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# The negative regulation of Mesp2 by mouse Ripply2 is required to establish the rostro-caudal patterning within a somite

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The Mesp2 transcription factor plays essential roles in segmental border formation and in the establishment of rostro-caudal patterning within a somite. A possible Mesp2 target gene, *Ripply2*, was identified by microarray as being downregulated in the *Mesp2*-null mouse. *Ripply2* encodes a putative transcriptional co-repressor containing a WRPW motif. We find that Mesp2 binds to the *Ripply2* gene enhancer, indicating that *Ripply2* is a direct target of Mesp2. We then examined whether Ripply2 is responsible for the repression of genes under the control of Mesp2 by generating a *Ripply2*-knockout mouse. Unexpectedly, *Ripply2*-null embryos show a rostralized phenotype, in contrast to *Mesp2*-null mice. Gene expression studies together with genetic analyses further revealed that Ripply2 is a negative regulator of Mesp2 and that the loss of the *Ripply2* gene results in the prolonged expression of Mesp2, leading to a rostralized phenotype via the suppression of Notch signaling. Our study demonstrates that a Ripply2-Mesp2 negative-feedback loop is essential for the periodic generation of the rostro-caudal polarity within a somite.

**KEY WORDS: Somitogenesis, Notch signaling, Presomitic mesoderm, Segmentation**

## INTRODUCTION

Somites are generated by sequential segregation of cell masses from the anterior part of the unsegmented presomitic mesoderm (PSM), in both a spatially and temporally coordinated manner every two hours (Iulianella et al., 2003; Pourquie, 2003; Saga and Takeda, 2001). The somites provide the basic axial structures that underlie the segmental architecture of not only the vertebra, ribs and muscles, which are all somite derivatives, but also of the vascular and nervous systems (Borycki and Emerson, 2000; Brand-Saberi and Christ, 2000; Monsoro-Burq and Le Douarin, 2000). Periodicity is generated by Notch signal oscillations linked to the segmentation clock (Bessho et al., 2001; Huppert et al., 2005; Morimoto et al., 2005; Rida et al., 2004). The temporal information that results from this is translated into spatial patterns in the anterior PSM, which is defined by the so-called determination front (Dubrulle and Pourquie, 2004).

The Mesp2 transcription factor plays important roles during somitogenesis (Saga et al., 1997), and its expression is periodically activated by cyclic Notch signaling and Tbx6 at the anterior PSM in the determination front (Yasuhiko et al., 2006). Mesp2 demarcates the next segmental boundary and defines the rostro-caudal identity of somites (Takahashi et al., 2000). It has been shown that *Mesp2*-null embryos fail to segment and that the resulting non-segmented somites show caudalized properties (Saga et al., 1997). Previously, we have shown that Mesp2 suppresses Notch activity via the activation of *Lfng*, which might function as a negative regulator of Notch signaling (Morimoto et al., 2005). In addition, Mesp2 acts as the transcriptional activator of *Epha4* in the anterior PSM (Nakajima et al., 2006). Mesp2 is also known to be a strong suppressor of genes such as *Dll1* and *Uncx4.1* that confer caudal properties upon the somitic cells via Notch signaling (Takahashi et al., 2000). However, the manner in which the caudal genes are suppressed is currently unknown. In our current study, which aimed to elucidate the molecular mechanisms underlying the regulation of somitogenesis by Mesp2, we have compared the gene expression patterns of $Mesp2^{+/-}$ and $Mesp2^{-/-}$ embryos, and found that several genes are affected by the *Mesp2* knockout. Among the downregulated genes that we identified in the *Mesp2*-null embryo, we focused on a putative transcriptional repressor. This gene turned out to be *Ripply2*, which was recently reported as a mouse homolog of zebrafish *ripply1* (Kawamura et al., 2005). Morpholino-mediated knockdown analysis revealed that *ripply1* is required for the proper transition from the PSM to somites. We generated a *Ripply2*-knockout mouse and now show that *Ripply2* is activated by Mesp2, but also functions negatively toward Mesp2 to regulate the levels of Notch signaling in the anterior PSM. This negative regulation is required for the periodic generation of the rostro-caudal patterning within a somite.

## MATERIALS AND METHODS

### GeneChip analysis

Total RNA was purified from cells corresponding to the S–1 to S2 somites and PSM of wild-type, *Mesp2-GFP* knock-in heterozygous and homozygous embryos at E10.5 using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. First-strand cDNAs were synthesized by incubating 5 μg of total RNA with 200 U SuperScript II reverse transcriptase (Invitrogen) and 100 pmol T7-$(dT)_{24}$ primer [5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3′]. After second-strand synthesis, the double-stranded cDNAs were purified using a GeneChip Sample Cleanup Module (Affymetrix), according to the manufacturer's instructions. Our detailed methods for the labeling of the

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double-stranded cDNAs and hybridization to a GeneChip Mouse Genome 430 2.0 Array (Affymetrix), and the subsequent washing, staining and data analysis have been described previously (Kanno et al., 2006). All of these data are also now available online at the National Institute of Health Sciences (http://www.nihs.go.jp/tox/TtgSubmitted.htm).

### Identification of the *Ripply2* somite enhancer

Highly conserved *Ripply2* upstream regions were identified using a cross-species DNA sequence comparison using the PipMaker website (http://pipmaker.bx.psu.edu/pipmaker/). We cloned a 5′ upstream genomic sequence of *Ripply2* from a bacterial artificial clone (RP23) contained in a mouse genomic library. A 1.5 kb DNA fragment containing the 171 bp highly conserved region was isolated by *Eco*RI and *Bam*HI digestion and then subcloned into the hsp-nlacZ reporter construct. Fertilized eggs from B6C3F1 female mice were collected for pronuclear injection and the injected eggs were then implanted into ICR female mice. Foster mothers were sacrificed at E10.5 and stained for β-galactosidase (β-gal) activity with X-Gal. The genotypes of the embryos were then identified by PCR using DNA prepared from the yolk sac.

### Luciferase assay

For luciferase reporter analysis under the control of the 1.5 kb *Ripply2* anterior-PSM enhancer (*Eco*RI-*Bam*HI) fragment (20 ng), reporter constructs were co-transfected with the expression vectors 3xFLAG-Mesp2 (0, 30, 100 ng) and 3xFLAG-E47 (0, 50 ng) into NIH3T3 cells ($0.25\times10^5$ cells per well in 24-multiwell plates) using Lipofectamine Plus (Invitrogen), following the manufacturer's instructions. The vector containing the *Renilla* luciferase gene under the control of the thymidine kinase promoter (1 ng) was co-transfected as an internal standard to normalize for transfection efficiency, and the amount of total plasmid was adjusted with pcDNA3.1. After 36 hours, luciferase activities were measured using a Dual Luciferase Assay Kit (Promega).

### Electrophoretic mobility shift assay (EMSA)

A 3xFLAG-Mesp2 protein was produced using the FreeStyle 293 Expression System (Invitrogen) and then collected via a nuclear extraction method. Double-stranded DNA oligonucleotide probes were end-labeled with DIG and protein-DNA complexes were detected using a DIG Gel Shift Kit (Roche). Binding reactions were carried out for 30 minutes on ice, and protein-DNA complexes were analyzed on 6% native polyacrylamide gels.

### *Ripply2* gene targeting strategy

The mouse *Ripply2* gene consists of four exons, the first of which harbors two putative in-frame translational initiation codons. We generated a targeting vector with a floxed *neo* cassette to remove a portion of exon 1, which would introduce a termination codon just after the second initiation codon and produce a null allele. The resulting linearized vector (25 μg) was then electroporated into TT2 ES cells (Yagi et al., 1993). G418-resistant cell clones were further selected by PCR. Correct homologous recombination was confirmed by Southern blotting, and targeted cell clones were aggregated with ICR 8-cells and then transferred to pseudopregnant female recipients. The resulting chimeric mice were bred with ICR females. Germline transmission of the targeted allele was confirmed by PCR. The floxed neomycin cassette was later removed by breeding with a CAG-Cre transgenic mouse (Sakai and Miyazaki, 1997).

### Gene expression and histochemical analysis

Methods for gene expression analysis by in situ hybridization of whole-mount samples and by skeletal staining have been described previously (Takahashi et al., 2000). The probes used in this study have been described previously (Takahashi et al., 2000; Takahashi et al., 2003; Nomura-Kitabayashi et al., 2002). For the *Ripply2* RNA probe, we used a full-length cDNA clone containing intron 1. Section in situ hybridization and immunohistochemical detection of proteins were performed as previously described (Morimoto et al., 2005). For whole-mount detection of Mesp2-venus, embryos were fixed with 4% paraformaldehyde in PBS overnight at 4°C, incubated with rabbit anti-GFP (MBL; 1:1000), followed by Alexa-488-conjugated goat anti-rabbit IgG (Molecular Probes; 1:400) and observed using a fluorescent microscope (Olympus BX61).

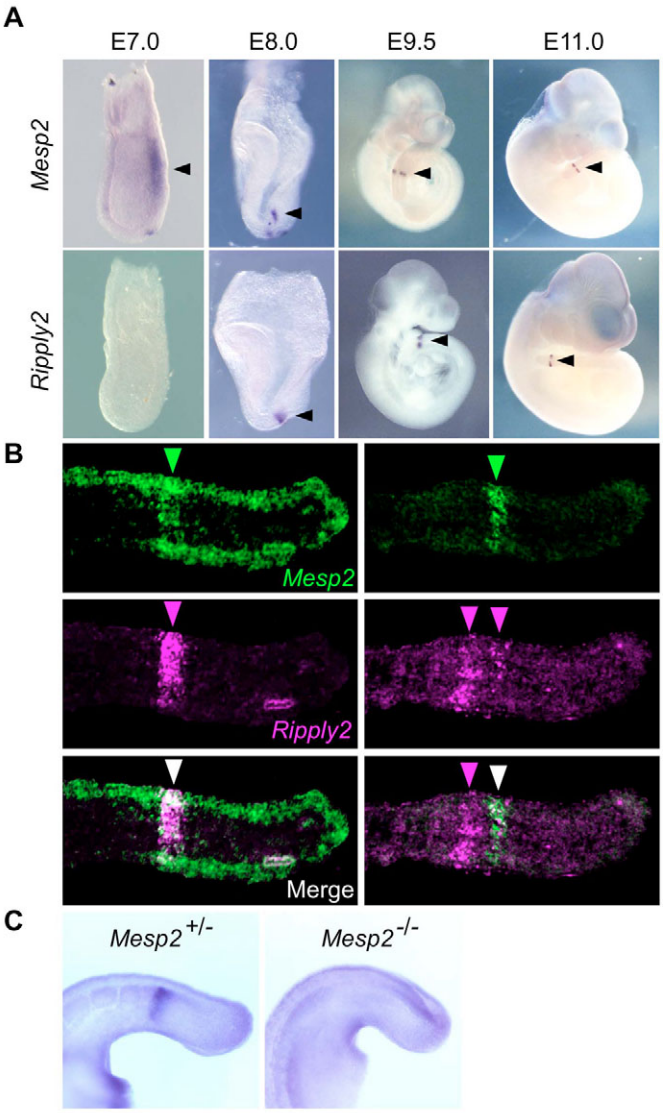


**Fig. 1. Analysis of the of *Ripply2* expression pattern.** (**A**) Comparison of the mRNA expression patterns of *Mesp2* and *Ripply2* during mouse development. Positive expression is indicated by an arrowhead. (**B**) Comparison of the spatial expression patterns of *Mesp2* and *Ripply2* as revealed by section double in situ hybridization. Two representative examples are shown for *Mesp2* (green) and *Ripply2* (magenta), and merged images of these expression patterns are shown beneath. The green signals in the periphery are artifacts and do not represent *Mesp2* expression. In some cases, only a single band could be observed for each gene, and these bands are merged in the image shown in the left-hand bottom panel. Two bands were sometimes visible for *Ripply2*, the posterior band of which merges with that of *Mesp2* (right-hand bottom panel). All samples were prepared from E10.5 embryos. (**C**) Whole-mount in situ hybridization showing that *Ripply2* expression is lost in the E9.5 *Mesp2*-null embryo.

## RESULTS

### *Ripply2* is a possible direct target of Mesp2

Mesp2 is known to function as a transcriptional activator of genes, such as *Epha4* and *Lfng*, which are expressed in the rostral half of the presumptive somite (Morimoto et al., 2005; Nakajima et al., 2006). Moreover, expression of the *Dll1* and *Uncx4.1* genes, which are expressed in the caudal half of the somite (Bettenhausen et al.,

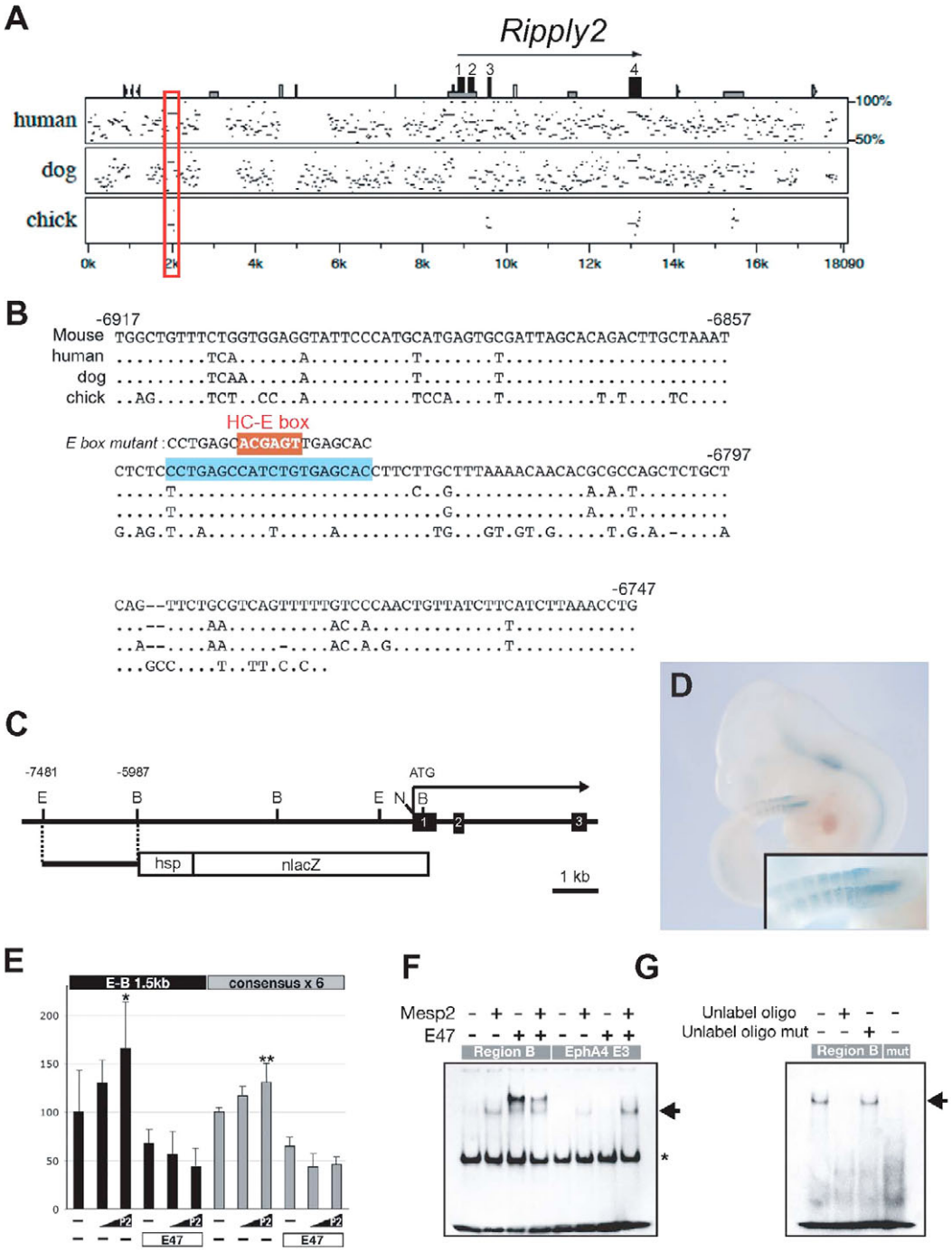


**Fig. 2. Mesp2 can directly bind to the enhancer element of the *Ripply2* gene and activate its transcription.** (**A**) Comparison of the genomic sequences around the *Ripply2* gene in mouse (top line) with those in human, dog and chick using MultiPipMaker sequence alignment software. A conserved region (framed in red) is evident across these species. (**B**) Sequence alignment of the 171 bp region conserved among the *Ripply2* genes, within which a highly conserved E-box is located. HC E-box, highly conserved E-box. (**C**) The genomic organization of the mouse *Ripply2* gene and the corresponding construct used in the transgenic analyses. A 1.5 kb DNA fragment containing this highly conserved 171 bp stretch (shown in A) of the *Ripply2* upstream region was ligated to a cassette composed of the *hsp* promoter and nlacZ (*lacZ* harboring a nuclear localization signal). E, *Eco*RI; B, *Bam*HI; N, *Nco*I. (**D**) The *Ripply2* enhancer drives *lacZ* reporter gene expression in somitic mesoderm cells at E11.0. The inset shows high magnification of the somitic region. (**E**) Luciferase reporter assay for *Mesp2* activation, with or without E47, using constructs harboring either the 1.5 kb *Ripply2* enhancer (left) or six repeats of the conserved 171 bp fragment (right). The addition of E47 had negative effects upon transactivation. The data represent the means±s.d. from four separate experiments. **P*<0.01, ***P*<0.04. (**F**) EMSA analyses revealing that a DNA fragment containing the conserved E-box (Region B, light-blue shading) from the *Ripply2* upstream region can bind Mesp2 in the absence of E47. This binding of Mesp2 thus appears to be different from its binding to the *Epha4* enhancer, which is dependant upon E47. Non-specific bands are indicated by the asterisk. (**G**) The binding specificity of Mesp2 was confirmed by successful competition with cold probe, but not with an E-box mutant probe (shown in B).

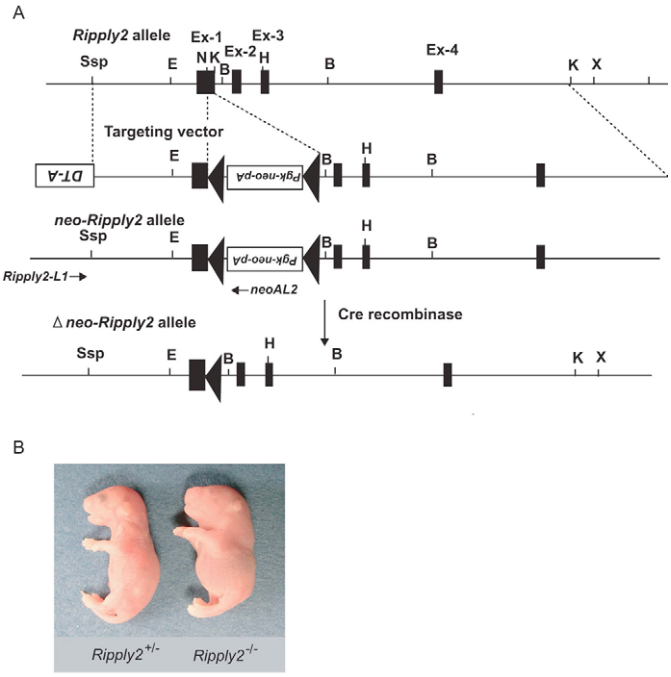


**Fig. 3. The targeting strategy used for the *Ripply2* gene and the external morphology of the resulting knockout mouse.** (**A**) The top line shows the genomic organization of the *Ripply2* gene, the second line represents the structure of the targeting vector, and the bottom two lines show the predicted structure of the *Ripply2* locus following homologous recombination. The first exon of *Ripply2* was partially deleted and replaced with a floxed *neo* cassette (the arrowheads on the line represent loxP sites). A germline chimeric mouse was then generated from recombinant ES cells containing the targeted allele and crossed with a CAG-Cre mouse to remove the *neo* cassette and establish the *Ripply2*-knockout mouse line. Ssp, *Ssp*I; E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; N, *Nco*I; K, *Kpn*I; X, *Xho*I. (**B**) The *Ripply2*-null mouse dies soon after birth and the external morphology at E17.5 is similar to those of segmentation-defective mutants, featuring a short trunk with rudimental tails.

1995; Leitges et al., 2000), is increased in the *Mesp2*-null mouse, indicating that Mesp2 is required for their suppression (Takahashi et al., 2000). However, the molecular mechanisms underlying this are unknown. To identify novel genes that operate downstream of Mesp2, we performed GeneChip analysis using RNAs prepared from both wild-type and *Mesp2*-null embryos. Among the genes that showed a reduction in expression in the *Mesp2*-null embryos (see Table S1 in the supplementary material), we selected a cDNA clone (corresponding to RIKEN cDNA C030002E08) that showed an identical expression pattern to that of *Mesp2* by in situ screening of 11.5 dpc embryos. This cDNA was subsequently revealed to be the mouse *Ripply2* gene recently reported by Kawamura et al. (Kawamura et al., 2005). The initial expression of *Mesp2* was found to be restricted to the nascent mesoderm at E7.0, but *Ripply2* expression appeared to be absent or very weak prior to somitogenesis (Fig. 1A). However, its expression became evident in the anterior PSM as a pair of bands by 8.0 dpc, similar to *Mesp2*

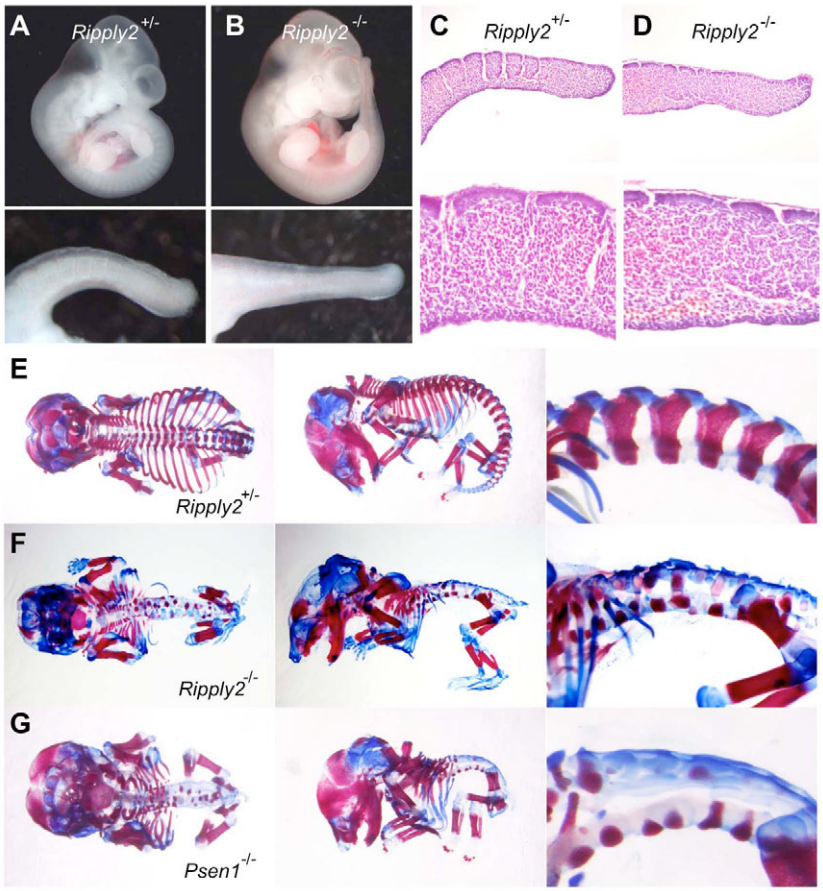


**Fig. 4. The *Ripply2*-knockout mouse exhibits segmentation defects.** (**A-D**) *Ripply2*$^{+/-}$ and *Ripply2*$^{-/-}$ embryos ($n$=3 at E10.5) were compared by external morphology (A,B) and by the Hematoxylin and Eosin staining of parasagittal sections of tail regions (C,D). *Ripply2*$^{-/-}$ embryos display irregularly sized myotomes, and an unclear segmental border. (**E-G**) Skeletal preparations at E17.5 stained with Alizarin Red-Alcian Blue reveal that the *Ripply2*$^{-/-}$ fetus harbors fewer pedicles of neural arches and lacks components of the proximal ribs (F; $n$=4), which is similar to the aberrant phenotype of the *Psen1*-null fetus (G; $n$=2).

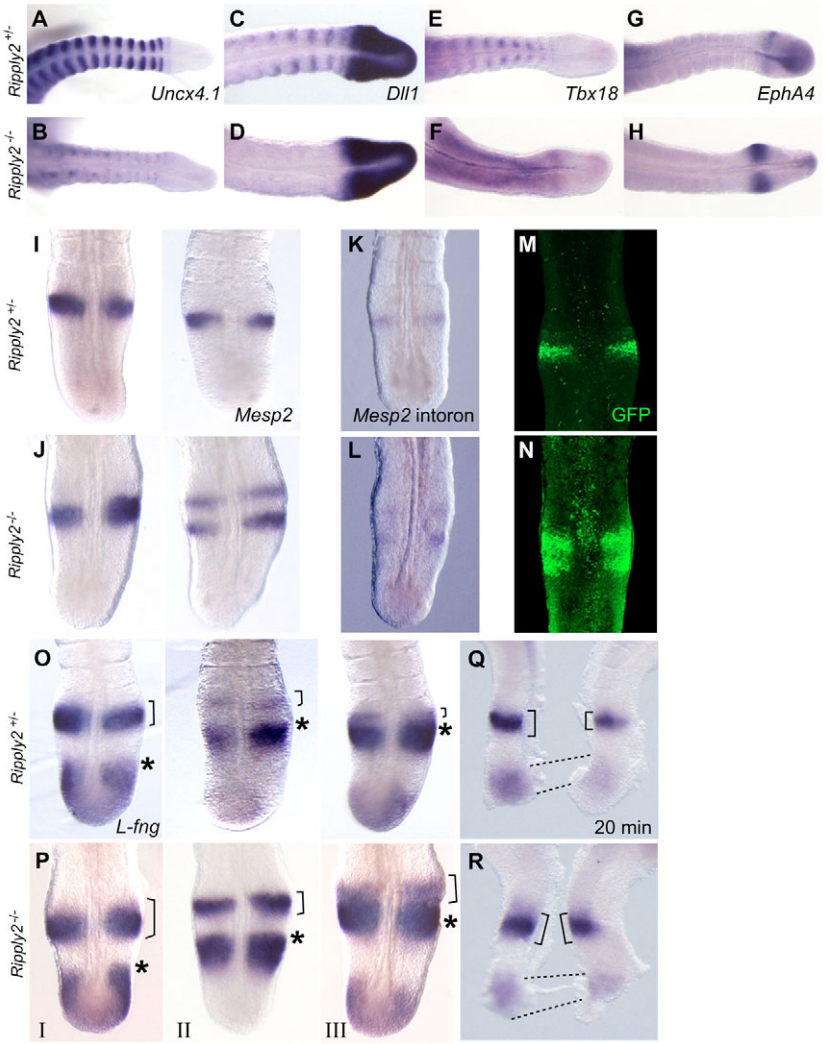


**Fig. 5. Altered gene expression in the *Ripply2*-null embryos.** Whole-mount in situ hybridizations were employed to characterize somitogenesis in the *Ripply2*$^{-/-}$ embryo. The expression of caudal genes such as *Uncx4.1* (**A**,**B**) and *Dll1* (**C**,**D**) was found to be reduced, whereas rostral genes such as *Tbx18* (**E**,**F**) and *Epha4* (**G**,**H**) show an expanded pattern in *Ripply2*$^{-/-}$ embryos at E11.5. (**I-N**) Comparisons of the expression patterns of *Mesp2* mRNA, detected by exon (I,J) and intron (K,L) probes, and protein levels (M,N), at E10.5. An additional *Mesp2* expression band appears rostrally in the *Ripply2*$^{-/-}$ embryos (J,L). Mesp2 protein expression, visualized by Mesp2-venus, was compared between the *Ripply2*$^{+/-}$ (M, *n*=2) and *Ripply2*$^{-/-}$ (N, *n*=3) genetic backgrounds. The confocal images were visualized by fluorescence, detected using anti-GFP antibodies. (**O**,**P**) Comparison of the *Lfng* expression patterns at different cyclic phases (indicated by I to III) at E10.5. The oscillatory expression of *Lfng* (asterisks) in the posterior PSM was unaffected, but the rostral-most expression bands (brackets) are slightly expanded in the *Ripply2*$^{-/-}$ embryos (P), as compared with the *Ripply2*$^{+/-}$ embryos (O). (**Q**,**R**) The prolonged expression of *Lfng* in the anterior PSM. The PSM of E10.5 *Ripply2*$^{+/-}$ (Q) and *Ripply2*$^{-/-}$ (R) embryos was separated into two halves, with one being fixed immediately and the other fixed after explant culturing for 20 minutes. Both were then analyzed for *Lfng* mRNA. The expression of *Lfng* in the anterior PSM is maintained for longer in the *Ripply2*$^{-/-}$ embryos.

(Fig. 1A). The expression of *Ripply2* then continued until 12.5 dpc, during the somite-forming period (Fig. 1A and data not shown). The expression domains of *Mesp2* and *Ripply2* were next compared by double in situ hybridization of embryonic tail sections. Two typical patterns are shown in Fig. 1B. One shows single bands that are completely merged, whereas the other is of a single *Mesp2* band and two *Ripply2* bands in which the caudal band is merged with a distinct *Mesp2* band. This observation indicates that *Mesp2* expression precedes that of *Ripply2*, but that *Ripply2* persists for longer. In addition, *Ripply2* expression was lost in the *Mesp2*-null embryo (Fig. 1C), as predicted from our GeneChip analysis. These data thus indicated that *Ripply2* might be a target of Mesp2.

To examine this possibility, we searched for possible cis-regulatory sequences in the *Ripply2* gene by comparing mouse, human, dog and chick genomic sequences using MultiPipMaker sequence alignment software (Fig. 2A). From these analyses, we identified a conserved region (–6917 to –6747, Fig. 2B). To investigate whether the 1.5 kb region containing this conserved 171 bp sequence (Fig. 2B) possessed enhancer activity, we performed transient transgenic analyses using a β-gal reporter (Fig. 2C). In five out of nine PCR-positive embryos, we detected specific β-gal expression in several segmented somites (Fig. 2D), which is a typical pattern for genes expressed in the anterior PSM, including *Mesp2* and *Epha4* (Haraguchi et al., 2001; Nakajima et al., 2006). We next employed a luciferase reporter assay system to ascertain whether the enhancer activity was dependant upon Mesp2. Two reporter constructs were generated – one containing the 1.5 kb genomic fragment and the other harboring six repeats of the 171 bp consensus sequence. Both constructs were activated by the addition of Mesp2, but not in conjunction with E47 (also known as Tcfe2a – Mouse Genome Informatics) (Fig. 2E). This result was different from the findings of our previous study of the *Epha4* enhancer (Nakajima et al., 2006), in which Mesp2 was observed to bind and transactivate the reporter activity only in the presence of E47, a possible heterodimeric partner. Since Mesp2 belongs to the bHLH-type transcription factor family, which is known to bind either to E-box or N-box motifs, we screened the 171 bp *Ripply2* gene consensus sequence for E-boxes, or for an N-box which is capable of binding to Mesp2 with or without E47. We identified a DNA fragment containing a highly conserved E-box CATCTG sequence, and confirmed that this binds to Mesp2, whereas a mutated form did not (Fig. 2F,G). E47 was also found to bind to this E-box, but this might not be functional binding as no associated activity was detectable by luciferase reporter assay. Furthermore, the binding of Mesp2 was weakened by the addition of E47. These results are consistent with the idea that Mesp2 binds to this E-box in the enhancer of the *Ripply2* gene, and that this enhancer does not require E47 for subsequent transactivation.

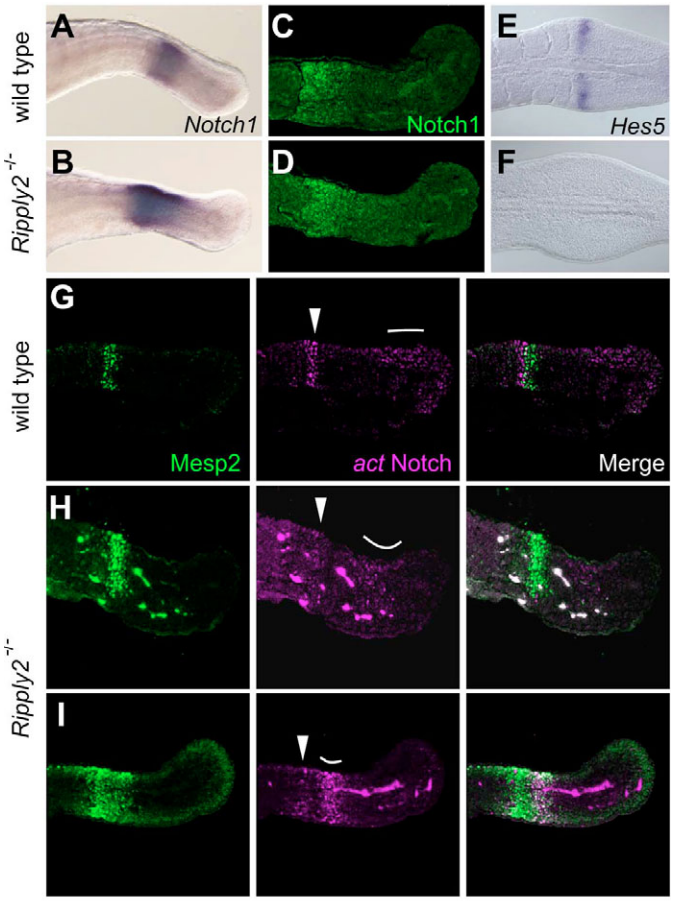


**Fig. 6. Notch signaling is reduced in the anterior PSM in the *Ripply2*$^{-/-}$ embryo.** (**A-F**) *Notch1* mRNA (A, *n*=2; B, *n*=2), Notch1 protein (C, *n*=2; D, *n*=2) and *Hes5* mRNA (E, *n*=2; F, *n*=4) expression patterns were compared between wild-type (A,C,E) and *Ripply2*$^{-/-}$ (B,D,F) embryos at E11.0. (**G-I**) Double immunostaining with anti-Mesp2 (green) and anti-active Notch1 (magenta; the white lines indicate activities in the anterior PSM) antibodies using sections of wild-type (G) and *Ripply2*$^{-/-}$ (H,I) E11.0 embryos. In the *Ripply2*$^{-/-}$ background, Mesp2 expression is upregulated but Notch activity is reduced.

## The *Ripply2*-knockout mouse exhibits a rostralized phenotype

Because Mesp2 confers rostral properties to the somites and is involved in the formation of the somite boundary, we speculated whether Ripply2 might function in this Mesp2 pathway during somitogenesis. To elucidate this possibility, we generated *Ripply2*-knockout mice using ES cell-mediated gene targeting (Fig. 3A). Since the heterozygous mice were found to be normal, we performed timed intercross matings to analyze the phenotypes of the homozygotes. As expected from the expression patterns, the *Ripply2*$^{-/-}$ embryos failed to proceed through normal somitogenesis and the embryos displayed no clear segmental borders (Fig. 4A-D). These homozygous mice also died soon after birth. The morphology of the 17.5-dpc fetus was found to be similar to that of the *Mesp2*-null embryo, with a short trunk and tail (Fig. 3B) (Saga et al., 1997). However, the vertebral phenotype of the *Ripply2*$^{-/-}$ embryos, as revealed by skeletal staining, differed from that of *Mesp2*-null embryos as it features extensive fusion of the pedicles in the neural arches owing to the caudalized characteristics of the somitic mesoderm (Saga et al., 1997).

The *Ripply2*$^{-/-}$ mouse fetus showed fewer pedicles of neural arches (Fig. 4E,F), and the phenotype resembled that of the presenilin 1 (*Psen1*)-null mouse (Fig. 4G), which lacks Notch signaling (Koizumi et al., 2001). The findings of our gene expression studies using both rostral and caudal molecular markers are consistent with these skeletal defects. In *Ripply2*$^{+/-}$ embryos, the expression of the caudal markers *Uncx4.1* and *Dll1* in the segmented somites was restricted to the caudal compartments of the somites (Fig. 5A,C). The expression of these genes is increased and more expansive in *Mesp2*-null embryos (Takahashi et al., 2000), but was greatly reduced in the *Ripply2*-null embryos, (Fig. 5B,D). In addition, no *Dll1* stripe could be observed within the somitic region or in the anterior PSM, although the expression in the posterior PSM was intact in the *Ripply2*$^{-/-}$ embryo (Fig. 5D). By contrast, the rostral markers were found to be present in the *Ripply2*$^{-/-}$ embryo (Fig. 5E-H). *Tbx18*, which is known to be involved in the maintenance of the rostral properties of the somites (Bussen et al., 2004; Kraus et al., 2001), was expressed in the rostral compartment of the segmented somites (Fig. 5E). In *Ripply2*$^{-/-}$ embryos, this expression was expanded throughout the entire somite region and no clear segmental pattern was evident (Fig. 5F). In addition, *Epha4* was expressed in the rostral compartment of S0 and S1 somites in the *Ripply2*$^{+/-}$ embryo (Fig. 5G), and this expression in the *Ripply2*$^{-/-}$ embryo was increased and the expression domain expanded as compared with the wild type (Fig. 5H). We thus conclude that the *Ripply2*-null mouse displays a rostralized phenotype.

## Ripply2 is a negative regulator of *Mesp2* expression

As we have previously reported, the rostro-caudal polarity of the somites is generated by the interaction between Mesp2 and the Notch signaling pathway in the anterior PSM (Morimoto et al., 2005). To identify the underlying cause of the rostralized phenotype in the *Ripply2*-null embryo, the *Mesp2* gene expression profile was examined. During somitogenesis in the anterior PSM, both wild-type and *Ripply2*$^{+/-}$ embryos generally showed either a single *Mesp2* expression band of variable width or no band, depending on the cyclic expression stage (Fig. 5I and data not shown). However, we observed that *Mesp2* is expressed in the *Ripply2*$^{-/-}$ embryo as either one or two bands (Fig. 5J). In other words, an additional band was frequently observed in the more-rostral region (four out of six examined). In addition, we did not observe any *Ripply2*$^{-/-}$ embryos without *Mesp2* expression, suggesting either that *Mesp2* expression is prolonged or that the *Mesp2* transcripts are stabilized in a *Ripply2*$^{-/-}$ background. To distinguish these possibilities, we performed in situ hybridization using an intron probe. Although the signal obtained was low, we frequently detected two bands in the *Ripply2*$^{-/-}$ embryos (Fig. 5L; in all three examined), but only one band in the *Ripply2*$^{+/-}$ embryos (Fig. 5K). Hence, the transcription of *Mesp2* appears to be prolonged in the absence of Ripply2, although the possibility that differences exist in their mRNA stability cannot yet be excluded.

We next examined how the expression of the Mesp2 protein is influenced in the *Ripply2*$^{-/-}$ background. As we have shown previously, Mesp2-venus can be used to visualize functional Mesp2 proteins in vivo because the homozygous knock-in mouse is viable and shows normal somitogenesis (Morimoto et al., 2005). In a typical case, a single Mesp2-venus band was detectable in the area just caudal to the next presumptive segmental border in the *Ripply2*$^{+/-}$ background (Fig. 5M). However, in the *Ripply2*$^{-/-}$ embryo, two broader and interconnected bands could be discerned (Fig. 5N). These data

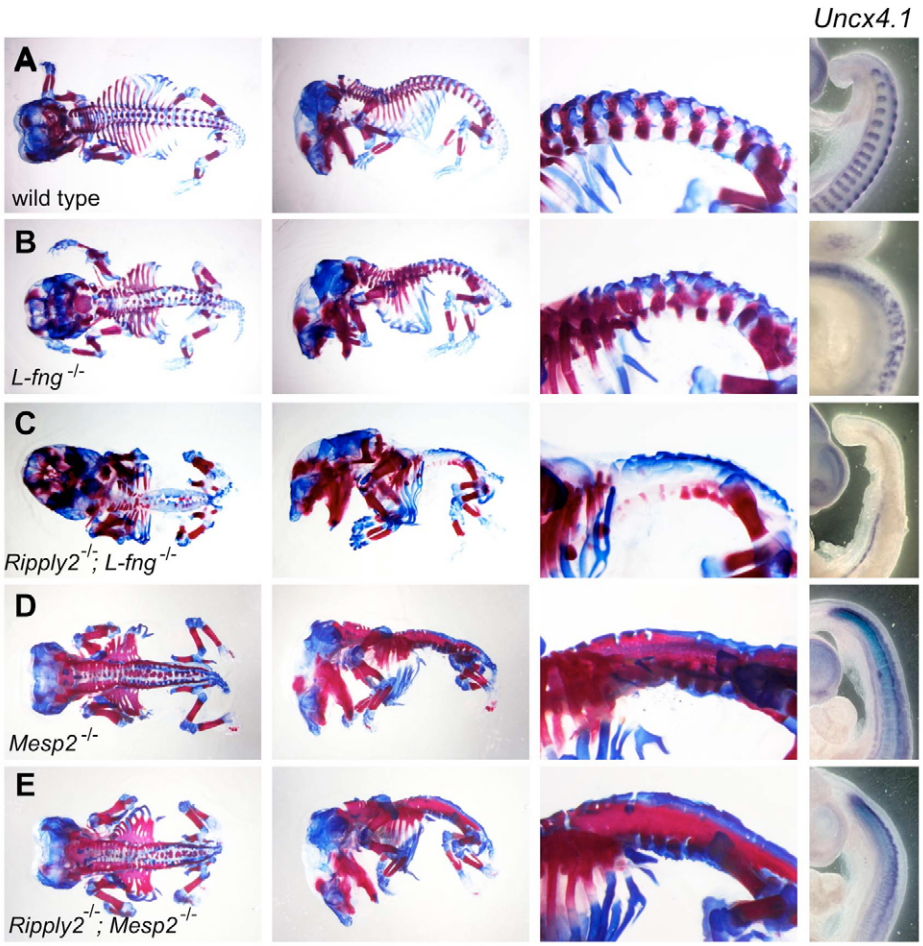


**Fig. 7. Genetic analyses using double-knockouts of *Ripply2* and either *Lfng* or *Mesp2*.** The skeletal morphologies and *Uncx4.1* expression patterns were compared among wild-type (**A**), *Lfng*-null (**B**), *Ripply2*/*Lfng* double-null (**C**), *Mesp2*-null (**D**) and *Ripply2*/*Mesp2* double-null (**E**) E17.5 fetuses or E9.5 embryos. The skeletal defects in the *Ripply2*$^{-/-}$ fetus were found to be further enhanced by the additional loss of *Lfng*, and the pedicles of the neural arches were almost completely absent in this compound-null fetus (C). By contrast, the *Ripply2*/*Mesp2* double-null fetus (E) shows a similar morphology to that of the *Mesp2* single-null fetus (D). The *Uncx4.1* expression pattern was independently examined at E10.5 (A, *n*=2; B, *n*=2; C, *n*=1) and E9.5 (A, *n*=4; B, *n*=2; C, *n*=2; D, *n*=4; E, *n*=2). Only representative images of E9.5 embryos are shown.

suggest that *Mesp2* is negatively regulated by Ripply2, and that these factors form a negative-feedback loop to restrict the levels of Mesp2.

We previously reported that *Lfng* expression is activated by Mesp2 in the anterior PSM and is subsequently involved in the suppression of Notch signaling. Moreover, *Lfng* expression shows a cyclic wave-like pattern in the posterior PSM, but its expression in the anterior PSM is similar to that of *Mesp2* in *Ripply2*$^{+/-}$ embryos. The width of this *Lfng* band becomes thinner before disappearing from the rostral end of the expression domain in the *Ripply2*$^{+/-}$ embryo (Fig. 5O). However, in the *Ripply2*$^{-/-}$ embryos, the anterior-most *Lfng* band was found to be wider and to persist for much longer as compared with the *Ripply2*$^{+/-}$ embryos (Fig. 5P). This persistent expression of *Lfng* was also evident from 20-minute explant culture experiments with a half-PSM (Fig. 5Q,R). These results suggest that Notch signaling might be suppressed, even in the presumptive caudal compartment of the somites, by prolonged Mesp2 and/or Lfng expression in the *Ripply2*$^{-/-}$ embryo.

## Mesp2, but not Lfng, is responsible for the Notch suppression necessary for rostro-caudal patterning

In somite-stage embryos, Notch activity oscillates in the posterior PSM and stabilizes as a clear stripe in the anterior PSM with elevated activity (Huppert et al., 2005; Morimoto et al., 2005). To further understand the molecular events operating in the anterior PSM of *Ripply2*$^{-/-}$ embryos, we first examined the expression of *Notch1* mRNA (Fig. 6A,B) and Notch1 protein (Fig. 6C,D) in these embryos. Interestingly, these expression patterns were found to be expanded in the anterior PSM in the *Ripply2*$^{-/-}$ embryo (Fig. 6B,D), but the Notch activity appeared to be lost as judged from the fact that the expression of *Hes5*, a Notch target gene (Ohtsuka et al., 1999), was absent (Fig. 6E,F). To further confirm this reduced Notch1 activity and its relationship to Mesp2 expression, we conducted double immunostaining analysis using anti-active Notch1 and anti-Mesp2 antibodies in both wild-type and *Ripply2*$^{-/-}$ embryos. In the wild-type embryos, the Notch activity in the anterior PSM exhibited a sharp boundary with Mesp2 that determines the next segmental boundary (Fig. 6G). In addition, the contrast between Notch activities leads to the generation of future rostral and caudal compartments of the somites, whereby the Notch active site becomes the future caudal compartment. In the *Ripply2*$^{-/-}$ embryo, the Notch1 signals oscillated normally in the posterior PSM (Fig. 6H,I). However, the elevation of Notch activity in the anterior PSM appeared to be repressed in these null embryos, whereas the Mesp2 expression banding was found to upregulated, as shown previously (Fig. 6H,I).

Since the expression of *Lfng* is under the control of Mesp2, we speculated that the suppression of Notch signaling might be the result of the prolonged activation of *Lfng* in the *Ripply2*$^{-/-}$ embryo. To test this possibility, we generated a *Ripply2*/*Lfng* double-knockout embryo from which we prepared skeletal specimens, and then examined the somite properties by analyzing the expression of the caudal molecular marker *Uncx4.1* (Fig. 7). Intriguingly, the vertebral morphology of the *Ripply2*/*Lfng*

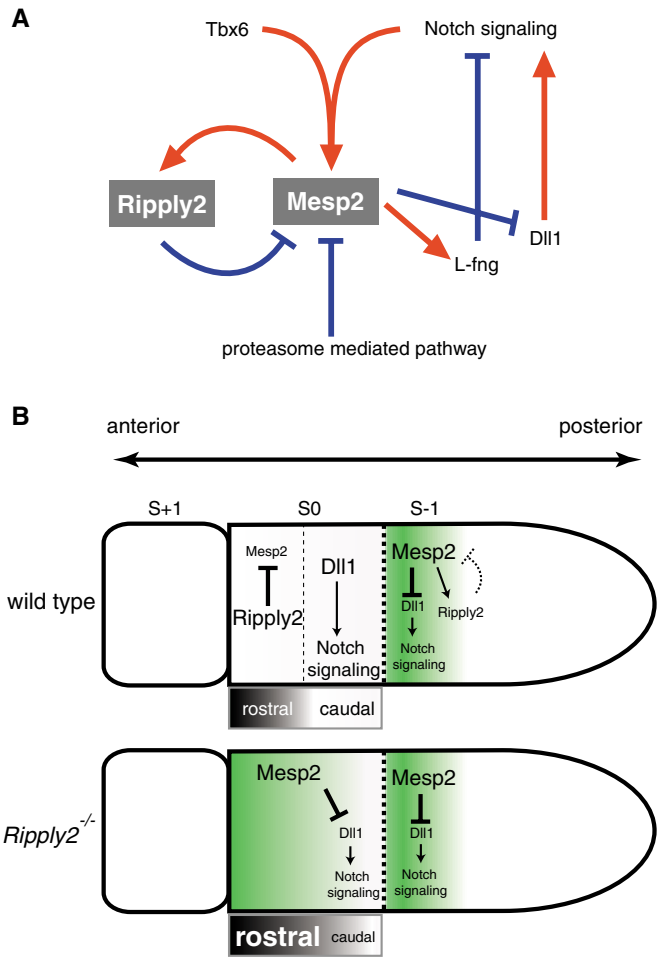


**Fig. 8. Genetic cascades in the anterior PSM regulating somitogenesis.** (**A**) Schematic of the positive (red line) and negative (blue line) regulation surrounding Mesp2. The transcription of *Mesp2* is enhanced by both Notch signaling and Tbx6. At the same time, Mesp2 suppresses Notch signaling by activating *Lfng* and suppressing *Dll1* expression. Mesp2 proteins are also rapidly degraded via a proteasome-dependent pathway. We herein propose a new negative regulatory system for Mesp2 via Ripply2. (**B**) Schematic illustrating how the rostro-caudal polarity is established or disrupted in the anterior PSM of the wild type and *Ripply2*$^{-/-}$ mutants. In the anterior PSM, Mesp2 is localized in the rostral compartment of S–1 and suppresses Notch signaling through the suppression of *Dll1*. By contrast, in the caudal compartment of S0, both *Dll1* expression and Notch signaling are retained because of the lack of Mesp2. In the *Ripply2*$^{-/-}$ embryo, Mesp2 expression persists for a longer period in both the rostral and caudal compartments, although the suppression on Notch signaling is incomplete. This results in the expansion of the rostral properties within the somites.

double-knockout mouse was not recovered, and was more rostralized as compared with either the *Ripply2*$^{-/-}$ (compare Fig. 4F with Fig. 7C) or *Lfng*-null fetus (Fig. 7B). The expression of *Uncx4.1* was also not recovered by the additional loss of Lfng (compare Fig. 5B with Fig. 7C), and was found to be completely diminished in the double-knockout embryos.

To determine whether the suppression of Notch signaling is mainly due to the function of Mesp2, we also generated *Mesp2*/*Ripply2* double-null mice and analyzed the resulting skeletal phenotypes. As expected, the vertebral morphology of these fetuses was found to be very similar to the *Mesp2* single-null fetus, and exhibited a caudalized phenotype (Fig. 7D,E). The expression of *Uncx4.1* was also upregulated to similar levels as in the *Mesp2*-null embryo (Fig. 7E). These results clearly showed that Mesp2 suppresses the expression of this gene independent of Ripply2, and that the defect observed in the *Ripply2*$^{-/-}$ mouse can be attributed to the function of Mesp2.

## DISCUSSION

Our current study establishes the hypothesis that the negative-feedback regulation of Mesp2 by Ripply2 constitutes a core component of the regulatory network involved in establishing rostro-caudal patterning. The periodicity of somitogenesis is established by mechanisms based on the negative regulation of several genes in the mouse posterior PSM (Rida et al., 2004), in which the clock genes *Hes7* and *Lfng* are negatively regulated by several mechanisms, including transcriptional suppression, protein degradation and destabilization of mRNA (Bessho et al., 2003; Chen et al., 2005; Cole et al., 2002; Hirata et al., 2004; Morales et al., 2002). In the anterior PSM, the levels of Mesp2 are strictly regulated to achieve the periodic suppression of Notch signaling, and also to establish the correct rostro-caudal polarity. During this activation step, the cooperation between Tbx6 and cyclic activated Notch-signaling is crucial for the periodic induction of *Mesp2* (Yasuhiko et al., 2006) (Fig. 8A). However, these processes must be regulated by both activation and inhibition. Previously, we reported that Mesp2 is regulated negatively by the proteasome pathway (Morimoto et al., 2006). In addition, our current study has identified Ripply2 as a potent negative regulator of *Mesp2* transcription, and as a factor that is required for the correct establishment of rostro-caudal patterning. In the absence of Ripply2, Mesp2 expression is maintained over a longer period and leads to the suppression of caudal properties (Fig. 8B). It is noteworthy in this regard that Ripply2 might function exclusively to negatively regulate Mesp2, because the phenotype of the *Ripply2*-knockout mouse is almost completely reversed by the additional loss of Mesp2.

The *Ripply2*-null mutant exhibits not only an expansion of rostral marker genes but also a reduction in the expression of caudal markers. Immunohistochemical analysis further revealed a decrease in the activated form of Notch1 in the anterior PSM in these *Ripply2*-null embryos. Previously, we have shown that Mesp2 suppresses Notch signaling to establish segmental boundaries via the activation of Lfng. However, Lfng appears not to be crucial for the suppression of Notch signaling in the *Ripply2*-null embryo as this suppression was not rescued by the additional loss of Lfng, and, in fact, this results in a further reduction in Notch signaling activity. We speculate that this is caused by the function of Lfng during Mesp2 distribution, based upon our observations of the Mesp2-venus knock-in mouse. In the wild-type embryo, the Mesp2-venus expression pattern shows a clear gradient, being higher in the presumptive rostral compartment. However, in the absence of Lfng, such a biased gradient is not generated, and the Mesp2-venus pattern shows a diffuse distribution in this background (our unpublished data). The phenotype of the *Ripply2*/*Lfng* double-knockout mouse appears also to reflect this distribution defect. In this double-null mouse, the expression of Mesp2 is prolonged owing to the lack of Ripply2, and is distributed across a much wider area along the anterior-posterior axis because of the lack of Lfng. This in turn enhances the function of Mesp2 that suppresses Notch signaling in the anterior PSM, and results in the somites becoming completely rostralized in these double mutants.

The mechanisms underlying the suppression of Mesp2 by Ripply2 are currently unknown. Ripply2 appears to be required for the termination of Mesp2 expression at an appropriate time. Moreover, because Ripply2 has no apparent DNA-binding domain, it is plausible to assume that it suppresses *Mesp2* by recruiting the Groucho homolog Tle1 and/or Tle3 via the WRPW motif, as revealed previously by in vitro assays in both zebrafish and mouse (Kawamura et al., 2005) (data not shown). *Tle1* and *Tle3* are known to be expressed in the PSM, but their expression patterns are not segmental (Dehni et al., 1995) (our unpublished data), and no loss-of-function studies have yet been reported. In the zebrafish, *ripply1* morphants also display upregulation of *mespb* in their somitic regions, and this is accompanied by the upregulation of *tbx24*, *deltaC* and *deltaD*. This might also account for the upregulation of *mespb* (Kawamura et al., 2005). We have previously identified a 300 bp upstream region of the *Mesp2* gene as a promoter-enhancer sequence required for the faithful expression of *Mesp2* in the anterior PSM where T-box factor binding in combination with Notch signaling is involved in the gene activation (Yasuhiko et al., 2006). However, Tbx6 expression is unchanged (data not shown) and the *Dll1* expression profile is somewhat decreased in the *Ripply2*-null embryos. Hence, although the impact of the loss of Ripply proteins upon Mesp gene expression appears to be similar between mouse and zebrafish, the underlying mechanisms might well be different.

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**Supplementary material**

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/8/1561/DC1

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