RESEARCH ARTICLE

Genetic basis of hindlimb loss in a naturally occurring vertebrate model

Emily K. Don1,2, Tanya A. de Jong-Curtain3, Karen Doggett3, Thomas E. Hall4, Benjamin Heng1, Andrew P. Badrock1, Claire Winnick1, Garth A. Nicholson1, Gilles J. Guillemin1, Peter D. Currie5, Daniel Hesselson6,7,*, Joan K. Heath3,*, and Nicholas J. Cole1,2,‡

ABSTRACT
Here we genetically characterise pelvic finless, a naturally occurring model of hindlimb loss in zebrafish that lacks pelvic fin structures, which are homologous to tetrapod hindlimbs, but displays no other abnormalities. Using a hybrid positional cloning and next generation sequencing approach, we identified mutations in the nuclear localisation signal (NLS) of T-box transcription factor 4 (Tbx4) that impair nuclear localisation of the protein, resulting in altered gene expression patterns during pelvic fin development and the failure of pelvic fin development. Using a TALEN-induced tbx4 knockout allele we confirm that mutations within the Tbx4 NLS (A78V; G79A) are sufficient to disrupt pelvic fin development. By combining histological, genetic, and cellular approaches we show that the hindlimb initiation gene tbx4 has an evolutionarily conserved, essential role in pelvic fin development. In addition, our novel viable model of hindlimb deficiency is likely to facilitate the elucidation of the detailed molecular mechanisms through which Tbx4 functions during pelvic fin and hindlimb development.

KEY WORDS: Pelvic fin, Development, TALENs, Hindlimb, Tbx4

INTRODUCTION

The study of limb development has relied heavily on mouse and chick embryos as models to understand the genetic mechanisms of limb induction, identity and outgrowth. We now describe a unique and viable pelvic finless zebrafish model of pelvic fin development and loss in a high-throughput, genetically tractable, model organism. The paired fins of modern fish species and tetrapod limbs share similar gene and protein expression patterns during limb and fin development as the forelimbs and hindlimbs of tetrapods are evolutionarily derived from fish pelvic fins, the pelvic fins of vertebrate forelimb initiation and early outgrowth (reviewed in Mercader, 2006). Despite the evolutionarily conserved role of Tbx4 in hindlimb/forelimb development Tbx5 shows dynamic localisation, being shown to be crucial for forelimb and hindlimb development, respectively (Ahn et al., 2002; Garrity et al., 2002; Ng et al., 2002; Rallis et al., 2003; Naiche and Papaioannou, 2003; Ruvinov et al., 2000; Tamura et al., 1999). While there is strong evidence for a crucial role for Tbx4 in hindlimb development, there is little known about how this transcription factor functions during their development. Tbx4 and Tbx5 proteins both contain a conserved DNA binding motif known as the T-box domain, which is a naturally occurring zebrafish strain in which the pelvic finless, a genetically tractable model of pelvic fin development, the mechanism through which Tbx4 functions during limb and fin development remains unknown. Here we show using a unique pelvic finless zebrafish model that not only is Tbx4 required for pelvic fin development, but also that the NLS of Tbx4 must be intact for Tbx4 to play its essential role in the induction of the apical ectodermal ridge and the outgrowth of the pelvic fin.

RESULTS AND DISCUSSION

Pelvic finless is a naturally occurring zebrafish strain in which the development of the pelvic fins (the teleost equivalent of hindlimbs)
fails (Don et al., 2011). These zebrafish are unique in that only the development of pelvic fins is altered and pelvic finless zebrafish are viable as adults as no other structures or developmental processes are affected. Pelvic finless zebrafish initiate pelvic fin development, evident by the 3-4 cell thick mesenchymal bulges that form in the pelvic regions around 3-4 weeks of development; however, these bulges do not form an apical ectodermal ridge and a subsequent loss of pelvic fin development is observed (Don et al., 2011).

We have now determined that polymorphisms in tbx4 are responsible for the specific and complete absence of pelvic fins in pelvic finless zebrafish (Fig. 1A-D). Using genetic mapping, we narrowed the genetic interval containing the pelvic finless locus to a 10 cM region of chromosome 15. Region-specific, targeted-enrichment next generation sequencing identified three nucleotide variations in codons 78 and 79 that encode two nonsynonymous amino acid mutations (A78V; G79A) in the NLS of Tbx4 that segregate invariably with the pelvic finless phenotype (n=122) (Fig. 1A-D).

Alignment of the Tbx4 NLS across all vertebrates for which an entire NLS sequence is available, shows that the motif is perfectly conserved in all species with hindlimbs or pelvic fins (Fig. 1D). Therefore, we hypothesised that the A78V; G79A mutations in Tbx4 cause the absence of pelvic fins in pelvic finless zebrafish. We have now determined that polymorphisms in tbx4 are responsible for the specific and complete absence of pelvic fins in pelvic finless zebrafish (Fig. 1A-D). Using genetic mapping, we narrowed the genetic interval containing the pelvic finless locus to a 10 cM region of chromosome 15. Region-specific, targeted-enrichment next generation sequencing identified three nucleotide variations in codons 78 and 79 that encode two nonsynonymous amino acid mutations (A78V; G79A) in the NLS of Tbx4 that segregate invariably with the pelvic finless phenotype (n=122) (Fig. 1A-D).

Alignment of the Tbx4 NLS across all vertebrates for which an entire NLS sequence is available, shows that the motif is perfectly conserved in all species with hindlimbs or pelvic fins (Fig. 1D). Therefore, we hypothesised that the A78V; G79A mutations in Tbx4 cause the absence of pelvic fins in pelvic finless zebrafish.

Fig. 1. The pelvic finless critical region maps to a region on chromosome 15 containing three SNPs in tbx4. (A) Low and intermediate resolution mapping reveals that the pelvic finless critical region lies between 54.1 and 63.7 cM on chromosome 15. (B) Region-specific, targeted-enrichment next generation sequencing filtering process revealed three SNPs in tbx4. (C) The three SNPs in exon 3 of tbx4 of pelvic finless zebrafish are predicted to cause two amino acid substitutions (A78V and G79A). (D) Schematic diagram of the conservation of vertebrate TBX4 nuclear localization signal (NLS). The NLS is located within the highly conserved T-box domain of the protein. The sequence of the TBX4 NLS is perfectly conserved amongst vertebrates with hindlimbs or pelvic fins, pelvic finless zebrafish exhibit variations in this motif.
mutation in the Tbx4 NLS underlies the loss of the pelvic fin development in zebrafish. To confirm whether mutations in the Tbx4 NLS are responsible for the developmental defects in pelvic finless zebrafish, we used genetic complementation, protein localization and in-situ hybridisation studies to explore the functional consequences of the naturally occurring A78V; G79A mutations.

Using Transcription Activator-Like Effector Nucleases (TALENs) directed to zebrafish tbx4, we introduced a frameshift mutation in exon 5 of tbx4 in wild-type zebrafish. Zebrafish tbx4 mutants (tbx4gi1/gi1) are viable and exhibit the identical pelvic fin loss seen in pelvic finless zebrafish (pflmq6/mq6) (Fig. 2B,D), while heterozygous animals (tbx4gi1/+) display normal pelvic fin development (Fig. 2C). We next performed a complementation test with the pelvic finless and tbx4 mutants. Compound heterozygotes (tbx4gi1/mq6) from a cross between homozygous pflmq6/mq6 and tbx4gi1/gi1 mutants do not develop pelvic fins and display an identical phenotype to pelvic finless zebrafish (pflmq6/mq6) (Fig. 2E,D), confirming that the pelvic finless mutation is allelic to tbx4 and that the mutations in the NLS of Tbx4 (A78V; G79A) result in tbx4 loss-of-function. To the best of our knowledge, these findings demonstrate for the first time that Tbx4 is essential for pelvic fin development and that mutations in the Tbx4 NLS are sufficient for pelvic fin loss in vivo.

We hypothesised that the NLS is essential for Tbx4 function during pelvic fin development as its paralog, Tbx5, requires nuclear localisation to perform its function during limb development (Zaragoza et al., 2004; Fan et al., 2003). To determine whether the A78V; G79A mutation identified in Tbx4 in pelvic finless zebrafish compromised nuclear localisation, we transfected C-terminal GFP-tagged Tbx4 constructs into HeLa cells and analysed the subcellular localization of the fluorescent proteins by confocal microscopy, due to an absence of specific zebrafish Tbx4 antibodies. We observed wild-type zebrafish Tbx4 (Tbx4-GFP) solely located in the nucleus of the majority of cells (70.53±3.9% nuclear only, n=110) (Fig. 3A,G). In contrast, the Tbx4 variant (A78V; G79A) mutated in pelvic finless zebrafish (zTbx4pfl-GFP) shows both nuclear and cytoplasmic localization (83.03±7.74% nuclear and cytoplasmic; n=83; Tbx4pfl-GFP vs Tbx4-GFP, P<0.0001) with a proportional reduction in the number of cells which exhibit solely nuclear localisation (Fig. 3D-F,G) suggesting that the conserved Tbx4 NLS sequence facilitates nuclear localisation of the protein. These results suggest that the conserved Tbx4 NLS is required for correct Tbx4 function and
that the naturally occurring mutations identified in pelvic finless zebrafish impair the function of the NLS, an outcome that is deleterious for Tbx4 function during early pelvic fin development.

To investigate the downstream consequences of the mutated Tbx4 NLS, we examined gene expression during pelvic fin development in wild-type and pelvic finless zebrafish by in-situ hybridisation. Expression of early pelvic fin development genes, pitx1 and tbx4, was observed in the mesenchyme of the developing pelvic fin buds of pelvic finless zebrafish in a similar pattern to wild-type zebrafish (Fig. 4A-D). We next examined the expression of fgf10a, a well characterised direct transcriptional target of Tbx4, which is required to induce and maintain the apical ectodermal ridge

Fig. 3. Mutations in the Tbx4 NLS sequence cause an impairment of nuclear localization of the protein in HeLa cells. (A-C) Wild-type Tbx4-GFP is located in the nucleus of the majority of cells (70.53±3.9% nuclear only, n=110) as shown by co-localisation with the nuclear stain, DAPI. (D-F) Mutations in the NLS of pelvic finless zebrafish Tbx4 (Tbx4\textit{pfl}-GFP) cause an impairment of nuclear localization of Tbx4 (83.03±7.74% nuclear and cytoplasmic, n=83). Scale bars: 10 µm. (G) Graph of the cellular location of zebrafish Tbx4-GFP and Tbx4\textit{pfl}-GFP. A two-way ANOVA with Tukey’s multiple comparisons test (***(P<0.0001). Error bars represent standard deviation from the mean (n=3).
during embryonic limb development (Min et al., 1998; Naiche and Papaioannou, 2003). Strikingly, we observed robust expression of fgf10a in the pelvic fin region of wild-type zebrafish, but fgf10a expression was completely absent from this region in homozygous pelvic finless mutants (Fig. 4E,F). In addition, we observed an altered expression of the apical ectodermal ridge marker, sp8 (Kawakami et al., 2004), in pelvic finless fish. Whilst sp8 expression was observed in the developing pelvic fin apical ectodermal thickening of wild-type zebrafish, sp8 expression was observed only in the apical ectodermal thickening precursor cells that have failed to accumulate in the dorsoventral boundary of the pelvic fin buds of pelvic finless fish (Fig. 4G,H). Collectively, these data lead us to conclude that mutations in the NLS of Tbx4 impair the function of the Tbx4 protein and compromise its ability to act as a transcriptional activator during early pelvic fin development.

Using a unique pelvic finless zebrafish model of hindlimb loss, we demonstrate that Tbx4 has an evolutionarily conserved, essential role in pelvic fin development. Pelvic finless zebrafish carrying a naturally occurring mutant version of Tbx4 (A78V; G79A) demonstrate complete pelvic fin loss. Zebrafish harbouring a TALEN-induced mutation in the tbx4 coding sequence confirm its crucial role in pelvic fin development as these fish also lack pelvic fins. Complementation crosses between these two fish lines demonstrate a striking specificity of pelvic fin loss since we do not observe defects in other structures or organ systems in fish with pelvic fin loss. The essential role for Tbx4 in pelvic fin development has previously been hypothesised, as a result of its crucial role in hindlimb development in mice (Naiche and Papaioannou, 2003) and its pelvic fin expression in zebrafish (Ruvinsky et al., 2000; Tamura et al., 1999). The limb-specific phenotype of our pelvic finless zebrafish model will allow for the investigation of the function of Tbx4 and downstream pathways during hindlimb development.

Pelvic finless and the TALEN-induced mutated tbx4 zebrafish described here are novel developmental models in which to examine the cellular function of Tbx4 in the hindlimb/pelvic fin developmental cascade. They will be useful for the functional characterisation of Tbx4 localisation and behaviour during hindlimb development. Because pelvic finless zebrafish exhibit specific loss of pelvic fins, with no other defects, pelvic finless zebrafish could represent a platform from which to investigate the genetic architecture of hindlimbs or pelvic fin loss in other species. This strategy has been successfully used to investigate the role of pitx1 (three spine stickleback fish, Cole et al., 2003; Shapiro et al., 2004; Chan et al., 2010) and hoxd9a (Fugu, Tanaka et al., 2005) in teleost pelvic fin development.

Our preliminary findings using these novel models show that the evolutionarily conserved Tbx4 NLS is necessary for pelvic fin development as the NLS mutations described in pelvic finless zebrafish impede the ability of the protein to function as a transcriptional activator. Our results suggest that the NLS mutations compromise the nuclear localisation of the Tbx4 protein. However, it is also possible that an intact NLS contributes to the DNA binding capacity of the protein. We propose that in the context of early pelvic fin development, the NLS of Tbx4 is necessary for the direct or indirect activation of fgf10a to complete pelvic fin bud induction and thus the impairment of Tbx4 NLS leads to pelvic fin loss. Our results suggest that the impairment of the NLS of Tbx4 results in a failure of pelvic fin development due to an inability to establish an apical ectodermal thickening.

Several lines of evidence indicate that the disruption of the apical ectodermal thickening (in fish) or the apical ectodermal ridge (in tetrapods) causes a failure of limb/fin development (Boulet et al., 2004; Crosseley et al., 1996; Fischer et al., 2003; Lewandoski et al., 2000; Moon and Capecchi, 2000; Barrow et al., 2003; Narita et al., 2005; Naiche and Papaioannou, 2003; Norton et al., 2005; Min et al., 1998; Sekine et al., 1999). Our findings suggest that during pelvic fin development, the impairment of the Tbx4 NLS results in a loss of fgf10a expression, which has been previously shown to result in the failure of the apical ectodermal thickening and the subsequent loss of limb/fin development in multiple animal models (Norton et al., 2005; Min et al., 1998; Sekine et al., 1999). Therefore, in pelvic finless zebrafish, impairment of the Tbx4 NLS impedes the ability of the protein to act as a transcription factor, resulting in the loss of the apical ectodermal thickening, and thwarting the development of the pelvic fins.

Our findings are consistent with previous studies of Tbx4 in other systems. Similar to pelvic finless or mutated tbx4 zebrafish, conditional knockout of Tbx4 in mouse models results in the loss of hindlimbs (Naiche and Papaioannou, 2003, 2007); however early knockout of Tbx4 results in embryonic lethality in these models. Results obtained from knockout studies of Tbx5, the forelimb paralog of Tbx4, have also produced similar results. Knockout of Tbx5 in mice results in the loss of forelimbs (Falhis et al., 2003) and in zebrafish the transient knockdown or knockout of txb5 leads to the loss of pectoral fins (Ahn et al., 2002; Garrity et al., 2002; Ng et al., 2002). In humans, mutations in TBX4 result in the lower limb development defects observed in small patella syndrome, however the mechanism by which the identified mutation cause the lower limb abnormalities remains unknown (Bongers et al., 2004). Mutations identified in Tbx5 have been shown to cause impaired nuclear localisation of the protein and have been identified as a molecular mechanism responsible for the upper limb and heart developmental defects of Holt–Oram syndrome (Basson et al., 1999; Fan et al., 2003; Li et al., 1997). Seven missense mutations linked to Holt–Oram syndrome (Q49K, 154T, G80R, G169R, R237Q, R237W and S252I) all showed a mislocalisation to the cytoplasm, caused by a nuclear trafficking defect, when transfected into HeLa cells (Fan et al., 2003). Of particular interest, the TBX5G80R mutation, which causes impaired nuclear localisation in Holt–Oram syndrome, corresponds to the Tbx4G79A residue which is mutated in pelvic finless zebrafish, suggesting a conserved role for this residue in limb development.

Utilising this naturally occurring vertebrate model of hindlimb loss we have demonstrated specific pelvic fin loss attributed to three single nucleotide variations in tbx4 cause impairment of the function of the NLS motif in the Tbx4 protein and we have confirmed the sufficiency of these mutations using genetic complementation with a TALEN-induced null allele. Unlike the embryonic lethality of Tbx4 null mice, the limb specificity of tbx4 mutations in zebrafish sets the stage of epistatic analysis of the genetic program underlying hindlimb development and is likely to facilitate the elucidation of the detailed molecular mechanisms through which Tbx4 functions in both the cytoplasm and nucleus during pelvic fin and hindlimb development.

**MATERIALS AND METHODS**

**Zebrafish**

The use and treatment of animals in this project were in accordance with and approved by the Animal Ethics Review Committee, University of Sydney, N.S.W., Australia (ARA: K031-201235665) and the Animal Ethics Committee, Macquarie University, N.S.W., Australia (ARA: 2013-006). Zebrafish (*Danio rerio*) were housed at 28°C, in a 13 h light and 11 h dark cycle. Embryos were collected by natural spawning and raised at 28°C in E3 solution according to standard protocols (Westerfield, 2000).
Positional cloning
Homozygous pelvic finless zebrafish (pf\textsuperscript{mp6/mp6}) were out-crossed to wild-type WIK zebrafish to generate polymorphic mapping strains. Bulk segregant analysis and rough mapping was performed using the MGH SSLP panel as described (Zhou and Zon, 2011) on 122 pelvic finless zebrafish and 40 wild-type siblings.

Region-specific, targeted-enrichment next generation sequencing and mutation analysis
Genomic DNA was extracted from individual pelvic finless and homozygous wild-type zebrafish as described (Gupta et al., 2010). Region-specific, targeted-enrichment was performed by the Beijing Genomics Institute (BGI) on the chromosomal region Chr15:24168360 - 36060966 (Ensembl Zv9), which contained the locus of the gene mutated in pelvic finless zebrafish as suggested by the mapping experiments, using hybrid array capture with Roche NimbleGen HD 11.8 MB capture array (Roche NimbleGen). Paired end sequencing was performed on a HiSeq2000 platform (Illumina). Raw image files were processed with Illumina basecalling Software 1.7 with default parameters and the sequences of each individual were generated as 90 bp pair-end reads. In the target region, 11,075,935 bp were sequenced to an average depth of 67× with a target region coverage of 95.04% in the pelvic finless sample and 93.83% in the wild-type sample. The fraction of unique mapped bases on, or near target was 88.70% for the pelvic finless sample and 89.63% for the wild-type sample. The captured region followed a Poisson distribution which revealed that the captured region was evenly sampled. Only mapped reads were used for subsequent analysis.

Sequence reads were generated by the Illumina HiSeq2000 platform and aligned to Zv9 zebrafish genome assembly using SOAPaligner (soap2.21) (Li et al., 2009b) (for subsequent SNP identification) and BWA v0.6.1 (Li and Durbin, 2009) (for insertion and deletion identification). SNP variants were called using SOAPsnp (Li et al., 2009a) and insertions and deletions were identified using GATK (McKenna et al., 2010). All variants were annotated by BGI. Filtering of coding variants was performed using dbSNP (release 138, https://www.ncbi.nlm.nih.gov/SNP/), prioritising by known gene function.

Validation and analysis of the \( \text{trx4} \) mutations was performed by direct DNA sequencing following PCR amplification of coding exons (ENSDART00000188603). PCR products were Sanger sequenced using Applied Biosystems 3730 and 3730xl capillary sequencers and Big Dye Terminator (Applied Biosystems) under standard cycling PCR conditions. The raw chromatogram trace files were analysed using Geneious\textsuperscript{®} 6.0.3 software (Biomatters).

Mutations
The mutations identified in pelvic finless zebrafish (pf\textsuperscript{mp6/mp6}) were identified as homozygous SNPs in exon 3 of \( \text{trx4} \) as follows:

- mRNA position 233, codon number 78, codon change G\(\text{C}A\)\(\rightarrow\)GTA, residue change A\(\rightarrow\)V (referred to as A78V in the text),
- mRNA position 236 and 237, codon number 79, codon change G\(\text{G}C\)\(\rightarrow\)GCA, residue change G\(\rightarrow\)A (referred to as G79A in the text).

TALENs
A pair of TALENs recognising exon 5 (aa121-169) of zebrafish \( \text{trx4} \) gene was designed using TAL Effector-Nucleotide Targeter and the TAL effector repeats were conformed by the ‘golden gate’ method as described previously (Cermak et al., 2011). TALEN mRNA was synthesised by 

- in vitro transcription using the SP6 mMESSAGE mMACHINE Kit (Ambion).

100 pg of mRNA encoding each TALEN heterodimer was injected into the cytoplasm of the cell of one cell-stage wild-type zebrafish embryos. One F1 line (\( \text{trx4}^\text{G1/G1} \)) derived from TALEN injected fish harbours a 7 bp deletion after mRNA position 492 resulting in a frameshift and a premature stop codon at position 164. In the text, individuals heterozygous for this mutation are referred to as \( \text{trx4}^\text{G1/G0} \) and homozygous individuals are referred to as \( \text{trx4}^\text{G1/G1} \) or TALEN-induced mutated \( \text{trx4} \) zebrafish.

Complementation crosses were performed by crossing a \( F_0 \) \( \text{trx4}^\text{G1/G1} \) founder harbouring the 7 bp deletion to homozygous pelvic finless zebrafish (pf\textsuperscript{mp6/mp6}) or homozygous wild-type zebrafish (\( \text{trx4}^\text{+/+} \)). Homozygous \( \text{trx4}^\text{G1/G1} \) zebrafish were obtained from crosses of a \( F_0 \) \( \text{trx4}^\text{G1/G1} \) founder to an identified \( F_1 \) \( \text{trx4}^\text{G1/G1} \) zebrafish. Offspring harbouring the 7 bp deletion were screened at 5 weeks post fertilisation for the presence or absence of pelvic fins. Selected fish were euthanized and imaged in 3% methyl cellulose on a Leica M165FC stereo dissection microscope.

Cellular localisation
For cellular localisation experiments, cDNAs encoding zebrafish wild-type (\( \text{tbx4}^\text{G0/G0} \)) and pelvic finless zebrafish (\( \text{tbx4}^\text{G1/G1} \)) C-terminal EGFP tagged sequences were generated by GeneArt (Invitrogen). cDNAs were subcloned into the BamHI and EcoRI sites of pCS2+ (Addgene). All constructs were verified by DNA sequencing.

HeLa cells were cultured in DMEM media (Life Technologies) containing 1% penicillin/streptomycin antibiotics and 10% FBS. Cells were maintained in a humidified 37°C incubator with 5% CO\( _2 \). For transfection, HeLa cells were seeded at a density of 0.3 \times 10^5 cells/well on poly-L-lysine 35 mm glass bottom culture dishes (MatTek). pCS2+ Tbx4-EGFP plasmids were introduced by transfection into cells using 1 µg of plasmid, 2 µl lipofectamine 2000 (Invitrogen) and 500 µl OPTI-MEM (Invitrogen) according to the manufacturer’s protocol. Transfection solution was removed and replaced with complete media with no antibiotics 6 h after transfection. Cells were fixed at 24 h with 4% paraformaldehyde in phosphate buffered saline (PBS) and cover-slipped with Prolong Gold Antifade reagent with DAPI (Invitrogen) to stain nuclei.

Confocal microscopy was performed using a Leica DM6000 upright laser scanning confocal microscope with Leica application suite advanced fluorescence software. Images were acquired with a 40× (1.4 NA) water immersion lens with DAPI and GFP channels using identical settings. Nuclear or cytoplasmic localization data was acquired from five random fields per coverslip. The number of cells with nuclear and/or cytoplasmic localization was counted and presented as a ratio of the total number of transfected cells in a visual field. Data were obtained from three independent experiments in biological triplicates. A two-way ANOVA with Tukey’s multiple comparison test was performed to determine significance between samples.

\textbf{In situ hybridisation}
Whole-mount \textit{in situ} hybridisations were carried out on pelvic region essentially as described (Westerfield, 2000). Plasmids containing fragments of \( \text{fgf10a} \) and \( \text{sp8} \) (Nagayoshi et al., 2008), \( \text{fgf}8 \) (Komisarcuk et al., 2009), \( \text{tbx4} \) (Tamura et al., 1999) were kindly donated for use in this project. A fragment of \( \text{pits1} \) was amplified using primers (Forward: 5’GGACTCAC- TTCACNACGCGCAGAG, Reverse: 5’TAGGCGTGGATGTCAVGGTC- CCCGTTAG) and cloned into pCR\textsuperscript{®}-TOPO\textsuperscript{®} vector. Diogoxin-labelled riboprobes were generated with SP6, T3 or T7 RNA polymerases (Roche) according the manufacturer’s instructions. Post staining, pelvic fins were dissected and mounted in 3% methyl cellulose and imaged using a Leica M165FC stereo dissection microscope. All experiments were performed in triplicate on pooled individuals (\( n=24 \)) from multiple spawnings.

\textbf{Acknowledgements}
We thank D. Lai and D. King of the Bosch Institute and K. Undheim for their technical assistance and the Becker Lab (Brain and Mind Research Institute, University of Sydney, Australia), Belmonte Lab (The Salk Institute for Biological Studies, San Diego, CA, USA) and Kawakami Lab (National Institute of Genetics, Shizuoka, Japan) for the kind donation of plasmids. The authors thank Dasha Syal for zebrafish care.
REFERENCES

Ahn, D.-G., Kourakis, M. J., Rohde, L. A., Silver, L. M. and Ho, R. K. (2002). T-box gene tbx5 is essential for formation of the pectoral limb bud. Nature 417, 754-757.

Alvarado, D. M., Aferol, H., Mccall, K., Huang, J. B., Techy, M., Buchan, J., Cady, Coates, M. I.

Chan, Y. F., Marks, M. E., Jones, F. C., Villarreal, G., Jr., Shapiro, M. D., Brady, Cermak, T., Doyle, E. L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, Cole, N. J., Tanaka, M., Prescott, A. and Tickle, C.

Collavoli, A., Hatcher, C. J., He, J., Okin, D., Schmutz, J. et al.

Ahn, D.-G., Kourakis, M. J., Rohde, L. A., Silver, L. M. and Ho, R. K. (2002). T-box gene tbx5 is essential for formation of the pectoral limb bud. Nature 417, 754-757.

Alvarado, D. M., Aferol, H., Mccall, K., Huang, J. B., Techy, M., Buchan, J., Cady, Coates, M. I.

Chan, Y. F., Marks, M. E., Jones, F. C., Villarreal, G., Jr., Shapiro, M. D., Brady, Cermak, T., Doyle, E. L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, Cole, N. J., Tanaka, M., Prescott, A. and Tickle, C.

Collavoli, A., Hatcher, C. J., He, J., Okin, D., Schmutz, J. et al.

Ahn, D.-G., Kourakis, M. J., Rohde, L. A., Silver, L. M. and Ho, R. K. (2002). T-box gene tbx5 is essential for formation of the pectoral limb bud. Nature 417, 754-757.

Alvarado, D. M., Aferol, H., Mccall, K., Huang, J. B., Techy, M., Buchan, J., Cady, Coates, M. I.

Chan, Y. F., Marks, M. E., Jones, F. C., Villarreal, G., Jr., Shapiro, M. D., Brady, Cermak, T., Doyle, E. L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, Cole, N. J., Tanaka, M., Prescott, A. and Tickle, C.

Collavoli, A., Hatcher, C. J., He, J., Okin, D., Schmutz, J. et al.

Ahn, D.-G., Kourakis, M. J., Rohde, L. A., Silver, L. M. and Ho, R. K. (2002). T-box gene tbx5 is essential for formation of the pectoral limb bud. Nature 417, 754-757.

Alvarado, D. M., Aferol, H., Mccall, K., Huang, J. B., Techy, M., Buchan, J., Cady, Coates, M. I.

Chan, Y. F., Marks, M. E., Jones, F. C., Villarreal, G., Jr., Shapiro, M. D., Brady, Cermak, T., Doyle, E. L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, Cole, N. J., Tanaka, M., Prescott, A. and Tickle, C.

Collavoli, A., Hatcher, C. J., He, J., Okin, D., Schmutz, J. et al.

Ahn, D.-G., Kourakis, M. J., Rohde, L. A., Silver, L. M. and Ho, R. K. (2002). T-box gene tbx5 is essential for formation of the pectoral limb bud. Nature 417, 754-757.

Alvarado, D. M., Aferol, H., Mccall, K., Huang, J. B., Techy, M., Buchan, J., Cady, Coates, M. I.

Chan, Y. F., Marks, M. E., Jones, F. C., Villarreal, G., Jr., Shapiro, M. D., Brady, Cermak, T., Doyle, E. L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, Cole, N. J., Tanaka, M., Prescott, A. and Tickle, C.

Collavoli, A., Hatcher, C. J., He, J., Okin, D., Schmutz, J. et al.

Ahn, D.-G., Kourakis, M. J., Rohde, L. A., Silver, L. M. and Ho, R. K. (2002). T-box gene tbx5 is essential for formation of the pectoral limb bud. Nature 417, 754-757.

Alvarado, D. M., Aferol, H., Mccall, K., Huang, J. B., Techy, M., Buchan, J., Cady, Coates, M. I.

Chan, Y. F., Marks, M. E., Jones, F. C., Villarreal, G., Jr., Shapiro, M. D., Brady, Cermak, T., Doyle, E. L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, Cole, N. J., Tanaka, M., Prescott, A. and Tickle, C.

Collavoli, A., Hatcher, C. J., He, J., Okin, D., Schmutz, J. et al.

Ahn, D.-G., Kourakis, M. J., Rohde, L. A., Silver, L. M. and Ho, R. K. (2002). T-box gene tbx5 is essential for formation of the pectoral limb bud. Nature 417, 754-757.
Papaioannou, V. E. (2001). T-box genes in development: from hydra to humans. *Int. Rev. Cytol.* 207, 1-70.

Rallis, C., Bruneau, B. G., Del Buono, J., Seidman, C. E., Seidman, J. G., Nissim, S., Tabin, C. J. and Logan, M. P. O. (2003). Tbx5 is required for forelimb bud formation and continued outgrowth. *Development* 130, 2741-2751.

Rodriguez-Esteban, C., Tsukui, T., Yonei, S., Magallon, J., Tamura, K. and Belmonte, J. C. I. (1999). The T-box genes Tbx4 and Tbx5 regulate limb outgrowth and identity. *Nature* 398, 814-818.

Ruvinsky, I., Oates, A. C., Silver, L. M. and Ho, R. K. (2000). The evolution of paired appendages in vertebrates: T-box genes in the zebrafish. *Dev. Genes Evol.* 210, 82-91.

Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N. et al. (1999). Fgf10 is essential for limb and lung formation. *Nat. Genet.* 21, 138-141.

Shapiro, M. D., Marks, M. E., Peichel, C. L., Blackman, B. K., Nereng, K. S., Jónsson, B., Schluter, D. and Kingsley, D. M. (2004). Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. *Nature* 428, 717-723.

Takeuchi, J. K., Koshiba-Takeuchi, K., Suzuki, T., Kamiyama, M., Ogura, K. and Ogura, T. (2003). Tbx5 and Tbx4 trigger limb initiation through activation of the Wnt/Fgf signaling cascade. *Development* 130, 2729-2739.

Tamura, K., Yonei-Tamura, S. and Belmonte, J. C. I. (1999). Differential expression of Tbx4 and Tbx5 in Zebrafish Fin buds. *Mech. Dev.* 87, 181-184.

Tanaka, M., Hale, L. A., Amores, A., Yan, Y.-L., Cresko, W. A., Suzuki, T. and Postlethwait, J. H. (2005). Developmental genetic basis for the evolution of pelvic fin loss in the pufferfish Takifugu rubripes. *Dev. Biol.* 281, 227-239.

Wang, K., Shi, D., Zhu, P., Dai, J., Zhu, L., Zhu, H., Lv, Y., Zhao, B. and Jiang, Q. (2010). Association of a single nucleotide polymorphism in Tbx4 with developmental dysplasia of the hip: a case-control study. *Osteoarthritis Cartilage* 18, 1592-1595.

Westerfield, M. (2000). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio).* Eugene: Univ. of Oregon Press.

Zaragoza, M. V., Lewis, L. E., Sun, G., Wang, E., Li, L., Said-Salman, I., Feucht, L. and Huang, T. (2004). Identification of the TBX5 transactivating domain and the nuclear localization signal. *Gene* 330, 9-18.

Zhou, Y. and Zon, L. I. (2011). The zon laboratory guide to positional cloning in zebrafish. *Methods Cell Biol.* 104, 287-309.