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The *Xenopus* Bowline/Ripply family proteins negatively regulate the transcriptional activity of T-box transcription factors

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ABSTRACT Bowline, which is a member of the *Xenopus* Bowline/Ripply family of proteins, represses the transcription of somitogenesis-related genes before somite segmentation, which makes Bowline indispensable for somitogenesis. Although there are three *bowline/Ripply* family genes in each vertebrate species, it is not known whether the Bowline/Ripply family proteins share a common role in development. To elucidate their developmental roles, we examined the expression patterns and functions of the *Xenopus* Bowline/Ripply family proteins Bowline, Ledgerline, and a novel member of this protein family, xRipply3. We found that the expression patterns of *bowline* and *ledgerline* overlapped in the presomitic mesoderm (PSM), whereas *ledgerline* was additionally expressed in the newly formed somites. In addition, we isolated *xRipply3*, which is expressed in the pharyngeal region. Co-immunoprecipitation assays revealed that Ledgerline and xRipply3 interacted with T-box proteins and the transcriptional co-repressor Groucho/TLE. In luciferase assays, xRipply3 weakly suppressed the transcriptional activity of Tbx1, while Ledgerline strongly suppressed that of Tbx6. In line with the repressive role of Ledgerline, knockdown of Ledgerline resulted in enlargement of expression regions of the somitogenesis-related-genes *mespb* and *Tbx6*. Inhibition of histone deacetylase activity increased the expression of *mespb*, as seen in the Bowline and Ledgerline knockdown experiments. These results suggest that the Groucho-HDAC complex is required for the repressive activity of Bowline/Ripply family proteins during *Xenopus* somitogenesis. We conclude that although the *Xenopus* Bowline/Ripply family proteins Bowline, Ledgerline and xRipply3 are expressed differentially, they all act as negative regulators of T-box proteins.

KEY WORDS: *somitogenesis, ripply, presomitic mesoderm, Tbx6, groucho*

Introduction

The T-box family genes, which encode transcription factors that contain the T-box DNA binding domain, are highly conserved in metazoans. T-box transcription factors play critical roles in the organogenesis of, for example, the heart, pituitary gland, and limbs. Thus, various developmental defects have been linked to mutations in T-box genes in humans (Naiche *et al.*, 2005). Tbx6, which is a member of the T-box family of transcription factors, functions as a transcriptional activator and is involved in the specification of the posterior paraxial mesoderm and the formation of the somite, which sequentially buds off from the paraxial, presomitic mesoderm (PSM). Mouse embryos with a null muta-

Abbreviations used in this paper: HDAC, histone deacetylase; PSM, presomitic mesoderm; Tbx, T-box; TLE, transducin-like Enhancer-of-split.

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tion in *Tbx6* are embryonically lethal at mid-gestation due to a deficiency of caudal somites (Chapman and Papaioannou, 1998). Moreover, mice that carry a hypomorphic allele of *Tbx6* show disruption of the anteroposterior polarity of their somites (Beckers *et al.*, 2000; Nacke *et al.*, 2000; Theiler and Varnum, 1985; Watabe-Rudolph *et al.*, 2002; White *et al.*, 2003). These findings underline the requirement of *Tbx6* for somite formation.

We and others previously identified a novel protein family, Bowline/Ripply, from the mouse, African clawed frog (*Xenopus laevis*), zebrafish, and amphioxus (Chan *et al.*, 2007; Chan *et al.*, 2006; Kawamura *et al.*, 2005; Kondow *et al.*, 2006; Li *et al.*, 2006; Morimoto *et al.*, 2007). In vertebrates, two *bowline/Ripply* family genes have been identified and characterized to date. The expression region of *bowline*, which is a member of the *bowline/Ripply* family gene in *Xenopus*, is restricted to the anterior PSM during somitogenesis (Kondow *et al.*, 2006). In *bowline*-knockdown embryos, somite boundary formation is disrupted, and the regions of *mespb* and *X-delta-2* expression, which are restricted to the anterior PSM during normal development, are expanded anteriorly (Kondow *et al.*, 2007). The corresponding defects in somites were observed in *Ripply1*-deficient embryos of zebrafish and in *Ripply2*-knockout mice (Chan *et al.*, 2007; Kawamura *et al.*, 2005; Morimoto *et al.*, 2007). Thus, Bowline/Ripply family proteins are essential for the proper formation of somite boundaries in vertebrates. Bowline/Ripply family proteins have two conserved domains: the WRPW tetrapeptide motif and the Ripply homology domain/Bowline-DSCR-Ledgerline conserved region (Ripply/BDLC domain). Through the WRPW-tetrapeptide motif, *Xenopus* Bowline and zebrafish Ripply1 interact with the transcriptional co-repressor Groucho/TLE (Kawamura *et al.*, 2005; Kondow *et al.*, 2006). We have previously shown that Bowline interacts with Tbx6 protein *via* the Ripply/BDLC domain and suppresses the transcriptional activity of Tbx6 (Kondow *et al.*, 2007). Similarly, in zebrafish, Ripply1 interacts with Tbx24, which is related to Tbx6, to suppress the transcriptional activity of Tbx24 (Kawamura *et al.*, 2008).

Previously, we isolated an additional *Xenopus bowline/Ripply* family gene, *ledgerline* (Chan *et al.*, 2006). Although *ledgerline* is also expressed in the anterior PSM during *Xenopus* somitogenesis, its molecular function in somitogenesis remains largely unknown. In the present report, we show that Ledgerline interacts with the Tbx6 protein and suppresses, to the same extent as Bowline, the transcriptional activity of Tbx6. Loss of function of *ledgerline* resulted in significant changes to the expression patterns of *mespb* and *Tbx6*.

We also show that a novel *Xenopus bowline/Ripply* family gene, *xRipply3*, is expressed in the pharyngeal region during *Xenopus* development. Furthermore, we demonstrate that xRipply3 interacts with the T-box protein Tbx1 and suppresses the transcriptional activity of Tbx1. Our findings reveal that *Xenopus* Bowline/Ripply family proteins share the common feature of acting as negative regulators of T-box proteins. In addition, our characterization of xRipply3 suggests the involvement of this novel Bowline/Ripply family protein in the development of the pharyngeal region.

Results

Expression profiles of Xenopus bowline/Ripply *genes in the presomitic mesoderm*

Ledgerline is one of the *bowline/Ripply* family genes in *Xenopus* and is expressed in the PSM (Chan *et al.*, 2006). However, the definitive expression pattern of *ledgerline* in the PSM has not been clarified. To define the spatial expression pattern of *ledgerline* in the PSM, we compared the mRNA distribution patterns of *ledgerline* and *bowline*, which is another *Xenopus bowline/Ripply* family gene. Serial sections of *Xenopus* embryos at stage 20 were prepared, and the *ledgerline* and *bowline* transcripts were detected by *in situ* hybridization (Fig. 1A-B). The expression of *bowline* was observed in the anterior halves of the S-I, S-II, and

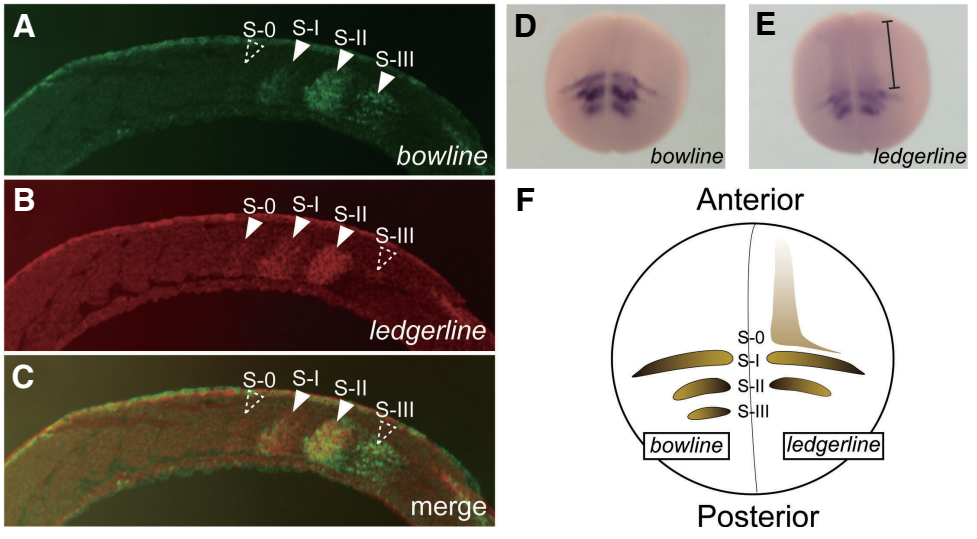

Fig. 1. Differential expression patterns of the *bowline* and *ledgerline* genes during *Xenopus* somitogenesis. (A,B) *Expression patterns of* bowline *and* ledgerline *in the* Xenopus *PSM. Longitudinal serial sections of a stage 20 embryo were stained for* bowline **(A)** *and* ledgerline **(B)**, *and the results are shown as a pseudocolor display. The white arrowheads in (A) and (B) represent the regions in which* bowline *and* ledgerline, *respectively, are expressed.* **(C)** *Merged image of (A,B). The white arrowheads in (C) represent the regions in which the expression patterns of* bowline *and* ledgerline *overlap. The sections are orientated so that the anterior part is to the left.* **(D,E)** *Whole-mount* in situ *hybridization for* bowline **(D)** *and* ledgerline **(E)**. *The expression of* bowline *is restricted to the somitomeres. Interestingly,* ledgerline *is expressed in the newly formed somites, as well as in the somitomeres. The black bar indicates* ledgerline *expression in the newly formed somites.* **(F)** *Schematic diagram of the* bowline *and* ledgerline *expression patterns.* Bowline *is expressed in the anterior halves of the S-I to S-III somitomeres (left side).* Ledgerline *is expressed in the newly formed somites, in addition to the anterior halves of the S-0 to S-II somitomeres (right side). The dorsal view is shown, with anterior being towards the top. The nomenclature used for the somitomeres follows that proposed earlier (Pourquie and Tam, 2001).*

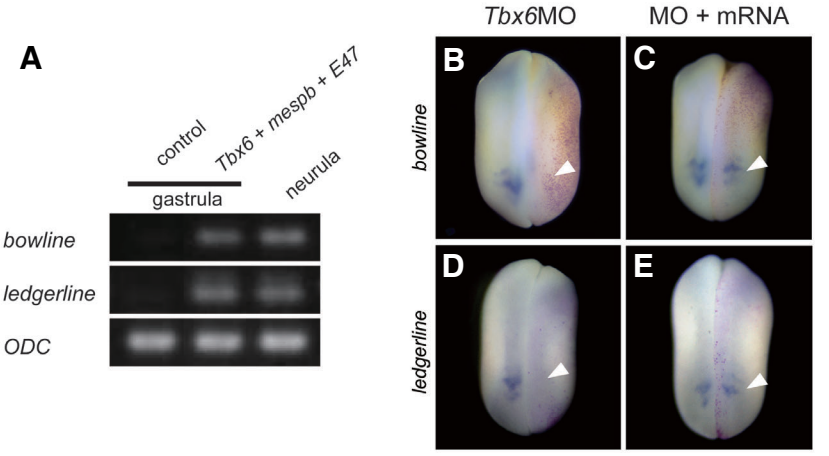

Fig. 2. Tbx6 is required for *bowline* and *ledgerline* expression during *Xenopus* development. (A) *The expression of* bowline *and* ledgerline *is induced by the synergistic effects of Tbx6, mespb, and E47. RT-PCR analysis shows that overexpression of the mixture of* Tbx6, mespb, *and* E47 *mRNA species induces ectopic* bowline *and* ledgerline *expression in gastrula-stage* Xenopus *embryos.* ODC *(*ornithine decarboxylase*) was used as an internal control.* **(B-E)** *Expression of* bowline *mRNA and* ledgerline *mRNA in Xtbx6 exint-MO-injected embryos. All the embryos were fixed at stage 19 for whole-mount* in situ *hybridization to detect the expression of* bowline **(B,C)** *and* ledgerline **(D,E)**. *Magenta-Gal staining indicates the injected side.* **(B,D)** *A representative embryo injected with 50 ng Xtbx6 exint-MO. Inhibition of the splicing of* Tbx6 *mRNA results in loss of expression of* bowline *and* ledgerline. *The white arrowheads in (B) and (D) indicate reduced* bowline *and* ledgerline *expression, respectively.* **(C,E)** *Representative embryos injected with 50 ng Xtbx6 exint-MO and 2 pg or 10 pg* Xenopus Tbx6 *mRNA, respectively. The white arrowheads in (C,E) indicate rescued* bowline *and* ledgerline *expression, respectively.*

S-III somitomeres, as previously reported (Kondow *et al.*, 2006), while the expression of *ledgerline* was observed in the anterior halves of the S0, S-I, and S-II somitomeres and the newly formed somites (Fig. 1A, B, D, E). The two gene transcripts co-localize in the anterior halves of the S-I and S-II somitomeres (indicated in yellow in the merged image; Fig. 1C). Thus, the spatial expression patterns of *bowline* and *ledgerline* are partially overlapped in the PSM, in which the *ledgerline* transcripts localize anteriorly with respect to the *bowline* transcripts (Fig. 1F).

Tbx6 is indispensable for the expression of both bowline *and* ledgerline

We have shown previously that the expression of *bowline* is activated by the combined effects of three transcriptional factors, Tbx6, mespb, and E47 (Hitachi *et al.*, 2008b). We examined whether the expression of *ledgerline* is also regulated by these three factors. The *in vitro*-transcribed mRNA species of *Tbx6*, *mespb*, and *E47* were injected into 2-cell stage embryos of *Xenopus*, and the *ledgerline* transcripts were detected by RT-PCR at the gastrula stage, at which point endogenous *ledgerline* transcripts are not detected during normal development. Induced expression of *ledgerline* was detected (Fig. 2A). This suggests that *ledgerline* expression is also activated by the combination of Tbx6, mespb, and E47.

To elucidate further the requirement of Tbx6 for endogenous *bowline* and *ledgerline* expression, we used antisense morpholino oligonucleotides (MO) to inhibit the function of Tbx6. The MO that targets the exon1/intron1 junction of *Xenopus Tbx6* (Xtbx6 exint-MO) specifically blocks Tbx6 function (Tazumi *et al.*, 2008). The Xtbx6 exint-MO (50 ng) was injected into the ventral marginal zone and dorsal lateral marginal zone of embryos at the 4-cell stage, with the uninjected side serving as a control. These embryos were cultured until stage 19, and the spatial expression patterns of *bowline* and *ledgerline* were examined by whole-mount *in situ* hybridization. The expression of *bowline* and *ledgerline* was lost in the MO-injected side (95%, n=38; and 100%, n=41, respectively) (Fig. 2B and D). The loss of *bowline* expression was rescued to a moderate extent by co-injection of Xtbx6 exint-MO with *Tbx6* mRNA (19%, n=37) (Fig. 2C). However, in the experiment looking at the rescue of *ledgerline* expression by co-injection of Xtbx6 exint-MO with *Tbx6* mRNA, *ledgerline* expression was rescued at a rate of only 1/37 (Fig. 2E). It is possible that the mechanism responsible for *ledgerline* expression regulation is different from that for *bowline* expression. Nevertheless, these results indicate that Tbx6 is indispensable for the expression of both *bowline* and *ledgerline* during *Xenopus* somitogenesis.

Bowline and Ledgerline suppress the transcriptional activity of Tbx6

Previously, we showed that Bowline acts synergistically with Grg4, which is a *Xenopus* Groucho/TLE protein, to repress *mespb* promoter activity activated by the Tbx6VP16 protein, which is the constitutively active form of Tbx6 (Kondow *et al.*, 2007). We examined whether Bowline suppresses the transcriptional activity of the wild-type Tbx6 protein. Transfection of wild-type *Tbx6* together with *NotchΔE*, which is a constitutively active component of Notch signaling, activated the *mespb* promoter in cultured COS7 cells (Fig. 3A), and this activation was repressed by Bowline (Fig. 3A). Thus, we confirmed that Bowline suppresses the transcriptional activity of wild-type Tbx6. Interestingly, Ledgerline, together with Grg4, repressed *mespb* promoter activity activated by Tbx6 and NotchΔE, or by Tbx6VP16 alone (Fig. 3B and C). In addition, Ledgerline interacted with Grg4 and the Tbx6 protein, as shown in the co-immunoprecipitation assay (Fig. 3D and 3E). These results indicate that Ledgerline is a negative regulator of Tbx6, as is the Bowline protein.

To examine the molecular function of Ledgerline during *Xenopus* somitogenesis, we blocked the translational initiation of Ledgerline using an antisense MO against *ledgerline*. Ledgerline-MO was unilaterally injected into 4-cell-stage embryos, and the spatial expression patterns of *mespb* and *Tbx6* were examined by whole-mount *in situ* hybridization. In the embryos injected with Ledgerline-MO, the regions of *mespb* and *Tbx6* expression were expanded anteriorly in comparison with the uninjected side (65%, n=20 and 93%, n=15, respectively) (Fig. 4A-D), which indicates that Ledgerline represses the expression of both *mespb* and *Tbx6* during *Xenopus* somitogenesis at the anterior PSM. Next, we investigated the involvement of Groucho/TLE protein in this repres-

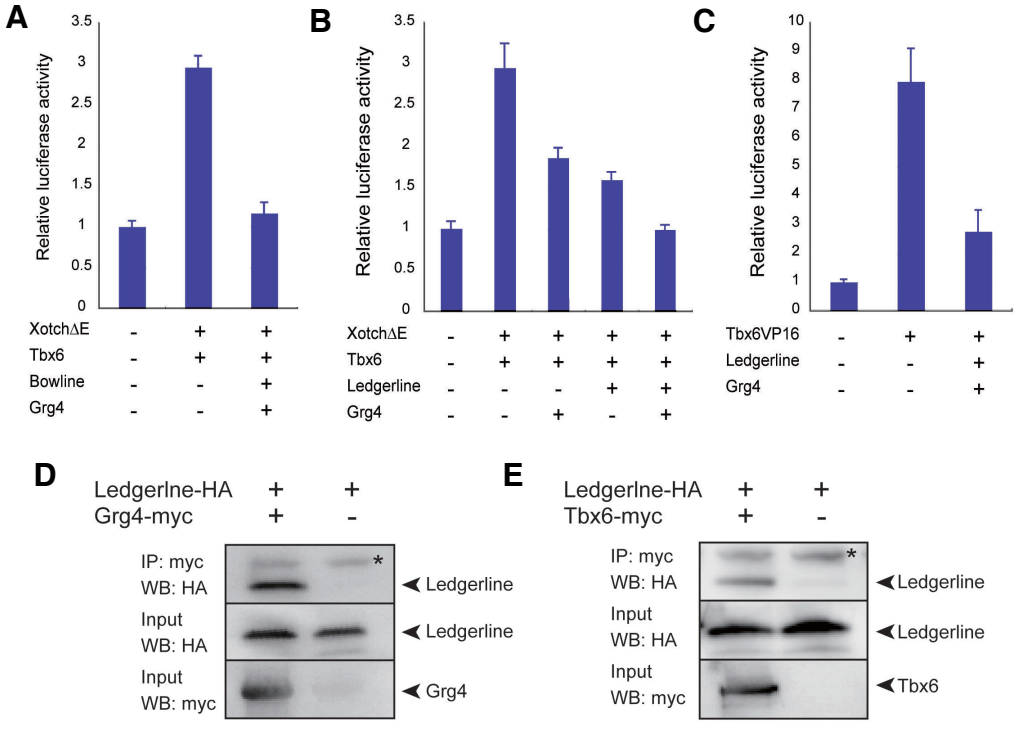

Fig. 3. Ledgerline suppresses the transcriptional activity of Tbx6. (A-C) *Luciferase assays of* mespb *promoter activity.* **(A)** *Bowline, in combination with Grg4, represses the activity of the* mespb *promoter activated by Tbx6 and XotchΔE. COS7 cells were transfected with the* pGL4.2Thy1 *(20 ng) and a combination of the indicated expression vectors, i.e., Tbx6 and XotchΔE (150 ng each) and Bowline and Grg4 (50 ng each).* **(B)** *Ledgerline also represses the activity of the* mespb *promoter activated by Tbx6 and XotchΔE. COS7 cells were transfected with the* pGL4.2Thy1 *(20 ng) and a combination of the indicated expression vectors, i.e., Tbx6 and XotchΔE (150 ng each) and Ledgerline and Grg4 (50 ng each).* **(C)** *Ledgerline, in combination with Grg4, represses the activity of the* mespb *promoter activated by Tbx6VP16. COS7 cells were transfected with* pGL4.2Thy1 *(20 ng) and a combination of the indicated expression vectors, i.e., Tbx6VP16 (200 ng) and Ledgerline and Grg4 (50 ng each). Error bars represent the SEM of three independent experiments.* **(D)** *Interaction between Ledgerline and Grg4 proteins in* Xenopus *embryos. Protein extracts of* Xenopus *embryos co-injected with* HA-ledgerline *mRNA and* myc-Grg4 *mRNA (500 pg each) were subjected to co-immunoprecipitation with the anti-myc antibody.* **(E)** *Interaction between Ledgerline and Tbx6 proteins in* Xenopus *embryos. Protein extracts of* Xenopus *embryos co-injected with* HA-ledgerline *mRNA and* myc-Tbx6 *mRNA (500 pg each) were subjected to immunoprecipitation with the anti-myc antibody. The asterisks indicate non-specific bands.*

sive effect *in vivo*. The repressive activity of Groucho/TLE protein is mediated by histone deacetylase (HDAC). Thus, we used trichostatin A to block the repressive activity of HDAC during *Xenopus* somitogenesis. Trichostatin A treatment induced the anterior expansion of *mespb* expression, as seen for knockdown of Ledgerline (100%, n=8) (Fig. 4E and F). This result suggests that the repressive activity of this Bowline/Ripply family protein is mediated by the Groucho/TLE-HDAC complex during *Xenopus* somitogenesis.

Isolation and characterization of a third Xenopus bowline/Ripply *gene,* xRipply3

The third *bowline/Ripply* gene, *Ripply3*, was found in the human, mouse, and zebrafish genomes. However, their expression patterns and molecular functions of these *Ripply3* genes have not been reported. We isolated the *Xenopus Ripply3* gene, designated as *xRipply3* (GenBank accession no. AB455086). This gene encodes 170 amino acids. The nucleotide sequences around the first in-frame methionine match the Kozak consensus sequence (Kozak, 1987) and most likely represent the translation initiation site.

Comparison of the amino acid sequences of the human, mouse, frog and zebrafish Ripply3 proteins revealed that the tetrapeptide WRPW, which acts as a Grouch/TLE recruitment motif, is completely conserved among these proteins (Fig. 5A). Moreover, the tetrapeptide FPVQ in the Ripply/BDLC region, which is essential for interactions with T-box proteins (Kawamura *et al.*, 2008) was also completely conserved (Fig. 5A). Phylogenetic analysis showed that Ripply3 forms a subfamily among the Bowline/Ripply family proteins (Fig. 5B). We next examined the subcellular localization of the

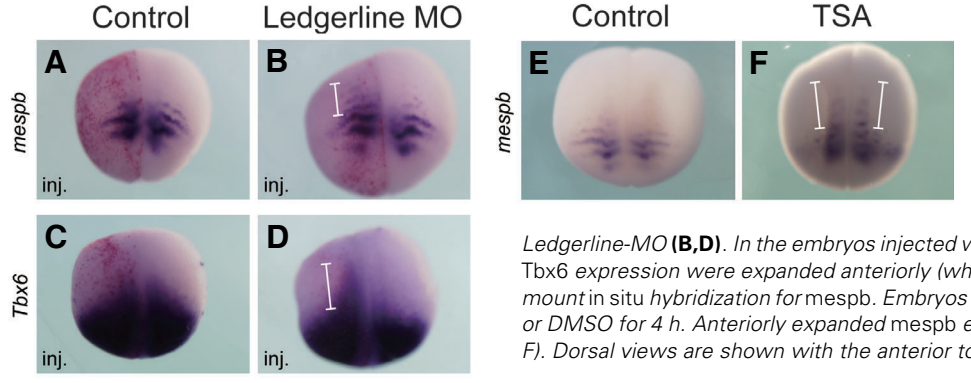

Fig. 4. Knockdown of Ledgerline causes abnormal expression of *mespb* and *Tbx6* during Xenopus somitogenesis. (A-D) *Whole-mount* in situ *hybridization for* mespb **(A,B)** *and* Tbx6 **(C,D)**. *Embryos at the 4-cell stage were injected with Control-MO* **(A,C)** *or Ledgerline-MO* **(B,D)**. *In the embryos injected with Ledgerline-MO, the regions of* mespb *and* Tbx6 *expression were expanded anteriorly (white bar in B and D, respectively).* **(E,F)** *Whole-mount* in situ *hybridization for* mespb. *Embryos were treated with 500 nM trichostatin A (TSA) or DMSO for 4 h. Anteriorly expanded* mespb *expression was induced by TSA (white bars in F). Dorsal views are shown with the anterior towards the top.*

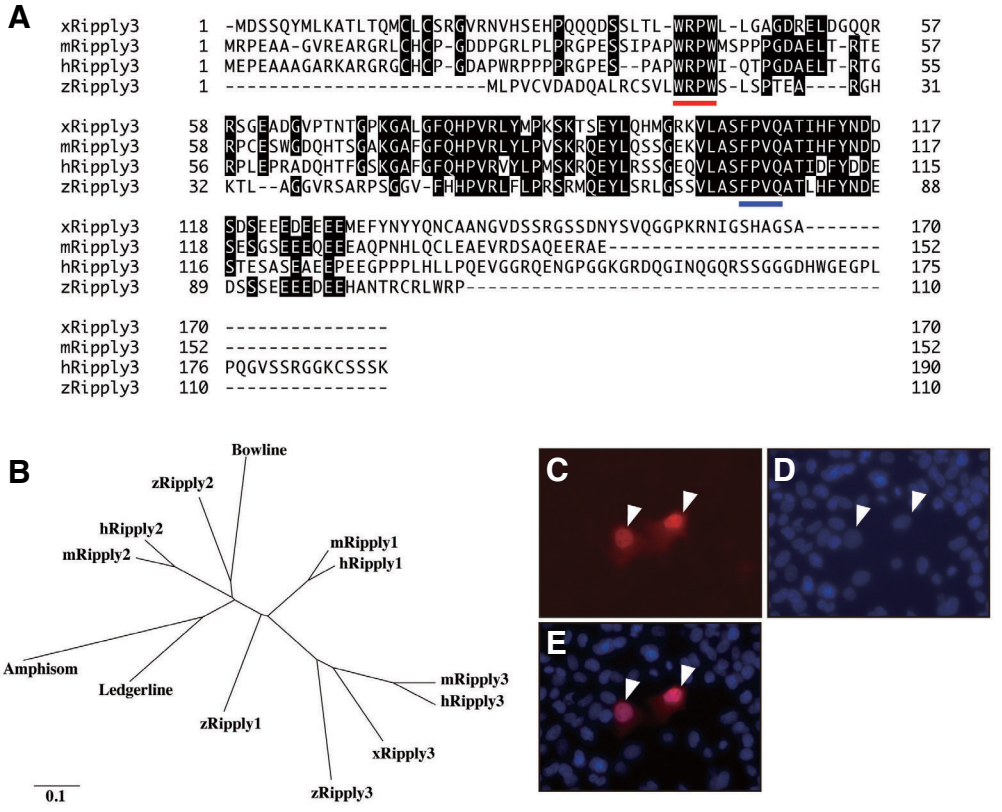

Fig. 5. Structure and subcellular localization of xRipply3. (A) *Comparison of the Ripply3 protein sequences in human, mouse, frog, and zebrafish. Identical amino acids are indicated by a black background. Conserved WRPW and FPVQ motifs are shown in red and blue underlines, respectively.* **(B)** *Phylogenetic tree of the Bowline/Ripply family proteins in human, mouse, frog, zebrafish, and amphioxus.* **(C-E)** *Subcellular localization of the xRipply3-HA protein in COS7 cells. Cultured cells were transfected with the HA-tagged* xRipply3 *plasmid and visualized by immunohistochemical staining with the anti-HA antibody. The localization of xRipply3-HA is mainly nuclear. Nuclei are counterstained with DAPI.*

xRipply3 protein. HA-tagged *xRipply3* was transfected into COS7 cells, and the HA-xRipply3 protein was localized by immunocytochemistry with an anti-HA antibody. In these COS7 cells, HA-xRipply3 was mainly localized to the nucleus (Fig. 5C-E). These results suggest that xRipply3 functions as a transcriptional modulator in the nucleus, as do other Bowline/Ripply family proteins.

Whole-mount *in situ* hybridization for *xRipply3* showed that *xRipply3* was expressed in the region close to the heart mesoderm at the neurula stage (Fig. 6A). At the tailbud stage, *xRipply3* was expressed in the pharyngeal region (Fig. 6B and C). Microscopic observations of sections of the *Xenopus* embryos hybridized with the *xRipply3* antisense probe confirmed that the expression of *xRipply3* was restricted to the pharyngeal region during *Xenopus* development (Fig. 6D). Similarly, the expression of murine *Ripply3* was also observed primarily in the pharyngeal region (Fig. 6E and F). These results indicate that *Ripply3* is expressed in the pharyngeal region, and reveal that the expression profiles of the *Xenopus* and murine *Ripply3* genes differ from those of already known *bowline/Ripply* family genes.

xRipply3 suppresses the transcriptional activity of Tbx1

Two of the Bowline/Ripply family proteins, Bowline and Ledgerline, suppressed the transcriptional activity of Tbx6. Therefore, we hypothesized that xRipply3 also suppresses the transcriptional activities of T-box proteins in the pharyngeal region. We examined whether xRipply3 suppresses the transcriptional activity of Tbx1, since Tbx1 is an activator-type T-box protein and plays a central role in the development of the pharyngeal apparatus (Baldini, 2005; Zoupa *et al.*, 2006). A reporter construct that carries tandem Tbx1 binding sites upstream of luciferase (pGL4-Tbox, Fig. 7A) was created, and assayed for luciferase activity in the absence or presence of Tbx1. Transfection of *Xenopus Tbx1* increased the luciferase activity of pGL4-Tbox 15- to 45-fold compared to that of the control (Fig. 7B). As expected, transfection of *xRipply3* decreased in a dose-dependent manner the luciferase activity of pGL4-Tbox activated by Tbx1 (Fig. 7C). These results suggest that xRipply3 functions as a negative regulator of Tbx1.

Previously, we showed that the repressive activity of Bowline is mediated by interaction with the Tbx6 and Groucho/TLE proteins (Kondow *et al.*, 2007). Therefore, we examined whether xRipply3 interacts with the Tbx1 and Groucho/TLE proteins in co-immunoprecipitation assays. In the presence of myc-tagged Tbx1, HA-tagged xRipply3 was immunoprecipitated by the anti-myc antibody (Fig. 7D). Similarly, the interaction between HA-tagged xRipply3 and myc-tagged Grg4 was revealed in a co-immunoprecipitation assay (Fig. 7E). These results suggest that the suppressive activity of xRipply3 is mediated by interactions with the Tbx1 and Groucho/TLE proteins.

Finally, to elucidate the relationship between xRipply3 and Tbx1 *in vivo*, we compared the expression patterns of *xRipply3*, *Tbx1*, and *Fgf8*, which is a downstream gene of *Tbx1*, in the *Xenopus* pharyngeal apparatus. Examinations of frontal sections showed that the *xRipply3*- and *Fgf8*-expressing region differed from the *Tbx1*-expressing region (Fig. 7F-H). It is reported that *Tbx1* is expressed in the pharyngeal arch region in *X. tropicalis* (Showell *et al.*, 2006). Thus, the *xRipply3*- and *Fgf8*-expressing regions are likely to be the pharyngeal pouches.

Discussion

A novel nuclear factor of the Bowline/Ripply family protein was isolated in the mouse, African clawed frog, zebrafish, and amphioxus (Chan *et al.*, 2007; Chan *et al.*, 2006; Kawamura *et al.*, 2005; Kondow *et al.*, 2006; Li *et al.*, 2006; Morimoto *et al.*, 2007). Recently, we reported that Bowline, one of the *Xenopus* Bowline/Ripply family proteins, functions as a negative regulator of Tbx6

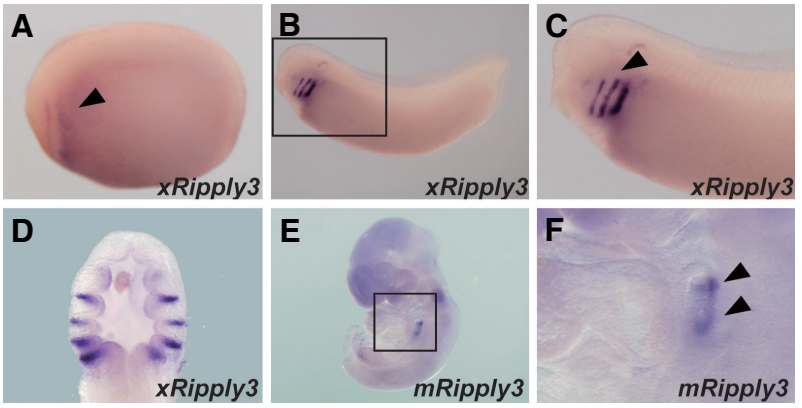

Fig. 6. Expression profile of *Ripply3* during X. *laevis* and mouse development. (A-C) *Whole-mount* in situ *hybridization for* xRipply3. **(A)** *At the late neurula stage,* xRipply3 *is expressed in the region near the heart mesoderm.* **(B)** *At the tailbud stage,* xRipply3 *expression is restricted to the pharyngeal region. Panel* **(C)** *is a magnified view of (B). Lateral views are shown with the anterior towards the left. The arrowheads in (A) and (C) indicate* xRipply3 *expression.* **(D)** *Tailbud-stage embryos stained with the* xRipply3 *probe were sliced frontally.* **(E,F)** *Whole-mount* in situ *hybridization for murine* Ripply3. **(E)** *Murine* Ripply3 *expression is observed in the pharyngeal region. Panel* **(F)** *is a magnified view of (E). The arrowheads in (F) indicate murine* Ripply3 *expression.*

proteins (Kondow *et al.*, 2007). In zebrafish somitogenesis, Ripply1 also negatively regulates the transcriptional activity of the Tbx24 protein (Kawamura *et al.*, 2008). However, whether the repressive activity for T-box proteins is a feature common to all Bowline/Ripply family proteins has not been revealed. In the present work, we addressed this question. We investigated the molecular function of Ledgerline, another *Xenopus* Bowline/Ripply family protein, in somitogenesis. Similar to the knockdown of Bowline, knockdown of Ledgerline resulted in the anterior expansion of the expression regions of several somitogenesis-related genes. We found that Ledgerline had suppressive activity against the transcriptional activity of Tbx6. In addition, we isolated a novel *Xenopus bowline/Ripply* family gene, *xRipply3*, and investigated its molecular functions. Unlike the already known *bowline/Ripply* family genes, *xRipply3* is expressed in the pharyngeal region. Moreover, xRipply3 suppresses the transcriptional activity of Tbx1. Therefore, our findings demonstrate that Bowline/Ripply family proteins have the common feature of being negative regulators of T-box proteins (Fig. 8).

A previous study showed that *ledgerline* is expressed in the anterior PSM during *Xenopus* somitogenesis (Chan *et al.*, 2006). However, the localization of *ledgerline* transcripts in the anterior PSM was not clearly defined. Our analysis of the region of *ledgerline* expression reveals that *ledgerline* is expressed in both the newly formed somites and the S0, S-I, and S-II somitomeres. *Bowline* is expressed in the S-I, S-II, and S-III somitomeres. Thus, there is overlapping expression of *ledgerline* and *bowline* in the S-I and S-II somitomeres. In addition, we reveal the transcription factors involved in the regulation of *ledgerline* expression. Tbx6, mespb, and E47 regulate *bowline* expression during *Xenopus* somitogenesis (Hitachi *et al.*, 2008b). Overexpression of *Tbx6*, *mespb*, and *E47* induced ectopic *ledgerline* expression, as well as *bowline* expression, in early-stage *Xenopus* embryos. Knockdown of Tbx6 by the Xtbx6 exint-MO abolished the expression of

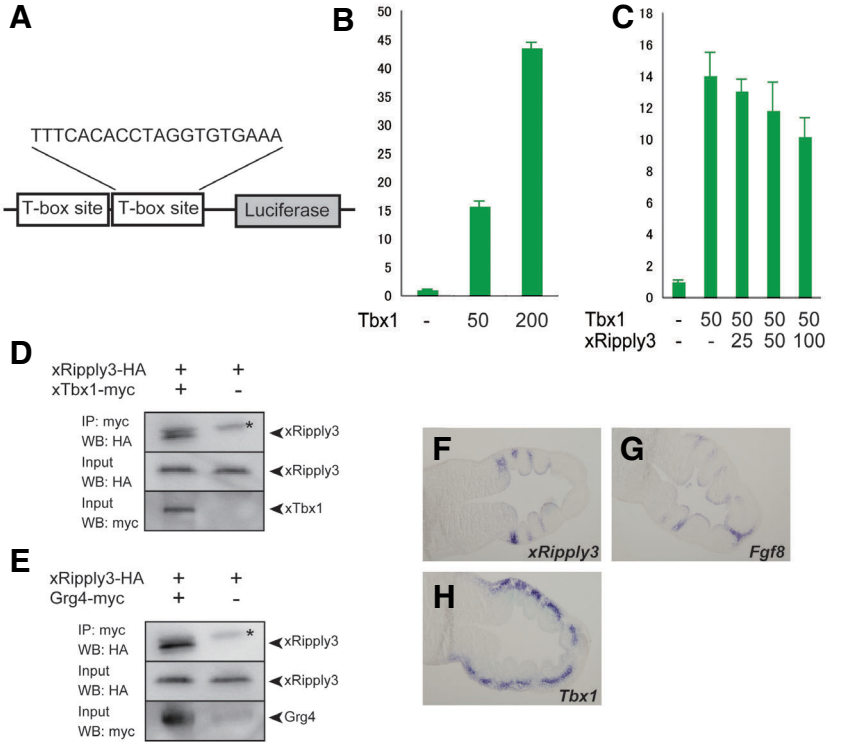

Fig. 7. xRipply3 suppresses the transcriptional activity of Tbx1. (A) *Schematic diagram of pGL4-Tbox, which contains tandem T-box binding sites.* **(B,C)** *Luciferase assays for the pGL4-Tbox construct.* **(B)** *Tbx1 dose-dependently increases the luciferase activity of pGL4-Tbox. COS7 cells were transfected with the* pGL4-Tbox *(100 ng) and the Tbx1 expression vector (50-200 ng).* **(C)** *xRipply3 represses the transcriptional activity of Tbx1 in the pGL4-Tbox construct. COS7 cells were transfected with* pGL4-Tbox *(100 ng) and a combination of the indicated expression vectors, Tbx1 (50 ng) and xRipply3 (25-100 ng). Error bars represent the SEM of three independent experiments.* **(D)** *Interaction between the xRipply3 and Tbx1 proteins in* Xenopus *embryos. Protein extracts of* Xenopus *embryos co-injected with* myc-Tbx1 *mRNA and* HA-xRipply3 *mRNA (500 pg each) were subjected to co-immunoprecipitation with the anti-myc antibody.* **(E)** *Interaction between the xRipply3 and Grg4 proteins in* Xenopus *embryos. Protein extracts of* Xenopus *embryos co-injected with* myc-Grg4 *mRNA and* HA-xRipply3 *mRNA (500 pg each) were subjected to co-immunoprecipitation with the anti-myc antibody. The asterisks indicate non-specific bands.* **(F-H)** *Frontal sections of* Xenopus *embryos were stained for* xRipply3 **(F)**, Fgf8 **(G)**, *and* Tbx1 **(H)** *transcripts. The regions of* xRipply3 *and* Fgf8 *expression differ from those of* Tbx1.

both *ledgerline* and *bowline* during *Xenopus* somitogenesis. Thus, it appears that Tbx6, mespb, and E47 are involved in the regulation of both *ledgerline* and *bowline*. Unexpectedly, although the decrease in *bowline* expression by Xtbx6 exint-MO was rescued by co-injection of Xtbx6 exint-MO with *Tbx6* mRNA, the decrease in *ledgerline* expression was scarcely rescued by co-injection of *Tbx6* mRNA. Taken together, our data suggest that the mechanism responsible for *ledgerline* regulation is not identical to that for *bowline* regulation, although Tbx6 works as common regulatory factor for *bowline* and *ledgerline* expression during *Xenopus* somitogenesis.

The molecular function of Ledgerline was found to be similar to that of Bowline. Ledgerline interacted with the Tbx6 and Groucho/TLE proteins, and suppressed the transcriptional activity of the Tbx6 protein. These findings suggest functional redundancy of Bowline and Ledgerline with respect to the negative regulation of Tbx6 during somitogenesis. This is supported by the finding that inhibition of both *Ripply1* and *Ripply2* induces dramatic up-regulation of *Mesp* genes in zebrafish (Moreno *et al.*, 2008). Furthermore, we found that treatment with the HDAC inhibitor TSA resulted in the anterior expansion of *mespb* expression region during *Xenopus* somitogenesis, as seen in embryos with knock-downs of Bowline and Ledgerline. Therefore, HDAC appears to be required for the suppressive activities of Bowline/Ripply proteins *in vivo*. Taken together, Bowline and Ledgerline may redundantly suppress the transcriptional activity of Tbx6, using HDAC to repress completely the transcription of somitogenesis-related-genes in the anterior PSM during *Xenopus* somitogenesis.

In the present study, we isolated a third *Xenopus bowline/Ripply* family gene and designated it as *xRipply3*. One remarkable property of *xRipply3* is its expression profile. The expression patterns of the known *bowline/Ripply* family genes are restricted to the anterior PSM, whereas that of *xRipply3* is observed in the pharyngeal region. Thus, *xRipply3* is assumed to be a novel *bowline/Ripply* family gene. xRipply3 interacted with Tbx1 protein in *Xenopus* embryos, and suppressed the transcriptional activity of Tbx1 in cultured cells. Considering the findings that (1) the expression patterns of *xRipply3* and *Tbx1* were not identical in *Xenopus* embryos, and (2) Ledgerline and Ripply1 limited the *Tbx6* and *Tbx24* expression in the PSM, respectively (Kawamura *et al.*, 2005), one possible function of xRipply3 in the pharyngeal apparatus is to limit the *Tbx1* expression in the pharyngeal arches by repressing its expression in the pharyngeal pouches.

In summary, we describe the expression profiles of two *Xenopus bowline/Ripply* family genes, *ledgerline* and *xRipply3*, in the anterior PSM and pharyngeal region, and reveal that they suppress the transcriptional activities of the T-box proteins. In future studies, it will be important to investigate the molecular mechanism(s) underlying the physiologic roles of xRipply3 during pharyngeal development.

Materials and Methods

Isolation of the Xenopus Ripply3 *gene*

The *xRipply3* cDNA was PCR-amplified from *Xenopus laevis* cDNA using the Phusion High-Fidelity polymerase (Finnzyme), and subcloned into the pBluescript II SK vector to generate pBS-Ripply3. The following primer set was used: 5'-GCAAACTAGGAATAAACAAG-3' and 5'-CAAAAACATACATATCTTTAATAAAT-3'. Rapid amplification of cDNA ends (RACE) was performed using the FirstChoice RLM-RACE kit (Ambion). The coding region of *xRipply3* was PCR-amplified and inserted into the pCS2+ vector, to generate pCS2-xRipply3.

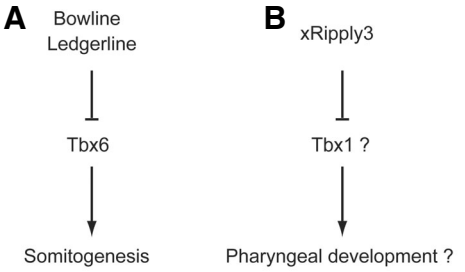

Fig. 8. Diagram illustrating the molecular functions of Bowline/Ripply family proteins in *Xenopus* development. (A) *Bowline and Ledgerline participate in somitogenesis as negative regulators of Tbx6 activity, since Bowline and Ledgerline suppress the transcriptional activity of Tbx6, which is required for the normal segmentation program of somites.* **(B)** *A third* Xenopus *Bowline/Ripply protein, xRipply3, has the potential to suppress the transcriptional activity of Tbx1 required for pharyngeal arch and pouch development, which suggests that xRipply3 functions as a negative regulator of Tbx1 activity in pharyngeal pouch development.*

Plasmid constructs

The coding regions of the *Xenopus Tbx1* and mouse *Ripply3* genes were PCR-amplified and subcloned into pBluescript SK-II, to create pBS-Tbx1 and pBS-mRipply3, respectively. The coding region of *ledgerline* was PCR-amplified and subcloned into the pCS2+ and HA-tagged pCS2+ vectors, to generate pCS2-Ledgerline and pCS2-HA-Ledgerline, respectively. The coding region of *Xenopus Tbx1* was PCR-amplified and subcloned into the myc-tagged pCS2+ vector, to generate pCS2-myc-Tbx1. To produce the pGL4-Tbox construct, the Tbx1 binding site (5'-TTTCACACCTAGGTGTGAAA-3'), as described previously (Ataliotis *et al.*, 2005), was tandemly inserted into the pGL4.23 vector (Promega). The pCS2-Bowline, pCS2-Grg4, pCS2-Tbx6-VP16, pCS2-XotchΔE, pCS2-mespb (previously referred to as pCS2-Thy1), pCS2-E47, pCS2-myc-Tbx6, and pCS2-myc-Grg4 plasmids have been described previously (Hitachi *et al.*, 2008b; Kondow *et al.*, 2006; Kondow *et al.*, 2007; Uchiyama *et al.*, 2001). The pGL4.2Thy1 construct, which contains the 5'-flanking region of *Xenopus mespb*, has also been described previously (Kondow *et al.*, 2007).

Embryo manipulation, micro-injection and whole-mount in situ *hybridization*

X. laevis embryos were obtained using a standard procedure (Abe *et al.*, 2004). The staging of embryos was according to the scheme of Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). *In vitro* transcription of mRNA was carried out according to the procedure described previously (Nitta *et al.*, 2007; Uchiyama *et al.*, 2001). The following antisense morpholinos were obtained from Gene Tools: Control-MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3'; Ledgerline-MO, 5'-AACTCCGCTGTTGATTCGGCTCCAT-3' (Chan *et al.*, 2006); and Xtbx6 exint-MO, 5'-TGCCCCAGTCACATACCTGAGTATC-3' (Tazumi *et al.*, 2008). Rescue experiments were performed by injecting embryos with Xtbx6 exint-MO (50 ng), together with *Xenopus Tbx6* mRNA (2 or 10 pg) and β-*gal* mRNA (200 pg). To inhibit endogenous HDAC activity, *Xenopus* embryos at stage 15 were treated with 500 nM trichostatin A (Wako Pure Chemicals) for 4 hrs at 23°C. Whole-mount *in situ* hybridization of *Xenopus* and mouse embryos was performed as described previously (Chan *et al.*, 2007; Harland, 1991; Sive *et al.*, 2000). *In situ* hybridization of sections was performed using the Discovery system (Ventana Medical Systems), as described previously (Hitachi *et al.*, 2008c). The templates

for the *bowline*, *mespb*, and *Tbx6* probes have been described (Hitachi *et al*., 2008b). The pCS2-Ledgerline, pBS-Tbx1, pBS-xRipply3, and pBS-mRipply3 constructs were used as templates for the probes. The template for *Fgf8* was kindly gifted by Dr. K. Tamura (Tohoku University). Galactosidase staining was carried out as described previously (Yabe *et al*., 2006), except that the chromogenic reaction was performed with Magenta-Gal as the substrate.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The procedures used for RT-PCR were as previously described (Suzawa *et al*., 2007). *Xenopus* embryos were injected with 500 pg of *Tbx6*, *mespb*, and *E47* mRNA. Total RNA was extracted from *Xenopus* stage-10 embryos using Isogen (Wako). The following primers were used: for *bowline*, forward 5'-CAAGTGGTTTGCCAAGTCCT-3' and reverse, 5'-AGCCAAGGTCTCCAGAACAA-3'; for *ledgerline*, forward 5'-CAATGTGGCAGTGTGGAGAC-3' and reverse, 5'-TGGAGGTTTCCCTTTGTCAT-3'; and for *Ornithine decarboxylase* (*ODC*), forward 5'-GTCAATGATGGAGTGTATGGATC-3', and reverse, 5'-TCCATTCCGCTCTCCTGAGCAC-3'.

Cell culturing, luciferase assays, subcellular localization and co-immunoprecipitation assay

Culturing of COS7 cells and the luciferase assay were performed as previously described (Hitachi *et al*., 2008a). The pCS2-Tbx6-VP16, pCS2-Ledgerline, pCS2-Grg4, pCS2-Bowline, pCS2-Tbx6, pCS2-xRipply3, and pCS2-myc-Tbx1 constructs were used as expression vectors. To normalize for transfection efficiency, 5 ng of the pGL-4.74 vector (Promega), which expresses the *Renilla* luciferase, was co-transfected in all the experiments. The data from each experiment were compared with the basal activity of the pGL4-Tbox or pGL4.2Thy1 construct, and are presented as n-fold increases over the respective activities of these plasmids. The experiments were performed at least twice in triplicate for each assay, and representative data are shown.

To examine the subcellular localizations of the xRipply3 proteins, COS7 cells were transfected with 200 ng of pCS2-HA-xRipply3, and immunocytochemistry was performed as described previously (Danno *et al*., 2008) with an anti-HA antibody (Y-11; Santa Cruz Biotechnology).

Xenopus embryos were microinjected with a mixture of *myc-Tbx6* mRNA and *HA-ledgerline* mRNA (500 pg each), *myc-Grg4* mRNA and *HA-ledgerline* mRNA (500 pg each), *myc-Tbx1* mRNA and *HA-xRipply3* mRNA (500 pg each) or *myc-Grg4* mRNA and *HA-xRipply3* mRNA (500 pg each), and immunoprecipitation was performed with an anti-myc antibody (9E10; Santa Cruz Biotechnology), as described previously (Kondow *et al*., 2007)

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References

ABE, T., FURUE, M., MYOISHI, Y., OKAMOTO, T., KONDOW, A. and ASASHIMA, M. (2004). Activin-like signaling activates Notch signaling during mesodermal induction. *Int J Dev Biol* 48: 327-332.

ATALIOTIS, P., IVINS, S., MOHUN, T.J. and SCAMBLER, P.J. (2005). XTbx1 is a transcriptional activator involved in head and pharyngeal arch development in *Xenopus laevis*. *Dev Dyn* 232: 979-991.

BALDINI, A. (2005). Dissecting contiguous gene defects: TBX1. *Curr Opin Genet Dev* 15: 279-284.

BECKERS, J., SCHLAUTMANN, N. and GOSSLER, A. (2000). The mouse rib-vertebrae mutation disrupts anterior-posterior somite patterning and genetically interacts with a Delta1 null allele. *Mech Dev* 95: 35-46.

CHAN, T., KONDOW, A., HOSOYA, A., HITACHI, K., YUKITA, A., OKABAYASHI, K., NAKAMURA, H., OZAWA, H., KIYONARI, H., MICHIUE, T. *et al.* (2007). Ripply2 is essential for precise somite formation during mouse early development. *FEBS Lett* 581: 2691-2696.

CHAN, T., SATOW, R., KITAGAWA, H., KATO, S. and ASASHIMA, M. (2006). Ledgerline, a novel *Xenopus laevis* gene, regulates differentiation of presomitic mesoderm during somitogenesis. *Zoolog Sci* 23: 689-697.

CHAPMAN, D.L. and PAPAIOANNOU, V.E. (1998). Three neural tubes in mouse embryos with mutations in the T-box gene Tbx6. *Nature* 391: 695-697.

DANNO, H., MICHIUE, T., HITACHI, K., YUKITA, A., ISHIURA, S. and ASASHIMA, M. (2008). Molecular links among the causative genes for ocular malformation: Otx2 and Sox2 coregulate Rax expression. *Proc Natl Acad Sci USA* 105: 5408-5413.

HARLAND, R.M. (1991). *In situ* hybridization: an improved whole-mount method for *Xenopus* embryos. *Methods Cell Biol* 36: 685-695.

HITACHI, K., DANNO, H., KONDOW, A., OHNUMA, K., UCHIYAMA, H., ISHIURA, S., KURISAKI, A. and ASASHIMA, M. (2008a). Physical interaction between Tbx6 and mespb is indispensable for the activation of bowline expression during *Xenopus* somitogenesis. *Biochem Biophys Res Commun* 372: 607-612.

HITACHI, K., KONDOW, A., DANNO, H., INUI, M., UCHIYAMA, H. and ASASHIMA, M. (2008b). Tbx6, Thylacine1, and E47 synergistically activate bowline expression in *Xenopus* somitogenesis. *Dev Biol* 313: 816-828.

HITACHI, K., KONDOW, A., DANNO, H., NISHIMURA, Y., OKABAYASHI, K. and ASASHIMA, M. (2008c). Molecular analyses of *Xenopus* Mesp-related genes. *Integrative Zoology* in press.

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.

KAWAMURA, A., KOSHIDA, S. and TAKADA, S. (2008). Activator-to-repressor conversion of T-box transcription factors by the Ripply family of Groucho/TLE-associated mediators. *Mol Cell Biol* 28: 3236-3244.

KONDOW, A., HITACHI, K., IKEGAME, T. and ASASHIMA, M. (2006). Bowline, a novel protein localized to the presomitic mesoderm, interacts with Groucho/TLE in *Xenopus*. *Int J Dev Biol* 50: 473-479.

KONDOW, A., HITACHI, K., OKABAYASHI, K., HAYASHI, N. and ASASHIMA, M. (2007). Bowline mediates association of the transcriptional corepressor XGrg-4 with Tbx6 during somitogenesis in *Xenopus*. *Biochem Biophys Res Commun* 359: 959-964.

KOZAK, M. (1987). At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J Mol Biol* 196: 947-950.

LI, X., ZHANG, W., CHEN, D., LIN, Y., HUANG, X., SHI, D. and ZHANG, H. (2006). Expression of a novel somite-formation-related gene, AmphiSom, during amphioxus development. *Dev Genes Evol* 216: 52-55.

MORENO, T.A., JAPPELLI, R., 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.

MORIMOTO, M., SASAKI, N., OGINUMA, M., KISO, M., IGARASHI, K., AIZAKI, K., KANNO, J. and SAGA, Y. (2007). The negative regulation of Mesp2 by mouse Ripply2 is required to establish the rostro-caudal patterning within a somite. *Development* 134: 1561-1569.

NACKE, S., SCHAFER, R., HABRE DE ANGELIS, M. and MUNDLOS, S. (2000). Mouse mutant «rib-vertebrae» (rv): a defect in somite polarity. *Dev Dyn* 219: 192-200.

NAICHE, L.A., HARRELSON, Z., KELLY, R.G. and PAPAIOANNOU, V.E. (2005). T-box genes in vertebrate development. *Annu Rev Genet* 39: 219-239.

NIEUWKOOP, P.D. and FABER, J. (1956). *Normal Table of Xenopus laevis (Daudin)*. North-Holland, Amsterdam.

NITTA, K.R., TAKAHASHI, S., HARAMOTO, Y., FUKUDA, M., TANEGASHIMA, K., ONUMA, Y. and ASASHIMA, M. (2007). The N-terminus zinc finger domain of *Xenopus* SIP1 is important for neural induction, but not for suppression of Xbra expression. *Int J Dev Biol* 51: 321-325.

POURQUIE, O. and TAM, P.P. (2001). A nomenclature for prospective somites and

phases of cyclic gene expression in the presomitic mesoderm. *Dev Cell* 1: 619-620.

SHOWELL, C., CHRISTINE, K.S., MANDEL, E.M. and CONLON, F.L. (2006). Developmental expression patterns of Tbx1, Tbx2, Tbx5, and Tbx20 in *Xenopus* tropicalis. *Dev Dyn* 235: 1623-1630.

SIVE, H.L., GRAINGER, R.M. and HARLAND, R.M. (2000). *Early Development of Xenopus laevis: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.

SUZAWA, K., YUKITA, A., HAYATA, T., GOTO, T., DANNO, H., MICHIUE, T., CHO, K.W. and ASASHIMA, M. (2007). *Xenopus* glucose transporter 1 (xGLUT1) is required for gastrulation movement in *Xenopus laevis*. *Int J Dev Biol* 51: 183-190.

TAZUMI, S., YABE, S., YOKOYAMA, J., AIHARA, Y. and UCHIYAMA, H. (2008). pMesogenin1 and 2 function directly downstream of Xtbx6 in *Xenopus* somitogenesis and myogenesis. *Dev Dyn* 237: 3749-3761.

THEILER, K. and VARNUM, D.S. (1985). Development of rib-vertebrae: a new mutation in the house mouse with accessory caudal duplications. *Anat Embryol (Berl)* 173: 111-116.

UCHIYAMA, H., KOBAYASHI, T., YAMASHITA, A., OHNO, S. and YABE, S. (2001). Cloning and characterization of the T-box gene Tbx6 in *Xenopus laevis*. *Dev Growth Differ* 43: 657-669.

WATABE-RUDOLPH, M., SCHLAUTMANN, N., PAPAIOANNOU, V.E. and GOSSLER, A. (2002). The mouse rib-vertebrae mutation is a hypomorphic Tbx6 allele. *Mech Dev* 119: 251-256.

WHITE, P.H., FARKAS, D.R., MCFADDEN, E.E. and CHAPMAN, D.L. (2003). Defective somite patterning in mouse embryos with reduced levels of Tbx6. *Development* 130: 1681-1690.

YABE, S., TAZUMI, S., YOKOYAMA, J. and UCHIYAMA, H. (2006). Xtbx6r, a novel T-box gene expressed in the paraxial mesoderm, has anterior neural-inducing activity. *Int J Dev Biol* 50: 681-689.

ZOUPA, M., SEPPALA, M., MITSIADIS, T. and COBOURNE, M.T. (2006). Tbx1 is expressed at multiple sites of epithelial-mesenchymal interaction during early development of the facial complex. *Int J Dev Biol* 50: 504-510.