. Genet.* **18**, 345-349.
Rohde, L. A. and Heisenberg, C. P. (2007). Zebrafish gastrulation: cell movements, signals, and mechanisms. *Int. Rev. Cytol.* **261**,159-192.
Schulte-Merker, S., Ho, R., Herrmann, B. and Nusslein-Volhard, C. (1992). The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116**, 1021-1032.
Shimizu, T., Yabe, T., Muraoka, O., Yonemura, S., Aramaki, S., Hatta, K., Bae, Y.-K., Nojima, H. and Hibi, M. (2005). E-cadherin is required for gastrulation cell movements in zebrafish. *Mech. Dev.* **122**, 747-763.
Solnica-Krezel, L. (2006). Gastrulation in zebrafish – all just about adhesion? *Curr. Opin. Genet. Dev.* **16**, 433-441.
Teruel, M. N., Blanpied, T. A., Shen, K., Augustine, G. J. and Meyer, T. (1999). A versatile microporation technique for the transfection of cultured CNS neurons. *J. Neurosci. Methods* **93**, 37-48.
Thiery, J. P. (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat. Rev. Cancer* **2**, 442-454.
Thisse, C., Thisse, B., Schilling, T. F. and Postlethwait, J. H. (1993). Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail embryos. *Development* **119**, 1203-1215.
Thisse, C., Thisse, B. and Postlethwait, J. H. (1995). Expression of snail2, a second member of the zebrafish snail family, in cephalic mesoderm and presumptive neural crest of wild-type and spadetail mutant embryos. *Dev. Biol.* **172**, 86-99.
Vogel, A. M. and Gerster, T. (1997). Expression of a zebrafish Cathepsin L gene in anterior mesendoderm and hatching gland. *Dev. Genes Evol.* **206**, 477-479.
Weinberg, E. S., Allende, M. L., Kelly, C. S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O. J., Grunwald, D. J. and Riggleman, B. (1996). Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos. *Development* **122**, 271-280.
Yamamoto, A., Amacher, S. L., Geissert, D., Kimmel, C. B. and De Robertis, E. M. (1998). Zebrafish paraxial protocadherin is a downstream target of *spadetail* involved in morphogenesis of gastrula mesoderm. *Development* **125**, 3389-3397.
Yamashita, S., Miyagi, C., Fukada, T., Kagara, N., Che, Y.-S. and Hirano, T. (2004). Zinc transporter LIVI controls epithelial-mesenchymal transition in zebrafish gastrula organizer. *Nature* **429**, 298-302.