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-
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.

In vertebrates, 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 S-III 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).
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 the Tbx6. The MO that targets the exon1/intron1 junction of *Xenopus Tbx6* (Xtbx6 extint-MO) specifically blocks Tbx6 function (Tazumi et al., 2008). The Xtbx6 extint-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 extint-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 extint-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...
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).
xRipply3 protein. HA-tagged xRipply3 was transfected into COS7 cells, and the HA-xRipply3 protein was visualized by immunohistochemical staining with the anti-HA antibody. 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...
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 extint-MO abolished the expression of...
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 a 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’-GCAAAGTACGTTAAACGGA-3’ and 5’-CAAAACATACATATCTTTATAAAT-3’. Rapid amplification of cDNA

Plasmid constructs

The coding regions of the Xenopus Tbx1 and mouse Ripply genes were PCR-amplified and subcloned into pBluescript SK-I1, 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’TTCACCTAGGTGAA-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-Notch3E, 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 Nieuwkop and Faber (Nieuwkop 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 antense morpholinos were obtained from Gene Tools: Control-MO, 5′-CCTCTTACCTCAGTTACAAATTATA-3’; Ledgerline-MO, 5′-AACCCTGGTGTGAATTCGGGATCACAC-3’ (Chan et al., 2006); and Xtbx6 exint-MO, 5′-TGGCCGCGTACACATACAGGAGT-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-galactosidase 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, mesp, 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, mesp, and E47 mRNA. Total RNA was extracted from Xenopus stage-10 embryos using Isogen (Wako). The following primers were used: for bowline, forward 5'-CAAGTGGTTTGGCAATGCTC-3' and reverse, 5'-AGCCAAAGGCTTCAAAACAA-3'; for ledgerline, forward 5'-CAATGTCGCCATGGTGAGAC-3' and reverse, 5'-TGAGGATCCTCTTCTTGCAT-3'; and for Ornithine decarboxylase (ODC), forward 5'-GTCATGTGAGGATGTAGTGAC-3', and reverse, 5'-TCCATCGCCTCTCTCTGAC-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, pBS-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 luciferase, was co-transfected with each of the above plasmids. To normalize for transfection efficiency, 5 ng of the pGL-4.74 vector (Promega), which expresses the luciferase, was co-transfected with each of the above plasmids. To normalize for transfection efficiency, 5 ng of the pGL-4.74 vector (Promega), which expresses the luciferase, was co-transfected with each of the above plasmids.

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