Distinct tissue-specific requirements for the zebrafish tbx5 genes during heart, retina and pectoral fin development

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1. Summary

The transcription factor Tbx5 is expressed in the developing heart, eyes and anterior appendages. Mutations in human TBX5 cause Holt–Oram syndrome, a condition characterized by heart and upper limb malformations. Tbx5-knockout mouse embryos have severely impaired forelimb and heart morphogenesis from the earliest stages of their development. However, zebrafish embryos with compromised tbx5 function show a complete absence of pectoral fins, while heart development is disturbed at significantly later developmental stages and eye development remains to be thoroughly analysed. We identified an overlap tbx5 gene in zebrafish—tbx5b—that is co-expressed with its paralogue, tbx5a, in the developing eye and heart and hypothesized that functional redundancy could be occurring in these organs in embryos with impaired tbx5a function. We have now investigated the consequences of tbx5a and/or tbx5b downregulation in zebrafish to reveal that tbx5 genes have essential roles in the establishment of cardiac laterality, dorsoventral retina axis organization and pectoral fin development. Our data show that distinct relationships between tbx5 paralogues are required in a tissue-specific manner to ensure the proper morphogenesis of the three organs in which they are expressed. Furthermore, we uncover a novel role for tbx5 genes in the establishment of correct heart asymmetry in zebrafish embryos.

2. Introduction

Tbx5 codes for a T-domain containing transcription factor that has been characterized in many vertebrate species, where it is widely expressed during the development of the heart, the eyes and the anterior set of appendages (tetrapod forelimbs and fish pectoral fins) [1–3]. Mutations in human TBX5 cause Holt–Oram syndrome (HOS; OMIM#142900), an autosomal-dominant ‘heart–hand’ condition characterized by heart and upper limb malformations [4,5]. Owing to its clinical relevance, several Tbx5 loss-of-function animal models have been developed to assess the role that this gene may play during the development of the vertebrate heart, limbs and eyes. These studies have shown that in mouse Tbx5-knockout embryos, both forelimb and heart development is severely impaired from the earliest stages of development [6–8]. Also, mis-expression of Tbx5 in the ventral retina produces altered projections of retinal ganglion cell (RGC) axons in chick embryos [9], consistent with a fundamental role for Tbx5 during eye morphogenesis.

The use of zebrafish as a model system has a series of advantages with respect to mouse, mainly their ease of embryo accessibility and the constant...
establishment of cardiac left–right asymmetry. A novel and fundamental role for these genes during the D-loop (right, normal), L-loop (left, reversed) and no-loop. Orientation of cardiac looping: Cle (v) and the atrium (a) parallel to each other (figure 1 b). The phenotypic similarities observed after ttx5a and ttx5b MO knock-down (figure 1d,e, respectively) in comparison to the characteristic looping observed in control morphants that results in the positioning of the ventricle (v) and the atrium (a) parallel to each other (figure 1a). However, in striking contrast to previous data, defects were observed not only in the degree of looping but also in the orientation of cardiac looping: ttx5 morphants can be classified into three distinguishable heart looping orientation groups (D-loop (right, normal), L-loop (left, reversed) and no-loop). After injection of a control MO, over 99% (n = 158) of the embryos displayed an S-shaped heart with the ventricle lying to the right-hand side of the embryo (D-loop; figure 1a,i). By contrast, 88% (n = 121) of the ttx5 MO-injected embryos had incomplete cardiac looping, and within these 57% showed D-loop, 24% showed L-loop and 19% showed no-looping at all (figure 1d,d′,i,j). Similarly, injection of ttx5b MO also caused heart looping defects (81%, n = 108), and these embryos displayed D-looped (52%), L-looped (16%) and no-looped (34%) cardiac morphologies (figure 1e,e′,i,j).

The phenotypic similarities observed after ttx5a or ttx5b knock-down prompted us to investigate whether these genes cooperatively regulate cardiac looping in contrast to what had been previously argued. To this end, we simultaneously knocked-down their function by co-injecting sub-optimal doses of ttx5a and ttx5b MOs (0.5 ng of each MO) which, when injected alone, did not affect cardiac looping (figure 1b,c,i; n = 75 for ttx5a MO and n = 71 for ttx5b MO). This showed that over 56% (n = 110) of the double-morphants had looping defects and the three D-loop, L-loop and no-loop phenotypes were detected (68%, 11% and 21%, respectively;

3. Results and discussion

To understand the unique and/or redundant roles that the ttx5 paralogues have during the development of the zebrafish heart, pectoral fin and eye fields, we have used MOs against ttx5a and ttx5b to downregulate their function during embryonic development either individually or in conjunction. Briefly, we used an anti-ttx5a MO oligonucleotide for the coding sequence [15], an anti-ttx5b oligonucleotide recognizing the 5′ UTR/coding sequence boundary—ttx5b(UTR)—as well as an anti-ttx5b oligonucleotide for the exon 3/intron 4 boundary (ttx5b(SP) MO). First of all, we characterized the functionality of our MOs by generating chimeric mRNAs containing the ttx5a or ttx5b(UTR) MO-recognition sites fused to enhanced green fluorescent protein (EGFP). Injection of these RNAs (100 pg) with or without their corresponding target MO (3 ng), showed that, indeed, co-injection of our ttx5a and ttx5b(UTR) MOs caused disappearance of EGFP signal (electronic supplementary material, figure S1a–d). In addition, to assess ttx5b gene knock-down efficiency, we performed RT-PCR experiments from embryos that had been injected with either a control MO or a ttx5b(SP) MO. This showed that an expected 215 bp (spliced) band was obtained in control embryos in contrast to the 791 bp (unspliced) band observed after injection of 2–4 ng of ttx5b(SP) MO (electronic supplementary material, figure S1e).
FIGURE 1. Knock-down of tbx5 genes causes cardiac looping defects. (a–c) Embryos injected with control MO or sub-optimal concentrations of tbx5a or tbx5b MOs. (d–g”) tbx5a morphant phenotypes. (e–e’) tbx5b-morphant phenotypes. (f–h”) Double knock-down of tbx5 genes (0.5 ng each MO (f–h”), 1.5 ng each MO (g–g”) and 3 ng each MO (h–h”)). (i) Quantification of the degree of looping phenotypes: wt, complete; phenotype, incomplete looping s.o., sub-optimal. (j) Quantification of the looping orientation phenotypes. A χ² statistic has been calculated to assess significant differences between groups (**p < 0.001, *p < 0.05). Images are frontal views of 48 hpf embryos, and myl7 expression is used to highlight the developing heart.

Figure 1f–f”,i,j) suggesting that, indeed, both genes act in the same pathway and cooperate with each other to ensure the completeness and orientation of looping of the zebrafish heart. Moreover, injection of increasing concentrations of both MOs caused an increase in the percentage of phenotypes, with 87% (n = 99) and 96% (n = 105) of double-morphants displaying looping phenotypes after injection with 1.5 and 3 ng of each MO, respectively. Although, in agreement with a previous report [17], the severity of the phenotype was not enhanced by double knock-down, downregulation of both genes increased the penetrance of the phenotype (figure 1i). Similarly to single and double-morphants injected with sub-optimal doses of the tbx5a and tbx5b MOs, these double-morphant embryos also exhibited looping orientation defects since the three orientation phenotypes were detected (figure 1g–g” h–h” j).

3.2. tbx5 morphants exhibit cardiac tube jogging defects

As heart looping orientation phenotypes are indicative of cardiac left–right asymmetry defects, we decided to examine whether heart jogging, the first morphologically evident break in the left–right symmetry of the zebrafish heart tube, was
Figure 2. *tbx5* morphants exhibit cardiac jogging and *lefty2* expression defects. (a–d’) *tbx5a* morphant phenotypes. (b–b’) *tbx5b* morphants. (c–e’) Left, right and middle jog phenotypes obtained by co-injection of *tbx5a* and *tbx5b* MOs at 0.5 ng (c–e’), 1.5 ng (d–d’) or 3 ng (e–e’) of each MO. (f) Control (ctrl) morphant. (g) Quantification of the phenotypes. (h–j) *lefty2* expression in control (h) and double-morphant (i,j) 22-somite stage embryos. (k) Quantification of the phenotypes. A $\chi^2$ test has been used to assess significant differences between groups (**p < 0.001). All images are dorsal views with anterior to the top, and *myl7* expression is used to highlight the developing heart tube in a–f.

and left of the ventricular cells due to their higher migration rates [19,20]. Second, and during the conserved process of cardiac looping, the ventricle is positioned to the right of the atrium. These two processes are defective in *tbx5a* and/or *tbx5b* morphants. Interestingly, known left–right cardiac determinants such as *Bmp4* have been isolated in screens aimed to find *tbx5*-induced genes [21], and bioinformatic approaches have highlighted the presence of *Bmp4*-binding sites in the vicinity of the *lefty2* locus (N. Mercader 2013, personal communication), another well-known left–right asymmetry determination factor. To address whether *tbx5* genes may be regulating *lefty2* expression in developing embryos, we assessed the expression of this gene after *tbx5* genes knock-down. In support of *tbx5* genes being upstream of *lefty2* expression, over half of the *tbx5a* and *tbx5b* double-morphants (52%, n = 106) showed no expression of *lefty2*, whereas 100% of control MO injected embryos (n = 43) had the characteristic left-sided expression of *lefty2* at the 22-somite stage (figure 2h–k). These experiments show that *tbx5* genes are upstream of *lefty2* expression, and hence the cardiac laterality phenotypes observed in *tbx5* morphants may be explained by this relationship between *tbx5* and the asymmetric gene *lefty2*. However, how bilaterally expressed genes such as *tbx5* can specifically regulate a left-side specific gene like *lefty2* remains unclear. Nevertheless, one can hypothesize that *lefty2* will only become activated in the left-hand side of the cardiac cone where *tbx5* acts with a co-activator that is only present in the left side of the developing embryo. Conversely, a repressor only found in the right side of the embryo may be inhibiting the activation of *lefty2* by *tbx5* genes in the right side of the cardiac cone.
The reasons for the discrepancies between our results, implicating the tbx5 genes in the asymmetry events the zebrafish heart undergoes (namely cardiac jogging first and looping later), and those of others [17] are unclear. One possibility is that we have used a MO against tbx5a but Moore et al [17] used the tbx5a mutant line hst to analyse the effects of this gene loss-of-function. The hst mutation introduces a premature STOP codon at residue 316 of the predicted open reading frame, which leaves the mutated protein with intact N-terminal and T-box (DNA binding) domains and part of the C-terminal domain. It is therefore possible that the hst mutation behaves as an hypomorphic allele with regard to the left–right asymmetric development of the heart. In agreement with this hypothesis, most of the Tbx5 mutations causing a clear HOS phenotype lie upstream of the predicted hst mutation. To test whether, indeed, the hst mutation behaves as an hypomorphic allele with regards to cardiac development, we developed an assay to assess whether the laterality phenotype of tbx5a heterozygous mutants could be rescued by introducing specific MO-insensitive forms of the tbx5a mRNA: (i) a full-length tbx5a mRNA that should be able to rescue the tbx5a MO-mediated phenotype, (ii) a tbx5a mRNA that is identical to that produced in hst mutant embryos and (iii) a severely truncated version of tbx5a that we have engineered by introducing a premature STOP codon within the T-box domain (figure 3a). Notably, both the full-length and the hst-like forms of tbx5a were able to partially rescue the cardiac jogging phenotype when co-injected with the tbx5a MO (figure 3b; n = 65 and n = 168, respectively). Similarly, a full-length tbx5b form was able to rescue the jogging phenotype of tbx5b morphants when co-injected with our tbx5b MO (figure 3b; n = 117). By contrast, the severely truncated form of tbx5a was not able to rescue the laterality phenotype (figure 3b; n = 79). Overall, these data demonstrate not only the specificity of the cardiac phenotypes caused by MO-mediated knock-down of tbx5a and/or tbx5b, but also that the hst mutation behaves as an hypomorphic allele with regard to cardiac laterality. We have ourselves analysed heart tube jogging in hst mutants (n = 38) and all of them displayed a normal leftward as visualized by myl7 expression in 26hpf embryos (figure 3c,d). These results underline the need for caution when using the hst mutation as a tbx5a loss-of-function allele.

3.3. tbx5 genes are essential for correct dorsoventral retina regionalization

Tbx5 genes are also conspicuously expressed in the dorsal retina, a feature conserved among vertebrates [1–3,22]. However, the consequences of Tbx5 loss-of-function in this domain have led to controversial results. Tbx5 gene function in the developing retina has been examined in developing chick embryos by mis-expressing this gene in the ventral domain, where it is not normally expressed. This caused dorsalization of the ventral retina as determined by upregulation of dorsal markers and downregulation of ventral ones, as well as altered projections of RGCs [9]. tbx5a knock-down in zebrafish led to downregulation of dorsal retina markers, while ventral markers did not seem to be affected and ganglion cell projections were not analysed [23]. We hypothesized that as tbx5a and tbx5b are co-expressed in the dorsal domain of the developing retina, functional redundancy may explain the controversial results found between different models. This prompted us to determine the consequences of the downregulation of tbx5 genes in this territory.

We analysed the expression of the dorsally expressed ephrin, efnb2a, and the ventrally expressed ephrin receptor, ephB2, because restricted ephrinB/ephiB expression along the dorsoventral axis has been shown to play a key role in retinotectal topographic map formation [24,25]. To quantify the extent of efnb2a expression, we measured the angle of expression of this gene by setting a ‘hinge’ in the centre of the lens (figure 4e). In control embryos, the efnb2a expression domain was measured to form an average angle of 63° (figure 4b,h). Knock-down of either tbx5a or tbx5b caused a reduction of the efnb2a angle of expression leading to an average angle of 54°, although this decrease was not found to be statistically significant (figure 4c,d,h). To investigate whether both paralogues function in conjunction to determine the extent of dorsal efnb2a expression, we co-injected sub-optimal doses of tbx5a and tbx5b MOs. Remarkably, the efnb2a
expression domain was greatly reduced to an average angle of 37°, a statistically significant 41% reduction compared with control embryos (figure 4e,h). Injection of increasing concentrations of both MOs caused slightly more severe effects than those observed after co-injection of sub-optimal doses of 

Moreover, injection of sub-optimal doses of 

tbx5a and tbx5b MOs similarly caused an increase in the expression extent of the ventral marker ephB2 (to an average of 167°, figure 4e,i). Again, injection of increasing concentrations of both MOs caused similar effects to those observed after co-injection of sub-optimal doses of 

tbx5a and tbx5b MOs (figure 4f–g,i). In contrast to the results obtained for the bilaterally symmetrical loss of expression of the dorsal marker ephb2a, statistically significant expansions of the ephB2 expression domain towards its ventro-nasal border were largely enough to explain the increase in the global domain of ephB2 expression in the different conditions (figure 4i).

Interestingly, the homeodomain transcription factor meis1 has been implicated in the establishment of the proper retino-tectal map of the developing zebrafish. meis1 knock-down causes a decrease of dorsal ephb2a expression and an increase of ventral ephB2, which is also associated with down-regulation of dorsal tbx5a expression [26]. We show that downregulation of 

tbx5a is enough to explain the defects in dorsoventral-restricted expression of the ephrinB/ephB and therefore propose a model by which meis1 acts upstream of tbx5 genes expression to ensure the correct dorsoventral expression of ephb2a and ephB2 in the developing retina.

Notably, dorsal ephb2a gene expression is not completely abolished after tbx5a and tbx5b knock-down, suggesting that other factors are acting with the tbx5 paralogues to maintain dorsal retina identity. Strikingly, T-box genes have been
shown to cooperatively interact in many developmental processes [27,28]. Regarding dorsal retina identity, several related T-box genes are co-expressed with tbx5 in this domain, namely the other three genes that, together with tbx5, form the Tbx2 subfamily of T-box genes, i.e. tbx2, tbx3 and tbx4 [29–31]. Functional redundancy between these genes may therefore explain the lack of complete downregulation of dorsal retina markers.

Finally, to assess whether altered ephb2a and ephb2 expression in tbx5a and/or tbx5b morphants altered the normal formation of the retinotectal map, we injected our MOs into one-cell stage atl5:gfp embryos that express the gfp transgene in RGCs under the regulation of the atl5 promoter, the zebrafish homologue of the Drosophila atonal gene [32]. By 48 hpf, RGCs have extended their axons to form the optic nerve that crosses the ventral midline to form the optic chiasm and project dorsally to the contralateral optic tectum (figure 4j). Injection of tbx5a and/or tbx5b MOs did not cause observable pathfinding errors (figure 4k–m). However, these experiments showed that the optic nerve of double-morphants was considerably thinner than that of control MO-injected siblings (figure 4m). Moreover, and in agreement with both tbx5 genes acting redundantly to ensure proper optic nerve formation, double-morphant embryos showed a significantly thinner optic nerve (figure 4n). It is noteworthy that space cadet mutants, fish that carry a mutation in the retinoblastoma gene rb1, exhibit thinner optic nerves than wild-type siblings [33] and that it has been shown that Tbx2 (closely related to Tbx5) molecularly interacts with Rb1 [34]. It is therefore tempting to speculate that, likewise, Tbx5 may be interacting with Rb1 to regulate the normal formation of the optic nerve in zebrafish embryos.

Altogether, our data show that knock-down of tbx5 genes causes an alteration of dorsoventral ephrinB2/ephB expression in the retina and the formation of a thinner optic nerve.

### 3.4. tbx5b knock-down causes a delay in pectoral fin growth

Although we had not previously observed tbx5b expression in developing pectoral fins [16], others have recently described it in the pectoral fin bud mesenchyme of 36 hpf embryos [17]. In agreement with tbx5b playing a role during zebrafish pectoral fin morphogenesis, tbx5b morphants had smaller pectoral fins when compared with control embryos at 3 dpf (figure 5a; [17]), which is reminiscent of the phenotypes observed upon subtle downregulation of tbx5a function [14,15] or downregulation of tbx5 target genes [35]. To get further insight into where in the limb developmental pathway tbx5b function is required, we used a series of markers to assess the state of the two tissues required for and involved in the process of fin outgrowth: the fin mesenchyme and the overlying fin ectoderm. Briefly, bi-directional fibroblast growth factor (FGF) signals emanate from and are received by both tissues, creating a positive feedback loop that is required to sustain pectoral fin outgrowth [10].

In control embryos, compacted pectoral fin territories can be observed by means of tbx5a expression at 26 hpf (figure 5b) and expression of the tbx5a target gene, fgf24, is activated in this territory, namely the pectoral fin mesenchyme (figure 5c–e). By contrast, tbx5a morphants failed to compact the tbx5a-labelled mesenchymal tissue (figure 5r) and fgf24 expression was never activated in the mesenchyme although its expression was readily detected in other tissues such as the branchial arches (figure 5s, arrow). Similar to control embryos, tbx5b morphants displayed compacted expression of tbx5a and fgf24 expression was activated in this territory (figure 5j–k). fgf24 expressed in the pectoral fin bud mesenchyme is required to activate the expression of another FGF, namely fgf10a [36]. Activation of fgf10a expression is
observed in both control and tbx5b morphants (figure 5d–d', l–l'), indicating that the fin bud outgrowth initiation programme is properly established in tbx5b-compromised embryos. However, in contrast to the similarities observed between control and tbx5b morphants regarding mesenchymal FGF expression, it was striking to note that at 36 hpf, when fgf24 expression has been downregulated in this tissue and activated in the overlaying fin ectoderm in control embryos, this did not occur after tbx5b depletion (figure 5e–e' n–n'). To determine whether, indeed, the only tissue with active FGF signalling was the fin bud mesenchyme and not the overlaying ectoderm of tbx5b morphants, we used pea3 expression, a direct read-out of cellular exposure to FGF, as a marker [37]. In control embryos, pea3 expression is detected in both mesenchymal and ectodermal compartments of the fin bud (figure 5f–f'), whereas only mesenchymal pea3 was detected after tbx5b knock-down (figure 5g–h').

Interestingly, ectodermal fgf24 was observed in 48 hpf tbx5b-morphant fins (figure 5o–o') and these fins resembled control 36 hpf ones (figure 5e'/e'). In agreement with FGF signalling being active in both mesenchymal and ectodermal tissues of tbx5b-depleted fins, pea3 expression was now evident in these two tissues (figure 5p–p'; the arrow marks expression in the tbx5b-morphant ectoderm), similarly to what is found in control MO-injected embryos (figure 5h–h').

Taken together, we show that pectoral fin development has a different requirement for each of the tbx5 paralogues: tbx5a function is required for the earliest steps of initiation of fin outgrowth, whereas tbx5b functions later to ensure properly timed and sustained fin outgrowth. Both these requirements are linked by the connection between tbx5 genes and the downstream regulation of FGF signalling. Briefly, during the initiation of pectoral fin outgrowth (figure 5(i)), tbx5a expressed prior to overt fin outgrowth is required to initiate fgf24 signalling in the pectoral fin mesenchyme, and hence in the absence of tbx5a the limb initiation programme is never established and pectoral fins fail to form (figure 3g, asterisks; [14,15]). Later, once the limb initiation programme has commenced and FGF signalling is active in the pectoral fin mesenchyme, tbx5b is required for the maintenance of pectoral fin growth. tbx5b morphants have a delay in pectoral fin growth: these embryos fail to downregulate fgf24 expression in the fin bud mesenchyme and activate this gene expression in the overlaying ectoderm at 36 hpf. Nevertheless, 12 h later, fgf24 expression is no longer detected in the mesenchyme and becomes clearly observed in the ectodermal tissue. Strikingly, 48 hpf tbx5b-morphant pectoral fins resemble younger (36 hpf) control fins, suggesting that tbx5b is required to setup a certain threshold of FGF activity in the mesenchyme that is necessary to (i) signal to the overlaying ectoderm and activate FGF signalling in this tissue and (ii) downregulate fgf24 expression in the mesenchyme (figure 5(t)). Thus, owing to this delay in FGF signalling activation in the ectoderm, tbx5b-deficient pectoral fins appear smaller than control fins (figure 5g,i). Given the critical requirement of tbx5a to establish the pectoral fin bud outgrowth initiation programme, it is not clear whether tbx5a may function, similarly to tbx5b, during these later stages of fin outgrowth. It is tempting to speculate that this is indeed the case, because, as mentioned before, the subtle downregulation of tbx5a function or its target genes is reminiscent of the tbx5b loss-of-function shown here.

3.5. Distinct tissue-specific requirements for tbx5a and tbx5b

Our characterization of the consequences of tbx5a and/or tbx5b knock-down in the three tissues where these genes are expressed demonstrates the existence of differential requirements for these paralogues in each tissue and distinct functional inter-relationships between the tbx5 genes.

Firstly, cardiac looping is affected in both single tbx5a and tbx5b morphants, and downregulation of both tbx5 genes does not increase the severity of the looping phenotype, indicating the essential function each of these genes plays to achieve the complete looping of the zebrafish heart. Moreover, we show that both genes act in the same pathway and cooperate with each other to ensure looping morphogenesis, because co-injection of sub-optimal concentrations of tbx5a and tbx5b MOs similarly causes looping phenotypes and increasing concentrations of both MOs caused an increase in the percentage of phenotypes (figure 1i). With regard to heart asymmetric development, we also show that both paralogues are essential for normal leftward heart tube jogging and consequent dextral looping to occur, because tbx5a and tbx5b morphants show jogging and looping orientation defects and co-inhibition of both genes does not result in either more severe or higher phenotypic penetrance (figures 1j and 2g). Secondly, a synergistic effect of tbx5a and tbx5b is necessary for proper efnb2a expression in the dorsal retina. efnb2a expression is not significantly affected in tbx5a or tbx5b morphants, whereas it is decreased by 50% in double tbx5a and tbx5b morphants, suggesting these two genes act together to guarantee the proper extent of efnb2a expression in this domain. Further, the optic nerves of double-morphants appear thinner than those of control siblings (figure 4). Finally, it is noteworthy that still another relationship between tbx5 genes is found regarding fin bud development, where tbx5a and tbx5b are differentially required to ensure the proper initiation of outgrowth first and maintenance of fin growth later, respectively (figure 5).

The tight regulation of Tbx5 gene dosage has been shown to be fundamental for many developmental processes to take place normally, because both the subtle upregulation and downregulation of its function has been shown to cause developmental defects [38–41]. Our data demonstrate that, moreover, tbx5 gene(s) dosage needs to be strongly controlled in a tissue-dependent manner to ensure the proper morphogenesis of the distinct tissues where this gene is most prominently expressed.

4. Material and methods

4.1. Animal welfare

The local ethics committee approved animal studies and all procedures conformed to the ethical rules and the current applicable legislation (Council Directive 86/609/EEC; Law 5/1995/GC; Order 214/1997/GC; Law 1201/2005/SG). Adult fish are kept in a designated fish facility with a designated manager and welfare officers. When animals need to be euthanized, an overdose of tricaine methane sulfonate (MS222, 200–300 mg l⁻¹) by prolonged immersion was used, which is a well-established humane method.
4.2. Animal maintenance

Adult zebrafish were bred under standard conditions and embryos obtained by natural spawning and incubated at 28.5°C in E3 medium [42]. They were further staged and fixed at specific time-points as described by Kimmel et al. [43]. Wild-type and ath5:GFP [32] fish were used in this study.

4.3. Morpholino oligonucleotides

MO oligonucleotides (Gene Tools LLC) were dissolved to 1 mM and 0.5–3 ng injected into one-cell stage embryos. MOs were co-injected with an anti-p53 MO (7.5 ng) to avoid off-target effects caused by toxicity, and all experiments were performed with at least three independent replicates. The MOs used were: a control MO; an ath5a MO for the coding sequence [15]; an ath5b MO against the 5' UTR coding sequence boundary—thx5b(LTR) MO—5' GGATCCGCATATTCCGTCGAGCT 3'; and an thx5b oligonucleotide for the exon 3/intron 4 boundary—thx5b(Sp) MO—5' TTAAAAAACACTGGCACTCCAGGGC 3'. To test the knock-down efficiency of the thx5b(Sp) MO, RT-PCR was performed using whole-embryo RNA from 24 hpf embryos that had been injected with either control or thx5b(Sp) MO. RNA was isolated using Trizol reagent (Invitrogen) and a reverse transcription reaction with SuperScript II RNase H—reverse transcriptase (Invitrogen) was then performed to generate cDNA following the manufacturer's instructions. The PCR was performed using the primers thx5b_ex3fwd 5' AGTATG GAGGGAATTAAAGTTTA 3' and thx5b_ex3rev 5' CATGTGTGTCCGTGGTAGGAGC 3' (present in the fourth exon of the thx5b gene) to detect spliced and un-spliced thx5b transcripts. As equivalent controls were obtained with both thx5b MOs, for most of the experiments results with the thx5b(LTR) MO are shown, unless otherwise indicated.

4.4. Morpholino functionality and specificity

To assess for the functionality of the MOs used, we generated chimeric mRNAs in which the tbx5a or thx5b(LTR) MO recognition sites (underlined) were fused to EGFP by PCR amplification using a plasmid containing EGFP-polyA as template. The following primers were used: thx5aMO_EGFP_fwd, 5' ATGGCGGACAGTGAAGACACCTTTCGGGTGAGCAAG 3'; thx5aMO_EGFP_rev, 5' ATGGAAAGTGAC 3'; thx5bMO_EGFP_fwd, 5' AGTATG GAGGGAATTAAAGTTTA 3' and thx5bMO_EGFP_rev, 5' CATGTGTGTCCGTGGTAGGAGC 3'. In conjunction with the reverse primer: FP_SV40rev 5' AAAGCTCAAATG 3'. The resulting products were cloned into the pGEM T-easy vector (Promega) and further transferred into the pCS2+ vector to obtain mRNAs. Eighty pigograms of mRNA with the corresponding MO (3 ng) were co-injected into one-cell stage embryos and heart laterality assessed at 26 hpf by means of myl7 expression.

4.5. Whole mount in situ hybridizations

The antisense RNA probes used were: myl7 [44], lefty2 (kindly provided by N. Mercader), ephb2a (kindly provided by J. Terriente), ephb2b (kindly provided by R. Dorsky), and 5′-3′ TCAACTCCAGGGC 3' and rev 5′-CTACAGTAGAGATGGG AG 3' and pax3 (obtained by RT-PCR with the primers fwd 5′ AGAAGAGCCGAGATCC 3' and rev 5′ TCCGTGTTGCCAGTCATATGGG 3'). Chromogenic whole mount in situ hybridizations were carried out as described by Albalat et al. [16]. Embryos were observed in an OLYMPUS MVX10 microscope and photographed with the OLYMPUS Cell^ software. Fluorescent whole mount in situ were carried out as described by Brent et al. [45]. Embryos were embedded in 1% low melting agarose (Sigma) dissolved in PBS and observed in a Leica SP2 confocal microscope. Acquired images are projections of z-stacks.

4.6. Quantification of retinal phenotypes

The extent of ephb2a and ephb2b expression domain was quantified by setting an imaginary hinge in the centre of the lens. The total angle of expression was sub-divided into nasal versus temporal by considering the choroid fissure as the ventral-most point. The Kruskal–Wallis test was used to assess statistical differences among experimental conditions.

4.7. Immunofluorescence

ath5:GFP 48 hpf embryos were fixed in 4% paraformaldehyde (PFA) at 4°C, washed with PBST (0.5% Triton), digested with 10 mg ml−1 proteinase K for 40 min and post-fixed in 4% PFA for 20 min. After PBST washes, embryos were blocked with 1% BSA and an anti-GFP antibody (Invitrogen, 1 : 600) was subsequently left overnight at 4°C. The antibody was washed out with 1% BSA washes before adding the secondary antibody (anti-rabbit Alexa488 1 : 200, Molecular Probes) and left overnight at 4°C. Secondary antibody washes were performed with PBST. The acquired images are projections of z-stacks taken with a Leica SP2 confocal microscope.

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