DEVELOPMENT AND DISEASE

The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome

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SUMMARY

Holt-Oram syndrome is one of the autosomal dominant human ‘heart-hand’ disorders, with a combination of upper limb malformations and cardiac defects. Holt-Oram syndrome is caused by mutations in the TBX5 gene, a member of a large family of T-box transcription factors that play important roles in cell-type specification and morphogenesis. In a screen for mutations affecting zebrafish cardiac function, we isolated the recessive lethal mutant heartstrings, which lacks pectoral fins and exhibits severe cardiac dysfunction, beginning with a slow heart rate and progressing to a stretched, non-functional heart.

We mapped and cloned the heartstrings mutation and find it to encode the zebrafish ortholog of the TBX5 gene. The heartstrings mutation causes premature termination at amino acid 316. Homozygous mutant embryos never develop pectoral fin buds and do not express several markers of early fin differentiation. The total absence of any fin bud differentiation distinguishes heartstrings from most other mutations that affect zebrafish fin development, suggesting that Tbx5 functions very early in the pectoral fin induction pathway. Moderate reduction of Tbx5 by morpholino causes fin malformations, revealing an additional early requirement for Tbx5 in coordinating the axes of fin outgrowth. The heart of heartstrings mutant embryos appears to form and function normally through the early heart tube stage, manifesting only a slight bradydcardia compared with wild-type siblings. However, the heart fails to loop and then progressively deteriorates, a process affecting the ventricle as well as the atrium.

Relative to mammals, fish require lower levels of Tbx5 to produce malformed appendages and display whole-heart rather than atrial-predominant cardiac defects. However, the syndromic deficiencies of tbx5 mutation are remarkably well retained between fish and mammals.

Key words: Holt-Oram syndrome, Zebrafish, T-box, Tbx5, Heart, Pectoral fin, Limb induction

INTRODUCTION

Members of the T-box family of transcription factors contain a highly conserved DNA binding motif of 180-190 amino acid residues (Murray, 2001; Simon, 1999; Tada and Smith, 2001). T-box genes are represented throughout metazoan evolution, and vertebrates alone include over 20 family members (Tada and Smith, 2001). T-box genes play key roles in cell-type specification and morphogenesis (Smith, 1999), and mutations in several T-box genes have been shown to result in human developmental disorders (Murray, 2001). For example, mutations in TBX5 cause human Holt-Oram syndrome, characterized by congenital defects in the heart and upper limbs (Basson et al., 1997; Li et al., 1997). TBX3 mutations produce ulnar-mammary syndrome (Bamshad et al., 1997; Bamshad et al., 1999). Mutations in TBX22 cause the rare syndrome CPX (X-linked cleft palate and tongue-tie) (Braybrook et al., 2001). Mutations in TBX1 are responsible for the DiGeorge syndrome (Lindsay et al., 2001; Merscher et al., 2001).

The function of tbx5 in the heart appears to be exquisitely sensitive to gene dosage, since either haploinsufficiency or gene duplication can produce the cardiac abnormalities associated with Holt-Oram syndrome (Dixon et al., 1993; Hatcher and Basson, 2001; McCorquodale et al., 1986; Melnyk et al., 1981). The sensitivity to dose may be explained in part by the synergistic interaction of Tbx5 with other transcription factors, including Nkx2.5 (Bruneau et al., 2001; Hiroi et al., 2001), and its activation or co-activation of multiple downstream targets including connexin40 and atrial natriuretic factor (Bruneau et al., 2001; Ghosh et al., 2001; Hiroi et al., 2001). Although tbx5 is expressed early in the cardiogenic mesoderm prior to heart tube formation (Bruneau et al., 1999; Chapman et al., 1996; Horb and Thomsen, 1999; Liberatore et al., 2000), specific functions in heart field specification have not been identified. Several tbx genes may play overlapping...
functions in early decisions of cardiac cell fate, as suggested by complete elimination of cardiac tissue by dominant negative effects of tbx5-engrailed constructs injected into X. embryo (Horb and Thomsen, 1999). The earliest described phenotypic effects in mice with a targeted mutation in tbx5 (tbx5−/−) mice include hypoplasia of the inflow tract, atria, and to a lesser extent, the left ventricle (Bruneau et al., 2001). Dominant effects observed in heterozygous human or Tbx5+− mice include atrial and ventricular septal defects, and electrophysiological defects, particularly atrioventricular block (Bruneau et al., 2001; Newbury-Ecob et al., 1996).

tbx5 is expressed in a dynamic pattern in the developing heart tube. At early stages, human (Hatcher et al., 2000a; Li et al., 1997), mouse (Bruneau et al., 1999; Chapman et al., 1996; Christoffers, 2000; Liberatore et al., 2000), chicken (Bruneau et al., 1999; Liberatore et al., 2000) and frog (Horb and Thompson, 1999) show somewhat different patterns of tbx5 expression, but by completion of cardiac looping all express tbx5 in a posterior-to-anterior gradient with highest levels of expression in the atria and absence of expression in the conotruncus or outflow tract. tbx5 is expressed in the heart of zebrafish (Begemann and Ingham, 2000; Ruvinsky et al., 2000), but its anteroposterior expression pattern was not described. Anteroposterior localization appears to be important, because engineered persistent expression of mouse tbx5 or Tbx4 in chick partially transforms the heart into an anterior heart tube morphogenesis (Liberatore et al., 2000).

Tbx5 also plays a role in the developing limb (Simon, 1999; Tamura et al., 2001). tbx5 is expressed in the forelimb, and tbx4 in the hindlimb, suggesting that tbx5 controls forelimb identity, and tbx4 hindlimb identity (Gibson-Brown et al., 1998; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998). Ectopic expression of Tbx5 or Tbx4 in chick partially transforms the identity of developing limbs, in accordance with this suggestion (Logan and Tabin, 1999; Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). The skeletal defects observed in patients with Holt-Oram syndrome, or experimentally, in limbs produced from ectopic buds in chick, indicate that Tbx5 also coordinates later limb outgrowth. Limb deformities in the Holt-Oram syndrome range from mild thumb deformities to a near absence of the arm (phocomelia) (Basson et al., 1994; Newbury-Ecob et al., 1996).

We here report the isolation of a recessive mutation that affects heart and pectoral fin formation in the zebrafish embryo. The pectoral fin is homologous to the tetrapod forelimb (Tamura et al., 2001). heartstrings (hst) is an unusual mutation in that even the earliest molecular evidence of pectoral fin bud generation is absent. The heart in hst mutant embryos forms relatively normally, but deteriorates during looping and later stages. We positionally cloned the hst locus, and show it encodes the zebrafish ortholog of tbx5. The hst mutation appears to eliminate Tbx5 function entirely, but we find Tbx5 function is markedly dose-dependent, especially in fin bud formation.

MATERIALS AND METHODS

Zebrafish lines
We identified the hst^m21 mutation in a screen for ENU-induced mutations on a TL background, as described previously (Chen et al., 2001). We maintained mutations by outcrossing to standard wild-type lines (WIK and TL). Embryos were raised and staged as described previously (Kimulc et al., 1995).

Meiotic and physical mapping of hst
We mapped the hst locus to the telomere of linkage group 5 using bulked segregant analysis (Michelmore et al., 1991) on a panel of 120 microsatellite markers from the zebrafish genetic map (Knapi et al., 1998). DNA of homozygous hst^m21 embryos (4974 meioses) was tested against individual microsatellite markers (Z markers) to refine the critical interval. BAC clones were identified by screening DNA pools by PCR (Incyte Genomics). Simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs) or simple sequence length polymorphisms (SSLPs) were defined in BAC end sequences and used to develop genetic markers for fine mapping. Genetic and physical maps were coordinated using the Goodfellow radiation hybrid (RH) panel (Geisler et al., 1999).

Identification of the hst mutation
RNA was extracted from wild-type or homozygous hst embryos aged 48 hours post-fertilization (hpf). RT-PCR of the tbx5 gene was performed using the Promega Access kit and primers tbxF4 (TTTGTGTGTTATAGGATTC) and tbxB2 (AGCAATAG-TTTGTTGCTT), followed by nested PCR with primers tbxF3 (GGAATTTAACGCTTGCTGA) and tbxB1 (TGATGTTGCAATGGCCTT). PCR products of four independent RNA samples were sequenced on both strands and aligned using the MacVector gene analysis program.

In situ hybridization, antibody, skeletal staining and histology
Whole-mount in situ hybridization and antibody staining was carried out as described previously (Thisse et al., 1993). Antibodies were obtained from Dr Frank Stockdale (MF-20 and S46). For skeletal stains, whole pectoral fins were excised from adults and fixed overnight in 4% paraformaldehyde. Alcian Blue/Alizarin Red staining was performed as described previously (Grandel and Schulte-Mmerker, 1998). For sectioning, embryos were mounted in JB-4 plastic medium (Polysciences), sectioned at 5 µm, stained with Methylene Blue/Fuchs in (Grandel and Schulte-Mmerker, 1998) and photographed with a Zeiss Axioscope.

Morpholino treatment
The morpholino antisense oligonucleotide “tbx-MO” (5’ GAAAG- GTGTCTTCACTGTCCGCAT 3’) was designed against the tbx5 translatable start site and purchased from Gene Tools, LLC. Wild-type or hst embryos primarily at the 1-cell stage with chori on intact were injected with ~2 nl of stocks ranging from 750 to 100 µM (12.4 to 1.7 ng) of morpholino diluted in Danieau’s solution.

RESULTS

hst is required for heart growth and pectoral fin induction
We identified the ENU-induced allele heartstrings^m21 (hst) in a large-scale screen for recessive lethal mutations that perturb cardiac function. Homozygous hst mutant embryos have severe cardiac and pectoral fin abnormalities, and die between 6 and 7 days post-fertilization (dpf). Heterozygous hst embryos have normal anatomy, and adults are viable and fertile.

The morphology of the initial heart tube of hst mutant
embryos at 24-26 hpf is indistinguishable from wild type. Both atrium and ventricle are evident and contract normally. The one subtle difference from wild type is heart rate. From the onset of cardiac contraction, hearts of hst mutant embryos beat slower than those of their wild-type siblings (Table 1). Measurements of heart rate (beats/minute) at 25 hpf, the earliest time-point at which regular heart rates could be scored, indicate the heart rate of hst mutant embryos is approximately 84.9% of wild-type siblings. By 2 dpf, the heart rate of hst mutant embryos is 77.6% of the wild-type siblings. Heart rates of heterozygote embryos are not different from wild type.

The first morphological defect in hearts of hst mutant embryos is failure to complete looping. The atrium completes the first step of situs formation (Chen et al., 1997), jogging to the left at 24 hpf. Thereafter, hst hearts remain central and linear (Fig. 1A,B). Circulation is vigorous in hst mutant embryos through 2 dpf. However, shortly thereafter hst hearts slowly stretch to a thin, ‘string-like’ morphology and circulation ceases by 3 or 4 dpf (Fig. 1C-E), as contractility progressively decreases. Pericardial edema is present at 2 dpf and frequently massive by 3 dpf (Fig. 1E). The extent of pericardial edema and degree of heart stretching varies with different WIK genetic backgrounds (Fig. 1D,E).

hst mutant embryos do not develop pectoral fins. Moreover, in contrast to nearly all zebrafish pectoral fin mutants described to date (Allende et al., 1996; Barresi et al., 2000; Begemann and Ingham, 2000; Begemann et al., 2001), hst mutant embryos do not produce any morphologically recognizable fin buds (Fig. 1F,G). In wild-type fins outgrowth begins around 26 hpf as mesodermal cells of the fin field assume a perpendicular arrangement with respect to the basement membrane of the epidermis, begin to proliferate, and push the overlying epidermis outward (Grandel and Schulte-Merker, 1998; van Eeden et al., 1996; Yelon et al., 2000). Small buds are apparent by 28 hpf. As buds grow out, the initially unstructured epidermal layer develops into the apical fold, analogous to the tetrapod apical ectodermal ridge (AER) (Neumann et al., 1999). In cross-sections of 48 hpf hst embryos, we observe small bilateral patches of dark-staining somatopleure mesodermal cells in the area from which wild-type buds emanate (Fig. 2A,B). By 3 dpf, these mesenchymal cells occasionally assume an orientation perpendicular to the basement membrane, but they do not proliferate further (Fig. 2C). No buds are evident in hst embryos examined up to day 6 (Fig. 1H,I), indicating that bud growth is not simply delayed.

The hst locus encodes the T-box transcription factor Tbx5

We identified the hst gene by positional cloning. We mapped the hst mutant locus to the telomere of LG5, between Z10827 and Z22208, and generated a physical contig bridging these two Z markers (Fig. 3A). Polymorphism in BAC-end sequences provided a means of generating SNP and SSCP markers subsequently used to map recombinants. Analysis of 4974 meiosis narrowed the critical interval to 0.08 cM (Fig. 3A). The tbx5 gene (AF185283) mapped within 2 cRad of the hst locus on the Goodfellow RH panel. tbx5 was an excellent candidate for hst, since mutations in human TBX5 result in congenital heart defects accompanied by malformations of the forelimb. We sequenced tbx5 cDNA generated by RT-PCR in hsm21 mutant embryos, and found a C to T transition at base pair 1356, which converts a glutamine to a premature stop codon (Q316Stop; Fig. 3B,C). The glutamine residue 316 is conserved in humans, chick, mouse and newts, but is not a site of previously described mutations.

The hst mutation lies approximately two-thirds of the way

### Table 1. Comparison of heart rate in hst and wild-type embryos

<table>
<thead>
<tr>
<th>Embryo age</th>
<th>hst</th>
<th>Wild type</th>
<th>% of wild type</th>
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<tr>
<td>25 hpf</td>
<td>65.19±12.00</td>
<td>76.77±10.32</td>
<td>84.92</td>
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<tr>
<td>48 hpf</td>
<td>116.43±12.66</td>
<td>150.03±15.21</td>
<td>77.60</td>
</tr>
<tr>
<td>72 hpf</td>
<td>110±24.03</td>
<td>172.68±16.20</td>
<td>64.03</td>
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Values are beats per minute ± standard deviation. At least 45 embryos were scored for every timepoint. hst and wild type values are significant (P<0.001) at every timepoint.
into the \textit{tbx5} open reading frame (Fig. 3C). If translated, the predicted mutant protein would contain the complete T-box binding domain and a portion of the carboxy-terminal region. The T-box domain of \textit{tbx5} modulates its DNA binding and protein dimerization activities, while the carboxyl-terminal region contains elements that positively regulate binding (Hiroi et al., 2001).

Mutant proteins that retain the ability to dimerize or to bind DNA without concomitant trans-activation have the potential to exert dominant negative activity (Veitia, 2002). Indeed, carboxy-terminal truncations in the T-box protein Brachyury with dominant negative activity have been described (Herrmann, 1991). However, we do not have genetic evidence for dominant effects of the \textit{hst} mutation, and \textit{hst} heterozygotes are phenotypically indistinguishable from wild-type embryos. The number of mutant offspring from \textit{hst} heterozygote incrosses is 25.8% (379/1396).

Premature nonsense codons can destabilize the transcripts leading to nonsense mediated decay (Nagy and Maquat, 1998). However, \textit{hst} transcripts are expressed in mutant embryos by in situ hybridization at levels comparable to wild type, suggesting that RNA stability is not markedly reduced by the mutation.

**Morpholino-mediated translational inhibition of Tbx5 phenocopies \textit{hst}**

To examine whether \textit{hst} defects are due to loss of Tbx5 function, we reduced Tbx5 levels by injection of morpholino-modified antisense oligonucleotides. Morpholinos inhibit the translation of specific RNA target molecules and have been shown to phenocopy a number of early zebrafish mutations (Heasman, 2002). We used a morpholino directed against the \textit{tbx5} translational start site (\textit{tbx5-MO}), and find injection of 12.4 ng produces a progression of heart and limb defects indistinguishable from those in \textit{hst} mutant embryos in 93% (272/292) of injected embryos (Fig. 4). At 25 hpf, the mean heart rate of morphant-injected embryos is 82.6% of uninjected embryos (\textit{n}=43 morphants, 44 uninjected controls), a rate similar to \textit{hst} embryos of the same age (Table 1). This progresses to failure of looping and cardiac dysfunction with a string-like heart. Fin buds do not form. We observed a milder version of the \textit{hst} phenotype (see below) in 2.1% (6/292) of injected embryos. 5.1% (15/292) of embryos displayed defects in trunk and tail development, or necrosis in the CNS, common non-specific defects noted with injection of other morpholinos delivered at high

![Fig. 2. Arrested pectoral fin bud induction in \textit{hst} mutant embryos. (A,B) Methylene Blue-stained cross sections through 48 hpf embryos. (A) Wild-type embryos have pectoral fin buds (arrows). (B) \textit{hst} mutant embryos retain small populations of presumptive fin mesenchymal cells (arrowheads) that fail to proliferate. (C) Methylene Blue-stained transverse sections of 3 dpf mutant embryos. Presumptive fin mesenchyme is usually present as a flat patch (arrowhead), but occasionally cells turn perpendicular to the basement membrane (double arrowhead), but do not proliferate further.](image)

![Fig. 3. Cloning and mapping of zebrafish \textit{hst}. (A) An integrated genetic and radiation hybrid (RH) panel map of the telomere of linkage group 5 (LG5), showing selected CA-repeat ‘Z’ markers, and the number of recombinant events detected from 4974 meioses. The position of \textit{tbx5} on the RH panel coincides with the critical genetic interval defined by linkage mapping of the \textit{hst} locus. (B) Sequencing of the \textit{tbx5} cDNA reveals a C to T change at base pair 1356 in \textit{hst} mutant embryos. This mutation changes residue 316 (glutamine) to a premature stop codon. (C) A schematic of the \textit{tbx5} cDNA indicating the extent of the T-box domain and the location of the \textit{hst} stop codon.](image)
Zebrafish heartstrings mutant is tbx5 (Ekker and Larson, 2001; Imai and Talbot, 2001). Lower doses of tbx5-MO (1.7 ng), phenocopy the mutation in 64% (76/118) of embryos, and cause a milder version with slight cardiac dysfunction, little or no pericardial edema, and undersized, delayed pectoral fin buds (Fig. 4E,H) in 30% (33/118) of embryos. Similar effects of tbx5 morpholinos on pectoral fin development were recently described by Ahn et al. (Ahn et al., 2002).

The phenocopy produced by tbx5-MO is not more severe than the hst phenotype, suggesting that hst<sup>m21</sup> is a strong allele, possibly null. To test this further, we injected tbx5-MO into offspring from two heterozygous hst parents. Tbx5-MO injection does not enhance the phenotype further in homozygous hst mutant embryos. Moreover, a low dose of tbx5-MO (1.7 ng) is sufficient to “convert” hst heterozygotes to a homozygous mutant phenotype, suggesting that hst mutation exerts its effects by affecting gene dose via loss-of-function rather than dominant-negative mechanisms.

**tbx5 expression pattern in the heart and fin buds**

tbx5 expression in the heart of hst mutant embryos is identical to wild type, when analyzed by RNA in situ hybridization. tbx5 transcripts appear as bilateral stripes in the lateral plate mesoderm (LPM) beginning at the 6- or 7-somite stage (Begemann and Ingham, 2000; Ruvinsky et al., 2000; Tamura et al., 1999; Yelon et al., 2000). The domain of tbx5 expression expands mediolaterally between the 10- and 15-somite stage. Thereafter, anterior (heart-forming) regions migrate medially, whereas the posterior (fin-forming) regions remain broadly spread across the yolk. At the 20-somite stage, when the heart tube is first assembled as a cone, tbx5 is expressed in the myocardial precursors as well as bilaterally in broad posterior regions that constrict by 26 hpf to form roughly circular fin fields.

Patterns of tbx5 expression in the heart differ among species, and have not previously been described in detail beyond 30 hpf in the zebrafish (Begemann and Ingham, 2000; Ruvinsky et al., 2000). At 26 hpf, expression in the presumptive

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**Fig. 4.** Translational inhibition by tbx5 morpholino. (A-K) Live embryos; (A,C,F,I) wild-type embryos; (B,D,G) embryos injected with 12.4 ng tbx5 morpholino (tbx5-MO); (E,H,J,K) embryos injected with 1.7 ng tbx5-MO. (A,B) Injection of 12.4 ng of tbx5-MO phenocopies heart stretching and pericardial edema of hst mutant embryos, and (C,D) eliminates bud formation at 48 hpf. (E) Injection of 1.7 ng of tbx5-MO delays the onset of bud formation to shortly before 48 hpf. (F,G) 12.4 ng tbx5-MO continues to inhibit bud formation at 3 dpf. (H) Buds of 1.7 ng-injected embryos grow slowly. (I-K) 1.7 ng-injected embryos in which the initial buds develop into shorter up-turned fins. (J) Some embryos develop only one bud and hence one fin. (A-E) Lateral views; (F-K) dorsal views. a, atrium; v, ventricle; arrowheads, developing pectoral fin buds; brackets, regions where fin bud failed to develop.

**Fig. 5.** tbx5 expression in the heart tube. (A,B) In situ hybridization of 26 hpf embryos. (A) At 26 hpf, tbx5 expression is detectable first in the presumptive atrium. (B) Upon longer exposure to substrate, tbx5 expression extends through presumptive atrium and ventricle. Heart tubes of wild-type (shown) and hst mutant embryos (not shown) look identical at this stage. (C,D) In situ hybridization of 48 hpf embryos. (C) wild type; (D, hst) tbx5 expression is strongest in the ventricle, and weaker in the atrium. a, atrium; v, ventricle; black arrowhead, venous (atrial) end of heart tube; white arrowhead, arterial (ventricular) end of heart tube.
atrium is slightly greater than in the presumptive ventricle (Fig. 5A,B). tbx5 expression in the heart tube of hst mutant embryos is identical to wild type at this and later stages, despite incomplete looping in hst mutant embryos. An atrial-enhanced pattern of tbx5 expression is noted in other species (Bruneau et al., 1999; Chapman et al., 1996; Christoffels et al., 2000; Hatcher et al., 2000b; Horb and Thomsen, 1999; Li et al., 1997; Liberatore et al., 2000). Surprisingly, in zebrafish the tbx5 expression gradient reverses in direction by 48 hpf, when looping is almost complete (Fig. 5C,D). At 48 hpf and beyond, tbx5 expression is strongest in the ventricle, weaker in the atrium, and absent in the inflow tract (Fig. 5C,D). We do not detect tbx5 expression in the outflow tract at any time. Thus, the pattern of tbx5 expression in the heart at later stages has polarity opposite to that of other vertebrate species.

Late onset cardiac deterioration in hst mutant embryos

hst mutant embryos show normal expression of nkh2.5, hand2, bmp4, cmcl2 and vhmc in the cardiac precursor regions at the 15-somite stage (Fig. 6). All of these markers are present in broad bilateral bands in the LPM, and show normal anteroposterior localization. These results suggest the heart field is properly demarcated and myocardial specification occurs normally in the hst mutant embryos.

In other species, tbx5 appears to play an important role in the assignment of atrial and ventricular cell fates in the heart (Bruneau et al., 2001; Liberatore et al., 2000). In zebrafish, atrial and ventricular lineages can be distinguished with molecular markers well before the chambers become morphologically discrete (Yelon et al., 1999; Yelon and Stainier, 1999). The presumptive ventricle expresses ventricular myosin heavy chain (vmhc) (Yelon et al., 1999), while the presumptive atrium expresses an atrial-specific myosin heavy chain recognized by the S46 antibody (Stainier and Fishman, 1992). Both vmhc and S46 are expressed in the heart of hst mutant embryos. At 33 hpf (not shown), wild-type and hst mutant embryos express versican broadly in the atrium and weakly in the ventricle. (E,F) 48 hpf; (G,H), 72 hpf; (E,G) wild-type embryos restrict versican expression to the AV boundary, but (F,H) hst mutant embryos fail to undergo this transition and continue to express versican predominantly in the atrium, with weak expression in the ventricle. (I-L) bmp4 expression in the heart. (I) Wild-type and (J) hst mutant embryos express bmp4 in the ventricle and inflow tract at 48 hpf. By 72 hpf (K) wild-type embryos restrict bmp4 to the AV junction, but (L) hst mutant embryos retain ventricle-enriched expression. (M-P) versican expression in the developing ear. At 48 hpf, (M) wild-type and (N) hst mutant embryos express versican broadly in the otic placode. By 72 hpf, (O) wild-type and (P) hst mutant embryos both restrict versican to the otoliths (Mowbray et al., 2001), suggesting the heart-specific defects in hst mutant embryos are not due to general developmental delay. a, atrium; av, atrioventricular boundary; i, inflow tract; op, otic placode; ot, otoliths; pf, pectoral fin bud; v, ventricle.
The pectoral fin field fails to differentiate in hst mutant embryos

We examined the stage at which the tbx5 mutation perturbs fin development. Normally, between the 10- and 15-somite stages, LPM fin field precursors spread mediolaterally (Yelon et al., 2000). tbx5 expression, present in the LPM from the 7-somite stage onward, becomes restricted to a dorsal patch coincident with the presumptive fin field by 26 hpf. tbx5 expression continues in the mesenchyme of the outgrowing fin bud from 48 hpf (Fig. 9A-C) through 5 dpf (Begemann and Ingham, 2000). In hst mutant embryos, the pattern of tbx5 expression in the LPM is normal up to the 20-somite stage. At 26 hpf, when fin bud outgrowth would normally initiate, tbx5 expression in the posterior LPM fails to condense into a circular patch and levels of transcript decrease greatly (Fig. 9A’). We detect only a few tbx5-positive cells by 32 hpf (Fig. 9B’), and no expression in the pectoral fin-forming region of the LPM at 48 hpf (Fig. 9C’).

Fig. 9. Fin bud development in hst mutant embryos. (A-I) Wild-type embryos, (A’-I’) hst mutant embryos. (A-C’) tbx5 expression in the developing pectoral fin field. At 26 hpf, (A) wild-type embryos restrict tbx5 expression to a circular patch, the presumptive fin field, in the dorsal LPM, but (A’) hst mutant embryos display a more dispersed, less abundant expression. (B,B’) At 32 hpf and (C,C’) at 48 hpf, (B,C) wild-type embryos continue tbx5 expression in the developing bud mesenchyme, but (B’,C’) in hst mutant embryos, tbx5 expression is greatly decreased (B’) at 32 hpf and (C’)) undetectable in the LPM thereafter. (D-F’) Expression of molecular markers of early limb bud induction. (D,D’) At 32 hpf, wild-type embryos express shh expression in the pectoral fin mesenchyme, but hst mutant embryos do not. (E,E’) Likewise, at 32 hpf dlx2 is expressed in the apical fold of wild-type but not hst mutant embryos. (F,F’) At 48 hpf, bmp4 is present in the apical fold of wild-type but not hst mutant embryos. (G-I’) hand2 expression in the developing fin field. (G,G’) At 24 hpf, wild-type embryos express hand2 broadly in the region of the LPM encompassing the fin field, but hst mutant embryos express hand2 faintly in only a few LPM cells. (H,H’) At 32 and (I,I’) at 48 hpf, wild-type embryos (H,I) express hand2 in the fin bud mesenchyme, but hst mutant embryos (H’,I’) have no detectable hand2 expression in the LPM. Arrows in H and G’ indicate cells weakly expressing hand2.

From 28 hpf, wild-type embryos express shh in the posterior mesenchyme (the ZPA; Fig. 9D) (Krauss et al., 1993; Neumann et al., 1999; Sordino et al., 1995), and dlx2 and bmp4 in the posterior LPM are normally expressed in the presumptive fin field by 26 hpf.
apical epidermis (Fig. 9E,F) (Akimenko et al., 1994; Martinez-Barbera et al., 1997; Neumann et al., 1999). hst mutant embryos never express these three markers in the dorsal LPM, although expression of these genes in other regions of the embryo is normal (Fig. 9D′-F′).

The bHLH factor hand2 normally is expressed in posterior LPM and developing fin (Fig. 9G-I). hst mutant embryos show normal hand2 expression up to the 20-somite stage, greatly reduced expression at 24 hours (Fig. 9G′) and no expression at 32 or 48 hpf (Fig. 9H′,I′). The expression of hand2 and tbx5 in the presumptive fin field prior to bud outgrowth suggests that some fin precursor cells differentiate in hst mutant embryos, but the buds fail to grow out. Thus, mutation of the hst gene reveals an early, essential requirement for Tbx5 in the process of fin field determination or fin bud induction.

**Pectoral fins anomalies caused by reduction in Tbx5**

The haploinsufficient effects of tbx5 mutations in human and mouse suggest that Tbx5 functions in the heart and limb are sensitive to dose. Clearly, the zebrafish is not affected by the same degree of Tbx5 deficiency, since hst heterozygous fish do not display a mutant phenotype. We progressively lowered levels of Tbx5 in the embryo by varying the concentrations of Tbx5 morpholino injected. Upon injection of 1.7 ng, we observe small pectoral fin buds in about 30% of injected embryos by 48 hpf (Fig. 4E). Although bud growth or initiation is delayed for several hours, all embryos that ultimately develop fins have a morphologically observable bud by 48 hpf.

Delayed buds continue to grow but typically produce abnormal embryonic fins that are shorter along the proximal-distal axis (Fig. 4J,K). Some embryos develop a bud on one side only, and subsequently grow a single fin (Fig. 4K). Some fins develop as stubby appendages, while other fins are overtly normal proximally, but the distal half of the fin folds upwards.

To assess the development of cartilaginous elements that support and shape the fin, we examined histological sections through the developing fins (Fig. 10A-D). Fins of hst mutant embryos are shorter, and sometimes severely stunted in growth. Endoskeletal development in fins is often normal proximally, but defective in the distal up-turned portion (Fig. 10B). In stubby appendages, the endoskeletal disc is several cell layers thick (Fig. 10C,D).

Because of the profound effect of Tbx5 haploinsufficiency in humans and mice (Basson et al., 1994; Bruneau et al., 2001; Newbury-Ecob et al., 1996), we wondered if protracted exposure to this degree of molecular deficiency would elicit fin defects in heterozygous adult fish. Therefore, we examined Alcian Blue/Alizarin Red histological preparations of whole adult pectoral fins (n=9 heterozygotes, 9 wild-type controls). We detected no defects in lepidotrichia formation, branching, or attachment of rays to the pectoral girdle at the base of the fin, or in overall fin shape (Fig. 10E,F). Thus, the effects of the hst mutation on zebrafish fin formation are fully recessive.

**DISCUSSION**

**The hst locus encodes Tbx5**

We here report a mutation in the zebrafish tbx5 ortholog as the cause of the heartstrings phenotype. In both the zebrafish hst mutant and in human Holt-Oram syndrome, a deficiency of Tbx5 perturbs heart and forelimb development. The cardiac defects of hst mutant embryos begin soon after the heart tube formation, as a subtle bradycardia, and progress by 3 dpf to stretching and functional failure of both chambers. The hst fin defect is quite pronounced, with failure to develop any molecular or histological evidence of a pectoral fin bud. Phenotypes that perturb fin outgrowth in ways more akin to human haploinsufficiency defects can be elicited by morpholino-induced lowering of the Tbx5 level. This suggests that the dominant nature of the tbx5 mutation described in humans may reflect a greater sensitivity to Tbx5 level rather than an essential difference in mechanism.

**Tbx5 is required for the maturation of cardiac function**

Clearly, Tbx5 is essential for normal growth of both chambers of the zebrafish heart. The timing of onset of phenotype well after heart tube formation suggests that the defect does not affect early cardiac progenitors, but rather subsequent growth.
and differentiation. The specific nature of the cellular pathophysiology remains to be determined.

In humans, the atrium is associated with the most severe and most penetrant aspects of Tbx5 mutation, although defects associated with the ventricle and other areas of the heart have been described (Bruneau et al., 1999; Bruneau et al., 2001; Hatcher et al., 2000a). In mice homozygous for Tbx5 deficiency, sinoatrial structures and the primitive left ventricle are severely hypoplastic (Bruneau et al., 2001). The inflow tract of hst mutant embryos appears normal, and cardiac defects include both atrium and ventricle. The particular sensitivity of the atrium in human and mouse may reflect, in part, the tbx5 expression gradient, which is higher in atrium than ventricle in those species. In the zebrafish, we find that tbx5 expression extends throughout both chambers, through the initial looping stages. At 48 hpf, the time when deterioration is clearly evident, tbx5 expression is greater in the ventricle than the atrium, i.e., a pattern reversed relative to other vertebrates.

tbx5 is believed to be important for atrial-ventricular patterning of the heart in other species. In mouse, engineered persistent expression of tbx5 in the entire heart tube perturbs chamber-specific gene expression and retards ventricular chamber morphogenesis (Liberatore et al., 2000). Overexpression of tbx5 in cultured human cardiomyocytes inhibits growth and proliferation, suggesting the function of TBX5 is to act as a brake on cellular proliferation (Hatcher and Basson, 2001). Thus, higher expression of tbx5 in atrial tissues may contribute to their development as thin-walled structures, as opposed to the thick-walled trabecular ventricular chambers (Hatcher and Basson, 2001). However, the expression patterns of markers of early cardiac mesoderm and chamber-specific markers are normal in hst mutant embryos, suggesting that the heartstrings mutation does not perturb atrial-ventricular patterning in zebrafish. In fact, the predominant cardiac defects of hst mutant embryos become evident well after the development of discrete morphological chambers that express chamber-specific markers and after the onset of circulation.

It is of interest, however, that hst mutant embryos display an early bradycardia. Pacemaking originates in the sinoatrial region, suggesting that it might be affected as in other species. Bradycardia may be an accompaniment to deficiencies throughout the heart (Warren et al., 2001). Alternatively, Tbx5 could affect the adhesive or conductive properties of cardiomyocytes by regulating the expression of downstream genes such as connexins. In mouse, TBX5 interacts with other transcription factors such as Nkx2.5 to regulate downstream targets with important roles in cardiac growth (ANF) and conduction (Cx40) (Bruneau et al., 2001).

Tbx5 is essential for pectoral fin induction

hst mutant embryos never generate pectoral fin buds, and do not maintain expression of tbx5 or hand2 within the differentiating fin field. hst mutant embryos do not express markers of the apical fold (dlx2, bmp4) or ZPA (shh), suggesting fin bud induction is blocked. Thus, the heartstrings mutant reveals an essential role for Tbx5 in the earliest stages of bud formation, either in fin bud induction itself or in the determination of the fin field as it coalesces within the lateral plate mesoderm. In other species, FGF10 is a strong candidate for the initial mesoderm-inducing signal. Targeted mutation of fgf10 or its receptor FGFR2 in mouse results in a complete or nearly complete elimination of both fore- and hind-limb buds (Min et al., 1998; Xu et al., 1998; Sekine et al., 1999). Proposed regulators of fgf10 include wnt-2b and wnt-8c (Kawakami et al., 2001). Regulatory loops between Tbx5 and the FGF, WNT and BMP signaling pathways have been proposed (Gibson-Brown et al., 1998; Ohuchi et al., 1998; Rodriguez-Esteban et al., 1999) but the precise relationships are not fully resolved. Once fgf10 and the relevant wnts are cloned in zebrafish, the hst mutant can be used to assess the role of Tbx5 in known pathways of limb/fin induction.

Low level inhibition of Tbx5 by morpholino perturbs fin shape, size and endoskeletal development, suggesting an additional role for Tbx5 in coordinating pectoral fin outgrowth. Similarly, in the chick, down-regulation of Tbx5 caused by blockage of FGF signaling in the limb causes loss of the radius and digits (Rodriguez-Esteban et al., 1999). In humans, tbx5 haploinsufficiency results in forelimb (arm) deformities ranging from subtle abnormalities of the thumb or carpel bones to severe shortening of the arm (OMIM#142900) (Newbury-Ecob et al., 1996). These limb defects in Holt-Oram syndrome are often bilateral but can be asymmetric (Basson et al., 1999). The defects from Tbx5 reduction in the zebrafish also can be asymmetric in severity, suggesting that there may quite sensitive thresholds for effects, as occurs for certain cell fates in response to morphogens (Gudon and Bourillot, 2001). Alternatively, mosaic distribution of morpholino could account for the asymmetry, but this seems less likely because we injected morpholino into one-celled eggs, and there is no clear left-right allocation of blastomeres to left or right sides in the zebrafish, as is the case in the frog (Abdelilah and Driever, 1997).

The essential elements of many organs, including heart (Kupperman et al., 2000; Rottbauer et al., 2001; Sehnert et al., 2002; Walsh and Stainier, 2001; Xu et al., 2002), blood (Childs et al., 2000; Liao et al., 2002; Lyons et al., 2002), eye (Hutson and Chien, 2002; Malicki et al., 2002; Vihitelc and Hyde, 2002), gut (Allende et al., 1996), kidney (Serluca et al., 2002; Sun and Hopkins, 2001) and bone (Popperl et al., 2000; Schilling et al., 1996) have proved decipherable by zebrafish mutational analysis. The essential genes and their mode of action for normal organ formation clearly are conserved through vertebrate evolution. Similarly, the organotypic elements perturbed in human genetic syndromes are mirrored in the fish with great fidelity.

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