Identification of hoxb1b downstream genes: hoxb1b as a regulatory factor controlling transcriptional networks and cell movement during zebrafish gastrulation

Akker, W.M. van den; Durston, A.J.; Spaink, H.P.

Int. J. Dev. Biol. 54: 55-62 (2010)
doi: 10.1387/ijdb.082678wv

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Identification of *hoxb1b* downstream genes: hoxb1b as a regulatory factor controlling transcriptional networks and cell movement during zebrafish gastrulation

WILLEM M.R. VAN DEN AKKER*[,1,2], ANTONY J. DURSTON[2,3] and HERMAN P. SPAINK[3]

[1]*Max Planck Institute for Biophysical Chemistry, Department of Molecular Cell Biology, Göttingen, Germany,* [2]*Hubrecht Institute, Developmental Biology and Stem Cell Research, Utrecht, The Netherlands and* [3]*Institute of Biology, Leiden University, Leiden, The Netherlands*

ABSTRACT Hox proteins are homeobox containing transcription factors that play important roles in patterning the presumptive central nervous system and the axial mesoderm in the early vertebrate embryo. *Hox* genes are first expressed during gastrula stages and recent studies suggest that their function goes beyond their role as patterning determinants. To improve our understanding of the role of Hox proteins during early vertebrate development, we designed a strategy to identify target genes of the zebrafish *hoxb1b* using overexpression and whole-genome microarray analysis. We directly compared the *hoxb1b* microarray data with those resulting from heterologous over-expression of the *Xenopus XhoxD1* gene in zebrafish embryos. Both genes are the first expressed *hox* genes in their respective native embryos and display similar spatial expression patterns. The zebrafish transcriptome was analysed prior to the start of the expression of the endogenous *hoxb1b* gene and we observed extensive overlap between the *hoxb1b* and *XhoxD1* putative downstream genes suggesting evolutionary functional conservation between these *hox* genes. Furthermore, genes encoding transcription factors and proteins that are known to be involved in cell adhesion and movement were over-represented among the candidate downstream genes, indicating the involvement of the developmentally earliest expressed *hox* genes in transcriptional networks and cell movement processes.

KEY WORDS: *hox gene, downstream target gene, gastrulation, zebrafish, microarray*

Introduction

During vertebrate gastrulation, the three definitive germ layers are formed and the anterior-posterior (A/P) axis of the embryo is determined. This complex process is characterised by extensive cell movements and patterning of tissues. Various lines of evidence have shown that *hox* genes, which encode homeobox transcription factors, play important roles in patterning during gastrulation (Iimura and Pourquie, 2006; Wacker *et al.*, 2004). It has recently also been suggested that they are involved in regulating cell migration (Iimura and Pourquie, 2006; Iimura and Pourquie, 2007).

In most vertebrates, *hox* genes are located in four clusters in the genome (Duboule, 2007; Garcia-Fernandez, 2005). Because of the whole-genome duplication event in the Actinopterygii (ray-finned fishes) leading to the teleost lineage (Hurley *et al.*, 2007), the zebrafish harbours eight *hox* clusters (Corredor-Adamez *et al.*, 2005; Postlethwait *et al.*, 2000) of which one is reduced to a single microRNA (Woltering and Durston, 2006). The generalised hypothesis is that the *hox* genes are expressed in a spatial and temporal fashion according to their position in a cluster ((Duboule and Dolle, 1989; Graham *et al.*, 1989; Izpisua-Belmonte *et al.*, 1991) reviewed by (Deschamps and van Nes, 2005)). In case of the frog *Xenopus laevis*, the first *hox* genes are expressed in the non-organiser mesoderm of the gastrula ((Wacker *et al.*, 2004) and references herein) and, upon involution of the mesoderm, transient *hox* expression seems to be stabilised via an interaction

Abbreviations used in this paper: A/P, anterior/posterior;

***Address correspondence to:** Willem M.R. van den Akker. Max Planck Institute for Biophysical Chemistry, Department of Molecular Cell Biology, Am Fassberg 9, D-37077, Göttingen, Germany. Fax: +49-551-201-1504. e-mail: wakker@gwdg.de

Supplementary Material for this paper (figure) is available at: http://dx.doi.org/10.1387/ijdb.082678wv

Accepted: 06 November 2008. *Final author corrected PDF published online:* 23 October 2009.

ISSN: Online 1696-3547, Print 0214-6282

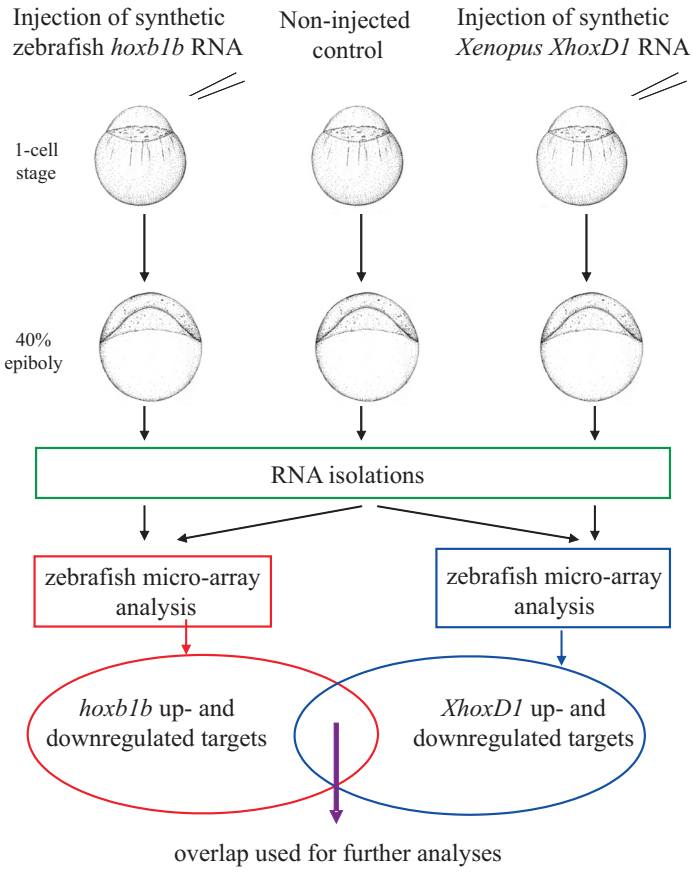


Fig. 1. Flow diagram of the experimental set-up of this study. *See text for details.*

with the Spemann organiser (the 'time-space translator model', (Jansen *et al.*, 2007; Wacker *et al.*, 2004)). The specific combination of expressed *hox* genes (the 'hox code', (Kessel and Gruss, 1991)) of the involuted mesoderm is transferred in a temporally and spatially specific manner to the overlying posterior neuroectoderm by which this tissue becomes patterned in the A/P axis of the embryo. Comparison with other model species suggests that this scenario has a broader significance as demonstrated by the behaviour of *hox* gene expression in for example mouse (Forlani *et al.*, 2003) and chicken (Iimura and Pourquie, 2006) embryos.

Recently, it was demonstrated that some *hox* genes might be actively involved in cell migratory events in the gastrula. Cells of the chicken epiblast layer in the anterior primitive streak region containing the artificially expressed *Hoxb1* gene, ingressed into the mesodermal layer and migrated anteriorly, while *HoxB9* expressing cells did not migrate or displayed only limited movements (Iimura and Pourquie, 2006). The molecular understanding of these various functions of *hox* genes during gastrulation is very limited. Since Hox proteins control the transcriptional activity of target genes, the identification of these downstream genes is an important step in elucidating the molecular *hox* functions. One strategy for identifying *hox* downstream genes is to analyse the transcriptosomal consequences of *hox* gene abrogation in a gastrula. This option is partially hampered by functional redundancy among *hox* genes (Greer *et al.*, 2000; McClintock *et al.*, 2002; Rossel and Capecchi, 1999; Studer *et al.*, 1998). Notably, *hox* genes having equivalent positions in different *hox* clusters and making up what is called a paralogue group (PG), can share particular functions, as becomes apparent when the simultaneous abrogation of two or three PG gene members displays a stronger phenotype than the additive effects of inactivation of individual members (e.g.(van den Akker *et al.*, 2001) (McNulty *et al.*, 2005)).

In the present study, we applied a gain-of-function approach to identify downstream genes of the developmentally first expressed zebrafish *hox* gene, *hoxb1b* (formerly *hoxa-1*, Alexandre *et al.*, 1996). To further select for *hox*-target gene interactions that are active during the earliest developmental stages, we made use of the supposed evolutionary conservation of the function of *hox* genes during gastrulation. For this we performed a direct comparison between the transcriptosomal changes upon the over-expression of the zebrafish *hoxb1b* gene and the heterologous over-expression of the first-expressed *Xenopus laevis hox* gene (*XhoxD1*) in zebrafish embryos. Four candidate target genes

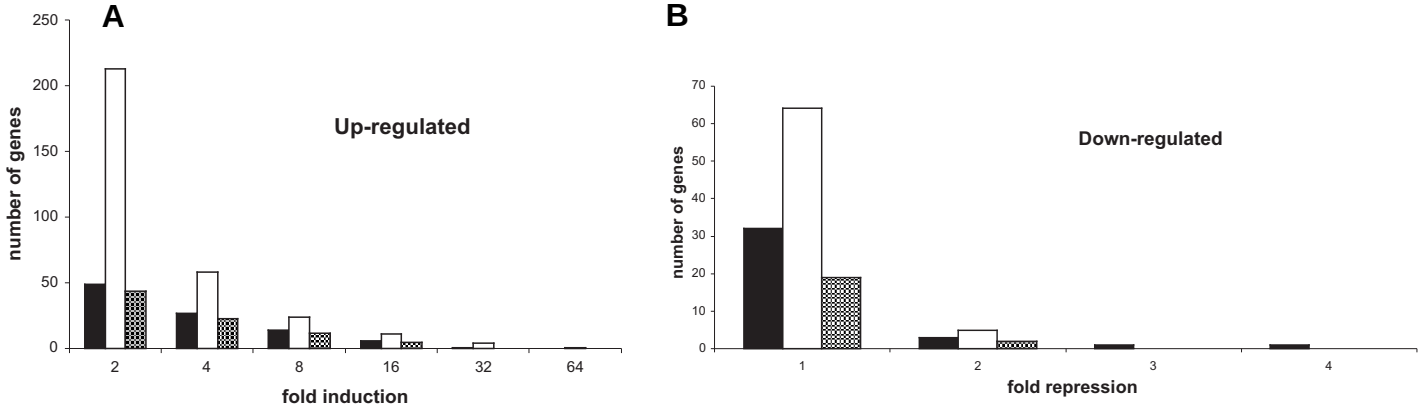


Fig. 2. Comparison of the number of identified *hoxb1b* and *XhoxD1* downstream genes and those identified as common to both *hox* genes. *Shown are the number of genes whose transcript levels were up-regulated* **(A)** *or down-regulated* **(B)** *by zebrafish* hoxb1b *(black bar) and* Xenopus XhoxD1 *(white bar) over-expression as determined by microarray analysis. The number of identified genes shared by both* hox *genes are shown by a blocked bar. Results are shown for common signature sets from all combined duplicate experiments and sorted according to their minimal fold of induction or repression using a P value filter of* e^{-5}.

TABLE 1

GENES UPREGULATED BY OVER-EXPRESSION OF ZEBRAFISH HOXB1B AND *XENOPUS* XHOXD1 IN ZEBRAFISH EMBRYOS AND ANALYSED AT 40% EPIBOLY

Accession number	Gene name or putative identity	Unigene	Function and/or process	Ratio	P-value
U40995	*hoxb1b; homeo box B1b*	Dr.83048	transcription factor	10.4*	7.E-10
BI879735	putative *neurogranin*	Dr.81838	signal transduction	6.6	5.E-19
AI878133	similar to *coronin, actin binding protein, 1c* (LOC562849)	Dr.76191	cytoskeletal regulation	3.0	3.E-30
BI671271	hypothetical LOC558422; similar to *lipin*	Dr.6402	nuclear phosphatase	2.6	2.E-07
AI601294	similar to *dynein, cytoplasmic 1, intermediate chain 1* (LOC799392)	Dr.77888	microtubule-based movement	7.1	4.E-15
BI866278	putative *brain-specific angiogenesis inhibitor 1 precursor*	Dr.81102	G-protein coupled receptor protein signalling pathway	8.3	2.E-21
BI981287	putative *cyclin-dependent kinase 5, regulatory subunit 1*	no entry	cell cycle regulation	3.8	3.E-20
BI840896	*bhlhb5; basic helix-loop-helix domain containing, class B, 5*	Dr.84568	transcription factor	17.4	3. E-43
AI721647	*nr2e1; nuclear receptor subfamily 2, group E, member 1*	Dr.78647	nuclear receptor	6.8	7.E-13
BM184118	*ndor1; nadph dependent diflavin oxidoreductase 1*	Dr.32954	oxidoreductase activity	2.3	3.E-17
AF007949	*ret1; receptor tyrosine kinase*	Dr.75763	signal transduction	5.0	4.E-11
AJ290391	*bcdo2l; beta-carotene 15, 15-dioxygenase 2, like*	Dr.79440	oxidoreductase activity	2.8	7.E-09
AF001909	*rx3*; *retinal homeobox gene 3*	Dr.540	transcription factor	7.0	8.E-12
U50563	*msxe; muscle segment homeobox e*	Dr.75086	transcription factor	3.0	2.E-11
BI473045	*sst3; somatostatin 3*	Dr.82638	regulation of cell migration	3.6	5.E-08
U67844	*dlx6a: distal-less homeobox gene 6a*	Dr.75092	transcription factor	2.8	6.E-09
AI793690	*dmbx1a; diencephalon/mesencephalon homeobox 1a*	Dr.78986	transcription factor	26.6	3. E-16
BG308656	*zgc:73361;* putative *reprimo-like*	Dr.27132	cell cycle	16.1	2.E-14
BI879533	*pcdh10b; protocadherin 10b*	Dr.82621	cell adhesion	5.8	9.E-17
BE015653	*gad1; glutamate decarboxylase 1*	Dr.81985	neurotransmitter biosynthetic process	5.1	2.E-07
BG306206	*phlda3*; *pleckstrin homology-like domain, family A, member 3*	Dr.82918	apoptosis	2.4	2.E-19
BM155603	putative *nkx6.2*	Dr.15365	transcription factor	18.9	4. E-11
AI641124	cxcl12b; *chemokine (C-X-C motif) ligand 12b* (*stromal cell-derived factor 1*)	Dr.105027	chemokine ligand	6.8	<1.E-50
AI793487	*sp8l; sp8 transcription factor-like* (*sp8a*)	Dr.29744	transcription factor	4.4	3.E-09
BE016629	putative *cytokine receptor-like factor 1*	Dr.32094	receptor activity	3.3	1.E-07
AF228334	*hand2; heart and neural crest derivatives expressed 2*	Dr.81423	transcription factor	14.5	2.E-13
L25273	*alcam; activated leukocyte cell adhesion molecule*	Dr.20912	cell adhesion	11.6	5.E-09
BI979064	*sp9: sp9 transcription factor*	Dr.86028	transcription factor	11.9	6. E-33
AI626641	putative *long-chain-fatty-acid-CoA ligase 6*	Dr.185	lipid biosynthesis	9.9	4.E-17
CO801543	*si:ch211-154o6.6; c type lectin domain family 4 member*	Dr.73909	carbohydrate binding	4.1	1.E-06

Data presented here are restricted to genes that exhibit enhanced transcript levels upon over-expression of both Hoxb1b and XhoxD1 as determined by microarray analysis. The complete datasets have been deposited in the MIAMExpress database. The given values represent mean values for all analysed data sets generated by the program Rosetta Resolver. Targets with ambiguous or unknown function are left out of the figure to improve legibility. The order of targets in this Table is such that it harmonizes with the data presented in Supplementary Figure S1.

* Since the injected *hoxb1b* plasmid contained the sequence of the oligonucleotide representing zebrafish *hoxb1b* on the microarray only data for *XhoxD1* are given.

were selected for further study by *in-situ* hybridization analysis and we confirmed that all of these genes are targets of the zebrafish *hoxb1b* gene as well as targets of the closely related paralogous *hoxb1a* gene. Our results are in agreement with the hypothesis that *hox* genes are involved both in controlling patterning events as well as in regulating cell movements during the earliest stages of vertebrate development. Moreover, the identification of a series of putative *hox*-downstream genes reported here provides a fertile basis for the further molecular understanding of the mechanisms underlying the early embryonic developmental events.

Results

Experimental rationale of this study

To gain insight into the functions of *hox* genes during early embryogenesis we set up a strategy to identify downstream genes of the developmentally first expressed *hox* gene in zebrafish, *hoxb1b*. For this, we combined *hox* over-expression in zebrafish embryos with whole-genome microarray analysis and compared the data obtained with those that were found when the first expressed *Xenopus hox* gene, *XhoxD1*, was heterologously expressed in zebrafish embryos (Fig. 1). Synthetic RNA of both *hox* genes was microinjected into 1-cell stage embryos and the total RNA of the developing embryos was harvested at 40% epiboly. This time point is just before the start of the endogenous transcription of *hoxb1b* at 50% epiboly (Alexandre *et al.*, 1996; Maves and Kimmel, 2005). The choice of this analysis time point eliminates the background effect of endogenously expressed *hoxb1b* while at the same time the embryonic environment is very closely related to that where *hoxb1b* is naturally first expressed. We anticipated that the direct comparison of the effects of *hoxb1b* and *XhoxD1* over-expression reduces the number of false positive target genes and offers the possibility to detect shared target genes which might expose evolutionarily conserved functions of the first expressed *hox* genes in developing embryos.

Extensive overlap between **hoxb1b** *and* **XhoxD1** *downstream genes*

Analysis of the microarray data (see Materials and Methods section for technical details) showed that there are significantly more zebrafish genes affected by the forced expression of the *XhoxD1* gene than by the zebrafish *hoxb1b* gene (Fig. 2). Of the 49 genes that are more than two-fold induced by *hoxb1b*, nearly 90% are also present among the collection of 213 *XhoxD1* induced genes. The percentage of common *hoxb1b/XhoxD1*

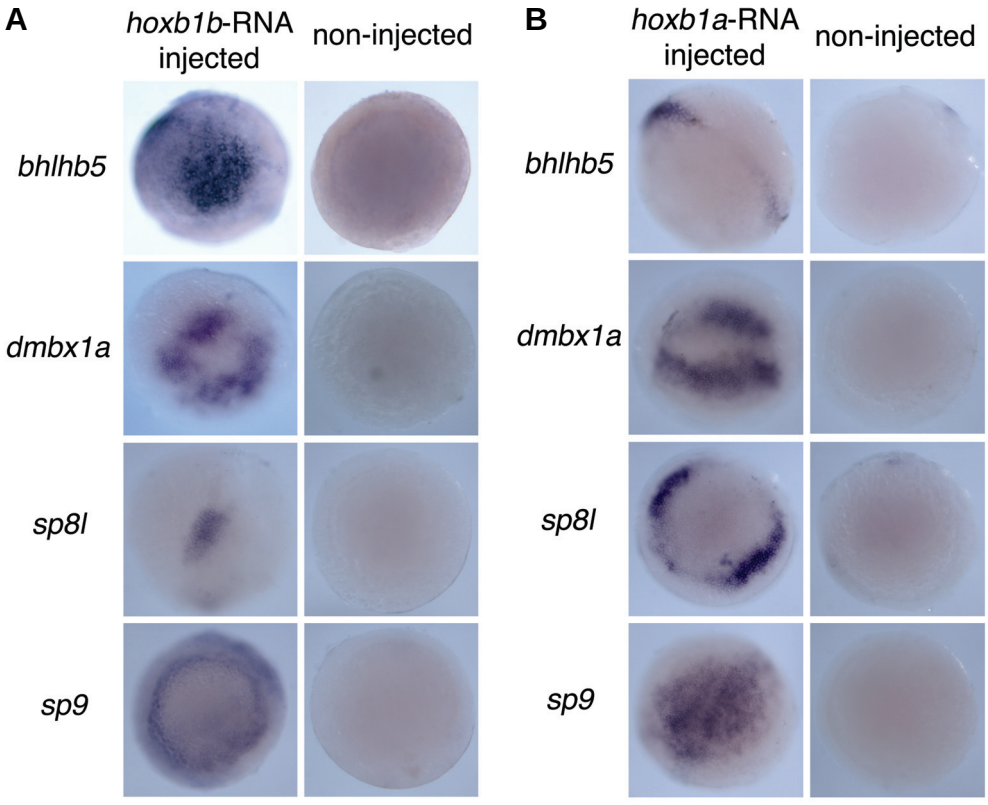


Fig. 3. Induced expression of transcription factors after the forced expression of *hoxb1b* and *hoxb1a* in zebrafish embryos. *Synthetic* hoxb1b *(150 pg)* **(A)** *or* hoxb1a *(50 pg)* **(B)** *RNA was microinjected into 1-cell stage embryos. At 50% epiboly, the embryos were analysed for the presence of* bhlhb5, dmbx1a, sp8l *and* sp9 *transcripts by* in-situ *hybridization. Non-injected hybridized control embryos are shown on the right sides.*

induced genes among the total number of *XhoxD1* up-regulated genes increases from 21% at 2-fold induction to 40% at 4-fold induction, and 50% at 8-fold induction. Similar results were obtained for the *hoxb1b* and *XhoxD1* down-regulated genes. Regarding the genes exhibiting at least two-fold reduced transcript levels about 60% of the 32 *hoxb1b* down-regulated genes are also identified in the collection of 64 genes down-regulated by *XhoxD1*. Therefore, it seems that the more strongly the expression level of a putative target gene is influenced by *hox*-overexpression, the more likely that it is a common target gene of both *hox* genes. Taken together, these data demonstrate that *hoxb1b* and *XhoxD1* have many downstream genes in common following their forced expression early during zebrafish development.

Endogenous expression of hoxb1b/XhoxD1 downstream genes

Since we have applied a gain-of-function approach in this study, it is of importance to check whether the identified potential hox-target genes are expressed under physiological (normal) conditions during early development. For this we have made use of the data obtained in the study of Mathavan and colleagues (Mathavan *et al*., 2005) who determined the temporal transcriptional expression levels during embryonic zebrafish development using the same microarray platform as applied in the present study, allowing direct comparison between data sets. The reliability of the Mathavan *et al*. dataset was recently confirmed in an independent study published by Ouyang and colleagues (Ouyang *et al*., 2008). The data presented in Supplementary Figure S1 visualise the endogenous temporal expression profiles of the up-regulated and down-regulated genes from the common *hoxb1b*/*XhoxD1* downstream gene target set. As expected, all down-regulated genes exhibit early native developmental expression, whereas several of the positively regulated genes are clearly expressed prematurely by the forced *hox* gene expression. It might be that some of

TABLE 2

GENES DOWN-REGULATED BY OVER-EXPRESSION OF ZEBRAFISH HOXB1B AND *XENOPUS* XHOXD1 IN ZEBRAFISH EMBRYOS AND ANALYSED AT 40% EPIBOLY

Accesión number	Gene name or putative identity	Unigene	Function and/or process	Ratio	P-value
X71845	*eve1*; *even-skipped-like1*	Dr.75074	transcription factor	-3.0	2.E-07
BI885265	putative Desmoglein	no entry	cell adhesion	-3.8	1.E-06
AI477935	*zgc:110712*, hypothetical protein LOC550250; Protein family: keratin type 1 cytoskeletal cytokeratin	Dr.33453	cytoskeleton	-3.6	2.E-10
AW077995	*ptgs2a*; *prostaglandin-endoperoxide synthase 2a*	Dr.113864	cell migration, prostaglandin biosynthesis	-2.6	8.E-08
BI850015	Hypothetical protein LOC794352; putative zinc finger protein 36 homolog	Dr.34611	transcription factor	-2.0	6.E-06
AI477963	*si:ch211-191a24.3*; putative tensin	Dr.77015	cell migration	-2.5	3.E-07
AW344023	CD44 homolog membrane glycoprotein precursor	Dr.122381	cell adhesion	-2.1	9.E-08
AF301264	*her9*; *hairy-related 9*	Dr.78757	transcription factor	-5.6	3.E-09
AW019321	*zgc:92414*; putative *urate oxidase*	Dr.106461	metabolism	-2.3	3.E-12
AI721923	*zgc:136396*; putative *collagenase 3 precursor*	Dr.78814	cell adhesion	-3.3	2.E-06

Data presented here are restricted to genes that exhibit reduced transcript levels upon over-expression of both Hoxb1b and XhoxD1 as determined by microarray analysis. The complete datasets have been deposited in the MIAMExpress database. The given values represent mean values for all analysed data sets generated by the program Rosetta Resolver. Targets with ambiguous or unknown function are left out of the figure to improve legibility. The order of targets in this Table is such that it harmonizes with the data presented in Supplementary Figure S1.

these latter interactions are functional during other stages of normal development (for example in the developing hindbrain) or during adulthood.

Spatially restricted expression of some* hoxb1b/hoxb1a *induced transcription factors

To validate the microarray data we tested the transcriptional activation of several identified putative downstream genes of *hoxb1b* and *XhoxD1* by zebrafish whole-mount in-situ hybridization. The four target genes selected, *sp8l* and'*sp9* (two members of the Sp1 family of zinc-finger transcription factors (Kawakami *et al*., 2004), *dmbx1a* (a homeobox-encoding gene, ((Kawahara *et al*., 2002); also known as *mbx*) and *bhlhb5* (a bHLH-encoding gene, (Adolf *et al*., 2004), all encode transcription factors and are all transcribed in presumptive rhombencephalic tissues. These tissues are patterned by transcription factor networks in which *hox* genes exert a central function. In addition, the structurally closely related *sp8l* and *sp9* genes as well as the other members of the *sp1*-family appear to be chromosomally linked to the various *hox* clusters, which might hint at a special relationship between *sp* and *hox* genes. Over-expression of *hoxb1b* early during zebrafish development did indeed induce the expression of all four putative target genes as expected from the microarray analyses (Fig. 3A). Moreover, these four genes were also induced by the ectopic expression of the zebrafish PG1 member *hoxb1a* (its endogenous expression starts around 9 hpf, at the transition of gastrulation to the segmentation stage (Maves and Kimmel, 2005)) as analyzed at the germ-ring stage of zebrafish development (Fig. 3B). This observation that different but related Hox proteins might have common downstream genes corroborates the proposal that the zebrafish *hoxb1b* and *hoxb1a* genes have partially redundant functions (McClintock *et al*., 2001). Interestingly, the induced expression domains of *bhlhb5*, *dmbx1a*, *sp8l* and *sp9* each display spatially restricted expression in the developing zebrafish embryo following ubiquitous over-expression of'*hoxb1b/hoxb1a* (Fig. 3). This indicates that the presence of PG1 *hox* genes is not the limiting factor in determining the localised expression of these downstream genes in the developing embryo at the stages studied, and it hints at the spatially restricted activities of factors that are positively or negatively involved in the transcriptional regulation of these downstream genes. In the case of *hox*-target genes whose transcriptional regulation is directly controlled by Hox proteins, spatial restriction of Hox-co-factors, like members of the Meis family, is a plausible explanation for the observed restricted expression domains of the target genes.

Relative abundance of morphogenetic regulators and transcription factors among target genes

Gene annotation of the *hoxb1b/XhoxD1* overlap group of positively and negatively regulated downstream genes shows an over-representation of genes encoding transcription factors and of genes that encode factors associated with cell adhesion and cell-movement processes (positively-regulated targets: 15/29 (52%); negatively-regulated targets: 9/10 (90%), Tables 1,2; Fig.4). These data support the idea that the first expressed *hox* genes have a role both in patterning and in regulating cell movements early during development.

Furthermore, we detected a strong up-regulation of *hoxb1b*

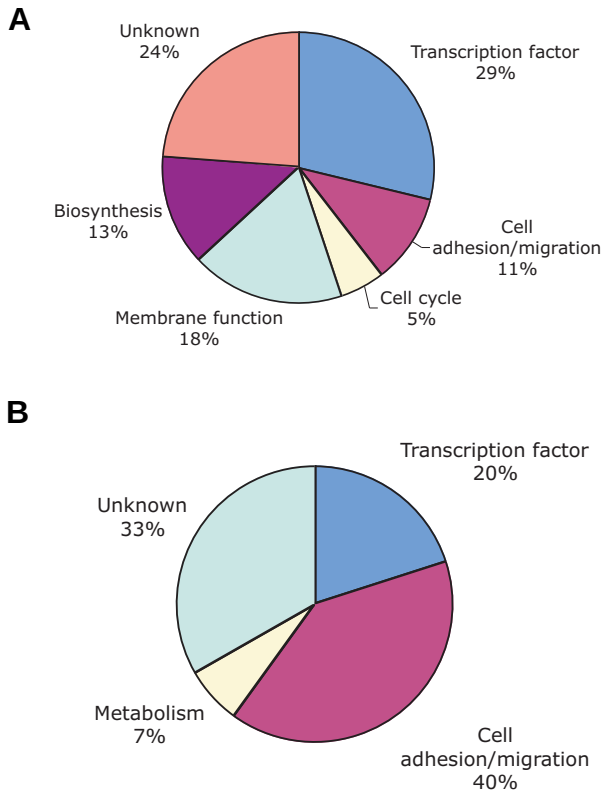


Fig. 4. Classification of the identified common *hoxb1b/XhoxD1* putative target genes. *The relative presence of both induced* **(A)** *and repressed* **(B)** *common* hoxb1b/XhoxD1 *target genes are grouped according to their supposed biological functions.*

upon over-expression of *XhoxD1*, possibly involving Hox-*hox* cross-regulation (or auto-regulation) (Hooiveld *et al*., 1999). Our data do not allow conclusions about hoxb1b-*hoxb1b* interactions since the oligonucleotide sequence on the array is present within the injected over-expression construct. Since no other *hox* genes are significantly induced in our experiments, we assume that the identified target genes are downstream genes of the first developmentally expressed *hox* genes and not (indirectly) induced via other *hox* genes.

Discussion

Gastrulation is a complex developmental process that all metazoan embryos have to go through. The identity of the *hox*-downstream genes identified in this study supports the idea that *hox* genes exert a role in patterning as well as in cell behavioural activities during this process. Comparison of zebrafish microarray gene expression profiles between the forced expression of zebrafish *hoxb1b* and *Xenopus XhoxD1*, revealed extensive overlap in their downstream genes indicating partial evolutionary conservation of Hox functions. A possible explanation for the incompleteness of this overlap is that particular Hox functions are differently distributed over PG1 *hox* members in diverse species. In this respect it is of interest that most effects of the simultaneous knock-down of all PG1 Hox members in *Xenopus laevis* could be overcome by *XhoxD1* over-expression in these *Xenopus*

morphants indicating a broad functional spectrum for this particular *hox* gene (McNulty *et al*., 2005). According to a similar line of reasoning, the lower number of identified *hoxb1b* downstream genes in comparison with those of *XhoxD1* might imply a more restricted function for *hoxb1b*. Alternatively, or additionally, evolutionary differences in the roles of the first expressed *hox* genes may exist between the fish and frog lineages which have an evolutionary divergence time of more than 400 million years (Prince and Pickett, 2002).

Comparison of the potential hox target genes identified here with their temporal endogenous expression profiles during embryogenesis (Mathavan *et al*., 2005) displays several apparent discrepancies. This is presumably an intrinsic consequence of the over-expression approach we applied. Firstly, the forced expression is performed before the endogenous expression of *hoxb1b*. Secondly, the forced expression of the *hox* genes is ubiquitous in the embryo and not restricted to the ventrolateral gastrula mesoderm in which tissue the first endogenous expression of *hox* genes takes place. So there seems to be a permissive environment for transcriptional regulation of various genes in which the artificially expressed *hox* gene is a limiting factor. This explains why several of the interactions identified are not necessarily functional during the earliest stages of normal development but may be during later developmental processes.

Members of the TALE subclass of homeodomain proteins, Meis and Pbx, have been reported to be functional in modulating the transcriptional activity of Hox proteins in vertebrates (Moens and Selleri, 2006; Pearson *et al.*, 2005), including zebrafish (Choe and Sagerstrom, 2005; Vlachakis *et al.*, 2001). Since the *meis* genes are expressed starting at 50% epiboly (Zebrafish Gene Expression Database, ZFIN), we do not expect that Meis proteins play a role in modulating the transcriptional activity of the Hox downstream genes identified here. Members of the *pbx* class (*pbx2, pbx3b, pbx4, pknox1.1* and *pknox1.2*), however, are ubiquitously expressed from the 1-cell stage onwards during early zebrafish development (Zebrafish Gene Expression Database, ZFIN) and are possibly involved in the transcriptional regulation of downstream targets of hoxb1b reported here. Our observations that some Hox target genes are not uniformly expressed in the embryo following forced expression might be explained by the spatially restricted activity of modulators of hox target gene expression at this stage of development or, alternatively, the result may imply that these downstream genes are not directly transcriptionally activated by Hoxb1b. Over the last few years, it has become evident that microRNAs exert important functions in gene regulation, including that of hox genes (reviewed by (Begemann, 2008)) Such control mechanisms might well be involved in the explanation of the spatial expression of hox-target genes that we report here. We can envisage a direct role of spatially-restricted microRNA mediated down regulation of the injected hox RNA, or otherwise regulation of the target gene itself (or an indirect effect by affecting a regulatory gene for this target gene).

The relative abundance of transcription factors and components that are involved in cell-cell interactions among the identified Hoxb1b target genes might indicate an involvement of this protein in patterning (specification) as well as cell in behavioural processes. A recent study in the invertebrate *Drosophila* indicates that the Hox target genes can be separated into directly regulated transcription factors and signalling molecules (regulator genes), and that most indirectly regulated genes (realisator genes) are involved in cell adhesion, polarity and cytoskeleton (Lovegrove *et al*., 2006). This fits with the hypothesis that Hox proteins play an active role in the complex cell movements taking place in the (pre-)gastrula of which epiboly and mesodermal ingression are the most prominent.

Recently, Rohrschneider and collegues (Rohrschneider *et al*., 2007) published a study on Hoxb1a downstream genes applying a loss-of-function approach using Morpholinos and focussing on the consequences for zebrafish rhombomere 4 characteristics at 19-20 hpf. It is striking that no overlap exists between their identified target genes of hoxb1a and our target genes (both studies made use of the same oligo-set for microarray analysis) and the target genes identified in the present study (both studies made use of the same oligo-set for microarray analysis). This might be explained in part by the differences between hoxb1a and hoxb1b, but we suppose that the temporal and tissue specific differences in the analyses are most crucial here, demonstrating the changing nature and the complexity of *hox* gene functions during development.

Conclusions

Despite the broad and strong interest in the functions of *hox* genes as transcriptional regulators, the identification of their downstream target genes appears to be quite resistant to being fully elucidated (Svingen and Tonissen, 2006). The large number of *hox* genes, their partially simultaneous expression and their partially redundant functions are only a few of the reasons making this a complex issue to investigate. Here, we report the identification of putative *hox*-downstream genes by the combination of forced expression early during development and microarray analyses. We were in part able to focus the range of identified *hox* downstream genes to the early developmental stages by the direct comparison of the effects of over-expression analysing two first expressed *hox* genes from distantly related organisms (zebrafish and *Xenopus*). This approach was chosen to select for evolutionary conserved mechanisms. Analysis of the categories of putative hox-target genes suggests that the earliest hox functions are related to processes involving patterning and cell movement, fitting well with the (pre-)gastrula stage processes in which *hox* genes are thought to be involved. The identification of putative downstream genes reported here is an important step in elucidating the molecular mechanisms of *hox* gene functions in the early stages of vertebrate development. Study of the individual candidate genes in relation to *hox* functions will further delineate the earliest steps in vertebrate development.

Materials and Methods

Overexpression of hox *genes in zebrafish embryos*

Fertilized zebrafish eggs were obtained from natural spawnings, raised under standard conditions (Westerfield, 1995) and staged according to Kimmel *et al*. (Kimmel *et al*., 1995). The zebrafish *pCS2hoxb1b* and *pCS2hoxb1a* constructs (McClintock *et al*., 2001) (a kind gift of V. Prince, Chicago) were used for over-expression experiments. Previously, we described the *Xenopus laevis XHoxD1* over-expression construct (McNulty *et al*., 2005). Capped RNA was synthesized *in vitro* using the mMessage mMachine kit (Ambion) according to the manufacturer's instructions. The

RNA was purified via a RNAeasy column (Qiagen) and microinjected into 1-cell stage embryos in a volume of 1 nl containing 50 or 150 pg RNA.

Zebrafish microarray analysis

Zebrafish microarray analysis was performed using 16,416 custom spotted 65-mer oligonucleotides designed by Compugen and synthesised by Sigma-Genosys (Mathavan *et al*., 2005). Additionally, a set of 340 custom-made oligonucleotides representing all zebrafish *hox* genes, various small GTPases, genes of the innate immune system and a set of lectin genes was spotted. A two-colour ratio methodology was applied to compare *hox*-RNA-injected embryos with non-injected ('control') embryos, essentially as described previously (Meijer *et al*., 2005). It is noted that the use of non-injected embryos as controls does not rule out a possible effect induced by the injection of RNA itself. Total RNA from 40% epiboly stage zebrafish embryos was isolated using the TRIzol reagent (Invitrogen) and purified as described previously (Meijer *et al*., 2005). For biological duplicate experiments, *hox* RNA was independently prepared and a different batch of zebrafish eggs was used. The reproducibility was demonstrated with *hoxb1b* RNA that gave similar results in the microarray analysis. The combined data sets from the three biological and several technical duplicates in these experiments were analyzed for the number of statistically significant up or down regulated genes with a threshold value P of e^{-5}. The microarray data were analyzed further with the Rosetta Resolver software package (version 6, Rosetta Impharmatics LLC). Selection of signature sequences was based on at least four replicates per experiment. Primary data sets have been deposited in the MIAMExpress database (accession number E-MEXP-1426).

Genomic and expression databases

Gene annotation of the microarray oligonucleotides was performed manually using the Ensemble zebrafish genome assembly version 6 (Zv6) and the Zebrafish Model Organism Database (ZFIN). In case of ambiguity of gene annotation these data were only included in the supplementary datasets. Classification of genes was based on the Unigene database (NCBI). Information about developmental gene expression was in part derived from the ZFIN database.

Whole-mount* in situ *hybridisation

For whole-mount *in situ* hybridisation (ISH) embryos were fixed in 4% paraformaldehyde and stored in methanol. ISH was performed essentially as described by Thisse and colleagues (Thisse *et al*., 2004). Anti-sense RNA *in-situ* probes were synthesized using T7 or Sp6 RNA polymerase and labelled with digoxigenin. The following zebrafish Expressed Sequence Tags (ESTs, provided by the IMAGE Consortium) were used for the preparation of *in-situ* antisense probes: *bhlhb5* [GenBank:BI840896], *dmbx1a* [GenBank:AI793690], *sp8l*(*sp8a*) [GenBank:CA470674] and *sp9* [GenBank:CA475616]. The identities of the ESTs were checked by sequencing. Linearization was done with *Sal*I (*bhlhb5* and *dmbx1a*), *Pvu*II (*sp8l*) and *Kpn*I (*sp9*), and transcription was performed using Sp6 (*bhlhb5* and *dmbx1a*) or T7 (*sp8l* and *sp9*). Detection of the anti-digoxigenin antibody-alkaline phosphatase conjugate was done using BM-Purple (Roche). Embryos were mounted in 90% glycerol and photographed using a compound microscope.

Acknowledgements

Dr. Victoria E. Prince is thanked for the generous gift of the zebrafish hox *over-expression constructs. Dr. Annemarie H. Meijer is acknowledged for technical advice. We thank the colleagues from the zebrafish facility at the Hubrecht Institute for their help. This work was financially supported by the European Commission 6th Framework Program (LSHG-CT-2003-503496, ZF-MODELS) and the European Union (LHSM-CT-2003-504468).*

References

ADOLF, B., BELLIPANNI, G., HUBER, V. and BALLY-CUIF, L. (2004). atoh1.2 and beta3.1 are two new bHLH-encoding genes expressed in selective precursor cells of the zebrafish anterior hindbrain. *Gene Expr Patterns* 5: 35-41.

ALEXANDRE, D., CLARKE, J.D., OXTOBY, E., YAN, Y.L., JOWETT, T. and HOLDER, N. (1996). Ectopic expression of Hoxa-1 in the zebrafish alters the fate of the mandibular arch neural crest and phenocopies a retinoic acid-induced phenotype. *Development* 122: 735-746.

BEGEMANN, G. (2008). MicroRNAs and RNA interference in zebrafish development. *Zebrafish* 5: 111-119.

CHOE, S.K. and SAGERSTROM, C.G. (2005). Variable Meis-dependence among paralog group-1 Hox proteins. *Biochem Biophys Res Commun* 331: 1384-1391.

CORREDOR-ADAMEZ, M., WELTEN, M.C., SPAINK, H.P., JEFFERY, J.E., SCHOON, R.T., DE BAKKER, M.A., BAGOWSKI, C.P., MEIJER, A.H., VERBEEK, F.J. and RICHARDSON, M.K. (2005). Genomic annotation and transcriptome analysis of the zebrafish (Danio rerio) hox complex with description of a novel member, hox b 13a. *Evol Dev* 7: 362-375.

DESCHAMPS, J. and VAN NES, J. (2005). Developmental regulation of the Hox genes during axial morphogenesis in the mouse. *Development* 132: 2931-2942.

DUBOULE, D. (2007). The rise and fall of Hox gene clusters. *Development* 134: 2549-2560.

DUBOULE, D. and DOLLE, P. (1989). The structural and functional organization of the murine HOX gene family resembles that of *Drosophila* homeotic genes. *EMBO J* 8: 1497-1505.

FORLANI, S., LAWSON, K.A. and DESCHAMPS, J. (2003). Acquisition of Hox codes during gastrulation and axial elongation in the mouse embryo. *Development* 130: 3807-3819.

GARCIA-FERNANDEZ, J. (2005). The genesis and evolution of homeobox gene clusters. *Nat Rev Genet* 6: 881-892.

GRAHAM, A., PAPALOPULU, N. and KRUMLAUF, R. (1989). The murine and *Drosophila* homeobox gene complexes have common features of organization and expression. *Cell* 57: 367-378.

GREER, J.M., PUETZ, J., THOMAS, K.R. and CAPECCHI, M.R. (2000). Maintenance of functional equivalence during paralogous Hox gene evolution. *Nature* 403: 661-665.

HOOIVELD, M.H., MORGAN, R., IN DER RIEDEN, P., HOUTZAGER, E., PANNESE, M., DAMEN, K., BONCINELLI, E. and DURSTON, A.J. (1999). Novel interactions between vertebrate Hox genes. *Int J Dev Biol* 43: 665-674.

HURLEY, I.A., MUELLER, R.L., DUNN, K.A., SCHMIDT, E.J., FRIEDMAN, M., HO, R.K., PRINCE, V.E., YANG, Z., THOMAS, M.G. and COATES, M.I. (2007). A new time-scale for ray-finned fish evolution. *Proc Biol Sci* 274: 489-498.

IIMURA, T. and POURQUIE, O. (2006). Collinear activation of Hoxb genes during gastrulation is linked to mesoderm cell ingression. *Nature* 442: 568-571.

IIMURA, T. and POURQUIE, O. (2007). Hox genes in time and space during vertebrate body formation. *Dev Growth Differ* 49: 265-275.

IZPISUA-BELMONTE, J.C., FALKENSTEIN, H., DOLLE, P., RENUCCI, A. and DUBOULE, D. (1991). Murine genes related to the *Drosophila* AbdB homeotic genes are sequentially expressed during development of the posterior part of the body. *EMBO J* 10: 2279-2289.

JANSEN, H.J., WACKER, S.A., BARDINE, N. and DURSTON, A.J. (2007). The role of the Spemann organizer in anterior-posterior patterning of the trunk. *Mech Dev* 124: 668-681.

KAWAHARA, A., CHIEN, C.B. and DAWID, I.B. (2002). The homeobox gene mbx is involved in eye and tectum development. *Dev Biol* 248: 107-117.

KAWAKAMI, Y., ESTEBAN, C.R., MATSUI, T., RODRIGUEZ-LEON, J., KATO, S. and BELMONTE, J.C. (2004). Sp8 and Sp9, two closely related buttonhead-like transcription factors, regulate Fgf8 expression and limb outgrowth in vertebrate embryos. *Development* 131: 4763-4774.

KESSEL, M. and GRUSS, P. (1991). Homeotic transformations of murine vertebrae and concomitant alteration of Hox codes induced by retinoic acid. *Cell* 67: 89-104.

KIMMEL, C.B., BALLARD, W.W., KIMMEL, S.R., ULLMANN, B. and SCHILLING, T.F. (1995). Stages of embryonic development of the zebrafish. *Dev Dyn* 203: 253-310.

LOVEGROVE, B., SIMOES, S., RIVAS, M.L., SOTILLOS, S., JOHNSON, K., KNUST, E., JACINTO, A. and HOMBRIA, J.C. (2006). Coordinated control of cell adhesion, polarity, and cytoskeleton underlies Hox-induced organogenesis

in *Drosophila*. *Curr Biol* 16: 2206-2216.

MATHAVAN, S., LEE, S.G., MAK, A., MILLER, L.D., MURTHY, K.R., GOVINDARAJAN, K.R., TONG, Y., WU, Y.L., LAM, S.H., YANG, H. *et al.* (2005). Transcriptome analysis of zebrafish embryogenesis using microarrays. *PLoS Genet* 1: 260-276.

MAVES, L. and KIMMEL, C.B. (2005). Dynamic and sequential patterning of the zebrafish posterior hindbrain by retinoic acid. *Dev Biol* 285: 593-605.

MCCLINTOCK, J.M., CARLSON, R., MANN, D.M. and PRINCE, V.E. (2001). Consequences of Hox gene duplication in the vertebrates: an investigation of the zebrafish Hox paralogue group 1 genes. *Development* 128: 2471-2484.

MCCLINTOCK, J.M., KHEIRBEK, M.A. and PRINCE, V.E. (2002). Knockdown of duplicated zebrafish hoxb1 genes reveals distinct roles in hindbrain patterning and a novel mechanism of duplicate gene retention. *Development* 129: 2339-2354.

MCNULTY, C.L., PERES, J.N., BARDINE, N., VAN DEN AKKER, W.M. and DURSTON, A.J. (2005). Knockdown of the complete Hox paralogous group 1 leads to dramatic hindbrain and neural crest defects. *Development* 132: 2861-2871.

MEIJER, A.H., VERBEEK, F.J., SALAS-VIDAL, E., CORREDOR-ADAMEZ, M., BUSSMAN, J., VAN DER SAR, A.M., OTTO, G.W., GEISLER, R. and SPAINK, H.P. (2005). Transcriptome profiling of adult zebrafish at the late stage of chronic tuberculosis due to Mycobacterium marinum infection. *Mol Immunol* 42: 1185-1203.

MOENS, C.B. and SELLERI, L. (2006). Hox cofactors in vertebrate development. *Dev Biol* 291: 193-206.

OUYANG, M., GARNETT, A.T., HAN, T.M., HAMA, K., LEE, A., DENG, Y., LEE, N., LIU, H.Y., AMACHER, S.L., FARBER, S.A. *et al.* (2008). A web based resource characterizing the zebrafish developmental profile of over 16,000 transcripts. *Gene Expr Patterns* 8: 171-180.

PEARSON, J.C., LEMONS, D. and MCGINNIS, W. (2005). Modulating Hox gene functions during animal body patterning. *Nat Rev Genet* 6: 893-904.

POSTLETHWAIT, J.H., WOODS, I.G., NGO-HAZELETT, P., YAN, Y.L., KELLY, P.D., CHU, F., HUANG, H., HILL-FORCE, A. and TALBOT, W.S. (2000). Zebrafish comparative genomics and the origins of vertebrate chromosomes. *Genome Res* 10: 1890-1902.

PRINCE, V.E. and PICKETT, F.B. (2002). Splitting pairs: the diverging fates of duplicated genes. *Nat Rev Genet* 3: 827-837.

ROHRSCHNEIDER, M.R., ELSEN, G.E. and PRINCE, V.E. (2007). Zebrafish Hoxb1a regulates multiple downstream genes including prickle1b. *Dev Biol* 309: 358-372.

ROSSEL, M. and CAPECCHI, M.R. (1999). Mice mutant for both Hoxa1 and Hoxb1 show extensive remodeling of the hindbrain and defects in craniofacial development. *Development* 126: 5027-5040.

STUDER, M., GAVALAS, A., MARSHALL, H., ARIZA-MCNAUGHTON, L., RIJLI, F.M., CHAMBON, P. and KRUMLAUF, R. (1998). Genetic interactions between Hoxa1 and Hoxb1 reveal new roles in regulation of early hindbrain patterning. *Development* 125: 1025-1036.

SVINGEN, T. and TONISSEN, K.F. (2006). Hox transcription factors and their elusive mammalian gene targets. *Heredity* 97: 88-96.

THISSE, B., HEYER, V., LUX, A., ALUNNI, V., DEGRAVE, A., SEILIEZ, I., KIRCHNER, J., PARKHILL, J.P. and THISSE, C. (2004). Spatial and temporal expression of the zebrafish genome by large-scale *in situ* hybridization screening. *Methods Cell Biol* 77: 505-519.

VAN DEN AKKER, E., FROMENTAL-RAMAIN, C., DE GRAAFF, W., LE MOUELLIC, H., BRULET, P., CHAMBON, P. and DESCHAMPS, J. (2001). Axial skeletal patterning in mice lacking all paralogous group 8 Hox genes. *Development* 128: 1911-1921.

VLACHAKIS, N., CHOE, S.K. and SAGERSTROM, C.G. (2001). Meis3 synergizes with Pbx4 and Hoxb1b in promoting hindbrain fates in the zebrafish. *Development* 128: 1299-1312.

WACKER, S.A., JANSEN, H.J., MCNULTY, C.L., HOUTZAGER, E. and DURSTON, A.J. (2004). Timed interactions between the Hox expressing non-organiser mesoderm and the Spemann organiser generate positional information during vertebrate gastrulation. *Dev Biol* 268: 207-219.

WESTERFIELD, M. (1995). *The zebrafish book: a guide for the laboratory use of zebrafish (Brachydanio rerio).* University of Oregon Press, Eugene.

WOLTERING, J.M. and DURSTON, A.J. (2006). The zebrafish hoxDb cluster has been reduced to a single microRNA. *Nat Genet* 38: 601-602.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

See our Special Issue ***Pattern Formation*** edited by Cheng-Ming Chuong and Michael K. Richardson at:
http://www.ijdb.ehu.es/web/contents.php?vol=53&issue=5-6

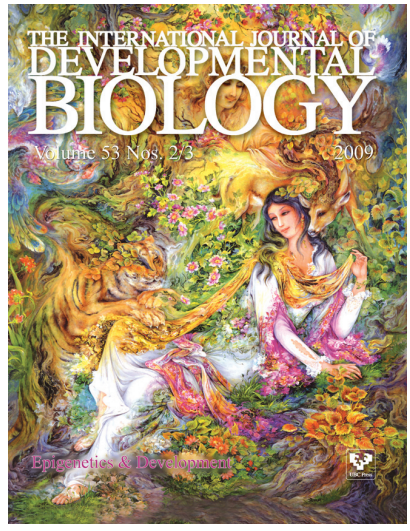
Function and specificity of Hox genes
David Foronda, Luis F. de Navas, Daniel L. Garaulet and Ernesto Sánchez-Herrero
Int. J. Dev. Biol. (2009): 1409-1419

PBX proteins: much more than Hox cofactors
Audrey Laurent, Réjane Bihan, Francis Omilli, Stéphane Deschamps and Isabelle Pellerin
Int. J. Dev. Biol. (2008) 52: 9-20

Pulling forces acting on Hox gene clusters cause expression collinearity
Spyros Papageorgiou
Int. J. Dev. Biol. (2006) 50: 301-308

Geometry and mechanics of teleost gastrulation and the formation of primary embryonic axes
Elena M. Cherdantseva and Vladimir G. Cherdantsev
Int. J. Dev. Biol. (2006) 50: 157-168

Transcriptional regulation and the evolution of development.
Gregory A Wray
Int. J. Dev. Biol. (2003) 47: 675-684



5 yr ISI Impact Factor (2008) = 3.271