

Hypomorphic *Mesp* allele distinguishes establishment of rostrocaudal polarity and segment border formation in somitogenesis

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SUMMARY

A bHLH-type transcription factor, *Mesp2*, plays an essential role in somite segmentation in mice. Zebrafish *mespb* (*mesp-b*), a putative homologue of mouse *Mesp2*, is transiently expressed in the rostral presomitic mesoderm similarly to *Mesp2*. To determine whether zebrafish *mespb* is a functional homologue of mouse *Mesp2*, zebrafish *mespb* was introduced into the mouse *Mesp2* locus by homologous recombination. Introduced *mespb* almost rescued the *Mesp2* deficiency in the homozygous *mespb* knockin mouse, indicating that *mespb* is a functional homologue of mouse *Mesp2*. Segmented somites were clearly observed although the partial fusion of the vertebral columns still occurred. Interestingly, however, the nature and dosage of the *mespb* gene affected the rescue event. A mouse line, which has a hypomorphic *Mesp2* allele generated by the introduction of

neo-mespb, gave rise to an epithelial somite without normal rostrocaudal (RC) polarity. RC polarity was also lacking in the presomitic mesoderm. The defects in RC polarity were determined by the altered expressions of *Uncx4.1* and *Dll1* in the segmented somites and presomitic mesoderm, respectively. In contrast, the expression of *EphA4* (*Epha4*), *lunatic fringe* or *protocadherin*, thought to be involved in segment border formation, was fairly normal in hypomorphic mutant embryos. These results suggest that the *Mesp* family of transcription factors is involved in both segment border formation and establishment of RC polarity through different genetic cascades.

Key words: Somitogenesis, *Mesp2*, *mespb*, Rostrocaudal polarity, Segment border, Resegmentation, Mouse, Zebrafish

INTRODUCTION

Somitogenesis is a dynamic morphogenetic process required for the generation of a metamer architecture in vertebrates. The paraxial mesoderm derived from the primitive streak or tailbud is aligned on both sides of the neural tube as the presomitic mesoderm (PSM). For formation of the metamer structure, mesenchymal PSM cells have to undergo two types of segmentation. One is initial segmentation, which is the segment border formation between the epithelial somites and PSM. The other is called resegmentation where individual vertebral units are formed. The initial segmentation process is accompanied by the mesenchymal-epithelial transition of PSM cells. Mesenchymal cells change their shape by epithelialization and are separated from caudal cells, which maintain mesenchymal morphology. The segment border formation occurs at fixed intervals and continues until the supply of paraxial mesoderm ends. Once the somites are formed, somitic cells start to differentiate, depending on their position within the somites. Cells facing the surface ectoderm differentiate into the dermomyotome, which then gives rise to the dermatome and myotome. The medial cells, under the

influence of the notochord, differentiate into the sclerotome. The second segmentation, called resegmentation, occurs only in the sclerotome, during which the rostral and caudal halves within somites are segregated and re-fused with the next neighboring halves to form vertebrae. This process proceeds because of a difference in the property of cells between the rostral and caudal compartments within the somite. Embryonic manipulation and gene expression analysis have revealed that the rostrocaudal (RC) polarity is established in the anterior PSM prior to initial segmentation (Tam et al., 2000).

Mesp2 is a member of the bHLH family of transcription factors. Expression is observed mainly during somitogenesis in the presomitic mesoderm, although a transient expression is also observed in nascent mesodermal cells at the onset of gastrulation. The *Mesp2*-null mouse shows two major defects in somitogenesis: the lack of the initial segment border, and the loss of rostral properties of the somite, resulting in the formation of a caudalized vertebrae (Saga et al., 1997). Genetic analysis revealed that *Mesp2* plays a critical role in the establishment of RC polarity within somite primordia by regulating *Dll1* expression through the Notch signaling pathway (Takahashi et al., 2000). To date, in any segmentation

mutant mouse, when RC polarity in PSM is disrupted, the segment border formation becomes disorganized. Therefore, establishment of RC polarity and initial segment border formation have not been genetically segregated.

Zebrafish *mespb* (formally known as *mesp-b*) was isolated on the basis of the homology of its bHLH region to that of *Mesp2* (Sawada et al., 2000). *mespb* is segmentally expressed in one to three stripes in the anterior part of somite primordia, corresponding to the expression domain of mouse *Mesp2*. Ectopic expression of *mespb* in zebrafish embryos causes a loss of the posterior identity within the somite primordia, leading to a segmentation defect (Sawada et al., 2000). The *Mesp2*-null mouse shows the opposite result. Therefore, it is most likely that *Mespb* is a functional homologue of *Mesp2*. However, the homology is observed only in the bHLH region (74% identity) and sequences outside this motif are variable. Furthermore, fish and amphibian somites consist mainly of the myotomal component, and sclerotomal cells differentiate at later stages of somitogenesis. Thus, no resegmentation process has been reported in these animals (van Eeden et al., 1996). In order to determine whether zebrafish *Mespb* has a function similar to *Mesp2* or whether these animals develop the *Mesp*-type transcription factor with a similar but distinct function, we examined the function of *Mespb* in mouse embryos using the gene knockin strategy.

During the course of the study, we generated the hypomorphic *mesp* allele in which endogenous *Mesp2* is replaced with the *mespb* or *neo-mespb* gene. In a series of *mespb*-knockin mice, we observed dosage-dependent defects in RC polarity of the somite, resulting in varying degrees of vertebral fusions. Interestingly, however, initial segmentation occurs in RC-defective mice although the segment border was not maintained in the matured somites. These results, together with gene expression analysis, indicate that the *Mesp* family gene is involved in the different genetic cascades, one leading to the somite border formation and the other to the establishment of RC polarity required for resegmentation.

MATERIALS AND METHODS

Gene targeting

A zebrafish *mespb* knockin vector was constructed to insert *mespb* cDNA containing the complete coding region, at the start site of the *Mesp2* coding region, using a common *NcoI* site at the ATG codon. The other parts, composed of the short and long arm regions of this vector, were almost the same as those in a targeting vector used for generating the *Mesp1* knockin mouse (Saga, 1998), except that a floxed neo cassette was used and the poly(A) signal was separated from *mespb* cDNA by the neo cassette. The vectors were linearized and electroporated into TT2 ES cells (Yagi et al., 1993). Correctly targeted clones were then aggregated with ICR embryos to generate chimeras, the mutant allele of which was transmitted through the germline. Subsequently, a *Mesp2^{neo-mespb/+}* mutant mouse was mated with a CAG-Cre mouse to excise the floxed neo cassette. The CAG-Cre mouse produces Cre-recombinase ubiquitously (Sakai and Miyazaki, 1997), thereby generating *Mesp2^{mespb/+}* mice.

Analyses of mutant embryos

Noon on the day when a vaginal plug was observed was counted as day 0.5 of gestation. The amnion DNA and the following allele-specific primers were employed for the PCR analysis. NeoAL: 5'-GAAAGAACCAGCTGGGGCTCGAG-3' and GR-3: 5'-GGAAG-

TTGAGTTCCTCATCACGATC-3' for the transgene, and P2-L3: 5'-CATCATGCCAGAGACTACAGCCTCA-3' and P2-R3: 5'-GTC-ACGGCATTAGCAAGGTTGAGAA-3' for the normal allele of *Mesp2^{neo-mespb/+}* chimeric mice. For Cre-excised *Mesp2^{mespb/+}* mice, *mespb*-L3: 5'-GTCTGTGAATGGAGGTTTGTGG-3' and pAR: 5'-CTCGAGCCCCAGCTGGTCTTTC-3' were used as primers.

The methods for whole-mount in situ hybridization, histological examination and skeletal staining have been described previously (Saga et al., 1996; Saga et al., 1997). For the detection of *mespb* mRNA by in situ hybridization, the 3' region of the bHLH domain of *mespb* cDNA was used as the RNA probe (Sawada et al., 2000).

RESULTS

Rescue of *Mesp2* deficiency by zebrafish *mespb* knocked into the *Mesp2* locus

Mesp-related family genes share highly homologous bHLH domains, while the sequences outside of this motif are more diverse (Fig. 1A), indicating a conserved functional relevance of the bHLH region (Saga et al., 1996; Saga et al., 1997; Sawada et al., 2000; Sparrow et al., 1998; Joseph and Cassetta, 1999). To explore the functional similarity and possible difference between mouse *Mesp2* and zebrafish *mespb* in somitogenesis, *Mesp2* exons were replaced with *mespb* cDNA by homologous recombination (Fig. 1B). The germline chimera of *Mesp2^{neo-mespb/+}* (hereafter we refer to this as *neo-mespb/+*) was established and was crossed with CAG-Cre mice to excise the floxed *neo* cassette for generating the *Mesp2^{mespb/+}* (hereafter we refer to this as *mespb/+*) mouse line. Southern blot analysis showed expected bands in chimera or Cre-excised *mespb/+* mice (Fig. 1C), indicating correct homologous recombination and subsequent excision of the floxed *neo* cassette by Cre-recombinase.

The heterozygous *mespb/+* mice appeared normal. *mespb/mespb* homozygous mice generated by intercrosses of *mespb/+* mice were viable and fertile but had kinked tails (Fig. 2A). The F₁ mice produced by the intercross of homozygous *mespb* mice also showed kinked tails. In situ hybridization using the *mespb*-specific probe revealed that *mespb* knocked into the *Mesp2* locus was expressed in a pattern similar to that of *Mesp2* (Fig. 2B,C). Furthermore, based on the external appearance, clearly segmented somites were observed in the *mespb/mespb* embryos, suggesting that an almost complete rescue of the segmentation defect was achieved by introduction of *mespb*.

Dosage of *Mespb* is critical for the rescue of resegmentation defects of *Mesp2*-null mice

In addition to the lack of formation of initial segmentation, the *Mesp2*-null mouse lacks the rostral property of somites preventing the resegmentation process. As a result of this resegmentation defect, the mice exhibit extensive fusion of the pedicle and the lamina of the neural arch of the vertebrae (Saga et al., 1997). The homozygous mice died shortly after birth, while heterozygous *Mesp2^{+/-}* (*p2* single dose) mice developed normally. By breeding a *mespb/+* mouse with a *Mesp2^{+/-}* (*+/-*) mouse, *mespb/-* mice were generated. These mice died shortly after birth, indicating that, unlike *Mesp2*, one copy of the *mespb* gene is not sufficient to rescue *Mesp2* deficiencies. Analyses of the skeletal phenotype of these mice revealed a fusion of both the rib and the vertebral column although the

severity was much milder than that observed in a *Mesp2*-null fetus (Fig. 2F-I). The result suggests a functional difference between *Mesp2* and *Mespb*.

Comparison among *mespb/mespb*, *mespb*^{-/-} and *-/-* fetuses clearly revealed the dosage effect of the *mespb* gene (Fig. 3A-F). Two copies of *mespb* resulted in a clearer separation of the pedicles and the lamina, although partial fusion remained, particularly in the pedicles (Fig. 3B). In contrast, a single copy of *mespb* only partially rescued the skeletal anomaly caused by *Mesp2* deficiency. Some separation of skeletal elements was generated within the fused vertebrae (Fig. 3C). For the *neo-mespb/neo-mespb* and *neo-mespb*^{-/-} fetuses, defects were more severe. The fusion of pedicle of the neural arch in the *neo-mespb/neo-mespb* or *neo-mespb*^{-/-} fetuses was more severe than that in *mespb*^{-/-} fetuses (Fig. 3D-E). In the *neo-mespb/neo-mespb* and *ne-mespb*^{-/-} embryos, *mespb* is expected to be transcribed in conjunction with a *neo* mRNA, which may affect

the stable translation of the *Mespb* protein. The conjunct *mespb-neo* transcripts were detected by RT-PCR (data not shown) and by in situ hybridization using the *mespb* probe (Fig. 2D).

To understand the phenotype at the molecular level, we first analyzed the effect of *Mespb* on the establishment of RC polarity, because the skeletal malformations are a result of the loss of this RC polarity within the somite. *Uncx4.1* serves as a good molecular marker for caudal half somites, and knockout mice of this gene lack the vertebral elements (especially pedicles) derived from the caudal sclerotome (Leitges et al., 2000; Mansouri et al., 2000). In wild-type embryos, *Uncx4.1* is exclusively expressed in the caudal half of the segmented somites (Fig. 3G). Consistent with the degree of fusion of the pedicles, the expression pattern of *Uncx4.1* in embryos with various *mespb* genotypes was affected in a dosage-dependent manner (Fig. 3H-K). In *neo-mespb*^{-/-} embryos that show the

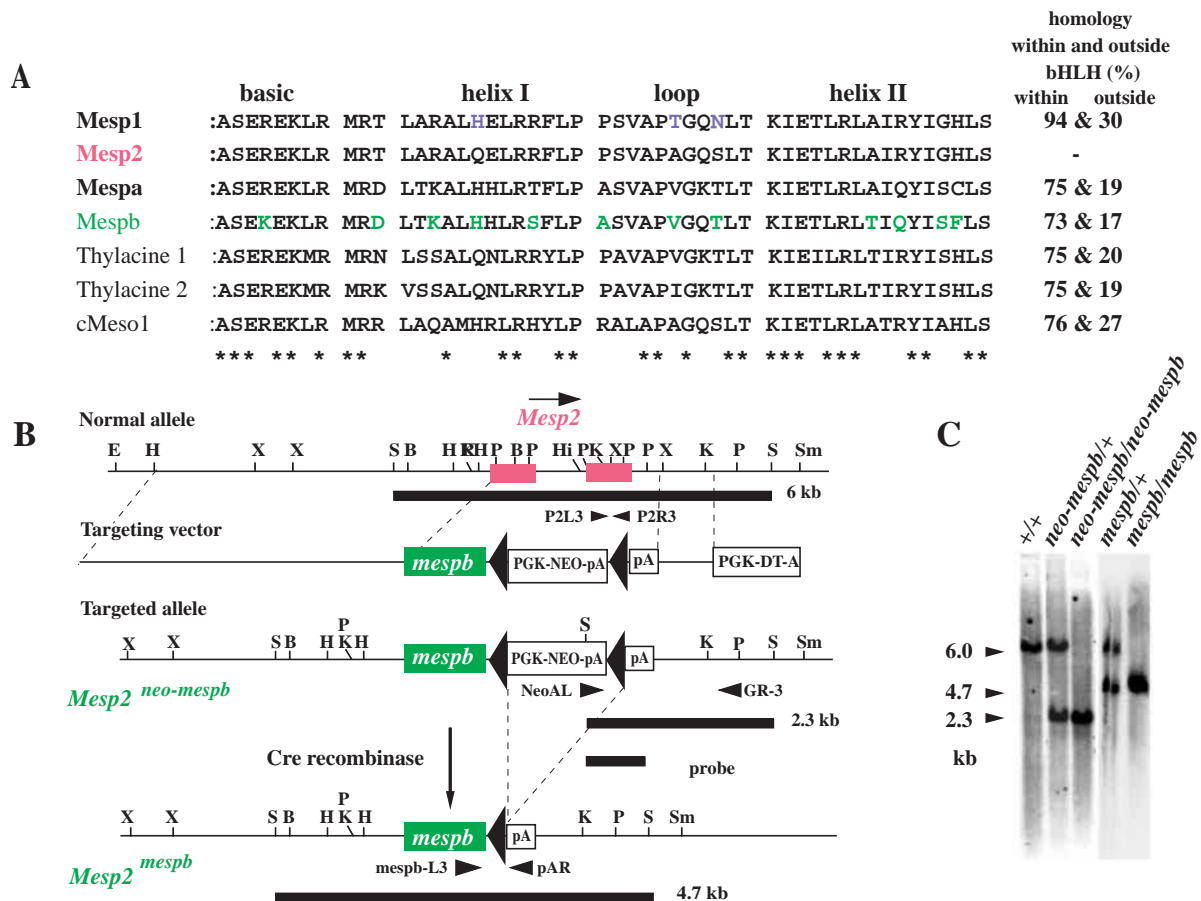
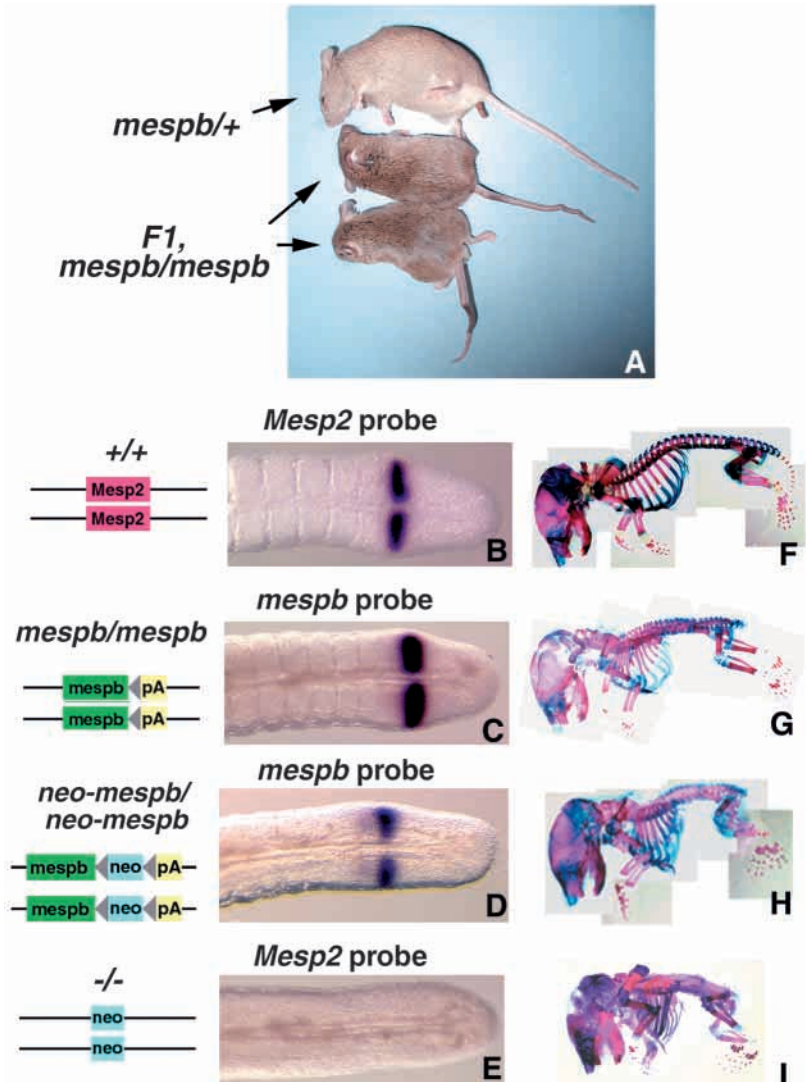


Fig. 1. Comparison among members of the *Mesp*-related gene family (A) and strategy of gene replacement of mouse *Mesp2* with zebrafish *mespb* (B,C). (A) Comparison of amino acid sequences in the bHLH motif. The percentage homology to *Mespb* within and outside the bHLH motif are shown. Differences are indicated in green (*Mespb*) and purple (*Mespb*). (B) Knock-in strategy. The top line shows the genomic organization of the *Mesp2* gene; the second shows the structure of the targeting vector; the third is the predicted structure of the *Mesp2* locus following homologous recombination. *Mesp2* exons (pink boxes) were completely deleted and replaced with the zebrafish *mespb* coding region flanked with floxed *neo* cassette and poly(A) signal (the arrowheads on the line represent loxP sites). Chimeric mice generated from recombinant ES cells containing targeted allele, *Mesp2*^{neo-mespb}, were mated with CAG-Cre mice to excise the floxed *neo* cassette, resulting in the generation of the *Mesp2*^{mespb} allele. The probe used for Southern blot analysis is indicated. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hinc*II; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sac*I; Sm, *Sma*I; X, *Xba*I. Arrows indicate PCR primers. (C) Genomic Southern blot analysis of *Sac*I-digested DNA from embryos with various *Mesp2* alleles. Arrowheads show the 6.0 kb fragment of the wild-type allele, the 2.3 kb targeted *Mesp2*^{neo-mespb}, and the 4.7 kb Cre-excised *Mesp2*^{mespb} allele. Genotypes of progeny are indicated at the top of each lane. All represent genotypes of the *Mesp2* allele.

Fig. 2. Characterization of *mespb*-knockin mice.

(A) Interbreeding of heterozygous *mespb*^{+/+} mice gives rise to viable *mespb*^{mespb} mice with kinked tails. In these *mespb*^{mespb} (C) and *neo-mespb*/*neo-mespb* (D) embryos, the *mespb* genes introduced are expressed in the expected region, which are similar to that of *Mesp2* (B). Segmented somites are observed in both *mespb*^{mespb} (C) and *neo-mespb*/*neo-mespb* embryos, but not in *Mesp2*^{-/-} embryos (E). However, the skeletal morphology (F-I) showed various defects in vertebrae formation in both *mespb*^{mespb} (G) and *neo-mespb*/*neo-mespb* (H) embryos. Embryo samples were prepared at 11.5 dpc. Skeletal specimens were prepared at 18.5 dpc. Anterior is to the left. Genotypes for various mice are schematically represented on the left. *Mesp2*, endogenous allele; neo, pgk-neo cassette replaced with *Mesp2* for gene targeting (ref); *mespb*, zebrafish *mespb* gene; gray arrowhead, lox sequence; pA, polyadenylation signal.



very severe fusion of the pedicles, the expression pattern of *Uncx4.1* was severely affected, expanding to the rostral half somites (Fig. 3K). However, as the dosage of the *mespb* gene increased, the expression of *Uncx4.1* shifted caudally, and an almost normal expression pattern was observed in *mespb*^{mespb} embryos (Fig. 3H). We have previously shown that the RC polarity of the somite is prefigured by the expression pattern of *Dll1* in the anterior PSM (Takahashi et al., 2000). In wild-type embryos, *Dll1* expression is uniform and intense in the caudal presomitic mesoderm (CPM) but it is markedly downregulated and localized in the caudal half somite primordia (Fig. 3M) (Bettenhausen et al., 1995). Because *Mesp2* suppresses *Dll1* expression in the presumptive rostral half of somite in PSM (Takahashi et al., 2000), *Dll1* expression in the PSM was expanded rostrally in *Mesp2*-null embryos (Fig. 3R). As expected, the degree of rostral expansion of *Dll1* expression was dependent on the dosage of *mespb* (Fig. 3N-Q). Thus *mespb*^{mespb} embryos showed an almost normal expression pattern (Fig. 3N), whereas the *neo-mespb*⁻ embryos exhibited an expanded *Dll1* expression similar to that in the *Mesp2*-null embryos (Fig. 3Q). The difference in the expression patterns of *Uncx4.1* and *Dll1* in mice with different *mespb* alleles indicates that the *mespb* dosage is essential to both establish RC polarity in anterior PSM and maintain this RC polarity in the somites.

Defective RC polarity reflects misregulation of *Mespb*

It has been shown that RC polarity within the somites is established by the autoregulated rostral restriction of *Mesp2* within the somite primordia (Takahashi et al., 2000). Thus, we examined whether the defective RC polarity in *mespb* mice is correlated with misregulation of *mespb* in the PSM. We used a *Mesp2*-*lacZ* mouse (*lacZ* gene knocked into the *Mesp2* locus). In this mouse line, the *Mesp2* expression pattern is properly reproduced by the expression of *lacZ* transcripts (Fig. 4A,D). The β -gal activity was sustained in the somitic region because of the stability of the enzyme (Fig. 4H). Since *Mesp2*

expression, once detected in one-somite width, is rostrally restricted (Takahashi et al., 2000), β -gal activity shows a rostrocaudal gradient within each somite (Fig. 4H). In the absence of *Mesp2*, *lacZ* expression is not restricted to be localized to the rostral compartment of the somite (Fig. 4G), resulting in uniform β -gal staining in the somitic region (Fig. 4K). In the case of the *mespb*^{+/L} embryos that contain one *mespb* allele in the genome, the *mespb* (Fig. 4B) and *lacZ* (Fig. 4E) expression patterns were similar to that of endogenous *Mesp2*; a single discrete band ranging from one-somite to half somite width due to the transcriptional suppression in the caudal half (Haraguchi et al., 2001). The graded β -gal staining in the somitic region was observed, although it was not as clear as that in the *Mesp2*^{+/L} embryos (Fig. 4F). In contrast, in the *neo-mespb*^{+/L} embryos containing one *neo-mespb* allele, the expression pattern of both *neo-mespb* (Fig. 4C) and *lacZ* (Fig. 4F) transcripts were different from *mespb* (Fig. 4B) and *lacZ* (Fig. 4E) of *mespb*^{+/L}; two bands were observed and the anterior one was not localized in the rostral compartment. This result indicates that *neo-mespb* expression is extended without localization in the rostral half, resulting in the uniform pattern of β -gal staining (Fig. 4J). The result indicates that the amount

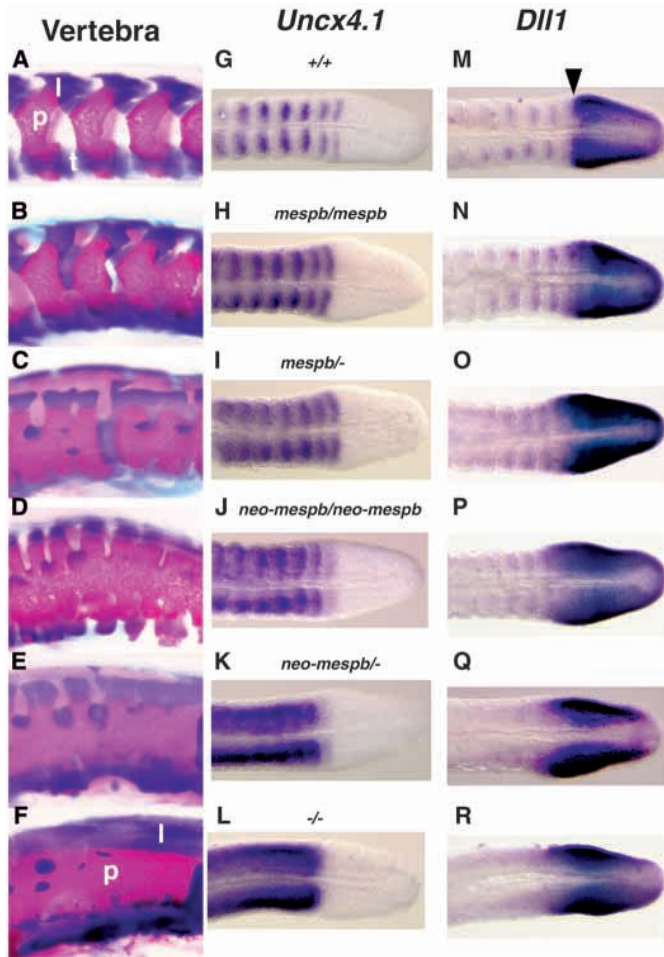


Fig. 3. *mespb* gene dosage effect revealed by the skeletal morphology at 18.5 dpc (A-F) and gene expressions at 11.5 dpc (G-R) reflecting RC polarity and subsequent resegmentation. (A-F) The lumbar regions of the vertebral columns stained with Alcian Blue-Alizarin Red. The wild-type embryo (A) exhibits clear separation of the lamina (l), pedicle (p) and transverse process (t). (B-E) Varying degrees of fusion of the pedicles and laminae are observed in the *mespb* or *neo-mespb* fetuses. (F) A *Mesp2*-null fetus with a completely fused pedicle and lamina. (G-R) The segmental pattern is prefigured by the expression pattern of *Uncx4.1* (G-L) in segmented somites and *Dll1* (M-R) in the anterior PSM. Expression of these genes, normally localized in the caudal half of each somite and CPM, is expanded rostrally in *mespb*, or *neo-mespb* embryos.

somites, resulting in the formation of the caudalized vertebrae as shown in Fig. 3.

Initial segmental border is formed in the hypomorphic mice

In spite of the loss of RC polarity and vertebral defects, the segmented somites appear to form in hypomorphic embryos, indicating segmentation without clear RC polarity. To confirm the segment border formation, the horizontal serial sections of embryonic tails at 11.5 dpc were compared among the various genotypes (Fig. 5). In sections of wild-type embryos, separated segmental borders were clearly observed between the somites (Fig. 5A). In contrast, neither segmental borders nor epithelial somites were observed in the *Mesp2*-null embryos (Fig. 5F). As expected from the external morphology, initial border formation was observed in *mespb/mespb* (Fig. 5B), *mespb/-* (Fig. 5C) and *neo-mespb/neo-mespb* (Fig. 5D) embryos. Moreover, in *neo-mespb/-* embryos, in which the segmented borders were not clear morphologically, the histological sections revealed that the initial segmental border is formed (Fig. 5E). However, in all hypomorphic embryos except for *mespb/mespb*, segregation is incomplete and somitic cells remain between newly formed somites. Furthermore, the segment borders tended not to be maintained. This was most obvious in *neo-mespb/-* embryos in which the somites finally fuse with each other. The somite fusions could be caused by

of *Mespb* protein provided by one *neo-mespb* locus is not sufficient to restrict *mespb* to the rostral compartment, a prerequisite for the establishment of RC polarity. In addition, the reduced amount of *Mespb* in *neo-mespb/-* or *neo-mespb/L* is unable to suppress *Dll1* expression in the caudal halves of

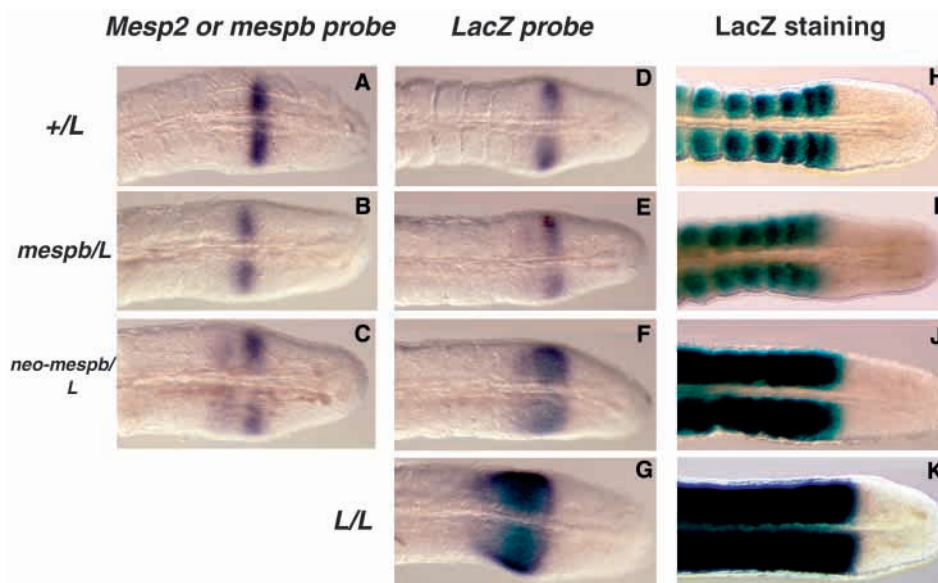


Fig. 4. Expression of *Mesp2* or *mespb* is autoregulated. *Mesp2*^{+/+}, *mespb*^{+/+} or *neo-mespb*^{+/+} mice were crossed with *Mesp2-lacZ*^{+/+} mice and the expression pattern of *Mesp2* or *mespb* was visualized. *Mesp2* (A), *mespb* (B-C) and *Mesp2-lacZ* (D-G) transcripts are shown by whole-mount in situ hybridization. The lack of autoregulation that results in the loss of *Mesp2-lacZ* restriction to the rostral compartment is revealed by the caudally extended expression pattern of β -gal activities (I-K), which are different from the striped expression pattern of *Mesp2*^{+/L} (H; *Mesp2* heterozygous embryo).

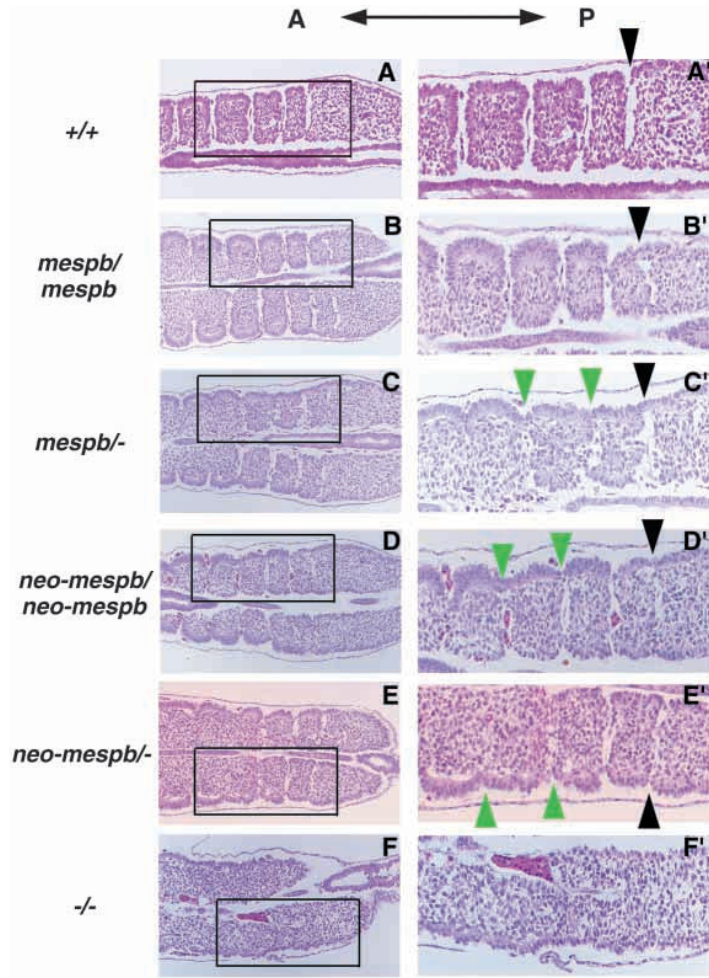


Fig. 5. Differential regulation between segment border formation and its maintenance. The border formation in nascent somites of embryos of various *mespb* genotypes was compared in serial horizontal sections. In all embryos (A-E, A'-E') except for the *Mesp2*-null embryo (F,F'), the initial segmental borders are generated. However, the borders are not maintained in the *neo-mespb*⁻ embryo (E,E'). Various levels of border fusion were observed in *mespb*⁻ and *neo-mespb/neo-mespb* embryos. The initial segmental borders are indicated by black arrowheads. Partial fusion between segmented epithelial somites is indicated by green arrowheads. All specimens were prepared at 11.5 d.p.c., but the AP level of these samples are not always same. (A'-F') Higher magnification of the boxed areas in A-F.

the loss of RC polarity (Durbin et al., 2000). These results suggest that the formation and maintenance of somite borders are regulated by distinct mechanisms.

***Lfng*, *EphA4* and *PAPC* expression appears normal in *mespb*-embryos**

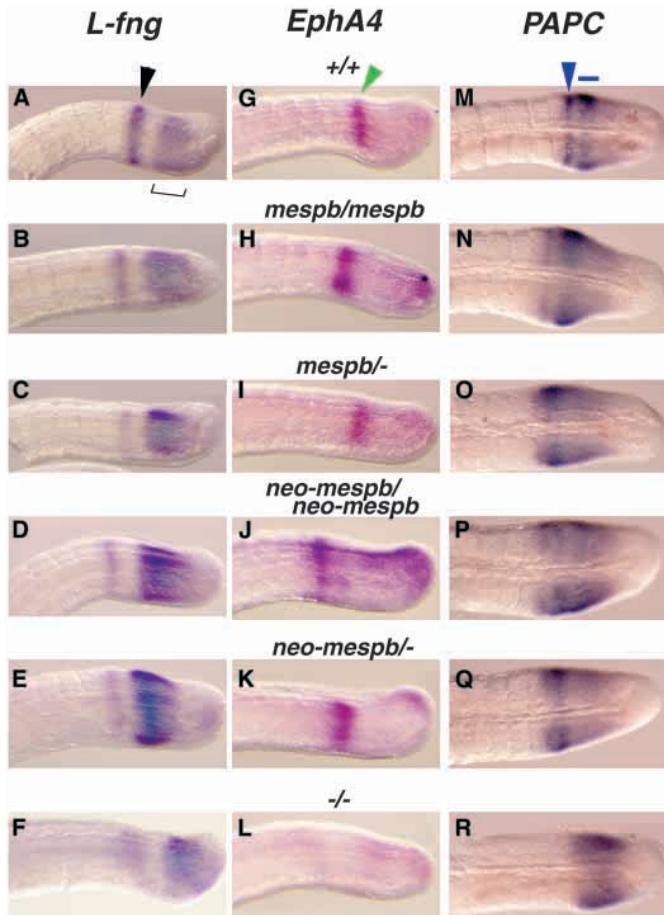
The above results led us to examine the expression pattern of *Lunatic fringe* (*Lfng*), *EphA4* (*Epha4*) and *protocadherin* (*PAPC*; *Pcdh8*), which have been implicated in segment border formation in the rostral PSM (Johnston et al., 1997; Durbin et al., 1998; Schmidt et al., 2001; Kim et al., 2000). In the PSM, various expression patterns of *Lfng* are observed even at a similar developmental stage, due to cyclical expression linked to a segmentation clock (McGrew et al., 1998; Forsberg et al., 1998). The expression domain of *Lfng* in the caudal PSM appears to travel to the rostral region (indicated by bracket in Fig. 6A), while the rostral stripe gradually becomes thinner and finally stays at the future segmentation point (Fig. 6A, indicated by black arrowhead). The Fringe protein, which modifies the Notch receptor in the fly wing disc, has been implicated in border formation (Moloney et al., 2000). Similarly, it has been shown to function in the vertebrate somitogenesis; a *Lfng*-deficient embryo cannot form a clear segmental boundary (Evrard et al., 1998; Zhang and Gridley, 1998). In *Mesp2*-null embryos, *Lfng* expression in the rostral PSM expanded anteriorly, while caudal expression remained

intact (Fig. 6F). To compare the expression patterns, we selected embryos that exhibited a similar expression profile with a thin band in the rostral PSM and a caudal broad band traveling rostrally (Fig. 6A). In all *mespb* embryos, namely, *mespb/mespb* (Fig. 6B), *mespb*⁻ (Fig. 6C), *neo-mespb/neo-mespb* (Fig. 6D) and *neo-mespb*⁻ (Fig. 6E), the definite bands of *Lfng* were detected in the rostral PSM close to the next segmental border. We then examined *EphA4*, which is normally expressed in a pattern similar to that of the rostral band of *Lfng* (Fig. 6G). *EphA4* has been implicated in segment border formation because its misexpression induces ectopic segment border formation in segmentless zebrafish *fss* mutant embryos (Durbin et al., 2000). Importantly, *EphA4* expression was severely downregulated in the *Mesp2*-null embryo (Fig. 6L). In contrast, relatively normal levels and patterns of *EphA4* expression were observed in all *mespb*-mice irrespective of the dosage and nature of *mespb* (Fig. 6H-K). Similar results were obtained in the expression pattern of *PAPC*, which is also known as a key molecule for segment border formation (Kim et al., 2000). In wild-type embryos (Fig. 6M), *PAPC* is typically expressed as one or two defined bands in the rostral PSM in addition to a diffuse caudal expression in the middle PSM. In *Mesp2*-null embryos (Fig. 6R), the rostral band is missing and only broad expression is observed caudally. In the *mespb*-knockin embryos, the rostral band tends to be rescued, accompanied by the recovery of segmental border formation (Fig. 6N-Q). In addition to the induction, it is noted that the expression pattern of *PAPC* appears to be affected by the *mespb* dosage, such that the rostral band showed a diffused pattern at low *mespb* dosage, reflecting the RC polarity as well. The above results strongly suggest that a small amount of *Mespb* is sufficient to induce rostral expression of *Lfng*, *EphA4* and *PAPC* and may drive segment border formation. The results also suggest that the rostral restriction of *mespb* expression is not necessarily required to elicit these gene expressions nor initiate segment border formation. This phenomenon is in contrast to that of the establishment of RC polarity, requiring a higher amount of *Mespb*, suggesting the presence of distinct pathways controlling the two events.

DISCUSSION

Establishment of hypomorphic allele

The aim of this work was to determine whether zebrafish



mespb and mouse *Mesp2*, which share 74% identity of the bHLH region and have similar expression patterns and functions in somite formation, are indeed functional homologues. The *mespb/mespb* mice developed normally and overcame most of the deficiencies caused by the loss of *Mesp2*. However, we still observed a partial fusion of the vertebrae and truncation of the trunk. In addition, *mespb/-* embryos showed a severe defect in RC polarity within the

Fig. 6. Gene expression implicated in the segmental border formation. The expressions of *Lfng* (A-F) *EphA4* (G-L) and *PAPC* (M-R) are compared in various *mespb* embryos at 11.5 dpc. *Lfng* is expressed in a highly dynamic manner. Therefore, 8-10 embryos of each genotype were analyzed by whole-mount in situ hybridization and we chose those with similar expression patterns for the comparison. The anterior thinner band of *Lfng* (black arrowhead) is rostrally extended in the *Mesp2*-null embryo. In contrast, the sharpness of the band was recovered in all *mespb* embryos. The rostral band of *EphA4* (green arrowhead) and *PAPC* (purple arrowhead) expression, which disappeared in the *Mesp2*-null embryo, is also present in *mespb* embryos.

somite, which was not observed in *Mesp2*^{-/-} embryos. This defect difference suggests a functional difference in either establishment or maintenance of RC polarity in the somite related with resegmentation between mouse *Mesp2* and zebrafish *Mespb*. The difference in the molecular nature could be attributable to the region outside the bHLH region since we previously observed a similar defect when *Mesp2* was replaced with *Mesp1*, the bHLH region of which has a 94% identity to that of *Mesp2* while the sequences outside this motif are diverse (Saga, 1998). However, we cannot rule out the possibility that a difference in the expression level and/or the stability of *mespb* mRNA and its protein may be the cause of this phenotype. Although transcriptional regulation is very important for the correct patterning of RC polarity, for technical reasons we have used the SV40 polyadenylation signal instead of the endogenous one in both *mespb* and *Mesp1*-knockin mouse, which may have affected the stability of the transcript.

Taking advantage of the nature of the *mespb* allele, we were able to generate a hypomorphic *Mesp* allele and analyze the dosage effect of the *Mesp* gene on somite formation. In various *mespb*-knockin mice, we observed the clear dosage effect on vertebral fusions. We think that this represents changes in the amount of the *Mespb* protein. In the *neo-mespb* embryo, *mespb* was transcribed with the *pgk-neo* cassette and the amount of neo-containing transcripts was comparable to that of *mespb* transcript in the *mespb* embryo. To date, we do not have a tool that can assess the translation

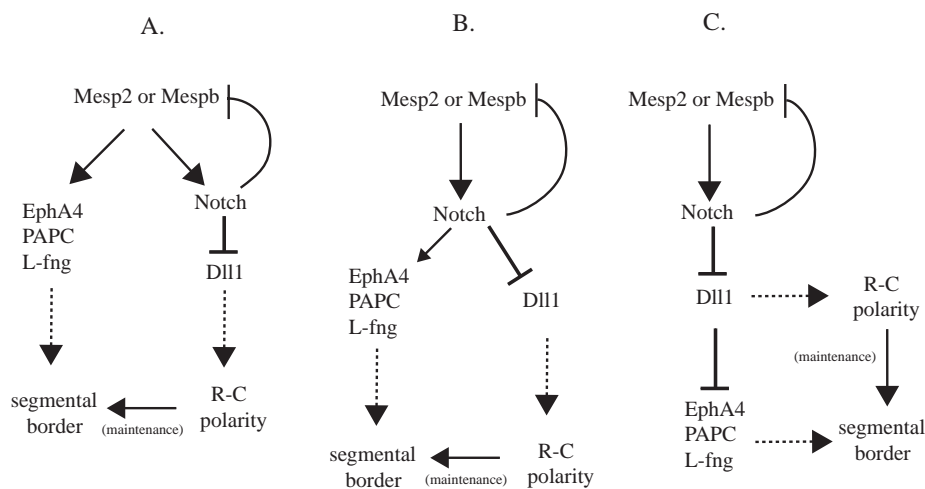


Fig. 7. Possible models of events leading to the somite border formation and the establishment of RC polarity. (A) *Mesp2* or *Mespb* might regulate these two events using different genetic pathways. *Mesp2* is known to suppress *Dll1* via the Notch signaling pathway. (B) However, the pathway might be important for the normal expression of *EphA4*, *Lfng* and *PAPC*, which is required for the border formation. (C) Finally the suppression of *Dll1* required for the establishment of RC polarity might play a role in the border formation. In all cases, Notch signaling is required for the autoregulation of *Mesp2*, and RC polarity is required for the maintenance of the segmental border. Only the anterior most bands of *Lfng* and *PAPC* are *Mesp*-dependent.

efficiency of the transcripts. However, when we compare the phenotypes and gene expressions between *mespb/mespb* and *mespb/-* mice or between *neo-mespb/neo-mespb* and *neo-mespb/-* mice, it is reasonable to conclude that the severity of the phenotype depends on the amount of the Mesp protein expressed.

RC polarity and segment border formation

One of the important findings in this study is that a hypomorphic embryo can form somite boundaries without clear RC polarity in the PSM, suggesting that segment border formation and establishment of RC polarity are genetically separate events. The two successive events are both affected in segmentation mutant mice including the *Mesp2*-null mouse (Harabe de Angelis et al., 1997; Koizumi et al., 2001; Evrard et al., 1998; Saga et al., 1997).

Experimentally, it is possible to separate segment border formation from RC polarity. In embryos from which the ectoderm is removed, normal RC polarity is established but no epithelial somites are generated indicating that the establishment of RC polarity is not directly linked to the border formation (Palmeirim et al., 1998; Correia and Conlon, 2000). Interestingly, no *EphA4* expression was induced in embryos from which ectoderm had been removed (Schmidt et al., 2001), suggesting a direct relationship between *EphA4* induction and segment formation. In our experiments, however, irrespective of defects in RC polarity, a relatively normal *EphA4* expression was induced in all hypomorphic embryos, supporting the idea that these two events are independent of each other. Considering the absence of *EphA4* expression in *Mesp2*-null embryos, *EphA4* expression requires both an ectodermal signal and *Mesp2*.

Previously, we have reported that *Mesp2* functions in generating RC polarity by suppressing *Dll1* expression in the rostral half of a presumptive somite. This suppression is mediated by the Notch signaling pathway (Takahashi et al., 2000). In the present study, *Dll1* expression was affected in *mespb*-knockin embryos in a dosage-dependent manner. Particularly in *neo-mespb/-* embryos, extensive expansion of *Dll1* expression was observed, indicating the lack of RC polarity. However, the initial segmental border was formed in the *neo-mespb/-* embryo, but not maintained. We believe that the failure to maintain the segmental border is due to the lack of RC polarity, since it was reported that the segmental border is maintained only when the rostral and caudal halves are confronted (Stern and Keynes, 1987). Therefore, the formation and maintenance of the segmental border must be regulated by different mechanisms: one is mediated by *Lfng*, *EphA4* and *PAPC* to generate the segment border, and the other by *Dll1* through the Notch signaling pathway to establish RC polarity (Fig. 7A). At the present, however, it is unclear at which level the two events bifurcate. It is also possible that the rostral localization of *Lfng*, *EphA4* and *PAPC* expression could be mediated by the Notch signaling pathway (Fig. 7B). A preliminary study using the *Mesp2-Notch1* mouse (*Notch1* knocked in the *Mesp2* locus) (Takahashi et al., 2000) suggests that *PAPC* expression is partly dependent on Notch signaling, indicating that other complicated pathways are involved in the regulation of these genes. Finally, it is formerly possible that the induction of *Lfng*, *EphA4* and *PAPC* requires RC polarity to some degree

(Fig. 7C) since a subtle and undetectable RC polarity may exist that is sufficient for initial segmentation but not for establishment of RC polarity.

Regulation of *Mesp* gene

The autoregulation of *Mesp2* transcription must be very important to establish RC polarity. Upon activation, *Mesp2* is initially expressed in an area approximately one somite wide, defining the anterior limit at its initial expression. Next the transcription is suppressed only in the caudal half, generating RC polarity within a somite primordium (Takahashi et al., 2000). Indeed in this study, we observed that misregulation of *mespb* in the caudal half resulted in misestablishment of RC polarity. This is most likely to be caused by the reduced amount of Mesp. Although the precise mechanism of the autoregulation is not yet known, Notch signaling has been implicated in this process, since no rostral restriction of *Mesp2* expression is observed in *Psen1*-null embryos (Koizumi et al., 2001). We have already shown genetically that *Psen1*-dependent Notch signaling is required for the induction of *Dll1* in the caudal half area of somite primordia (Takahashi et al., 2000). Thus, it is possible to speculate that this signaling also functions in suppressing *Mesp2* in the caudal half leading to establish RC polarity.

At present, little is known about the direct targets of *Mesp2* or *Mespb*, nor when and how long *Mesp* protein functions. Future studies should be focused on visualizing the protein molecules involved in the regulatory network to clarify the functional molecular cascade.

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