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T-box gene *tbx5* is essential for formation of the pectoral limb bud

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The T-box genes *Tbx4* and *Tbx5* have been shown to have key functions in the specification of the identity of the vertebrate forelimb (*Tbx5*) and hindlimb (*Tbx4*)[1,2]. Here we show that in zebrafish, *Tbx5* has an additional early function that precedes the formation of the limb bud itself. Functional knockdown of zebrafish *tbx5* through the use of an antisense oligonucleotide resulted in a failure to initiate fin bud formation, leading to the complete loss of pectoral fins. The function of the *tbx5* gene in the development of zebrafish forelimbs seems to involve the directed migration of individual lateral-plate mesodermal cells into the future limb-bud-producing region. The primary defect seen in the *tbx5*-knockdown phenotype is similar to the primary defects described in known T-box-gene mutants such as the *spadetail* mutant of zebrafish[3,4] and the *Brachyury* mutant of the mouse[5], which both similarly exhibit an altered migration of mesodermal cells. A common function for many of the T-box genes might therefore be in mediating the proper migration and/or changes in adhesive properties of early embryonic cells.

The formation of vertebrate limbs involves a complex series of morphogenetic events, including the specification of limb fields within the lateral-plate mesoderm, induction of the limb buds at appropriate axial levels, and the initiation and patterning of distal limb outgrowth[6,7]. During early stages of vertebrate limb morphogenesis, *Tbx5* is strongly expressed within the forelimb buds of a variety of vertebrate species[8–13]. Recent misexpression studies have shown that this gene and a closely related gene, *Tbx4*, which is expressed within the hindlimb bud, are crucial in the determination of limb identity and the regulation of limb outgrowth[1,2]. However, the initiation of *Tbx5* expression within the anterior lateral-plate mesoderm, which supplies the forelimb progenitor cells, precedes the emergence of visible forelimb buds[8–11,13], indicating that this gene might have additional early functions in forelimb development. To investigate the possibility of an earlier function for the *Tbx5* gene during vertebrate forelimb development, we generated a knockdown phenotype in zebrafish, using antisense oligonucleotides containing morpholino moieties in their backbones. These 'morpholino' oligonucleotides are thought to exert their inhibitory effects through physical blocking of the translational initiation of target messenger RNAs, and also have been shown to exhibit a low toxicity and high specificity in a variety of *in vivo* systems[14], including the fertilized eggs of zebrafish[15].

We designed two different morpholino oligonucleotides for the *tbx5* gene of zebrafish, one recognizing the first 25 bases of the coding sequence, the other targeting a sequence of a similar size but located within the 5′ untranslated region (UTR), ten nucleotides upstream of the initiation codon. The effectiveness of these oligonucleotides in inhibiting the translation of target mRNAs was first examined by an assay *in vivo* using chimaeric mRNAs in which the coding sequence of the gene encoding enhanced green fluorescent protein replaced the coding sequence of *tbx5* in frame after the first 27 bases. Both oligonucleotides were able to block the translation of the green fluorescent protein when injected together with the test mRNA into the early-stage embryo, whereas the control oligonucleotide designed for the 5′ UTR sequence of the zebrafish *tbx4* gene

was not, indicating that both oligonucleotides are capable of recognizing their targets in a sequence-specific manner under *in vivo* conditions (see Supplementary Information). In addition, when injected into early-stage embryos, both morpholino oligonucleotides generated indistinguishable phenotypic outcomes for the pectoral fins that were not seen in embryos injected with the control oligonucleotide (Fig. 1), again confirming the specific action of these oligonucleotides.

Introduction of 5 ng or more of either morpholino oligonucleotide by microinjection into the embryo at the one-cell or two-cell stage resulted in a complete lack of pectoral fins in all of the injected embryos at 3 days of development ($n = 500$; Fig. 1a, b). Fish examined at 4 days of development were found to lack all of the skeletal elements of the pectoral fins lying distal to the cleithrum, including the scapulocoracoid and the endoskeletal disc (Fig. 1c–f), which give rise to the girdle and the endoskeletal supports of the pectoral fins of the juvenile and adult fish[16]. These results indicate that the inhibition of translation of *tbx5* mRNAs by morpholino oligonucleotides has led to the loss of all pectoral limb-bud-derived structures. Our observations also indicate that the knockdown of *tbx5* function might directly affect limb-bud formation at a very early stage, as a pectoral fin bud was never visibly apparent at any stage of development in *tbx5* morpholino-injected embryos (Fig. 1g, h).

In both tetrapod and fish embryos, limb development begins with the formation of a local condensation of mesenchyme cells within the prospective limb field. Signals from the mesenchyme then instruct the overlying ectoderm to form the apical ectodermal ridge (AER), which in turn has a function in initiating and maintaining a cascade of events, including the specification of the zone of polarizing activity (ZPA) in the posterior part of the limb-bud mesenchyme[6,7]. Members of the *Hox* cluster genes are also expressed very early in the limb bud, some of which appear prior to and independently of the establishment of the ZPA and/or AER[17–20]. In *tbx5* morpholino-treated embryos, expression of known early markers of fin/limb-bud formation, such as the AER-specific marker *dlx2*, the ZPA-specific marker *shh* and the fin/limb-specific *hoxa* and *hoxd* cluster genes, is never observed (Fig. 2), indicating that, in the absence of *tbx5* function, several of the events characterizing early periods of pectoral fin development were not initiated. These results are consistent with the normal onset of expression of *tbx5* within the anterior lateral-plate mesoderm before the initiation of pectoral fin-bud formation in zebrafish[13,21], indicating that *tbx5* might begin to function very early during pectoral fin development, possibly even before the initial formation of the mesenchymal core of the fin bud.

The normal morphogenesis of the *tbx5*-positive cells in the lateral-plate mesoderm of a control embryo is shown in Fig. 3a, c, e, g. In the 10-somite-stage embryo (14 h post fertilization (hpf); Fig. 3a), the *tbx5*-positive lateral-plate cells are arranged in two bilateral stripes extending from the level of the posterior midbrain to the second somite in the trunk. By the 20-somite stage (19 hpf; Fig. 3c), the *tbx5*-expressing cells are no longer arranged in a

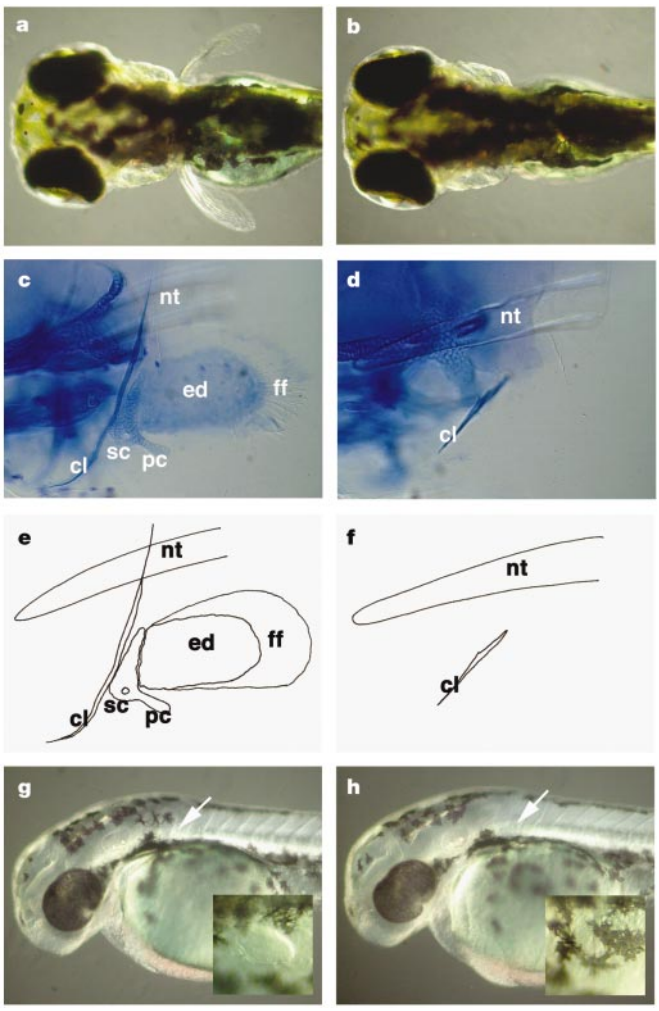

Figure 1 Defects in pectoral fin formation in zebrafish embryos injected with anti-*tbx5* morpholino oligonucleotides. **a**, **c**, **e**, **g**, Embryos injected with the control oligonucleotide (5 ng per embryo). **b**, **d**, **f**, **h**, Embryos injected with an anti-*tbx5* oligonucleotide (5 ng per embryo). **a**, **b**, Dorsal view of 3-day-old embryos; **c**, **d**, lateral views of pectoral fin skeletons of 4-day-old larval fish stained with Alcian blue[16]; **e**, **f**, line drawings of the notochord and the major skeletal elements of the pectoral fins shown in **c** and **d**; **g**, **h**, lateral views of 36-hour-old embryos. Insets show the dorsal views of the prospective pectoral fin regions on the left side (marked by arrows) at a high magnification. Note that at this concentration no gross abnormality was noted in either group of embryos except for the complete lack of pectoral fins in embryos injected with an anti-*tbx5* morpholino oligonucleotide. nt, notochord; cl, cleithrum; sc, scapulocoracoid; pc, postcoracoid process; ed, endoskeletal disc; ff, fin fold.

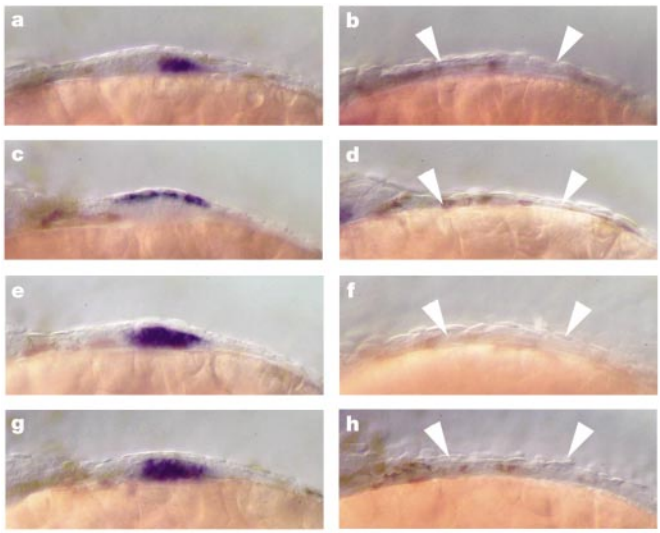

Figure 2 Expression of marker genes in embryos injected with the control oligonucleotide (**a**, **c**, **e**, **g**) (5 ng per embryo) and an anti-*tbx5* morpholino oligonucleotide (**b**, **d**, **f**, **h**) (5 ng per embryo). **a**, **b**, *shh*; **c**, **d**, *dlx2*; **e**, **f**, *hoxa9b*; **g**, **h**, *hoxd9a*. Embryos at 30 hpf. Arrowheads in **b**, **d**, **f** and **h** mark the prospective pectoral fin bud region in *tbx5* morpholino-injected embryos. Note the flat appearance of the pectoral fin region in embryos injected with the anti-*tbx5* morpholino oligonucleotide.

contiguous stripe but are broken up into an anterior group of cells (asterisk), which seems later to contribute to the heart primordia, and a more posterior group of cells (arrow) at the position of the future pectoral fin bud. The pattern of *tbx5* expression within this posterior group of cells further condenses by 24–30 hpf such that most of the *tbx5*-expressing cells become located within the mesenchymal region of the forming pectoral fin buds (Fig. 3e, g).

In embryos injected with the *tbx5* morpholino antisense oligonucleotide, the initiation of *tbx5* expression within the anterior lateral-plate mesoderm (Fig. 3b) as well as the subsequent elaboration of the *tbx5* expression domains into the anterior heart and posterior pectoral fin primordia (Fig. 3d) seem to occur normally, although the overall arrangement of the expressing cells seems somewhat more loosely organized in these embryos than in control embryos. However, by 24 hpf, when the control embryos begin to initiate the formation of pectoral fins as evidenced by the presence of a dense local population of *tbx5*-expressing cells (Fig. 3e, arrow), in the morpholino-injected embryos, the *tbx5*-expressing mesenchyme cells in the prospective fin field remain dispersed (Fig. 3f), and

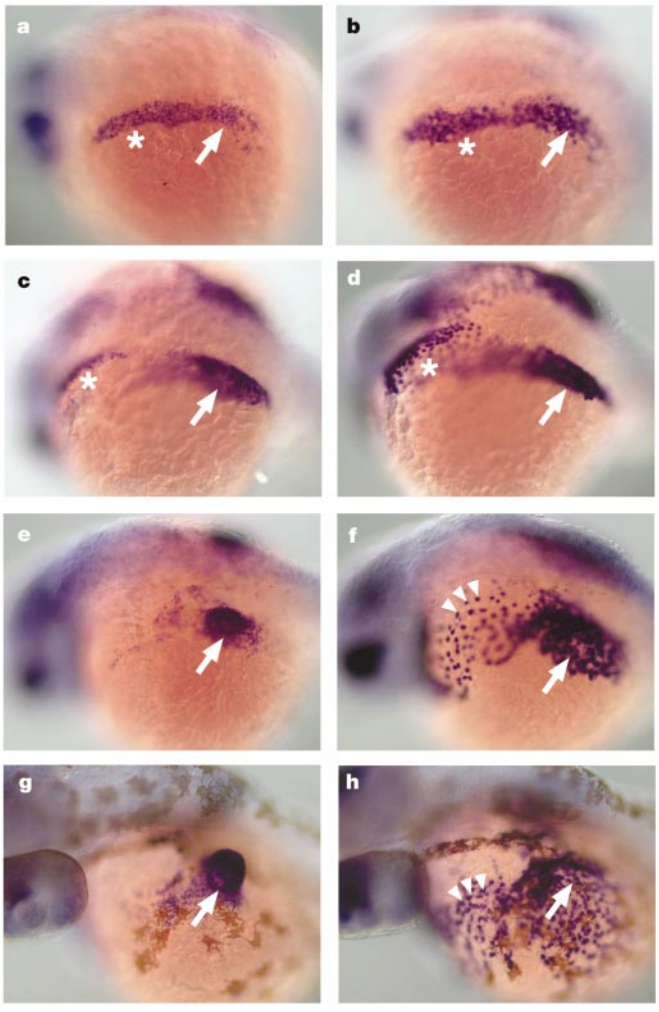

Figure 3 Expression of *tbx5* in embryos injected with the control oligonucleotide (**a**, **c**, **e**, **g**) (5 ng per embryo) and an anti-*tbx5* morpholino oligonucleotide (**b**, **d**, **f**, **h**) (5 ng per embryo). **a**, **b**, Ten-somite stage; **c**, **d**, 20-somite stage; **e**, **f**, 24 hpf; **g**, **h**, 36 hpf. Oblique dorsal view of the left side of the embryo, with the anterior to the left and dorsal to the top in all panels. Arrows and asterisks mark the pectoral fin and heart primordia, respectively. Small arrowheads in **f** and **h** mark the cells of the pericardium in *tbx5* morpholino-injected embryos showing high levels of *tbx5* expression, which normally express *tbx5* at this stage and earlier, but only weakly (D.A., unpublished observation).

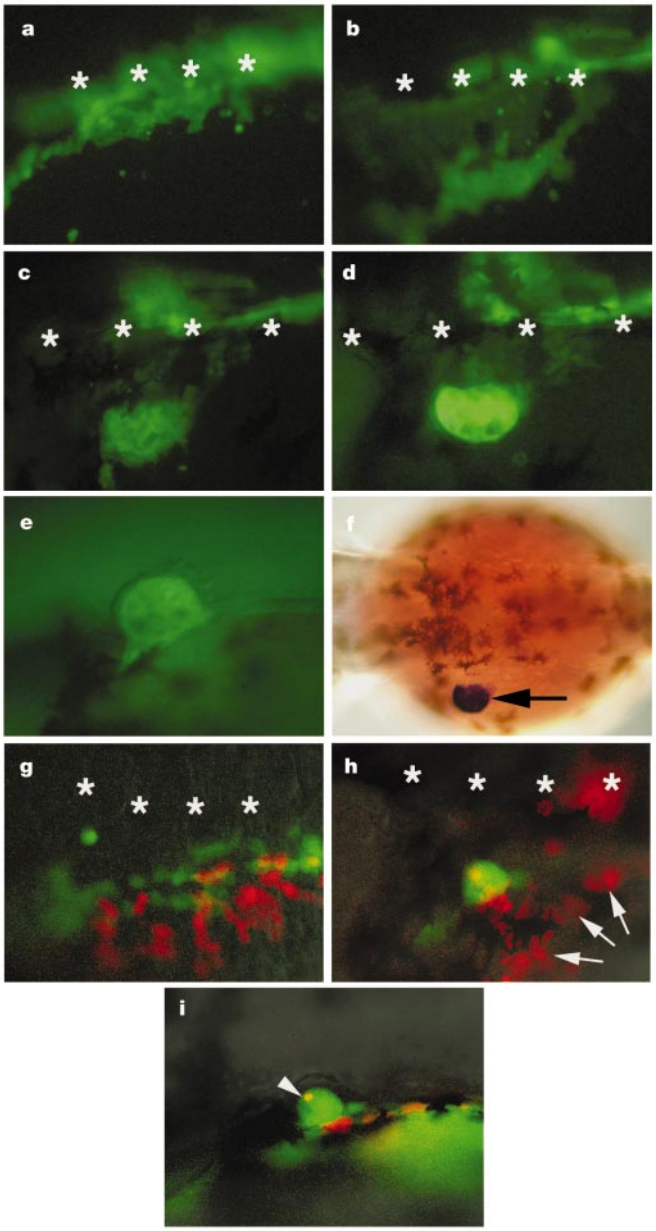

Figure 4 Rescued formation of a pectoral fin bud by transplanted wild-type cells in a *tbx5* morpholino-injected embryo. **a**–**e**, Images of the prospective pectoral fin fields taken at different time points during the development of a *tbx5* morpholino-injected host carrying transplanted wild-type cells (green). **f**, Dorsal view of the same specimen after whole-mount *in situ* hybridization with the *hoxa9b* probe, a marker for the pectoral fin mesenchyme cells[17,18]. Note the strong expression of *hoxa9b* within the rescued fin bud (arrow), which is not seen in the contralateral side that did not receive wild-type cells. **a**, Ten-somite stage; **b**, 26-somite stage (22 hpf); **c**, 30 hpf; **d**–**f**, 36 hpf. Fluorescent (**a**–**d**), composite (**e**) or white-light (**f**) images. In all rescues, the congregation of the wild-type cells always occurred at the correct axial position for normal pectoral fin outgrowth (in the area adjacent to the second somite). No rescues were seen in host embryos in which wild-type cells were located outside the lateral-plate mesoderm at the level of the first three somites ($n = 63$). **g**–**i**, Differential contribution of wild-type (green) and *tbx5* morpholino-treated (red) cells to the formation of a pectoral fin bud in a *tbx5* morpholino-injected host. **g**, Ten-somite stage; **h**, **i**, 36 hpf. Note the sharp boundary separating the wild-type cells (green) from the underlying layer of morpholino-treated cells (red). The single red spot in **i** (arrowhead) is a dead cell fragment, presumably swept into the fin bud by the migration of wild-type cells. In the remaining cases of rescue ($n = 4$), morpholino-injected cells were excluded from the mesenchymal layers of the fin bud. Oblique dorsal (**a**–**d**, **g**, **h**), lateral (**e**, **i**) and dorsal (**f**) views, with the anterior to the left and dorsal to the top in all panels. Asterisks in **a**–**d**, **g**, **h** mark the anterior and posterior ends of the ventral borders of the first three somites, with the distance between the marks covering the width of each somite (~50 μm).

eventually are scattered over the yolk away from the normal position of the pectoral fin bud (Fig. 3h).

The patterns of *tbx5* expression in both control and experimental embryos indicate that one of the functions of the *Tbx5* gene is to mediate the correct movement of lateral-plate mesoderm cells into the future pectoral limb-bud-producing region. In support of this possibility, we found that wild-type cells, if transplanted into the anterior lateral-plate region of *tbx5* morpholino-treated embryos, can rescue pectoral fin buds through a directed migration. As illustrated in Fig. 4a–e, which is a series of photographs taken at various time points from one of the rescued embryos, we observed that fluorescently labelled wild-type cells within a region adjacent to the first three somites (the area that normally contributes cells to the pectoral fin buds (D.A., unpublished observation)) were reproducibly able to undergo cell movements (Fig. 4a, b) and condense (Fig. 4c, d) to form a pectoral fin bud (Fig. 4e, f) within morpholino-treated host embryos ($n = 7$). Furthermore, when we simultaneously transplanted cells from a wild-type donor embryo and from a morpholino-treated donor embryo into a morpholino-treated host embryo, we found that only the wild-type cells (labelled green in Fig. 4g–i) were capable of forming and populating the rescued fin bud structure, whereas morpholino-treated cells (labelled red in Fig. 4g–i) remained outside the fin bud in a single layer of variably scattered cells around the limb-producing region (Fig. 4h, arrows; $n = 5$). These results indicate that *tbx5* function is required autonomously to allow cells to populate and form the mesenchymal core of the developing pectoral fin bud.

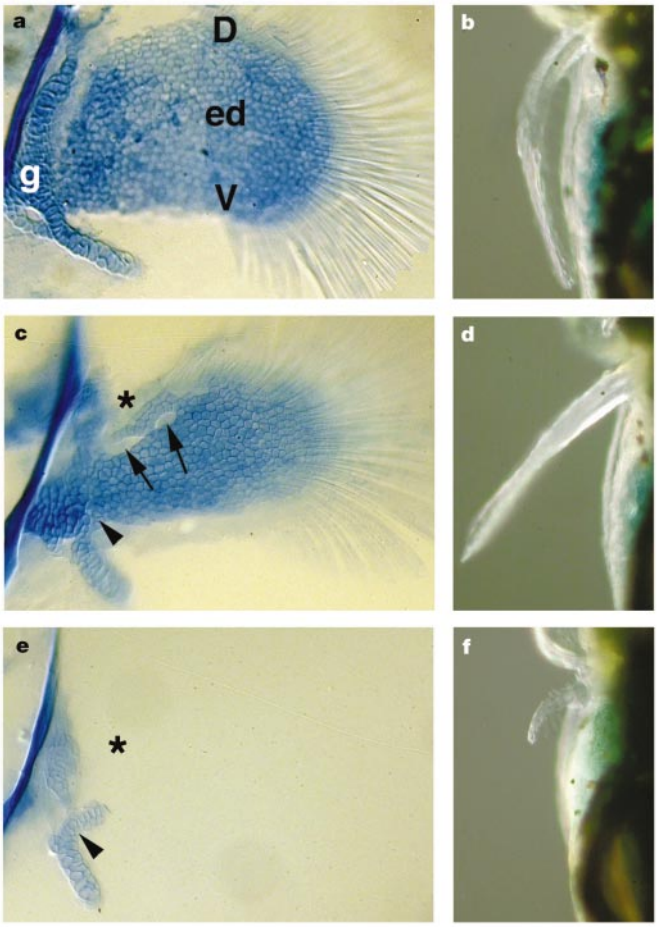

Figure 5 Reduction in pectoral fin size and defects in cartilage formation in 5-day-old larval zebrafish injected with low concentrations of anti-*tbx5* morpholino oligonucleotide (1–3 ng per embryo). **a**, **b**, A normal pectoral fin from embryos injected with the lowest dose of morpholino (1 ng per embryo). **c**–**f**, Smaller pectoral fins from embryos injected with a medium dose of *tbx5* morpholino oligonucleotides (2–3 ng per embryo) showing an abnormal development of cartilage structures. These include a partial fusion between the girdle (g) and the endoskeletal disc (ed) (arrowheads in **c** and **e**), which contributes to the abnormal angle of articulation between the pectoral fin and the girdle (as seen in **d**), an incomplete formation (arrows in **c**) or severe reduction of the dorsal portion of the endoskeletal disc (marked by asterisks in **c** and **e**) that accompanies the reduction in disc size, and a malformation (**c**) or splitting (**e**) of the pectoral girdle. Note that, because of the rotation of fin buds during the morphogenesis of pectoral fins in zebrafish, the dorsal portion of larval pectoral fins actually derives from the anterior half of the fin bud[16]. D, dorsal; V, ventral.

It has been suggested from studies of known genetic mutants in T-box genes, such as the *Brachyury* or *T* mutant in mouse[5] and the *spadetail* mutant in zebrafish, which carries a defect in the *tbx16* gene[4], that a general function of many of the T-box family genes might be in mediating the correct migration of cells, perhaps through changes in adhesive properties, to their proper positions in the developing embryo[3–5,22]. For example, the primary defect described in the *spadetail* mutant is a cell-autonomous failure of marginal mesodermal cells to converge correctly to the dorsal midline during gastrulation[3], resulting in an embryo lacking somitic trunk mesoderm. Likewise, deficiencies and abnormal morphogenesis of mesodermal derivatives in *Brachyury*-homozygous mutants have been shown to be due to the inability of mesodermal cells to move correctly through the primitive streak during gastrulation in mouse embryos[5]. The failure of *tbx5* morpholino-treated lateral-plate cells to form mesenchymal aggregates, coupled with the normal behaviours of wild-type cells observed within morpholino-treated host embryos, indicates that the *tbx5* gene in zebrafish embryos might similarly be acting through the regulation of migration and/or adhesive properties of limb precursor cells, either before or during the formation of the pectoral fin bud.

In contrast to the drastic effects of *tbx5* knockdowns on the development of pectoral fins, we saw only subtle and late defects in patterning of the hearts in morpholino-injected embryos, despite the fact that *tbx5* is also prominently expressed in the heart primordia during zebrafish development[13,21]. In 5-day-old zebrafish, the completion of heart 'looping' normally positions the atrium dorsally to the ventricle. However, in *tbx5* morpholino-injected embryos, the heart fails to undergo this late phase of heart looping, leading to the placement of both heart chambers at the same dorsoventral level with the atrium positioned posteriorly to the ventricle (see Supplementary Information). In spite of the failure to complete looping, the beating and unidirectional flow of blood are not significantly impaired in the hearts of a *tbx5*-knockdown fish.

The heart phenotype observed in the *tbx5*-knockdown zebrafish is mild compared with the phenotype reported in mammals, in which the loss or reduction of *Tbx5* function has been shown to lead to much more severe defects, such as hypoplasia of the posterior segment of the heart tissue and abnormalities in cardiac septation[23]. The differences in heart phenotypes between *tbx5*-knockdown fish and *Tbx5*-knockout mice could be due to limitations in the morpholino antisense technique that we have used in this study. Alternatively, it is possible that the differences in phenotype might reflect differential requirements for *Tbx5* function in heart development between higher and lower vertebrates.

However, we have found that by using a smaller amount of morpholino oligonucleotides (less than 3 ng per embryo) a series of intermediate phenotypes could be generated for the pectoral fins (Fig. 5a–f), which indicates a possible conservation of *Tbx5* dose dependence in pectoral limb development between fish and tetrapods. We also found that in these instances the reduction in fin size was accompanied by structural defects, such as large tissue gaps, only on the dorsal side of the endoskeletal discs (Fig. 5a, c, e). Similar asymmetries in limb defects have previously been docu-

mented to occur in Holt–Oram syndrome patients, who possess only one good copy of the *Tbx5* gene[24,25]. At present, however, it is not clear whether such similarities in phenotypes are due to similarities in underlying mechanisms. For instance, here we have reported our findings showing that lateral-plate mesoderm cells in zebrafish seem to migrate extensively during formation of the limb bud. However, in the mouse and chick, *Tbx5*-expressing cells do not seem to undergo similar early migration movements[8–11]. Future studies on the precise functions of *Tbx5* genes in both higher and lower vertebrates, including fate-mapping work and the isolation of downstream target genes, will be instructive in determining the degree of conservation and divergence of *Tbx5* functions in vertebrate limb development and evolution. □

Methods

Morpholino oligonucleotides were purchased from Gene Tools LLC with the following sequences: control oligonucleotide (anti-*tbx4* 5′ UTR), 5′-CCAGAACGCAGTAATTTGTCCACTT-3′; anti-*tbx5* oligonucleotide for the coding sequence, 5′-GAAAGGTGTCTTCACTGTCCGCCAT-3′; anti-*tbx5* oligonucleotide for the 5′ UTR sequence, 5′-CCTGTACGATGTCTACCGTGAGGC-3′. Solubilization and injection of oligonucleotides were performed as described[15]. Unless indicated otherwise, the results presented here are based on the anti-*tbx5* oligonucleotide for the 5′ UTR sequence. Alcian blue staining of the cartilages in larval fish and whole-mount *in situ* hybridization on fixed embryos were performed with the procedures described in refs 16 and 26, respectively. Transplantation experiments were performed as described[3]. In brief, for single transplantation experiments, wild-type donor embryos were first labelled with fluorescein–dextran (relative molecular mass 40,000 (M_r 40K)) at the one-cell or two-cell stage. When the donor embryos reached late blastula stage, about 10–20 cells were taken out of each donor embryo and were subsequently introduced into the marginal zones of early gastrula-stage host embryos that had been preinjected with 5 ng anti-*tbx5* oligonucleotide at the one-cell to four-cell stage. Double-transplantation experiments were performed with the same procedure, except for the simultaneous use of both wild-type donor embryos (labelled with M_r 40K fluorescein–dextran, green) and *tbx5* morpholino-injected donor embryos (labelled with M_r 10K rhodamine–dextran, red).

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Competing interests statement

The authors declare that they have no competing financial interests

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An abundant erythroid protein that stabilizes free α-haemoglobin

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The development of red blood cells (erythrocytes) is distinguished by high-level production of the oxygen carrier, haemoglobin A (HbA), a heterotetramer of α- and β-haemoglobin subunits. HbA synthesis is coordinated to minimize the accumulation of free subunits that form cytotoxic precipitates[1–3]. Molecular chaperones that regulate globin subunit stability, folding or assembly have been proposed to exist but have never been identified. Here we identify a protein stabilizing free α-haemoglobin by using a screen for genes induced by the essential erythroid transcription factor GATA-1 (refs 4, 5). Alpha Haemoglobin Stabilizing Protein (AHSP) is an abundant, erythroid-specific protein that forms a stable complex with free α-haemoglobin but not with β-haemoglobin or haemoglobin A ($\alpha_2\beta_2$). Moreover, AHSP specifically protects free α-haemoglobin from precipitation in solution and in live cells. AHSP-gene-ablated mice exhibit reticulocytosis and abnormal erythrocyte morphology with intracellular inclusion bodies that stain positively for denatured haemoglobins. Hence, AHSP is required for normal erythropoiesis, probably acting to block the deleterious effects of free α-haemoglobin precipitation. Accordingly, AHSP gene dosage is predicted to modulate pathological states of α-haemoglobin excess, such as β-thalassaemia.