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Identification and expression analysis of two novel members of the Mesp family in zebrafish

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ABSTRACT Mesp proteins play crucial roles in the formation of heart, vasculature and somites during vertebrate embryogenesis. We have used phylogenetic and genomic analysis, combined with qRT-PCR and *in situ* hybridization, to characterize two novel additional *mesp* genes in zebrafish, *mesp-ab* and *mesp-bb*, and describe their expression pattern in wild type and segmentation mutants. Both *mesp-ab* and *mesp-bb* are expressed in early mesoderm with *mesp-ab* expression starting during late blastula stages and *mesp-bb* expression initiating later, at the end of gastrulation. During somitogenesis, both *mesp* genes are expressed dynamically in the anterior presomitic mesoderm. *mesp-ab* is expressed in presumptive somites S-I and S-II, while *mesp-bb* is detected in S-I, S-II and S0, with expression restricted to the rostral compartment of presumptive somites. We show that the segmentation clock program regulates expression of these newly identified zebrafish *mesp* genes in a similar manner to their ohnologs, *mesp-aa* and *mesp-ba*. We also present evidence that zebrafish, minnow and salmon retained these additional *mesp* genes after the teleost whole genome duplication, while medaka, stickleback, fugu and tetraodon did not. Finally we show that although expression and regulation of zebrafish *mesp* genes appears highly comparable, there is no conservation in non-coding regions with other teleosts. In this study we have completed the description of the Mesp family in zebrafish, which will enable correct genome annotation and facilitate further functional studies on the role of these proteins in zebrafish.

KEY WORDS: *mesp, zebrafish, presomitic mesoderm, conserved regulation*

Introduction

mesp genes, which encode basic helix-loop-helix (bHLH) transcription factors, play important roles in formation of mesodermal tissues during development. In humans, mutations in *MESP2* are associated with skeletal defects (Whittock *et al.*, 2004). *Mesp2* knockout mice also show severe axial skeletal defects that result from problems in the formation of the somites, which are the building blocks of the axial skeletal elements of the vertebrate body. In particular, Mesp2 was shown to be important for somite border formation and their rostral-caudal identity (Morimoto *et al.*, 2005, Saga *et al.*, 1997, Takahashi *et al.*, 2000). This crucial role in somitogenesis is conserved in all non-mammalian species examined, such as chick, *Xenopus* and zebrafish (Saga and Takahashi, 2008). Knockout of *Mesp1* in mouse, on the other hand, leads to defects in heart formation, probably through perturbed migration of progenitor cells during gastrulation (Saga *et al.*, 1999). Interestingly, it has also been shown that Mesp1 is able to influence cardiovascular cell fate: overexpression in mouse embryonic stem cells leads to differentiation of cardiovascular cell types (Bondue *et al.*, 2008, David *et al.*, 2008, Lindsley *et al.*, 2008). However, as shown by double knockout and rescue studies in mouse, Mesp1 and Mesp2 together play partially redundant roles not only in cardiac progenitor migration but also in somite formation (Kitajima *et al.*, 2000, Saga, 1998). Interestingly, the single *Ciona mesp* gene has also

Abbreviations used in this paper: bHLH, basic helix-loop-helix; HCNE, highly conserved non-coding element; PSM, presomitic mesoderm; RACE, rapid amplification of cDNA ends; WGD, whole genome duplication; WISH, whole mount *in situ* hybridization.

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been shown to have a role in heart formation (Davidson *et al.*, 2005, Satou *et al.*, 2004), suggesting a conserved ancestral role for *mesp* genes.

In zebrafish, two *mesp* genes, *mesp-a* and *mesp-b* arranged head-to-tail on chromosome 7 were characterized previously (Durbin *et al.*, 2000, Sawada *et al.*, 2000). Zebrafish *mesp-a*, for instance, is expressed in the germ ring during gastrulation and was shown to be required for initiation of haematopoiesis (Hart *et al.*, 2007, Sawada *et al.*, 2000). Later in development, *mesp-a* is expressed in a dynamic manner in the anterior presomitic mesoderm (PSM), from where the somites will form, and plays a role in rostral-caudal somite polarity and boundary formation (Lee *et al.*, 2009, Sawada *et al.*, 2000). *mesp-b* is also expressed in the anterior PSM and knockdown studies in zebrafish suggest that it has a conserved role with *Mesp2* during somite segmentation, being involved in the rostro-caudal polarity of the somites and their epithelialisation (Kawamura *et al.*, 2005, Lee *et al.*, 2009, Sawada *et al.*, 2000). In addition, when zebrafish *mesp-b* is knocked into the mouse *Mesp2* locus it is able to mostly rescue the *Mesp2* deficiency, suggesting *mesp-b* is functionally homologous to *Mesp2* (Nomura-Kitabayashi *et al.*, 2002). Interestingly, the regulatory regions that govern expression in the anterior PSM also appear to be conserved between fish *mesp-b* and mammalian *Mesp2*, since a medaka *mesp-b* cis-regulatory module is able to functionally replace the mouse *Mesp2* PSM enhancer (Yasuhiko *et al.*, 2008).

Here we report the existence of two additional *mesp* genes, *mesp-ab* and *mesp-bb* on zebrafish chromosome 25. These additional genes likely have arisen as a result of the teleost-specific whole genome duplication (WGD) event that occurred ~350 million years ago (Crow *et al.*, 2006, Hoegg *et al.*, 2004). From our genomic analysis and in accordance with the zebrafish rules we propose a new nomenclature of *mesp* genes in zebrafish. We also describe the spatio-temporal expression of these genes during embryogenesis and in segmentation mutants. We show that expression of all four zebrafish *mesp* genes (*mesp-aa*, *mesp-ba*, and the newly identified mesp-ab and mesp-bb) is very similar, and that T-box and RBPJ-κ binding sites can be found in the genomic sequence surrounding these genes, suggesting that core regulatory elements have been conserved.

Results

Isolation of mesp-ab *and* mesp-bb

We scanned databases for cDNA, EST, protein and genomic sequences related to *mesp-aa* and *mesp-ba* and found two genes which map to zebrafish chr.25. We then isolated full-length cDNA for these genes using PCR and RACE (Supplementary Table S1). Based on sequence, phylogenetic and synteny analysis presented below and in consultation with the zebrafish nomenclature committee we have named these genes *mesp-ab* and *mesp-bb*. The two *mesp* genes previously identified on chr.7 are now named *mesp-aa* and *mesp-ba.*

Full-length *mesp-ab* encodes a protein of 240 amino acids (compared to 223 aa encoded by *mesp-aa*), whilst full-length *mesp-bb* encodes a protein of 244 amino acids (compared to 236 aa encoded by *mesp-ba*). It has been noted previously that Mesp-related proteins are highly conserved in the bHLH domain, but share little sequence similarity in the rest of the protein, and this is also the case for zebrafish Mesp-ab and Mesp-bb (Supplementary Table S2). For instance Mesp-ab is only 41.2% identical to Mesp-aa overall but across the bHLH they share 87% identity, whilst Mesp-bb is 42.2% identical to Mesp-ba, with 88.9% identity in the bHLH domain. When compared to human MESP1 and MESP2, Mesp-ab and Mesp-bb share between 73.5% - 83.3% identity across the bHLH domains but very little similarity outside this domain (Supplementary Table S2).

Phylogenetic analysis of Mesp proteins

We next searched databases for cDNA, EST, protein and genomic sequence for evidence of *mesp*-related genes in four other teleost species (medaka, fugu, stickleback and tetraodon). In addition to the two published medaka sequences (Terasaki *et al.*, 2006) we found sequences in fugu, tetraodon and stickleback, which encode *mesp-a* and *mesp-b* genes based on the sequence and phylogeny analysis presented here (Fig. 1A; Supplementary Table S1). A neighbour joining tree of these *mesp*-related genes is shown on Fig. 1A and indicates that *mesp-ab* is mostly closely related to *mesp-aa* and *mesp-bb* to *mesp-ba*. Interestingly we were only able to find evidence for one *mesp-a* and one *mesp-b* in fugu, medaka, tetraodon and stickleback (see below and discussion). This could be explained by two scenarios: either the *mesp-a/mesp-b* locus duplication only occured in zebrafish or it was specifically lost in the other four fish species after the teleost WGD. However, if we could find other teleost fish containing a *mesp* locus duplication, then this would argue for the second scenario-retention in zebrafish and loss in the other fish. To test this we further searched trace archives and found sequences related to *mesps* in minnows *(Pimephales promelas* and *Poeciliopsis turneri)* and atlantic salmon *(Salmo salar)*. The genomes and EST projects of these fish are still preliminary and we were not able to identify all likely *mesp* genes in these fish. However our analysis indicates that the *mesp-bb* duplication is not unique to zebrafish, but is also present in fathead minnow (*Pimephales promelas*), suggesting that fugu, medaka, tetraodon and stickleback may have lost this extra set of *mesp* genes (Fig. 1B). Salmon, which has undergone a 4th round of genome duplication after the teleost WGD, also show a *mesp* locus duplication (Fig. 1B).

Syntenic analysis of Mesp proteins

Zebrafish *mesp-aa* and *mesp-ba* are found in tandem on chr.7 flanked by an *sv2b* homologue and an *anpep* homologue (ENSDARG00000089706) and in a landscape containing *akap13* and *igf1rb*. We find *mesp-ab* and *mesp-bb* in a similar landscape on chr.25 in Zv9 (Fig. 1C). We also find fugu, medaka, tetraodon and stickleback *mesps* surrounded by genes homologous to *svb2, anpep, akap13* and *igf1r* in addition to *ap3s2*. Interestingly this syntheny is partially maintained in tetrapods, with the Mesp locus being surrounded by *Anpep* and *Ap3s2* to one side, but *Wdr93* and *Gnrhr2* on the other side.

Medaka chr.3 and chr.6 share a common ancestor with zebrafish chr.7 and chr.25 (Kasahara *et al.*, 2007, Woods *et al.*, 2005) and since medaka *mesp-a* and *mesp-b* are mapped to chr.3 we also looked at chr.6 for evidence of additional *mesp*-related genes that would support the hypothesis that *mesp-ab* and *mesp-bb* arose during the teleost WGD. Although we were able to find similar surrounding genes *(sv2b* and *anpep)* on the medaka chr.6 assembly we were unable to find any *mesp*-related gene sequence in this region (Fig. 1C). However the genomic sequence in this area is

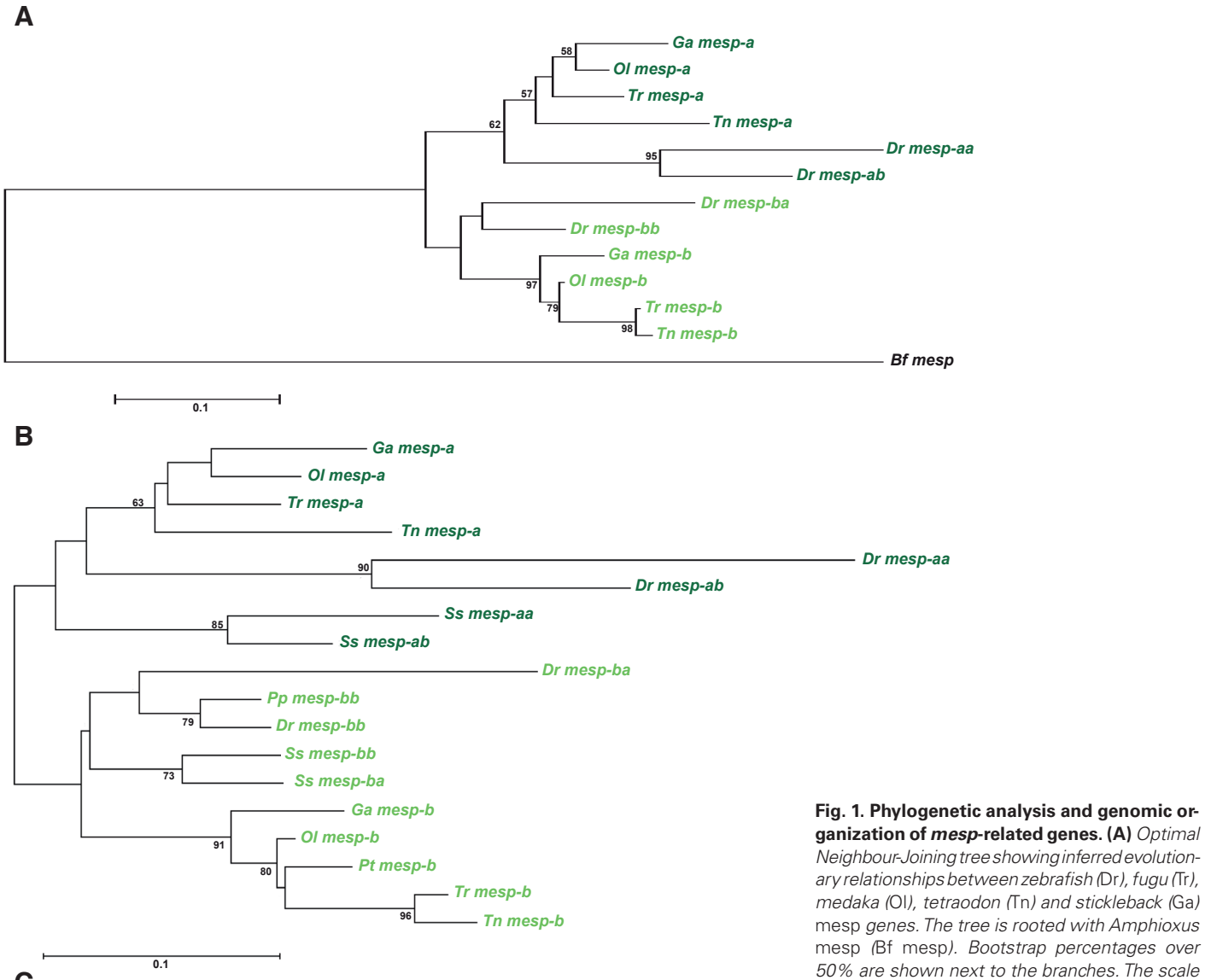
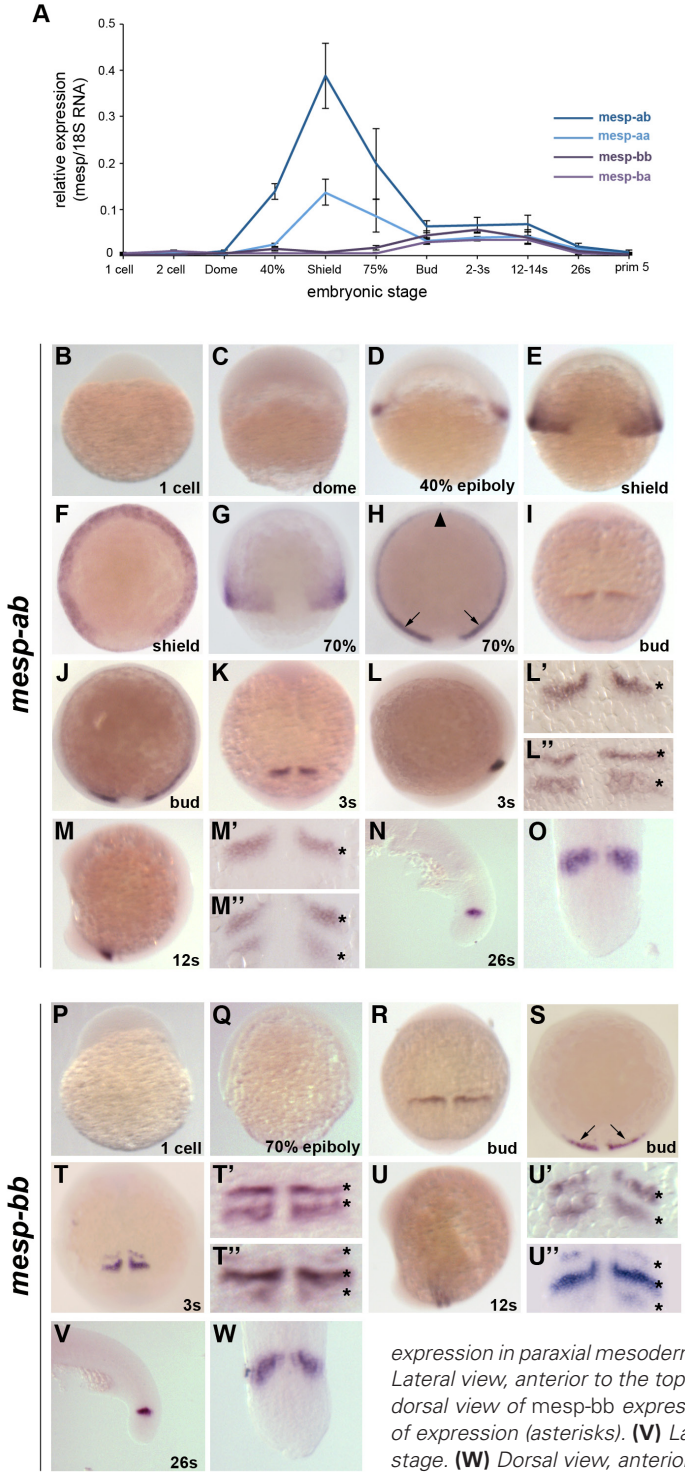


Fig. 1. Phylogenetic analysis and genomic organization of *mesp*-related genes. (A) *Optimal Neighbour-Joining tree showing inferred evolutionary relationships between zebrafish (*Dr*), fugu (*Tr*), medaka (*Ol*), tetraodon (*Tn*) and stickleback (*Ga*)* mesp *genes. The tree is rooted with Amphioxus* mesp *(*Bf mesp*). Bootstrap percentages over 50% are shown next to the branches. The scale bar infers the number of amino acid substitutions per site.* mesp-a *genes are shown in dark green,* mesp-b *genes in light green.* **(B)** *Neighbour-joining tree including minnow (*Pm, Pp*) and salmon (*Ss*) sequences.* **(C)** *Arrangement of genes surrounding* mesp*-related genes in zebrafish, medaka, fugu, tetraodon and stickleback.* mesp *genes are found in tandem and surrounded by* akap13, sv2b, anpep, ap3s2 *and* igf1r *in all fish (except zebrafish chr. 7 which does not have* ap3s2 *in this region). Homologous genes are shown in the same colour across species, and annotated with gene name or gene identifier. In the case of* zgc:73349 *homologues of this gene are not annotated in medaka and stickleback but inspection of the genomic suggests the sequence is present. Additional inserted genes on zebrafish chr25 are shown in grey. Dotted line indicates incomplete genomic sequence. Genomic coordinates are given for each region shown based on genome sequence versions Zebrafish Zv9, MEDAKA1, FUGU4, TETRAODON8, Stickleback BROADS1.*

incomplete and thus we can not rule out that additional *mesp* genes exist in medaka. A search for additional *sv2b, anpep,* or other flanking genes in fugu, tetraodon and stickleback did not reveal any evidence of a duplicated *mesp*-containing region in these species. Although more comprehensive genomic sequence for other teleosts will be needed to confirm it, the results presented above support a view that *mesp-ab* and *mesp-bb* are the result of a duplication of an ancestral chromosomal region, which now lies on chr.7 and chr.25 in zebrafish.



Expression of mesp*-related genes in wild-type embryos*

We next characterized the temporal and spatial expression of the newly identified *mesp-ab* and *mesp-bb* genes in early zebrafish embryos. qPCR analysis indicates that *mesp-ab* and *mesp-bb* have very similar temporal expression profiles to *mesp-aa* and *mesp-ba*, respectively (Fig. 2A). The expression of both *mesp-aa* and *mesp-ab* initiates at around 40% epiboly stage, with *mesp-ab* being more highly expressed than *mesp-aa*. Expression of both genes peaks at shield stage and continues at low levels until 26-somite stage. In contrast the expression of *mesp-ba* and *mesp-bb* initiates later in development at bud stage, and continues at similar levels until late somite stages. Expression of all four genes is gone by 24 hours-post-fertilization (hpf; prim 5) when somitogenesis completes.

To analyse spatial expression we generated whole mount *in situ* hybridization (WISH) probes, which recognize the 3' end of each RNA (in order to avoid the bHLH domain which may cross-react between paralogues) and compared these with probes which recognize the full-length RNA. We found no difference in expression patterns between full-length and bHLH-free probes (Supplementary Fig. S1 and data not shown) and since the full-length probe gave a stronger signal we used these for the following analyses (Figs. 2-4).

Fig. 2. Embryonic expression of *mesp-ab and mesp-bb*. **(A)** *qPCR time course of* mesp *gene expression from 1-cell to prim-5 (24 hours-post-fertilization). Expression is shown in units relative to total 18S RNA expression at the stage shown.* **(B,C)** *Expression of* mesp-ab *is not detected at 1-cell or dome stage. Animal to the top.* **(D,E)** mesp-ab *expression initiates in the ventral and lateral margin at 40% epiboly and persists in a similar pattern at shield stage. Dorsal view, animal to the top.* **(F)** *Animal view of shield stage* mesp-ab *expression, ventral to the top.* **(G,H)** *As gastrulation proceeds and mesoderm ingresses, expression persists in the lateral mesoderm (arrows), and is down-regulated on the ventral side (arrowhead).* **(G)** *Shows dorsal view, animal to the top,* **(H)** *shows animal view, ventral to the top.* **(I)** *At the end of gastrulation stripes are seen in the paraxial mesoderm. Dorsal view, anterior to the top.* **(J)** *Anterior view, ventral to the top, of bud stage* mesp-ab *expression in paraxial meosderm.* **(K-O)** mesp-ab *expression persists in paraxial mesoderm during somitogenesis.* **(K)** *Dorsal view, anterior to the top, at 3-somite stage showing one stripe of expression.* **(L)** *Lateral view, anterior to the top left, at 3-somite stage showing one stripe of expression.* **(L'-L")** *Enlarged dorsal view of* mesp-ab *expression in paraxial mesoderm at 3-somite stage showing one or two stripes of expression (asterisks).* **(M)** *Lateral view, anterior to the top left, at 12-somite stage.* **(M'-M")** *Enlarged dorsal view of* mesp-ab *expression in paraxial mesoderm at 12-somite stage showing one or two stripes of expression (asterisks).* **(N)** *Lateral view, anterior to the left, of* mesp-ab *expression in the tail at 26 somite stage.* **(O)** *Dorsal view, anterior to the top, of* mesp-ab *expression in the tail at 26-somite stage.* **(P,Q)** *Expression of* mesp-bb *is not detected at 1-cell stage or throughout early development; 70% epiboly shown as an example. Animal to the top.* **(R)** mesp-bb *expression is first detected at bud stage when stripes are seen in the paraxial mesoderm. Dorsal view, animal to the top.* **(S)** *Animal view, ventral to the top, of bud stage* mesp-bb *expression in the paraxial mesoderm (arrows). (T-W)* mesp-bb *expression persists in paraxial mesoderm during somitogenesis.* **(T)** *Dorsal view, anterior to the top, at 3-somite stage showing two stripes of expression.* **(T'-T")** *Enlarged dorsal view of* mesp-bb *expression in paraxial mesoderm at 3-somite stage showing two or three stripes of expression (asterisks).* **(U)** *Lateral view, anterior to the top left, at 12-somite stage showing two stripes of expression.* **(U'-U")** *Enlarged dorsal view of* mesp-bb *expression in paraxial mesoderm at 8-12-somite stage showing one or two stripes of expression (asterisks).* **(V)** *Lateral view, anterior to the left, of* mesp-bb *expression in the tail at 26-somite stage.* **(W)** *Dorsal view, anterior to the top, of* mesp-bb *expression in the tail at 26-somite stage.*

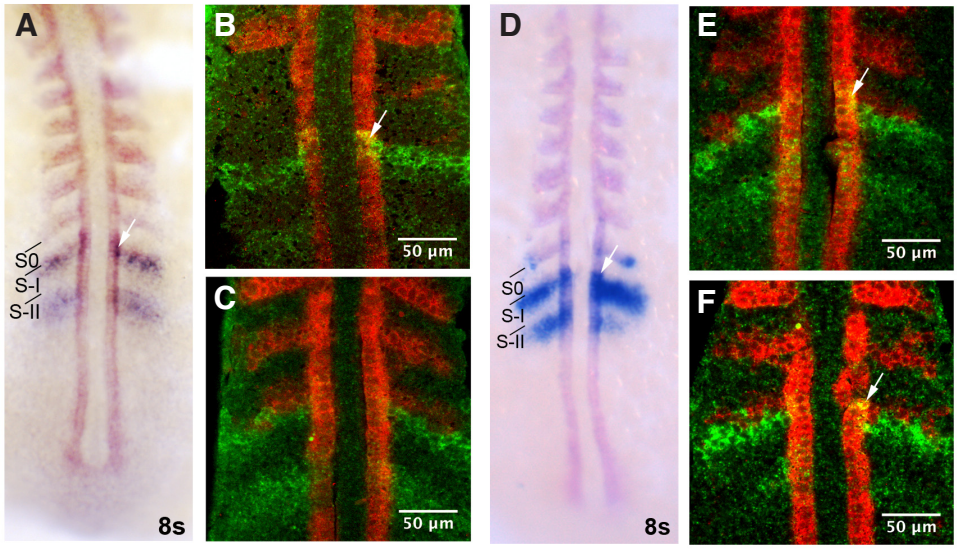


Fig. 3. Expression of *mesp-ab* and *mesp-bb* in the anterior presomitic mesoderm. (A) *Double* in situ *showing expression of* myod1 *(red) and* mesp-ab *(blue) at 8-somite stage.* **(B,C)** *Fluorescent double* in situ *showing expression of* myod1 *(red) and* mesp-ab *(green) at 8-somite stage. The most anterior stripe of* mesp-ab *in S-I is immediately adjacent to the caudal* myod1 *expression in S0. Dynamic* mesp-ab *expression also overlaps with* myod1 *expression in the adaxial cells (white arrows).* **(D)** *Double* in situ *showing expression of* myod1 *(purple) and* mesp-bb *(blue) at 8-somite stage.* **(E,F)** *Fluorescent double* in situ *showing expression of* myod1 *(red) and* mesp-bb *(green) at 8-somite stage. Expression of* mesp-bb *in S-I abuts* myod1 *expression in S0. Dynamic* mesp-bb *expression also overlaps with* myod1 *expression in the adaxial cells (white arrows). S0, S-I and S-II mark the position of presumptive somites. All embryos were flat-mounted and are shown in dorsal views, anterior to the top.*

Spatially *mesp-ab* expression also closely resembles *mesp-aa* expression (Durbin *et al.*, 2000, Sawada *et al.*, 2000). In accordance with our qPCR results, *mesp-ab* is not detected maternally or at early blastula stages by WISH (Fig. 2 B,C) but is first detected at 40% epiboly in the blastoderm margin where it is excluded from the dorsal region (Fig. 2D). At shield stage expression continues to be seen in the ventral-lateral margin (Fig. 2 E,F). At 70% epiboly expression is seen in the mesodermal layer of cells (Fig. 2 G,H; arrows), with expression begining to recede from the ventral region (Fig. 2H; arrowhead). From tailbud stage through somite formation stages transverse stripes of expression are seen in the anterior region of the PSM (Fig. 2 I-O). In the PSM, *mesp-ab* expression is dynamic and either one or two sets of stripes can be observed in embryos of the same stage until near the end of somitogenesis (26-somite stage), when only one stripe is observed (Fig. 2 K-O).

Similarly *mesp-bb* expression closely resembles *mesp-ba* expression (Sawada *et al.*, 2000). In accordance with our qPCR results, we find *mesp-bb* is not detectable by WISH maternally or during blastula and gastrula stages (Fig. 2 A,P-Q). Expression is detected at bud stage when transverse stripes are seen in the PSM (Fig. 2 R-S; arrows). These stripes are expressed dynamically and at bud stage either one, two or three stripes are observed, while either two or three stripes persist throughout the majority of somitogenesis (Fig. 2 T-U''). However, by 26-somite stage, as somite formation is finishing, only one stripe of *mesp-bb* expression is seen (Fig. 2 V-Y).

Previous studies have shown that *mesp-aa* is expressed at the anterior PSM in a territory that corresponds to the prospective somites S-I (restricted to the rostral compartment) and S-II (Durbin *et al.*, 2000, Sawada *et al.*, 2000). To define the precise location of *mesp-ab* expression within the prospective somites we compared its expression with *mesp-aa* and with the caudal somite compartment marker *myod1,* using double WISH. This analysis revealed that *mesp-aa* and *mesp-ab* expression overlaps during somitogenesis (Supplementary Fig. S2). Moreover, when compared with *myod1* we see that the most anterior *mesp-ab* stripe is positioned immediately adjacent and posterior to the last *myod1* stripe present in prospective somite S0 (Fig. 3A-C). Thus *mesp-ab* expression is located in the rostral compartment of prospective somites in the anterior PSM.

Comparison of *mesp-bb* expression with *mesp-ba* and *myod1* (Fig. 3D-F; Supplementary Fig. S2) also confirms that *mesp-bb*

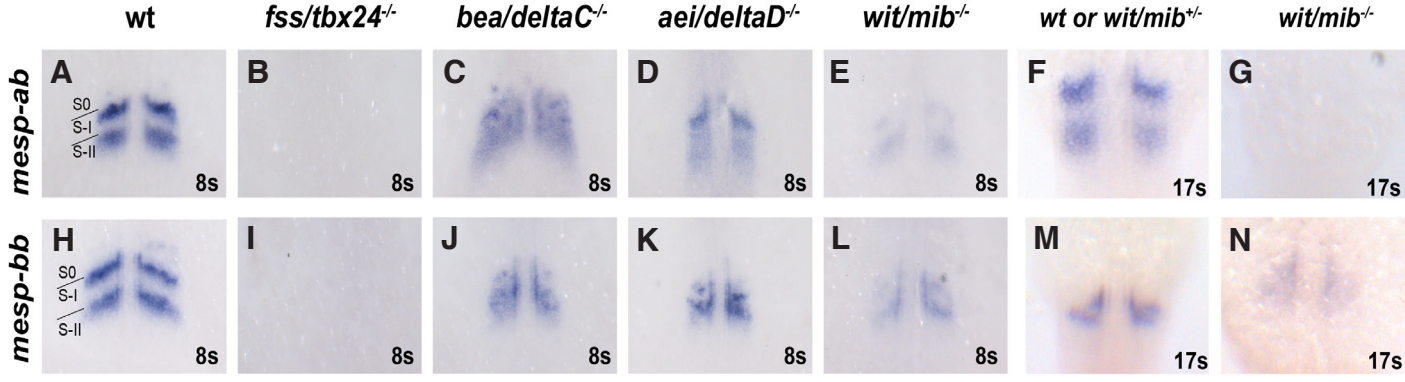


Fig. 4. Expression of *mesp-ab* and *mesp-bb* in segmentation mutants. (A,H) *Wild-type,* **(B,I)** fss/tbx24$^{-/-}$, **(C,J)** bea/deltaC$^{-/-}$, **(D,K)** aei/deltaD$^{-/-}$, **(E,L)** wit/mib$^{-/-}$ *embryos at the 8-somite stage hybridized with* mesp-ab **(A-E)** *and* mesp-bb **(H-L)** in situ *probes.* **(G,N)** wit/mib$^{-/-}$ *and* **(F,M)** *wild-type or* wit/mib$^{+/-}$ *sibling embryos at 17-somite stage hybridized with* mesp-ab **(F, G)** *and* mesp-bb **(M,N)**. *S0, S-I and S-II mark the position of presumptive somites. All embryos were flat-mounted and are shown in dorsal views, anterior to the top.*

expression, similarly to *mesp-ba* is found at the rostral compartment of prospective somites S0, S-I and S-II (Sawada *et al.*, 2000; Fig. 3D-F).

The similarity in expression patterns between all *mesp* zebrafish genes suggest that, although their coding sequences have changed since duplication, the regulatory elements are more constrained and have remained alike.

The new mesp *genes are also downstream of the segmentation program*

It was shown previously that *mesp* genes are downstream of the segmentation program (Durbin *et al.*, 2000, Sasaki *et al.*, 2011, Sawada *et al.*, 2000, Yasuhiko *et al.*, 2006). In zebrafish two classes of mutants that show a striking segmentation phenotype were identified in the large scale genetic screens: *fused somites* (*fss*), in which the T-box family transcription factor *tbx24* is mutated, and several Notch pathway genes (Holley, 2007). In *fss/tbx24*$^{-/-}$ mutants no somitic boundaries are formed, while in Notch signalling mutants such as *bea/deltaC*$^{-/-}$, *aei/deltaD-/-* and *wit/mib-/-* the segmentation clock is desynchronized and irregular somitic boundaries are made (reviewed in Lewis *et al.*, 2009).

Our results and others show the expression of *mesp-aa* is absent from *fss/tbx24*$^{-/-}$ mutants and very weakly expressed in *bea/deltaC*$^{-/-}$, *aei/deltaD-/-* and *wit/mib-/-* mutants (Supplementary Fig.S3 B-E; Durbin *et al.*, 2000, Sawada *et al.*, 2000). In addition *mesp-ba* expression is also absent in *fss/tbx24*$^{-/-}$ mutants, while expressed in a diffuse pattern in the *bea/deltaC*$^{-/-}$, *aei/deltaD-/-* and *wit/mib-/-* mutants (Supplementary Fig.S3 G-J; Sawada *et al.*, 2000). These expression data suggest that while *mesp-aa* activation is shared by Tbx24 and Notch signalling, *mesp-ba* expression, although clearly synchronized by the Notch pathway to make perfect stripes, is primarily dependent on Tbx24 activity.

The newly identified members *mesp-ab* and *mesp-bb* show a similar expression regulation by the segmentation program to their ohnologues. Similar to *mesp-aa*, the expression of *mesp-ab* is absent in *fss/tbx24*$^{-/-}$ mutants (Fig. 4B) and very weak or absent in the strongest Notch signalling mutant the *wit/mib-/-* (Fig. 4 E-G). In the mutants for the Notch ligands *bea/deltaC*$^{-/-}$ and *aei/deltaD-/-*, the expression of *mesp-ab* is weak and diffuse (Fig. 4 C,D), suggesting a redundant requirement of the two ligands. The expression of *mesp-bb* is also absent in *fss/tbx24*$^{-/-}$ mutants (Fig. 4I) and weak and diffuse in the Notch signalling mutants *bea/deltaC*$^{-/-}$, *aei/deltaD-/-* and *wit/mib-/-* (Fig. 4 J-N). In contrast to *mesp-ab* and similar to its ohnologue *mesp-ba*, the expression in the strongest Notch signalling mutant *wit/mib-/-* is still detected (Fig. 4N), suggesting that the *mesp-b* group is mainly regulated by Tbx24.

T-box and RBPJ-κ binding sites are present in the mesp locus regulatory regions

Previous studies have shown T-box and RBPJ-κ binding sites are important in regulating somite expression of *mesp* genes in medaka and mouse (Terasaki *et al.*, 2006, Yasuhiko *et al.*, 2006, Yasuhiko *et al.*, 2008). Since the expression patterns of the *mesps* are highly similar we asked whether these binding sites are present in teleost *mesp* loci using the matrix-scan program within RSAT (Thomas-Chollier *et al.*, 2008, Turatsinze *et al.*, 2008; Fig. 5). This revealed many T-box and RBPJ-κ binding sites in all teleost species, and identified the previously discovered T-box and RBPJ-κ binding sites upstream of medaka *mesp-b* which regulate gene expression in the somites (Terasaki *et al.*, 2006, Yasuhiko *et al.*, 2008; Fig. 5). Clusters of T-box and RBPJ-κ binding sites are also seen upstream of the teleost *mesp-a* genes, except in stickleback

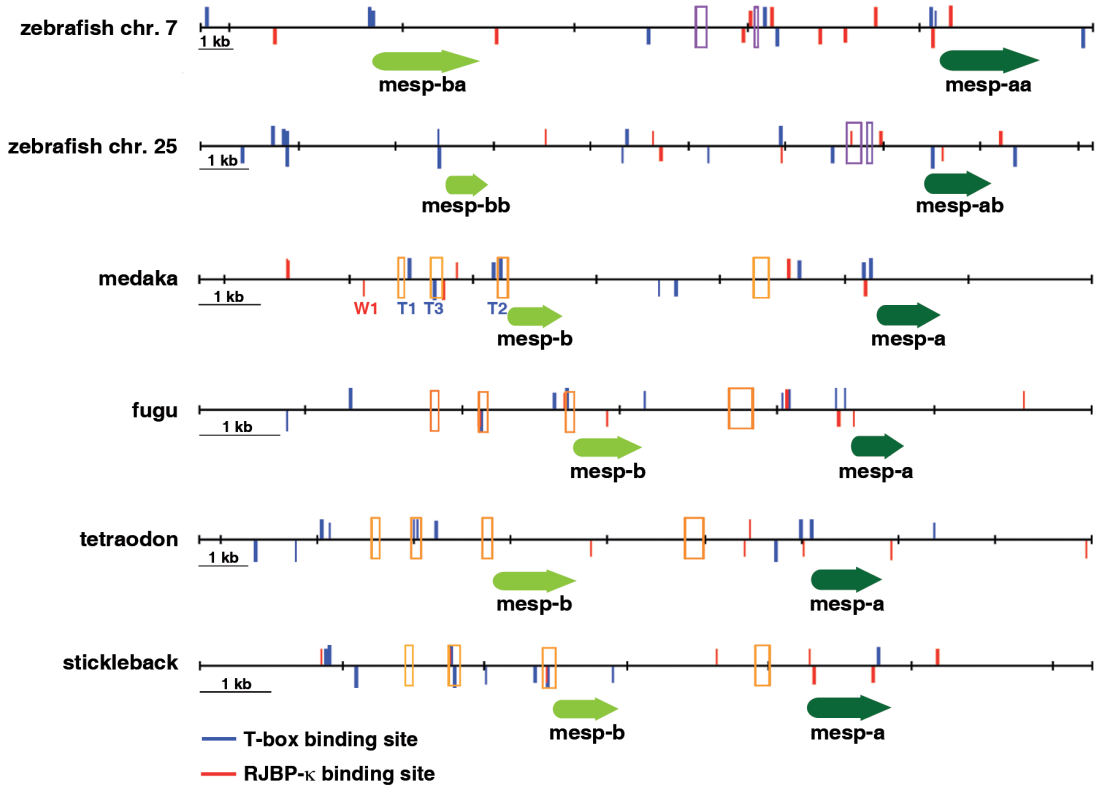


Fig. 5. Analysis of regulatory regions across teleost *mesp* loci. *Regions encompassing 5 kb upstream of* mesp-b *to 2 kb downstream of* mesp-a *in the* mesp *loci of zebrafish chromosome 7 and 25, medaka, fugu, tetraodon and stickleback are shown with T-box (blue) and RJBP-κ binding sites (red). Previously identified T-box (T1, T2, T3) and RJBP-κ (W1) binding sites that regulate medaka* mespb *expression in somites are indicated. Regions boxed in purple are conserved between the two zebrafish loci while regions boxed in orange are conserved between medaka, fugu, tetraodon and stickleback. See Supplementary Fig. S4 for conservation details.*

which appears to lack T-box binding sites in this region.

We also compared these genomic regions using VISTA which detects regions of high conservation (HCNEs; Frazer *et al.*, 2004; Supplementary Fig. S4). This analysis revealed limited conservation in non-coding regions between the two zebrafish loci, other than in the intron of *mesp-ab*/*bb,* in the intergenic region between the two *mesp* genes, and downstream of *mesp-aa/ab*. Surprisingly there was no homology in non-coding regions between zebrafish and the *mesp* loci in other teleosts, suggesting that if regulatory sites are conserved between zebrafish and other teleosts they do not fall in a region of high conservation (Supplementary Fig. S4A). When medaka was compared to the other teleosts conservation in non-coding regions was seen between fugu, tetraodon and stickleback, but not zebrafish, in the region upstream of *mesp-b* and between the two *mesp* genes (Supplementary Fig. S4B). Interestingly the regulatory T-box (T1-3) and RBPJ-κ (W1) binding sites identified upstream of medaka *mesp-b* fall in or close to these regions of conservation, suggesting this regulation may be conserved in percomorphs (Supplementary Fig. S3B; Fig. 5). Short regions of high conservation were also found upstream of the *mesp-a* genes, although these regions were not conserved between zebrafish and other teleosts (Fig. 5; Supplementary Fig. S4).

These results indicate that genomic sequence within the *mesp* loci has also diverged, although T-box binding sites upstream of *mesp-b* genes have remained in a conserved region in medaka, fugu, tetraodon and stickle back, but not zebrafish.

Discussion

We have isolated two *mesp*-related genes in zebrafish, *mesp-ab* and *mesp-bb*. The phylogenetic and syntenic analysis presented here suggests that these are ohnologues of *mesp-aa* and *mesp-ba*, generated by the teleost whole genome duplication. This duplication appears to have been retained in zebrafish and at least one other cyprinid (fathead minnow) but lost in percomorphs (medaka, fugu, tetraodon and stickleback). However more complete EST and genomic sequences in other fish will be needed to examine this issue fully.

After genome duplication there are several hypotheses for the fates of the new pair of genes: one of the new pair can either be lost; can take on a new function (neo-functionalization); may share the function with the ancestral gene (sub-functionalization); or a combination of these fates (neo-sub-functionalization). Functional change may be brought about by changes in protein coding sequence, or through changes in regulatory elements that cause divergence in spatio-temporal expression of duplicated genes (Jimenez-Delgado *et al.*, 2009).

Our expression analysis suggests that *mesp-ab* and *mesp-bb* have close to identical expression patterns with *mesp-aa* and *mesp-ba*, respectively. We do not believe these similar expression patterns are due to probe cross-reactivity between the bHLH domains of the *mesp-a* genes (or *mesp-b* genes) because, for instance, the bHLH sequence between *mesp-aa* and *mesp-ba* is more similar than between the two *mesp-a* genes (80.2% vs 75.3% identity respectively; Supplementary Table S2) yet *mesp-aa* and *mesp-ba* give different temporal and spatial expression patterns (Sawada *et al.*, 2000; data not shown). This suggests that differences seen in expression are specific to that gene.

The similarity in expression patterns between the ohnologues suggests that the core regulatory elements of these genes have yet to evolve in zebrafish, which is highly unusual for ohnologues (Kassahn *et al.*, 2009) and suggests these regulatory sequences are in someway constrained. Another explanation would be that these genes are not ohnologues, but the result of more recent duplication which would mean regulatory sequence has not yet had time to evolve. However, we did not find a great deal of conservation in non-coding regions between the two zebrafish loci, which would argue against this possibility. However since the *mesp* regulatory sequences in zebrafish are yet to be mapped, it is not known if these fall within the short HCNEs that were found upstream of zebrafish *mesp-aa/ab*. Intriguingly though, *mesp* expression in the anterior PSM is very similar in mouse, *Xenopus*, zebrafish and medaka which suggests that the enhancers regions for the *mesp* genes in all vertebrates may be conserved. In support of this, enhancer analysis has shown that a 350 bp region upstream of mouse *Mesp2*, a 2.8 kb region upstream of medaka *mesp-b*, a 2.9 kb region upstream of fugu *mesp-b* and a 3.5 kb region upstream of *Xenopus laevis mesp-b* are able to drive expression of a reporter in anterior PSM in a pattern that recapitulates endogenous *Mesp2*/*mesp-b* expression (Haraguchi *et al.*, 2001, Moreno *et al.*, 2008, Moreno and Kintner, 2004, Terasaki *et al.*, 2006). In addition, the medaka 2.8 kb *mesp-b* enhancer can substitute for the mouse *Mesp2* 350 bp enhancer, suggesting the *mesp-b* anterior PSM enhancer is conserved in vertebrates despite differences in size (Yasuhiko *et al.*, 2008). In *Ciona* T box binding sites found upstream of *mesp* are critical for expression of a reporter in the heart/muscle progenitor cells (Davidson *et al.*, 2005), whilst in medaka and mouse T-box sites upstream of *mesp-b*/*Mesp2* are also required for reporter expression in stripes at the anterior PSM (Terasaki *et al.*, 2006, Yasuhiko *et al.*, 2008). Our binding site analysis indicates that clusters of T-box sites are present upstream of all *mesp-b*s, and that in medaka, fugu, tetraodon and stickleback these sites fall within or close to regions of high conservation. Consistent with this we found that *mesp-b* expression was dependent on Tbx24 activity. RJBP-κ sites upstream of medaka *mesp-b* and mouse *Mesp2* have been shown to act together with T-box sites to enhance reporter gene expression but are not required for activation (Terasaki *et al.*, 2006, Yasuhiko *et al.*, 2008); again this is consistent with our observations which indicate *mesp-ba/bb* expression in zebrafish is not dependent on Notch signalling. The regulatory regions that govern *mesp-a* expression have not been investigated, but our segmentation mutant analysis suggests both Notch signaling and Tbx24 are required in a non-redundant manner for normal expression in the anterior PSM. Interestingly clusters of T-box and RJBP-κ sites are found upstream of *mesp-a* genes, except in stickleback, and may suggest that these sites are also active in regulating expression from the *mesp* locus.

We also note that the expression levels of *mesp-ab* mRNA are higher than *mesp-aa* during gastrulation and somitogenesis, and similarly *mesp-bb* expression is higher than *mesp-ba* during somitogenesis (Fig. 2A). While it is tempting to speculate that *mesp-ab* and *mesp-bb* could therefore play a greater role in development than their counterparts on chromosome 7, until protein levels are determined and functional studies performed on these two new genes this can not be determined.

Although the spatio-temporal regulation of *mesp* gene expression is highly similar the protein sequences are quite dissimilar, particularly in the region outside the central bHLH domain (Supple-

mentary Table S2). The bHLH domain is involved in DNA binding but the function of the N- and C-terminal domains of these proteins is less well understood. Recently however, the N-terminal domain of Mesp2 has been shown to regulate Notch signalling through interacting with and destabilizing the Mastermind-like 1 protein (Sasaki *et al.*, 2011). Despite differences in the sequence of Mesp proteins they seem to have similar functions in the embryo since different Mesp proteins are able to compensate for each other. For instance, although zebrafish *mesp-ba* and mouse *Mesp2* are only 24.8% identical overall (but 73.5% over the bHLH domain) *mesp-ba* is able to rescue a *Mesp2* knockout mouse. In addition, *Mesp1* is able to rescue the *Mesp2* knockout in mouse, suggesting that these proteins also function in a similar fashion (Saga, 1998). Despite this there are some differences in function between different Mesp proteins as illustrated by a study which showed mouse Mesp2 and *Xl*-mesp-b are able to activate a *lunatic fringe* promoter reporter construct more strongly than either mouse Mesp1 or *Xl*-mesp-a (Hitachi *et al.*, 2009).

In conclusion, the expression of *mesp-ab* and *mesp-bb* appears conserved with their homologues in fish and other vertebrates during early mesoderm formation and somitogenesis, suggesting they may also function in a conserved way in mesoderm and somite patterning.

Materials and Methods

Zebrafish lines

AB wild type and mutant (*fss*/*tbx24*^{te314a}, *bea*/*deltaC*^{tm98}, *aei*/*deltaD*^{fr233} and *wit*/*mib*^{ta52b}) embryos were staged according to Kimmel *et al.*, 1995.

Isolation of mesp-ab *and* mesp-bb

PCR primers were designed against reported cDNA sequence for *mesp-ab* (previously known as *mesp2*) and coding genomic sequence for *mesp-bb* and used to amplify cDNA from a mixed stage sample consisting of 75%, bud, 4-6 somite stage and 9-11 somite stage. Products were cloned in to pGEM-T Easy vector (Promega) and verified by sequencing. Primer sequences are reported in Supplementary Table S2. 5' and 3' RACE was then used to identify full-length cDNA at 4-6 somite stage. Briefly for 5' RACE, total RNA was reverse transcribed with a gene specific primer and GoScript reverse transcriptase (Promega). A homopolymeric dC tail was added to the 5' end. For 3' RACE, total RNA was primed with 3'RACE adaptor primer and reverse transcribed as above. UTR sequences were obtained by PCR with primers gene specific and adaptor primers, cloned in to pGEM-T Easy vector and verified by sequencing. Full-length sequences are provided in Supplementary Table S1 and have been submitted to Genbank with accession numbers JN084105 (*mesp-ab*) and JN084106 (*mesp-bb*).

Sequence retrieval and phylogenetic analysis

Where possible full-length cDNA and protein sequences were downloaded from Ensembl or Genbank. For zebrafish *mesp-ab* and *mesp-bb* we used the full-length sequences identified in this paper. For medaka *mesp-a* and *mesp-b* we used sequences from Terasaki *et al.*, 2006. For fugu *mesp-a*, fugu *mesp-b* and tetraodon *mesp-a* sequence analysis suggested the sequences available in the databases were not full length and so we used additional genomic sequence, alignment with known full-length transcripts and conserved exon/intron boundaries to add 5' and 3' sequence to the putative coding transcript. For stickleback and tetraodon *mesp-b* sequences, which have not been annotated, we searched the genomic sequence between *mesp-a* and *sv2b* and found evidence for a *mesp-b*-like bHLH coding sequence. The alignment of this sequence with other fish *mesp-bs* and conserved exon/intron structure allowed us to identify a putative coding sequence. Searches of databases at NCBI did not uncover any additional sequences for these fish. For salmon and minnow searches of EST, genome and trace archive sequences were performed. All sequences used here are reported in Supplementary Table S1.

Sequence alignment and phylogenetic tree analysis was performed in MEGA5 (Tamura *et al.*, 2011). MUSCLE was used to align sequences and then improved manually; analyses used alignments of the bHLH domain plus some flanking amino acid sequence. Both Neighbour Joining and Maximum Likelihood analyses were performed and gave similar results. 1000 bootstrap replicates were performed. Evolutionary distances were computed using the JTT matrix-based method.

Syntenic analysis

We analysed the region around *mesp-aa/mesp-ba* and *mesp-ab/mesp-bb* in zebrafish and in other species manually and using Genomicus (Muffato *et al.*, 2010). The region from 11646289 to 11178241 on chr.25 (Zv9) is shown in Fig. 1, but it should be noted that annotations for another *mesp*-related gene, *sv2b* and *anpep* can also be found in the interval 11129393-11051080 on chr.25. However the quality of sequence and assembly over this region of chr.25 is poor and this apparent duplication could be due to an assembly error (see pgpviewer.ensembl.org).

Conservation and binding site analysis

Genomic sequence of the *mesp* locus from medaka, fugu, tetraodon, stickleback and zebrafish chr. 7 and chr. 25, including 5kb upstream of *mespb* and 2kb downstream of *mespa* was analysed using MLAGAN in VISTA (Frazer *et al.*, 2004). The same sequence was then analysed to discover T-box and RBPJ-κ binding sites using RSAT (Thomas-Chollier *et al.*, 2008, Turatsinze *et al.*, 2008). Previously identified position weight matrixes for Brachyury and Tbx16 (Bryne *et al.*, 2008, Garnett *et al.*, 2009) and RBPJk (Krejci *et al.*, 2009, Tun *et al.*, 1994) where used. Sites with a p value of < 0.0001 were considered binding sites. RSAT provides a graphical output of these sites which was used in Fig. 5 then further annotated with the *mesp* genes and conserved regions identified in VISTA.

qPCR

Total RNA was extracted using Trizol reagent (Invitrogen) and treated with RQ1 DNAse. The total RNA was retrotranscribed with GoScript reverse transcriptase (Promega) using random hexamers and oligodT_{15}. Products were purified using Qiaquick PCR purification kit (Qiagen). Reactions were performed using SYBR Green I Master kit (Roche) on a Stratagene MX3005P and data analyzed using MxPro software. Cycling parameters were as follows, 95°C 10 min for 1 cycle then 95°C 30 sec, 60°C 30 sec, 72°C 30 sec for 30 cycles then 95°C 60 sec, 60°C 30 sec, 95°C 30 sec for all primer sets. Primer sequences used can be found in Supplementary Table S3. For each replicate data were normalized to an 18S rRNA endogenous control. The mean expression level with standard error of the mean of three replicates are reported.

In situ *hybridization*

mesp-aa and *mesp-ba* plasmids were a kind gift of Hiroyuki Takeda (Sawada *et al.*, 2000). For *mesp-ab* and *mesp-bb* we amplified full-length sequence from cDNA and cloned these into pGEM-T Easy vector. For probes without bHLH domain we used PCR to isolate the 3' end of the transcript. For primers details see Table S3. Whole-mount *in situ* hybridizations were performed according to standard protocols (Nusslein-Volhard and Dahm, 2002). Whole-mount double fluorescent *in situ* hybridizations were performed as described previously (Julich *et al.*, 2005) with minor modifications: the red signal was developed with FAST RED (Roche AP substrate) and the green with Tyramide FITC (POD substrate).

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References

BONDUE, A., LAPOUGE, G., PAULISSEN, C., SEMERARO, C., IACOVINO, M., KYBA, M. and BLANPAIN, C. (2008). Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. *Cell Stem Cell* 3: 69-84.

BRYNE, J.C., VALEN, E., TANG, M.H., MARSTRAND, T., WINTHER, O., DA PIEDADE, I., KROGH, A., LENHARD, B. and SANDELIN, A. (2008). JASPAR, the open access database of transcription factor-binding profiles: new content and tools in the 2008 update. *Nucleic acid res.* 36: D102-106.

CROW, K.D., STADLER, P.F., LYNCH, V.J., AMEMIYA, C. and WAGNER, G.P. (2006). The "fish-specific" Hox cluster duplication is coincident with the origin of teleosts. *Mol. Biol. Evol.* 23: 121-136.

DAVID, R., BRENNER, C., STIEBER, J., SCHWARZ, F., BRUNNER, S., VOLLMER, M., MENTELE, E., MULLER-HOCKER, J., KITAJIMA, S., LICKERT, H. *et al.,* (2008). MesP1 drives vertebrate cardiovascular differentiation through Dkk-1-mediated blockade of Wnt-signalling. *Nat Cell Biol* 10: 338-345.

DAVIDSON, B., SHI, W. and LEVINE, M. (2005). Uncoupling heart cell specification and migration in the simple chordate Ciona intestinalis. *Development* 132: 4811-4818.

DURBIN, L., SORDINO, P., BARRIOS, A., GERING, M., THISSE, C., THISSE, B., BRENNAN, C., GREEN, A., WILSON, S. and HOLDER, N. (2000). Anteroposterior patterning is required within segments for somite boundary formation in developing zebrafish. *Development* 127: 1703-1713.

FRAZER, K.A., PACHTER, L., POLIAKOV, A., RUBIN, E.M. and DUBCHAK, I. (2004). VISTA: computational tools for comparative genomics. *Nucleic acid res.* 32: W273-279.

GARNETT, A.T., HAN, T.M., GILCHRIST, M.J., SMITH, J.C., EISEN, M.B., WARDLE, F.C. and AMACHER, S.L. (2009). Identification of direct T-box target genes in the developing zebrafish mesoderm. *Development* 136: 749-760.

HARAGUCHI, S., KITAJIMA, S., TAKAGI, A., TAKEDA, H., INOUE, T. and SAGA, Y. (2001). Transcriptional regulation of Mesp1 and Mesp2 genes: differential usage of enhancers during development. *Mech. Dev.* 108: 59-69.

HART, D.O., RAHA, T., LAWSON, N.D. and GREEN, M.R. (2007). Initiation of zebrafish haematopoiesis by the TATA-box-binding protein-related factor Trf3. *Nature* 450: 1082-1085.

HITACHI, K., KONDOW, A., DANNO, H., NISHIMURA, Y., OKABAYASHI, K. and ASASHIMA, M. (2009). Molecular analyses of *Xenopus laevis* Mesp-related genes. *Integr. Zool.* 4: 387-394.

HOEGG, S., BRINKMANN, H., TAYLOR, J.S. and MEYER, A. (2004). Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. *J. Mol. Evol.* 59: 190-203.

HOLLEY, S.A. (2007). The genetics and embryology of zebrafish metamerism. *Dev Dyn* 236: 1422-1449.

JIMENEZ-DELGADO, S., PASCUAL-ANAYA, J. and GARCIA-FERNANDEZ, J. (2009). Implications of duplicated cis-regulatory elements in the evolution of metazoans: the DDI model or how simplicity begets novelty. *Brief Funct Genomic Proteomic* 8: 266-275.

JULICH, D., HWEE LIM, C., ROUND, J., NICOLAIJE, C., SCHROEDER, J., DAVIES, A., GEISLER, R., LEWIS, J., JIANG, Y.J. and HOLLEY, S.A. (2005). beamter/deltaC and the role of Notch ligands in the zebrafish somite segmentation, hindbrain neurogenesis and hypochord differentiation. *Dev Biol* 286: 391-404.

KASAHARA, M., NARUSE, K., SASAKI, S., NAKATANI, Y., QU, W., AHSAN, B., YAMADA, T., NAGAYASU, Y., DOI, K., KASAI, Y. *et al.,* (2007). The medaka draft genome and insights into vertebrate genome evolution. *Nature* 447: 714-719.

KASSAHN, K.S., DANG, V.T., WILKINS, S.J., PERKINS, A.C. and RAGAN, M.A. (2009). Evolution of gene function and regulatory control after whole-genome duplication: comparative analyses in vertebrates. *Genome res.* 19: 1404-1418.

KAWAMURA, A., KOSHIDA, S., HIJIKATA, H., OHBAYASHI, A., KONDOH, H. and TAKADA, S. (2005). Groucho-associated transcriptional repressor ripply1 is required for proper transition from the presomitic mesoderm to somites. *Dev. Cell* 9: 735-744.

KIMMEL, C.B., BALLARD, W.W., KIMMEL, S.R., ULLMANN, B. and SCHILLING, T.F. (1995). Stages of embryonic development of the zebrafish. *Developmental dynamics: an official publication of the American Association of Anatomists* 203: 253-310.

KITAJIMA, S., TAKAGI, A., INOUE, T. and SAGA, Y. (2000). MesP1 and MesP2 are essential for the development of cardiac mesoderm. *Development* 127: 3215-3226.

KREJCI, A., BERNARD, F., HOUSDEN, B.E., COLLINS, S. and BRAY, S.J. (2009). Direct response to Notch activation: signaling crosstalk and incoherent logic. *Sci. Signal.* 2: ra1.

LEE, H.C., TSENG, W.A., LO, F.Y., LIU, T.M. and TSAI, H.J. (2009). FoxD5 mediates anterior-posterior polarity through upstream modulator Fgf signaling during zebrafish somitogenesis. *Dev. Biol.* 336: 232-245.

LEWIS, J., HANISCH, A. and HOLDER, M. (2009). Notch signaling, the segmentation clock, and the patterning of vertebrate somites. *J. Biol.* 8: 44.

LINDSLEY, R.C., GILL, J.G., MURPHY, T.L., LANGER, E.M., CAI, M., MASHAYEKHI, M., WANG, W., NIWA, N., NERBONNE, J.M., KYBA, M. *et al.,* (2008). Mesp1 coordinately regulates cardiovascular fate restriction and epithelial-mesenchymal transition in differentiating ESCs. *Cell Stem Cell* 3: 55-68.

MORENO, T.A., JAPPELLI, R., IZPISUA BELMONTE, J.C. and KINTNER, C. (2008). Retinoic acid regulation of the Mesp-Ripply feedback loop during vertebrate segmental patterning. *Dev. Biol.* 315: 317-330.

MORENO, T.A. and KINTNER, C. (2004). Regulation of segmental patterning by retinoic acid signaling during *Xenopus* somitogenesis. *Dev. Cell* 6: 205-218.

MORIMOTO, M., TAKAHASHI, Y., ENDO, M. and SAGA, Y. (2005). The Mesp2 transcription factor establishes segmental borders by suppressing Notch activity. *Nature* 435: 354-359.

MUFFATO, M., LOUIS, A., POISNEL, C.E. and ROEST CROLLIUS, H. (2010). Genomicus: a database and a browser to study gene synteny in modern and ancestral genomes. *Bioinformatics* 26: 1119-1121.

NOMURA-KITABAYASHI, A., TAKAHASHI, Y., KITAJIMA, S., INOUE, T., TAKEDA, H. and SAGA, Y. (2002). Hypomorphic Mesp allele distinguishes establishment of rostrocaudal polarity and segment border formation in somitogenesis. *Development* 129: 2473-2481.

NUSSLIN-VOLHARD, C. and DAHM, R. (2002). *Zebrafish*. Oxford University Press, Oxford.

SAGA, Y. (1998). Genetic rescue of segmentation defect in MesP2-deficient mice by MesP1 gene replacement. *Mech Dev* 75: 53-66.

SAGA, Y., HATA, N., KOSEKI, H. and TAKETO, M.M. (1997). Mesp2: a novel mouse gene expressed in the presegmented mesoderm and essential for segmentation initiation. *Genes Dev* 11: 1827-1839.

SAGA, Y., MIYAGAWA-TOMITA, S., TAKAGI, A., KITAJIMA, S., MIYAZAKI, J. and INOUE, T. (1999). MesP1 is expressed in the heart precursor cells and required for the formation of a single heart tube. *Development* 126: 3437-3447.

SAGA, Y. and TAKAHASHI, Y. (2008). Mesp-family genes are required for segmental patterning and segmental border formation. *Adv. Exp. Med. Biol.* 638: 113-123.

SASAKI, N., KISO, M., KITAGAWA, M. and SAGA, Y. (2011). The repression of Notch signaling occurs via the destabilization of mastermind-like 1 by Mesp2 and is essential for somitogenesis. *Development* 138: 55-64.

SATOU, Y., IMAI, K.S. and SATOH, N. (2004). The ascidian Mesp gene specifies heart precursor cells. *Development* 131: 2533-2541.

SAWADA, A., FRITZ, A., JIANG, Y.J., YAMAMOTO, A., YAMASU, K., KUROIWA, A., SAGA, Y. and TAKEDA, H. (2000). Zebrafish Mesp family genes, mesp-a and mesp-b are segmentally expressed in the presomitic mesoderm, and Mesp-b confers the anterior identity to the developing somites. *Development* 127: 1691-1702.

TAKAHASHI, Y., KOIZUMI, K., TAKAGI, A., KITAJIMA, S., INOUE, T., KOSEKI, H. and SAGA, Y. (2000). Mesp2 initiates somite segmentation through the Notch signalling pathway. *Nat. Genet.* 25: 390-396.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. and KUMAR, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.* . 28:2731-2739

TERASAKI, H., MURAKAMI, R., YASUHIKO, Y., SHIN, I.T., KOHARA, Y., SAGA, Y. and TAKEDA, H. (2006). Transgenic analysis of the medaka mesp-b enhancer in somitogenesis. *Dev. Growth Differ.* 48: 153-168.

THOMAS-CHOLLIER, M., SAND, O., TURATSINZE, J.V., JANKY, R., DEFRANCE, M., VERVISCH, E., BROHEE, S. and VAN HELDEN, J. (2008). RSAT: regulatory sequence analysis tools. *Nucleic acid res.* 36: W119-27.

TUN, T., HAMAGUCHI, Y., MATSUNAMI, N., FURUKAWA, T., HONJO, T. and KAWAICHI, M. (1994). Recognition sequence of a highly conserved DNA binding

protein RBP-J kappa. *Nucleic acid res.* 22: 965-971.

TURATSINZE, J.V., THOMAS-CHOLLIER, M., DEFRANCE, M. and VAN HELDEN, J. (2008). Using RSAT to scan genome sequences for transcription factor binding sites and cis-regulatory modules. *Nat Protoc* 3: 1578-1588.

WHITTOCK, N.V., SPARROW, D.B., WOUTERS, M.A., SILLENCE, D., ELLARD, S., DUNWOODIE, S.L. and TURNPENNY, P.D. (2004). Mutated MESP2 causes spondylocostal dysostosis in humans. *Am J Hum Genet* 74: 1249-1254.

WOODS, I.G., WILSON, C., FRIEDLANDER, B., CHANG, P., REYES, D.K., NIX, R., KELLY, P.D., CHU, F., POSTLETHWAIT, J.H. and TALBOT, W.S. (2005). The zebrafish gene map defines ancestral vertebrate chromosomes. *Genome res.* 15: 1307-1314.

YASUHIKO, Y., HARAGUCHI, S., KITAJIMA, S., TAKAHASHI, Y., KANNO, J. and SAGA, Y. (2006). Tbx6-mediated Notch signaling controls somite-specific Mesp2 expression. *Proceedings of the National Academy of Sciences of the United States of America* 103: 3651-3656.

YASUHIKO, Y., KITAJIMA, S., TAKAHASHI, Y., OGINUMA, M., KAGIWADA, H., KANNO, J. and SAGA, Y. (2008). Functional importance of evolutionally conserved Tbx6 binding sites in the presomitic mesoderm-specific enhancer of Mesp2. *Development* 135: 3511-3519.