Activator-to-repressor conversion of T-box transcription factors by the Ripply family of Groucho/TLE-associated mediators

Running title: Mediator in the repression by T-box proteins

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ABSTRACT

The T-box family of transcription factors, defined by a conserved DNA binding domain called the T-box, regulates various aspects of embryogenesis by activating and/or repressing downstream genes. In spite of the biological significance of the T-box proteins, how they regulate transcription remains to be elucidated. Here, we show that the Groucho/TLE-associated protein, Ripply, converts T-box proteins from activators to repressors. In cultured cells, zebrafish Ripply1, an essential component in somite segmentation, and its structural relatives, Ripply2 and 3, suppress the transcriptional activation mediated by the T-box protein Tbx24, which is coexpressed with *ripply1* during segmentation. Ripply1 associates with Tbx24 and converts it to a repressor. Ripply1 also antagonizes the transcriptional activation of another T-box protein, No tail (Ntl), the zebrafish ortholog of Brachyury. Furthermore, injection of high dosage of *ripply1* mRNA into zebrafish eggs causes defective development of the posterior trunk, similar to the phenotype observed in homozygous mutants of *ntl*. A mutant form of Ripply1, defective in association with Tbx24, also lacks the activity in zebrafish embryos. These results indicate that the intrinsic transcriptional property of T-box proteins is controlled by Ripply family proteins, which act as specific adaptors that recruit the global corepressor Groucho/TLE to T-box proteins.

INTRODUCTION

T-box proteins play many crucial roles in development by activating and/or repressing the transcription of their target genes (23, 32). For instance, reduced function of *T/Brachyury*, the founder member of this family, causes truncated tail in the mouse (10). The importance of T-box transcription factors is also shown by the facts that mutations in human T-box genes cause severe congenital disorders, such as DiGeorge, Ulnar-mammary, and Holt-Oram syndromes (26). Furthermore, the T-box family is evolutionally conserved from *C. elegans* to insects and vertebrates showing remarkable functional conservation across species. However, in spite of the biological significance of the T-box proteins, how they regulate transcription remains to be elucidated.

One of the processes in which T-box proteins play crucial roles is the somite segmentation in vertebrates (3, 4, 24). Somitogenesis is the sequential subdivision of segmented precursors of the vertebral column and musculature from the presomitic mesoderm (PSM) (27, 29). Prior to morphological subdivision, a segmental prepattern is established in the anterior PSM. Genetic analyses of the mouse and the zebrafish indicate that several Tbx genes are required for distinct processes of this prepattern formation (11, 24, 36), which also involves Notch signaling and Mesp2, a basic helix-loop-helix transcription factor (21, 33). For instance, Tbx6 induces the expression of *Mesp2* and is required for proper patterning of somites of the mouse (36). Similarly, *tbx24/fused somites*, structurally related to mouse *Tbx6*, is expressed in the anterior PSM of the zebrafish embryo, and required for the formation of somite boundaries and the expression of *mesp-b*, a zebrafish homolog of *Mesp2* (24, 30), suggesting that Tbx24 is involved in the transcriptional regulation of the *mesp-b* locus in zebrafish. Because *Mesp2,* as well as *mesp-b,* is expressed in only a portion of Tbx-expressing cells (24, 28, 36), however, another molecule also seems to be involved in Tbx-mediated transcriptional regulation of *mesp* genes in the anterior PSM.

In a previous study, we showed that Ripply1, which is associated with the global transcriptional corepressor Groucho/TLE, terminates the expression of segmentation genes in the anterior PSM of zebrafish embryos (16). In *ripply1*-deficient embryos, the expression of *mesp-b*, is upregulated in a cell-autonomous manner, whereas, in embryos injected with *ripply1*mRNA, the expression of *mesp-b* is highly suppressed in the anterior PSM (16). These results suggest that Ripply1 regulates the proper expression of *mesp-b* in the anterior PSM. Taking it into account that the expression of *mesp-b* could be induced by Tbx, we can speculate that Ripply1 may antagonize the function of Tbx in the transcription of *mesp* genes. Therefore, we examined precisely the relationship between Tbx24 and Ripply1 in the transcription of *mesp-b*. We showed that Ripply1 is coprecipitated with Tbx24 and converts it from an activator to a repressor in culture cells. The other members of the Ripply family, Ripply2 and Ripply3, can also antagonize the transcriptional activation mediated by Tbx24. On the other hand, Ripply1 also antagonizes the transcriptional activation of another T-box protein, No tail (Ntl), both *in vitro* and *in vivo*. These results reveal a molecular mechanism underlying dynamic conversion of the regulatory property of Tbx proteins in the transcription of their targets.

MATERIALS AND METHODS

Plasmid Constructions. The *mesp-b* 2.5-kb luciferase reporter plasmid was constructed by inserting a 2.5-kb genomic fragment upstream of the translational initiation site of zebrafish *mesp-b* fused to the firefly luciferase gene in the pGL3 Basic vector (Promega). For pBP-TbxX2 or pBP-mutTbxX2, a T-box site (5'-AATTCACACCT-3') or a mutT-box sequence (5'-AATTCAgACCT-3'), respectively, was inserted into the interferon-β basal promoter fused to the luciferase gene.

Luciferase Assay and TSA Treatment. Human embryonic kidney 293T cells were cultured at 37^{O}C in DMEM medium supplemented with 10% fetal calf serum and antibiotics. For luciferase assay, 293T cells (1.0 x10^4/well) were seeded into 24-well collagen-coated plates (Iwaki). The cells were transiently transfected with the reporter gene plasmids along with the indicated expression vector using fugene6 reagent (Roche). The total amount of DNA in each transfection was kept the same by supplementing with empty vector, pCS2+. After incubation for 24 h, the cells were washed with PBS(-) and lysed, and luciferase activity was assayed by using a Dual-Luciferase reporter assay system (Promega). For normalizing the transfection efficiencies, pRL-tk-Luc (Promega), *Renilla* luciferase reporter gene under the control of the thymidine kinase promoter, was cotransfected. The average normalized firefly luciferase with mock alone was set at 100%, and the error bar represents the standard deviation. All transfections were performed in triplicate, and similar results were obtained in at least duplicate experiments. For TSA treatment, the cells were first incubated with DMEDM for 12 h post-transfection with DMEM medium containing 300nM TSA (Sigma). After incubation for another 24 h, the cells were lysed for analysis of luciferase activity.

Co-immunoprecipitation. Whole cell extracts were prepared from 293T cells transfected with the various expression vectors and incubated with either anti-Myc (4A6; Upstate Biotechnology) or anti-FLAG (D-8; Santa Cruz Biotechnology) antibody for 1 h at 4°C. The antigen-antibody complexes were collected using protein

G-Sepharose (GE Healthcare) and washed several times. Precipitated proteins were separated by SDS-PAGE and electrophoretically transferred to PVDF membranes. Western blotting was carried out according to the standard procedure by using rabbit polyclonal anti-FLAG or mouse monoclonal anti-Myc antibodies.

ChIP Experiments. ChIP experiments were performed by using some modifications of a previously described method (20). Briefly, 293T cells (2.0 $x10^6$/10-cm dish) were transfected with linearized *mesp-b* 2.5-kb luciferase gene and each expression vector. After a 40-h incubation, the cells were cross-linked with formaldehyde, and the genomic DNA was sheared to an average size of 400-bp by sonication. Sonicated samples were reacted with α-myc antibody (9E10; Santa Cruz Biotechnology) or with α-FLAG antibody (D9, SantaCruz), collected with protein G-Sepharose (GE Healthcare), and washed several times. After reversal of the crosslink, the DNA isolated by ChIP was amplified by PCR using the following primers: T-site containing region-s, 5'-CAACAAACACAAAAAGCACACGTT-3'; T-site containing region-as, 5'-GGTGAAAGGAGGATGGAGGTTTAT-3'; Proximal-region-s, 5'-GCATCTCTATTGACGATATC-3'; Proximal-region -as, 5'-CATTTCATTGCTCTAGCTCA-3'; Distal-region-s, 5'-TTTCAGAGCACAACAAAAGCAGTG-3'; Distal-region-as,

5’-TTATGTATGCATGAACGCAGATGG-3’.

Fish and Microinjection. Zebrafish TL2 inbred line was used for the experiments (18). Capped mRNA encoding the entire open reading frame of *ripply1* was synthesized form linearlized pCS2+ripply1 (8) by using the mMessage mMachine Sp6 kit (Ambion). The sequences of MOs used in the study are followed: *ripply1* MO 5'-CATCGTCACTGTGTTTTTCGTTTTG-3'; *tbx24* MO 5’-CATTTCCACACCCAGCATGTCTCGG-3’; *deltaD* MO 5'-AACAGCTATCATTAGTCGTCCCATG-3'.

Synthesized mRNA or MO was injected into the yolk of fertilized embryos at the 1-cell stage.

Whole-mount *in situ* hybridization. Whole-mount *in situ* hybridization was performed as described previously (17). Antisense RNA probe was transcribed according to the standard procedure.

RESULTS

Up-regulation of *mesp-b* mRNA in *ripply1*-deficient embryos is dependent on Tbx24. In *ripply1*-deficient embryos, the expression of *mesp-b* was earlier shown to be up-regulated in the anterior PSM and extended anteriorly (16). The expression pattern of *mesp-b* in the anterior PSM is also known to be perturbed in zebrafish embryos defective in either Notch signaling or the transcription factor Tbx24 (12, 24, 30). Thus, as a first approach to address how Ripply1 regulates the *mesp-b* expression, we investigated the epistatic relationship between *ripply1* and *tbx24*or *deltaD,* a Notch ligand. As previously described, the expression of *mesp-b* is significantly reduced in *tbx24* MO-injected embryos (Fig. 1A, *n*=12; 100% affected), suggesting the activator function of Tbx24 (24, 30), but is up-regulated in *ripply1* MO-injected embryos (*n*=15; 100% affected) (16). In embryos injected with both *tbx24* MO and *ripply1* MO, the *mesp-b* mRNA is almost completely suppressed as in the *tbx24* MO-injected embryos (Fig. 1A, *n*=7; 100% affected). In contrast, the anteriorly expanded expression of *mesp-b* caused by the *ripply1* MO injection is not reduced in the embryos injected with *deltaD* MO and *ripply1* MO; although its expression in the PSM is perturbed, showing scattered salt-and-pepper pattern (Fig. 1B, *n*=9; 100% affected). These results suggest that the transcriptional suppression of *mesp-b* mRNA by Ripply1 depends on the function of Tbx24 in zebrafish embryos.

Ripply1, as well as Ripply 2 and 3, antagonizes the transcriptional activation of Tbx24 through the T-box binding site. To investigate the relationship between Tbx24 and Ripply1 in the transcription of *mesp-b* more precisely, we next carried out *in vitro* luciferase reporter assays in which the reporter gene was under the control of the 2.5-kb genomic fragment upstream of the *mesp-b* initiation codon (Fig. 2A). We found that Tbx24 significantly activated the luciferase activity in a dose-dependent manner (Fig. 2B). This Tbx24-mediated activation of the reporter gene, however, was completely prevented by cotransfection with Ripply1 (Fig. 2C). In contrast, Ripply1 did not significantly affect the enhancement of reporter activity by the Notch intracellular domain of *notch1a* (Fig. 2D), which is a constitutively active form of the Notch receptor (34). These results indicate that Ripply1 specifically suppresses transcriptional activation by Tbx24.

To identify the regulatory element targeted by Ripply1, we next constructed a series of reporter plasmids containing progressive 5'-deletions of the 2.5 kb *mesp-b* fragment fused to the luciferase gene. As summarized in Fig. 3A, reporter genes containing at least 82 bp upstream of *mesp-b* could be activated by Tbx24 and suppressed by Ripply1, but those with 56 bp upstream of *mesp-b* were not affected by either Tbx24 or Ripply1. Because a putative T-box binding site (T-site) exists between 82 and 56 bp upstream of *mesp-b*, we next determined whether the T-site (5'-AATTCACACCT-3') is sufficient for the suppression of Tbx24 activity by Ripply1. The suppression by Ripply1 was also recapitulated by pBP-TbxX2, which contains two tandem T-sites upstream of the interferon-β basal promoter and the firefly luciferase gene (Fig. 3B). This suppression by Ripply1 was abolished by substitution of a single nucleotide within the T-site that is crucial for T-box binding (mutT) (8, 22), suggesting that the regulatory element targeted by Ripply1 cannot be separated from the T-site targeted by Tbx24.

In addition to Ripply1, zebrafish Ripply2 and Ripply3 also antagonized Tbx24 activation in dose-response manners (Fig. 3C). Thus, the Ripply family proteins can commonly antagonize the transcriptional activation by Tbx24.

Ripply1-Tbx24 complex associates with T-box binding site. The inability to separate the regulatory element for Ripply from the Tbx-binding element indicated that Ripply1 might inhibit Tbx24 transcriptional activation *via* a physical interaction. To examine this possibility, we generated Myc-tagged Rippy1 and FLAG-tagged Tbx24 proteins. We confirmed that both proteins were functional in the luciferase assay (See Fig. S1 in the supplemental material). The two proteins were predominantly co-localized in the nuclei (Fig. 4A), suggesting that the suppression by Ripply1 is not due to removal of Tbx24 from the nucleus. Rather, co-immunoprecipitation assays showed preferential association of Tbx24-FLAG with Myc-Ripply1 and *vice versa* in 293T cell lysates (Fig. 4B). To examine whether this Tbx24-Ripply1 complex is associated with DNA at the T-site, a chromatin immunoprecipitation (ChIP) assay was performed in 293T cells. Myc-Ripply1 is specifically associated with the DNA region containing T-sites (T-region), but not with another two regions in the 5' upstream of the *mesp-b*, only in the presence of Tbx24-FLAG (Fig. 4C). On the other hand, Tbx-24-FLAG is consistently associated with the T-region independently of Ripply1 (Fig. 4C). These results strongly suggest that Ripply1 represses the function of Tbx24 by forming a complex with Tbx24 that binds to the T-site.

T-domain and Ripply homology domain are indispensable for the interactions between Tbx24 and Ripply1. To get further mechanistic insights into the interactions between Tbx24 and Ripply1, we next prepared several deletion or amino acid-substituted constructs of Tbx24 and Ripply1 (Fig 5A). By conducting immunoprecicipitation assays, we found that the T-domain alone, which is highly conserved among the Tbx family proteins, is sufficient for the association with Ripply1 but that other region of Tbx24 is dispensable for the interactions (Fig. 5D).

On the other hand, a ~50 amino acid length sequence at the carboxyl terminus, called the Ripply homology domain, is conserved among the Ripply family proteins. Especially, highly conserved sequences exist in this domain (Fig. 5B). Substitution of some of these sequences to an alanine stretch (Ripply1-mutFPVQ) results in a significant reduction in the transcriptional repression of the pBP-TbxX2 reporter construct (Fig. 5C), indicating that this conserved sequence is required for Ripply-mediated transcriptional repression. This is at least caused by a significantly decreased affinity of Ripply1-mutFPVQ for Tbx24, because the immunoprecipitation assay revealed that Ripply1-mutFPVQ was not efficiently precipitated with Tbx24 compared with the co-precipitation with wild-type Ripply1 (Fig 5E).

Ripply1 converts the transcriptional property of Tbx24 from activator to repressor by recruiting the transcriptional corepressor Groucho/TLE. Ripply1 interacts with the transcriptional corepressor, Groucho/TLE, through the WRPW motif (7, 16). To determine whether Groucho/TLE is involved in the suppression of Tbx24 by Ripply, we next examined the effect of removing the WRPW motif. Compared with the wild-type Ripply1 protein, Ripply1 lacking the WRPW tetrapeptide did not efficiently suppress Tbx24-mediated enhancement of luciferase activity (Fig. 6A). Because multimeric Groucho/TLE complexes contain histone deacetylases (HDACs) in *Drosophila* and mammals (5, 7), we next examined the effect of trichostatin A (TSA), a chemical inhibitor of HDACs (37). We found that TSA partially rescued the suppression of luciferase activity by Ripply1 (Fig. 6B). These results suggest that Ripply1 counteracts the transcriptional activity of Tbx24 by recruiting the Groucho/TLE complex *via* the WRPW motif. Specifically, Ripply acts as an adaptor that converts Tbx24 from an activator to a repressor. In fact, whereas Tbx24 activates the reporter genes in the absence of Ripply1 in 293T cells, it acts as a transcriptional repressor when there is an excess of Ripply1 (Fig. 6C). Thus, Ripply1 controls the characteristics of transcriptional regulation by Tbx24 from activator to repressor.

Ripply1 can antagonize the transcriptional activation of another T-box gene, *no tail*, *in vivo* and *in vitro*. The result that the T-domain, which is conserved among the all of Tbx family proteins, is necessary and sufficient for the association between Ripply1 and Tbx24 suggests that Ripply acts on other T-box transcription factors (Fig. 3C). Indeed, Ripply1 suppressed transcriptional activation by No tail (Ntl), a zebrafish ortholog of the mouse T-box protein T/Brachyury (9, 31) (Fig. 7A). Thus, Ripply proteins appear to suppress the function of various T-box trans-activators *in vitro*.

In addition, we investigated the ability of Ripply1 to antagonize T-box proteins *in vivo* by injecting *ripply1* mRNA into one-cell stage eggs. As previously reported, injection of wild type *ripply1* mRNA at low dosage caused segmental disruption of somites and suppression of *mesp-b* expression in the PSM. On the other hand, misexpression of *ripply1* mRNA at higher dosage resulted in truncation of the posterior body and the absence of a notochord and horizontal myoseptum (Fig. 7B). Although the altered somite patterning is similar to the phenotype observed in *fuses somites*/*tbx24* mutants (24), the posterior truncation and the absence of a differentiated notochord and horizontal myoseptum, regardless of the presence of a neural tube and somites (Fig. 7C, *n*=3; 100% affected), is strikingly similar to *ntl* homozygous mutants (9). Furthermore, as observed in *ntl* homozygous mutants (1, 9, 35), in the *ripply1* mRNA-injected embryos, the expression of *myod* in the adaxial cells, which is induced by notochord, was missing at the one-somite stage (Fig. 7D). In a later stage, bilateral expression of *myod* is fused in the posterior trunk, as observed in *ntl* homozygous mutants (Fig. 7E). In contrast, the expression of *mesp-a* was normal in the *ripply1* mRNA-injected embryos (Fig. 7D). Of note, the expression of *ntl* itself was also unchanged in the *ripply1* mRNA-injected embryos (Fig. 7F). Interestingly, conversion of the *Xenopus* homolog of *T/Brachyury* into a transcriptional repressor by fusion with the En1 repressor domain also produces a similar phenotype as *ntl* homozygous mutants (6). Therefore, the morphological defect observed in *ripply*-injected embryos could be consistent with the expected defect when Ntl is converted into a repressor. Collectively, our results suggest that Ripply proteins are regulators of a wide range of T-box proteins.

Association with T-box protein is required for the function of Ripply in embryos.

Finally, to see whether interaction with T-box proteins is required for Ripply activities *in vivo*, we injected mRNA encoding either *ripply1* or *ripply1*-mutFPVQ into zebrafish embryos. As previously described, injection of the wild type *ripply1* mRNA results in reduced expression of *mesp-b*, but not *mesp-a* (Fig. 5F). In contrast, the injection of this mutated *ripply1* mRNA did not cause an apparent decrease in *mesp-b* expression in the anterior PSM (Fig. 5F). Thus, the interaction between Ripply and T-box protein is required for the activity of Ripply1 even in embryos.

DISCUSSION

Previously, we showed that zebrafish *ripply1* plays roles in the repression of gene expression in the anterior PSM. Although we suggested that the Groucho/TLE transcriptional co-repressor is involved in this suppression, the molecular mechanism underlying this suppression has mostly remained. Here, we have provided evidence to support a model by which Ripply proteins convert the transcriptional property of T-box proteins from activators to repressors. During the preparation of this manuscript, Kondow et al. reported that Bowline, a *Xenopus* counterpart of Ripply, suppresses the activity of an activated form of Tbx6 fused with VP16 in cultured cells (19). Although this report implies potential significance between Ripply/Bowline and a T-box protein, it still remains unclear whether Ripply/Bowline can actually suppress the activity of intact T-box proteins. In contrast, our results clearly indicate that the activity of the intact Tbx24 molecule is modulated by zebrafish Ripply1 and that the property of Tbx24 can be actually converted from activator to repressor by Ripply1. Furthermore, treatment with an inhibitor specific for HDAC activity strongly suggests that HDAC, which is known to be associated with Groucho/TLE, is actually involved in the Ripply-mediated suppression. We also show that Ripply1 is actually recruited to the T-box protein-binding DNA sequence through Tbx24. In addition, the T-domain and a highly conserved amino acid stretch in the Ripply homology domain are indispensable for the interactions between Tbx24 and Ripply1; and, as anticipated from this result, other members of either the Ripply or Tbx family are also interactive with Tbx24 or Ripply1, respectively. Finally, we show that this interaction is required for the activity of Ripply in embryos. Based on these results, we conclude that T-box proteins are converted from activators to repressors through specific interactions with Ripply proteins, which recruit the global corepressor Groucho/TLE.

The spatiotemporal regulation of gene expression, which is critical for proper development, is managed by a number of molecular processes, including the replacement of coactivator and corepressor complexes. For instance, external signals, such as Wnt and Notch, displace corepressor complexes, which maintain “default repression” of these signals’ targets, from DNA-binding transcriptional regulators (2, 13-15). Here, we show that the spatiotemporal gene regulation is also accomplished by another fashion of replacement of corepressor complexes. Our study provides evidence that T-box proteins are converted from activators to repressors through specific interactions with Ripply proteins, which recruit the global corepressor Groucho/TLE-HDAC complex. During somite segmentation in zebrafish, the expression of *mesp-b*, a target of Tbx24, is dynamically regulated to yield the characteristic stripe pattern in the anterior PSM (30), even though Tbx24 is constantly expressed in this region (24). Considering that *ripply1* is expressed in a stripe fashion in the anterior PSM (16), Ripply1 appears to provide conversion of the transcriptional property of Tbx24 in a stripe fashion in this region, and this conversion generates the characteristic expression pattern of *mesp-b*. Because defective function of Ripply1 or Mesp-b results in abnormal antero-posteror compartmentation within a single somite unit in zebrafish embryos, the Ripply1-mediated transcriptional control of *mesp-b* appears to be crucial for proper segmentation of somites. Thus, the regulatory recruitment of corepressor complexes to T-box proteins, established by the dynamic expression of *ripply1,* is likely to be a key process to establish the segmental gene expression during somite development.

The identification of Ripply1 as a switching molecule for T-box genes suggests a novel transcriptional mechanism that could participate in other aspects of development, although the relationship between the Ripply family and T-box factors besides Tbx24 and Ntl remains to be elucidated. The overlapping expression of *ripply* and T-box genes, observed in various developing tissues and organs (A.K and S.T, unpublished data), supports the idea that the two proteins cooperate in development. Further understanding of the cooperative transcriptional regulation by Ripply and T-box proteins could help elucidate the mechanisms underlying the disproportionate activation or repression found in human genetic disorders associated with mutations in T-box genes.

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Legends for figures

FIG. 1. Tbx24-dependent transcriptional suppression of *mesp-b* mRNA by Ripply1 in zebrafish embryos. Whole-mount *in situ* hybridization. The stained embryos are flat-mounted by removing the yolk (dorsal view and anterior to the top). The MOs were injected into the fertilized embryos at the appropriate concentration as previous described. (A) Ectopic expression of *mesp-b* mRNA is not detectable in the *tbx24* and *ripply1* MO-injected embryos. The embryos were simultaneously stained with the probes for *no tail* (notochord and tailbud) and *mesp-b* (segmental expression in the anterior PSM). Expression of *mesp-b*, which is normally restricted to the anterior PSM, is significantly decreased in *tbx24* MO-injected embryos. In *ripply1* MO-injected embryos, *mesp-b* mRNA is ectopically expressed in the presumptive somatic regions. In both the *tbx24* and *ripply1* MO-injected embryos, *mesp-b* mRNA is not ectopically expressed in the somatic regions, which is totally different from those observed in *ripply1* MO-injected embryos. Staining of *no tail* appears comparable in either case. (B) Ectopic expression of *mesp-b* mRNA is detected in the *deltaD* and *ripply1* MO-injected embryos. The embryos was stained with the probe for *mesp-b*. Expression of *mesp-b* mRNA is scatted in the anterior PSM of *deltaD* MO-injected embryos. In *deltaD* and *ripply1* MO-injected embryos, expression of *mesp-b* mRNA is not properly terminated in the anterior PSM in a similar manner to those in *ripply1* MO-injected embryos.

FIG. 2. Ripply1 antagonizes the luciferase reporter gene activation mediated by Tbx24. Luciferase assays in 293T cells were conducted to examine the transcriptional relationship between Ripply1 and Tbx24. The amounts of plasmids used for transfection are shown under each bar. The average normalized firefly luciferase activity with

pCS2+ alone was set at 100%, and error bars represent the standard deviations. (A) Schematic representation of the *mesp-b* 2.5-kb luciferase reporter construct. Two T-sites and RBP-Jκ-binding sequences are emphasized with boxes, and the number shows their location relative to the *mesp-b* initiation codon. (B) Tbx24 significantly activates reporter gene activity in 293T cells. (C) Ripply1 completely suppresses the enhancement of Tbx24 reporter activity. (D) Ripply1 does not suppress the transcriptional up-regulation of the reporter gene by the Notch intracellular domain (NICD).

FIG. 3. Ripply1 antagonizes the transcriptional activation of Tbx24 through the Tbx binding site. (A) Deletion constructs of the *mesp-b* luciferase reporter gene. The effects of Tbx24 and Ripply1 on each reporter gene are summarized on the right side of the panel. (B) The T-site is sufficient for the transcriptional suppression of Tbx24 by Ripply1. Sequence of each T-site or mutT-site inserted upstream of interferon-β basal promoter of luciferase reporter gene is shown. (C) Using the pBP-TbxX2 reporter gene, zebrafish Ripply1, as well as Ripply2 and 3, antagonize the transcriptional activation of Tbx24 through the T-site in dose-dependent manners.

FIG. 4. The Tbx24-Ripply1 protein complex associates with the T-site. (A) Colocalization of Myc-Ripply1 and Tbx24-FLAG proteins in the nucleus of 293T cells. (B) Myc-Ripply1 and Tbx24-FLAG proteins preferentially coimmunoprecipitate from lysates of 293T cells. (C) ChIP analysis reveals that Myc-Ripply1 and Tbx24-FLAG complex but not Myc-Ripply1 alone preferentially associate with the DNA sequence containing the T-sites. The DNA region containing the T sites (T-region) is

coprecipitated with Myc-Ripply1 in the presence of Tbx24 while a DNA region close to the T-sites (Proximal region), which includes potential binding sequences to Pax, Hox, TCF/Lef1, and E47 transcription factors, as well as another region around ~2.0-kb upstream from the T-sites (Distal region), are not immunoprecipitated. The length of the amplified band is ~250-bp in each case. On the other hand, Tbx24-FLAG is consistently associated with the T-region in the presence or absence of Ripply1.

FIG. 5. Ripply homology domain is required for the interactions with T-domain of Tbx24 and the suppression of *mesp-b* mRNA in zebrafish embryo. (A) Schematic representation of modified FLAG-tagged Tbx24 and myc-tagged Ripply1 variants used in the study. Conserved domains of Tbx24 and Ripply1 are emphasized with different colors. (B) Comparisons of Ripply homology domain in zebrafish Ripply proteins. Identical amino acid residues are shown on a black background and similar residues are on a grey background. The four-amino acid stretch (the 97th-100th amino acid stretches) is substituted to alanine stretch (emphasized with *). This variant is designated ripply1-mutFPVQ. (C) Luciferase reporter assay. The assay employed a luciferase reporter in which two T-sites were placed upstream of the basal promoter used in Figure 3B. In contrast to wild type Ripply1, substitution of the highly conserved sequences in the Ripply homology domain to a stretch of 4 alanines results in significant reduction of the transcriptional repression of the pBP-TbxX2 reporter construct. (D) Immunoprecipitation assay. T-domain alone (173 amino acid length) preferentially coimmunoprecipitates with Myc-Ripply1. (E) Substitution of four amino acid residues highly conserved in Ripply homology domain results in the significant reduction of affinity with Tbx24. (F) Whole-mount in situ staining with *mesp-b* and

mesp-a probes. Injection of *ripply1* mRNA results in a significant reduction of *mesp-b*, but not *mesp-a*, mRNA in the anterior PSM (arrowheads) at the 3-somite stage. However, the same amount of substituted *ripply1*-mutFPVQ mRNA does not cause such suppression of *mesp-b* mRNA. Stained embryos are viewed from the dorsal side and anterior to the top.

FIG. 6. Ripply1 exerts Tbx24-dependent transcriptional repression by recruiting the Groucho/TLE-HDAC complex. (A) Suppression of Tbx24 transcriptional activity depends on the Groucho/TLE-binding motif WRPW of Ripply1. (B) Transcriptional suppression of Tbx24 by Ripply1 involves the HDAC activity. (C) Transcriptional property of Tbx24 proteins is converted from activator to repressor by Ripply1. The assay employed a luciferase reporter in which two T-sites were placed upstream of the thymidine kinase promoter and the luciferase gene of pST0/TLN (25). Although Tbx24 normally acts as a transcriptional activator, Tbx24 acts as a repressor in the presence of Ripply1 (100ng).

FIG. 7. Transcriptional activation of Ntl, a zebrafish ortholog of T/Brachyury, is antagonized by Ripply1. (A) Luciferase assays in 293T cells. Zebrafish Ripply1 suppresses the transcriptional activation of Ntl proteins. (B) Injection of *ripply1* mRNA causes the truncation of the posterior body, similar to *ntl* homozygous mutants (9). Synthetic *ripply1* mRNA was injected into fertilized embryos at a concentration of 10 ng/μl. Shown is the lateral view 36 hour post-fertilization (hpf). (C) The notochord (N) and horizontal myoseptum (arrowhead) are absent in the *ripply1* mRNA-injected

embryos. Transverse sections (7μm) at the trunk regions above the yolk tube of 36-hpf embryos. The sections were briefly stained with hematoxylin. DA, dorsal aorta. (D) Expression of *myod* in the adaxial cells, induced by the presence of the notochord, is absent from the *ripply1* mRNA-injected embryos at the one-somite stage. In contrast, expression of *mesp-a* in the anterior PSM appears indistinguishable between the control and *ripply1* mRNA-injected embryos. (E) Bilateral expression of *myod* is fused in the posterior trunk (*), as observed in *ntl* homozygous mutants (1, 35). (F) Expression of *ntl* appears indistinguishable between the control and *ripply1* mRNA-injected embryos. Animal pole view at the shield stage.

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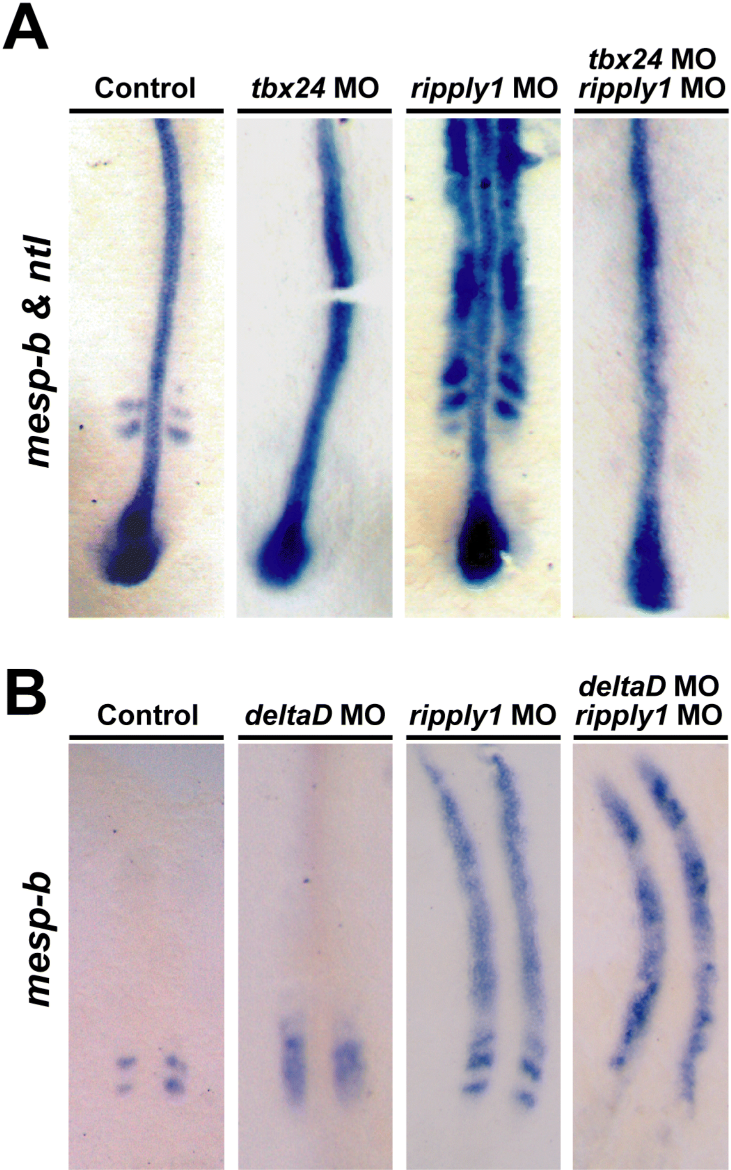
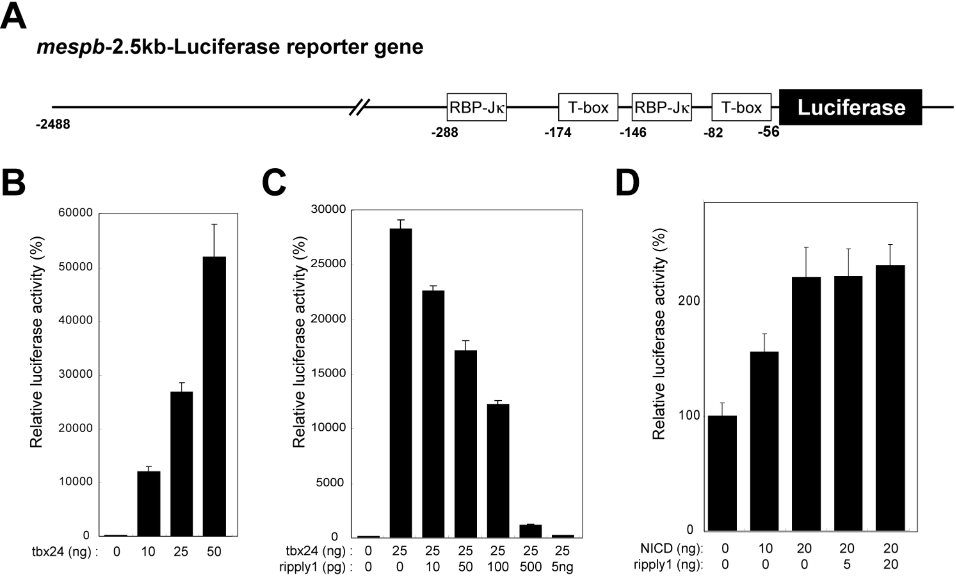
Figure 1

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Relative luciferase activity (%)
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Relative luciferase activity (%)
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15000
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tbx24 (ng) : 0 25 25 25 25 25 25
ripply1 (pg) : 0 0 10 50 100 500 5ng
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100
0
NICD (ng): 0 10 20 20 20
ripply1 (ng): 0 0 0 5 20

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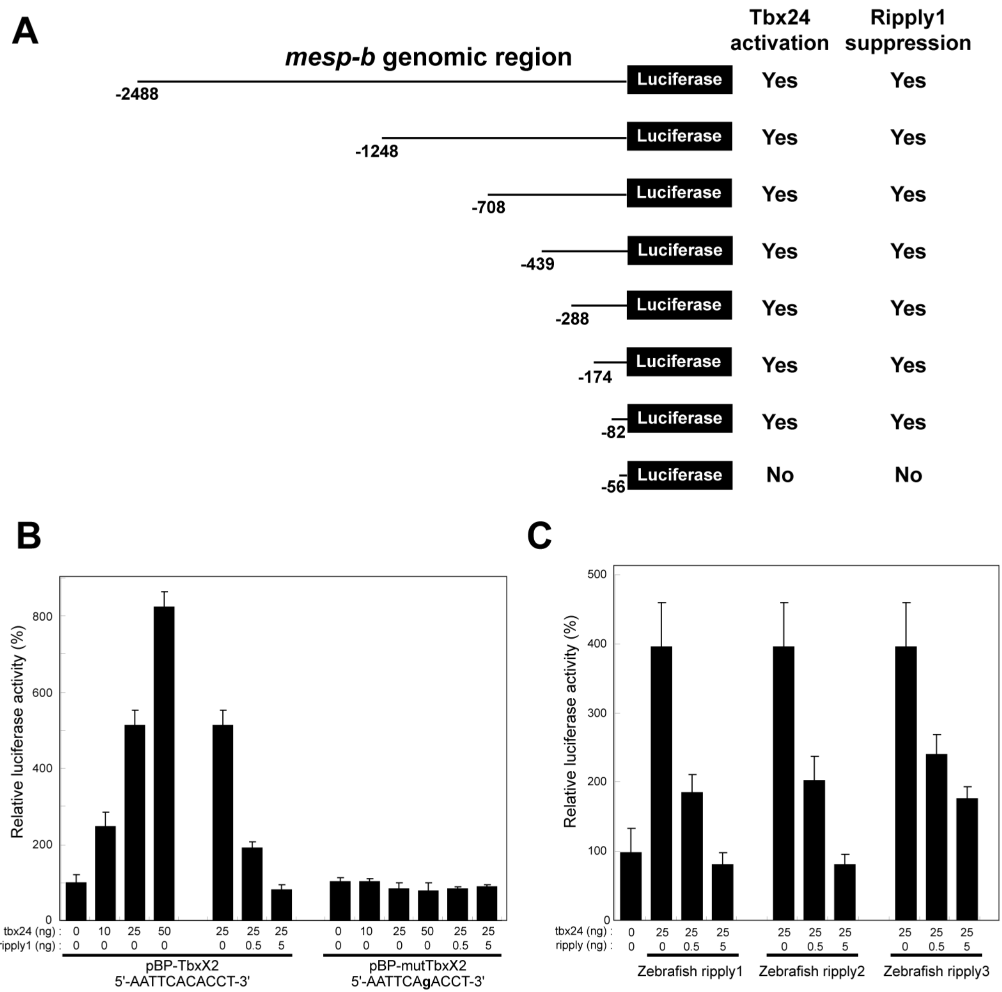
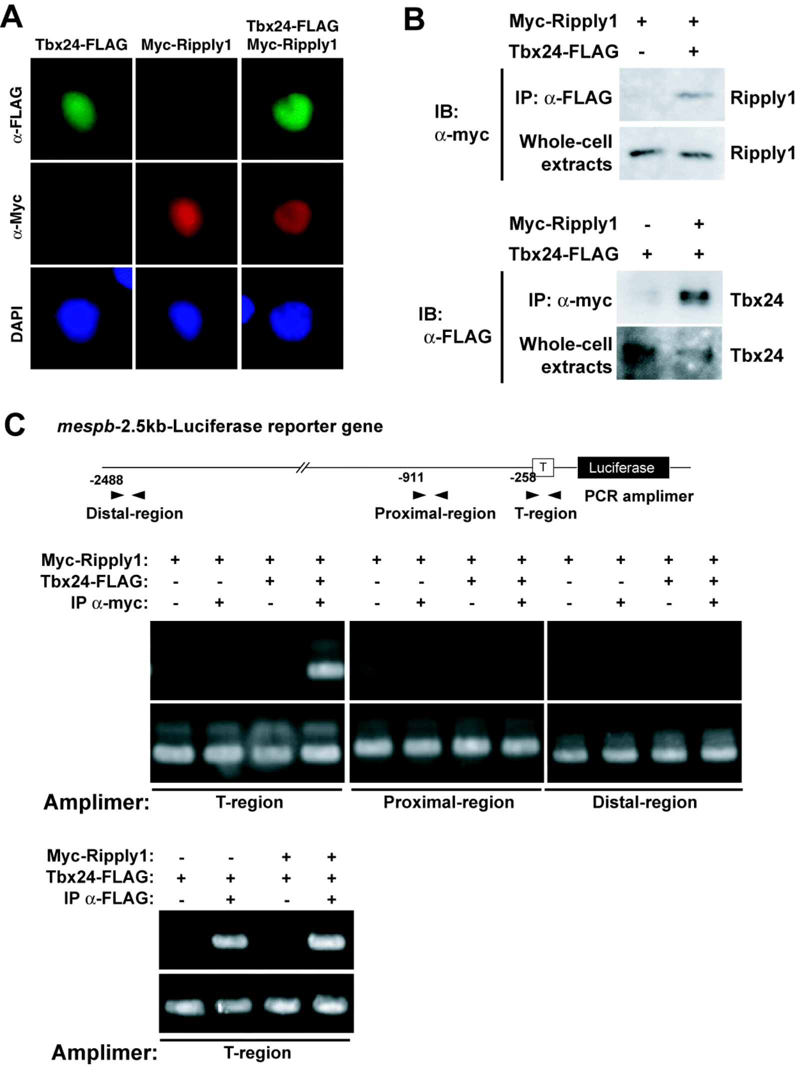
Figure 3

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IB: α-myc
IP: α-FLAG
Ripply1
Whole-cell extracts
Ripply1
Myc-Ripply1 - +
Tbx24-FLAG + +
IB: α-FLAG
IP: α-myc
Tbx24
Whole-cell extracts
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C
mespb-2.5kb-Luciferase reporter gene
T
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T-region
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IP α-myc:
Amplimer:
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Figure 5

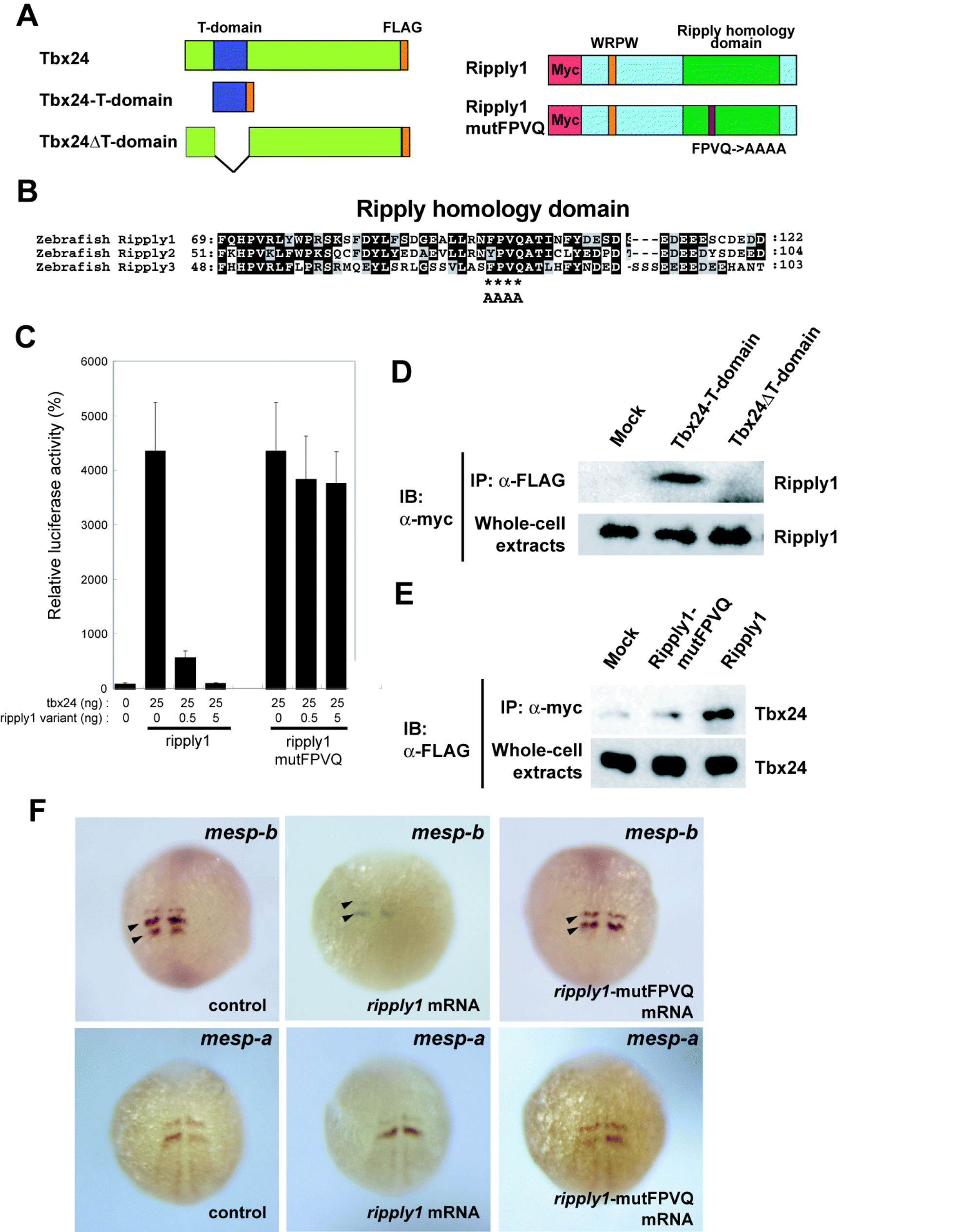

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Figure 6

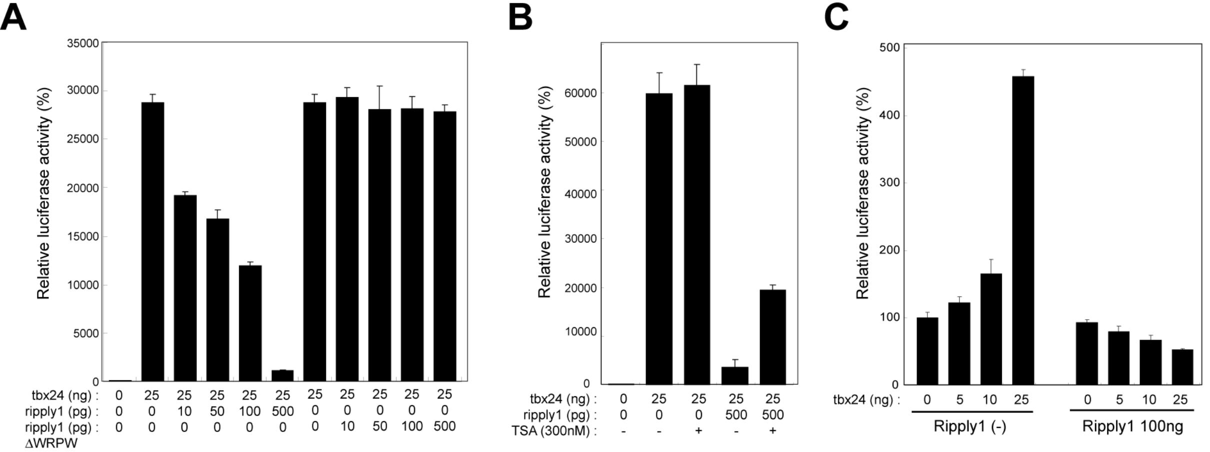

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Figure 7