STUDY OF MOLECULAR INTERACTIONS UNDERLYING SOMITE BOUNDARY POSITIONING IN ZEBRAFISH

by

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Abstract

Somitogenesis is the sequential formation of epithelial blocks of structures called somites at the anterior pre-somitic mesoderm in vertebrates, in an anterior to posterior direction. Somites are precursors to vertebrae, skeletal muscles, etc and are arrange on both sides of notochord. A “Clock and Wavefront” model is used to explain the complex mechanism of somite generation. A certain clock, controlled by the Notch/Delta signaling pathway, activates oscillating genes from posterior towards the anterior pre-somitic mesoderm, where the temporal information of the clock is converted to spatial patterning by the activation of genes such as *Mesp*. This region is defined by the wavefront of FGF signaling, where a subset of cells are released from FGF signaling and become responsive to Notch signaling leading to formation of the presumptive somite boundary.

Previous studies with mouse embryos revealed that the presumptive somite boundary is periodically created at the anterior border of the expression domain of Tbx6 protein at the anterior PSM. This Tbx6 anterior domain is also important for *Mesp2* activation, and subsequent degradation of this domain is important for proper positioning of the somite boundary. *Ripply1* and *Ripply2* are required for the determination of the Tbx6 protein border as mouse defective of *Ripply1/2* genes showed anterior expansion of Tbx6 protein expression. *Ripply* deficient embryos also showed increased expression of Mesp2, indicating Ripply1/2 may act downstream of Mesp2 in Tbx6 protein degradation. These findings indicate

that Ripply1/2 are more direct mediators of Tbx6 degradation than Mesp2. However, the mechanism by which this Tbx6 domain is regulated remains unclear. Interestingly, studies in zebrafish and frog showed that *Ripplys* are able to suppress Tbx6 function at the transcription level, raising questions whether Ripply-mediated mechanism of Tbx6 regulation is conserved among different species.

In this study, to test the generality of Tbx6 protein-mediated process in somite segmentation and to further examine the mechanism of regulation of Tbx6 protein, antibody against zebrafish Tbx6/Fss, previously referred to as Tbx24, was generated. Consistent with the findings in mice, the anterior border of Tbx6 domain also coincides with the presumptive somite boundary and the *tbx6* mRNA domain was located far anterior to its protein domain, indicating the possibility of post-transcriptional regulation. Interestingly, the anterior Tbx6 domain showed periodic expression with a temporary upper band, which coincided with the *mesp* expression, indicating Tbx6 is also important for *mesp* expression. When *ripply1* was knockdown using morpholinos, somite boundaries were lost and Tbx6 protein expression domain was expanded anteriorly, but in a graded manner. In contrast, no significant phenotype and no expansion of Tbx6 domain were observed in *ripply2* single morphants. However, when both *ripply1* and *ripply2* were knockdown, somite boundaries disappear similar to the *ripply1* single morphants, but the Tbx6 domain was ubiquitously expanded anteriorly, indicating the redundancy in the function of *ripply1* and *ripply2*. The *tbx6* mRNA was also

expanded but not so severely as the Tbx6 protein.

I further demonstrated that Ripply could directly reduce the expression level of Tbx6 protein by co-injecting either zebrafish or mouse *Tbx6* mRNA with zebrafish or mouse *Ripply* mRNA into zebrafish embryos and detecting the protein level. This Tbx6 reduction depends on physical interaction between Ripply and Tbx6, as zebrafish Ripply1 mutated at FPVQ amino acid site or mouse Ripply2 mutated at FPIQ site, important for interaction with Tbx proteins, failed to co-immunoprecipitate with either zebrafish or mouse Tbx6.

In mouse, Mesp2 is required for expression of both *Ripply2* and *Ripply1*, so I speculated the possibility of similar regulation or *ripply* in zebrafish. Using the hsp-Gal4/UAS-*mesp-ba* transgenic line, I over expressed *mesp-ba* and analyzed the expressions of *ripply1* and *rippy2*. Interestingly, no significant change was observed in *ripply2* expression, however, *ripply1* was highly upregulated at least at the anterior pre-somitic mesoderm and somite regions, but not posteriorly. In addition, anterior domain of Tbx6 was highly reduced. This reduction was rescued when *ripply1* and *ripply2* were both knockdown in these embryos, suggesting that *mesp* mediates *ripply* reduction of Tbx6. Expectedly, the onset of *ripply1* and *ripply2* expression occurred after reduction of FGF signaling at the anterior PSM in normal zebrafish embryos, but this expression was initiated much earlier in embryos treated with SU5402, an FGF inhibitor, indicating FGF is required for posterior inhibition of *ripply*.

My study, for the first time, reveals the expression patterns of zebrafish Tbx6

protein, and the importance of the Tbx6 anterior domain for somite boundary positioning in zebrafish. Most importantly, I showed that Ripply is a direct regulator of the Tbx6 reduction at the protein level, and this reduction is mediated by mesp. However, mesp is not sufficient to induce *ripply* unlike mouse, indicating some other factor apart from mesp may be involved in somite boundary formation in zebrafish.

Introduction

Somites are epithelial blocks of segmental structures generated periodically on both sides of the neural tube from the presomitic mesoderm (PSM), in an anterior to posterior fashion (Figure 1A), and are precursors of important tissues like the vertebrae, dermis of the back, skeletal muscles, etc. This periodic generation is achieved by a complex and dynamic mechanism operating in the PSM (Pourquie 2011, Maroto et al., 2012, Oates et al., 2012, Kageyama et al. 2012, Saga 2012). In 1976, Cooke and Zeeman developed a "Clock and Wavefront" model where they postulated the existence of a positional information gradient, "Wavefront", along the anterior-posterior axis, which interacts with an oscillating "Clock" to set the times at which cells undergo a catastrophic changes in adhesive and migratory behavior resulting in formation of somites (Cooke and Zeeman, 1976, Baker et al., 2008). The so-called segmentation "Clock" (Figure 1A) is generated from the posterior PSM and creates oscillatory expression of genes in the PSM cells. Because the phase of oscillation among PSM cells is gradually delayed in a posterior-to-anterior direction, a wave of the oscillation appears to move in a posterior-to-anterior fashion, where they get arrested on interaction with the "Wavefront" and result in formation of somites. The segmental pattern of somites is primarily defined by positioning of presumptive intersomitic boundaries and the position of each boundary is repeatedly established in an anterior-to-posterior

order in accordance with posterior elongation of body length. The period of oscillation is almost consistent during somitogenesis, for instance, 120 min in the mouse and 20 to 30 min in the zebrafish. Furthermore, the time interval of this boundary formation is coupled with the time period of the segmentation clock. Thus, during the process of the boundary formation, the oscillatory gene expression is converted into a spatial pattern with periodicity. The FGF signaling has been considered important for spatial patterning of temporal Notch information and determining the position of this boundary formation (Sawada et al., 2001, Dubrulle et al., 2001, Niwa et al., 2011, Akiyama et al., 2014). A wavefront is generated by the FGF gradient from posterior to anterior PSM, which gradually moves posteriorly in correspondence to body elongation. The anterior limit of this gradient, which determines the position of the next/future somite boundary, shifts posteriorly permitting the cells at the anterior PSM to respond to Notch signaling thereby activating segmentation genes such as *Mesp.* Previous studies showed that the downstream of FGF, pERK (phosphorylated ERK), demonstrates a dynamic oscillatory expression from posterior to anterior PSM in mouse (Niwa et al., 2011, Figure 1A). However, in zebrafish, pERK showed no oscillatory expression and is strongly expressed from the posterior PSM towards the intermediate PSM but absent in anterior PSM, and closely resembles its *fgf8* mRNA (Figure 1A, Sawada et a., 2001, Akiyama et al., 2014). Recent study in zebrafish also indicated that pERK generates ‘ON’ and ‘OFF’ status as seen in Xenopus, and undergoes a stepwise change pattern within the PSM and correlates

with the future somite boundary (Akiyama et al., 2014).

Thus, a number of transcription factors and cell-to-cell signaling molecules are involved in this temporal to spatial conversion (Dubrulle et al., 2001, Sawada et al., 2001, Aulehla et al., 2003, Aulehla et al. 2008, Gibb et al., 2009, Aulehla and Pourquie 2010, Niwa et al. 2011, Akiyama et al., 2014, Bajard et al., 2014). For instance, the oscillatory changes in FGF and Notch signalings determine the onset of expression of the *Mesp2,* a transcription factor involved in the spatial patterning of somites, at the anterior PSM in the mouse embryos (Oginuma et al. 2008, Niwa et al., 2011, Figure 1A). Then, *Mesp2* expression defines the spatial pattern of Tbx6, which plays another critical role in the positioning of the segmentation boundary (Nikaido et al., 2002, White et al., 2003, Morimoto et al., 2005, Yasuhiko et al. 2006, Oginuma et al. 2008). The presumptive segmentation boundary is generated at the anterior border of the expression domain of Tbx6 protein, which is posteriorly shifted by 1 segment length during the time period of 1 segmentation cycle (Oginuma et al. 2008). Conversely, Tbx6 is indispensable for the PSM expression of *Mesp2*, indicating that Tbx6 and Mesp2 are mutually regulated. This feedback loop between Mesp2 and Tbx6 appears to regulate the periodical shift of the anterior border of the expression domain of Tbx6 protein, which is referred to as “Tbx6 domain”, hereinafter (Saga 2012).

Importantly, the anterior border of the Tbx6 domain, is not consistent with that of *Tbx6* mRNA, but rather regulated by a proteasome-mediated mechanism (Oginuma et al. 2008). Although the molecules directly executing this proteolysis

are still unclear, studies with knockout mice indicate that *Ripply1* and *Ripply2*, as well as *Mesp2*, are required for the down-regulation of Tbx6 proteins (Takahashi et al. 2010). In addition, considering that the expression of *Ripply1* and *Ripply2* in the PSM is lost in *Mesp2*-deficient mouse embryos, segmentation in mouse can be explained in three phases (Takahashi et al., 2010, Saga, 2012, Figure 1A): The Notch active domain and the pERK signaling are seen traveling from posterior PSM to anterior PSM, and The Tbx6 protein is spread from tailbud to anterior PSM. In the phase I, Notch active domain (dark metallic gold) on reaching the anterior PSM, activates the expression of *Mesp2* at the Tbx6 positive and pERK negative region. In phase II, Mesp2 protein then activates *Ripply1/2*. In phase III, Ripply suppressed Tbx6 expression at the anterior PSM in the Mesp2 expressing domain.

Zebrafish defective for *tbx6/fss*, previously referred to as *tbx24*, exhibit defective boundary formation as in the case of its mouse counterpart (van Eeden et al., 1996). However, in contrast to the analysis with mouse mutants, previous studies with zebrafish and Xenopus Ripply suggested another function of Ripply in the regulation of Tbx6 (Kawamura et al., 2005, Kawamura et al., 2008, Kondow et al. 2006, Kondow et al., 2007, Hitachi et al. 2009). In cultured cells, Ripply1, Ripply2 and Ripply3 suppress the transcriptional activation mediated by Tbx6. Ripply1 associates with Tbx6 and converts it to a repressor. A mutant form of Ripply1, defective in association with Tbx6, lacks this activity in zebrafish embryos. These results indicate that the intrinsic transcriptional property of T-box

proteins is also controlled by Ripply family proteins, which act as specific adaptors that recruit the global co-repressor Groucho/TLE to T-box proteins in this context. Thus, it is still unclear whether Ripply regulates Tbx6 proteins at the protein level even in other animals apart from the mouse. The molecular interaction between Notch, Tbx6, Ripply and Mesps is demonstrated in a simple model (Figure 1B, Takahashi et al., 2010). However, this model must be validated in several different ways, one for instance, is by elucidating whether Ripply1 and/or Ripply2 can actually suppress the protein level of Tbx6.

For a better understanding of the mechanism of Tbx6-mediated patterning of somites, in this present study I examined whether the expression pattern of Tbx6 proteins correlate with the presumptive somite boundary positioning in the zebrafish by generating antibody specific for zebrafish Tbx6 (previously called Tbx24), and whether *ripply* is also required for reduction of Tbx6 proteins in zebrafish. My results show that *ripply*-dependent regulation of Tbx6 protein in the positioning of somite boundary is significantly common in the zebrafish and the mouse, and that Ripply was able to significantly reduce the level of Tbx6 proteins in zebrafish as confirm by co-injection of *Tbx6* mRNA and *Ripply* mRNA into zebrafish eggs.

Mouse studies also showed that Mesp2 regulates Ripply1 and Ripply2 (Morimoto et al., 2007, Takahashi et al., 2010). Similarly, I also identified that in zebrafish, ripply is regulated by mesp at least in the anterior PSM while it may be regulated by some other factor at the posterior PSM. Finally, I examined the

relationship between *ripply* expression and FGF signaling. Inhibition of FGF by SU5402 causes the posterior shift of the newest band of *ripply* expression indicating FGF represses *ripply* at the posterior PSM for proper somite boundary formation. These results strongly suggest that Ripply is a critical regulator of the Tbx6 protein level in the establishment of intersomitic boundaries and that this mechanism is conserved among vertebrates.

Materials and Methods

Fish and embryos

Zebrafish were maintained at 28°C on a 14-h light/10-h dark cycle. All studies on wild-type fishes were performed using the TL2 inbred line (Kishimoto et al., 2004).

In situ hybridization

Whole-mount *in situ* hybridization of zebrafish embryos was carried out according to the protocol previously described (Nikaido et al., 1997). Probes were synthesized for *mesp-aa/ba* (Sawada et al., 2000), *tbx6* (Nikaido et al., 2002), *ripply1/2* (Kawamura et al., 2005) by using a standard protocol. The fragments of *mesp-ab/bb* were amplified by PCR and cloned into pBS-SK+ or pGEM-T easy vector respectively to synthesize the RNA probes. For fluorescence *in situ* hybridization, the probes were labeled with digoxigenin and color was detected by using TSA Plus-Fluorescein Solution (Jülich et al., 2005).

Antibody preparation and whole mount immunostaining

For immunostaining of zebrafish Tbx6 protein, anti-rabbit antibody against zebrafish Tbx6 was generated. The immunogen was prepared from *E. coli* expressing a fragment of the zebrafish Tbx6, ranging from the 561st to the 874th position in its amino-acid sequence. Purified proteins electroeluted from

poly-acrylamide gel were used to immunize 2 rabbits. After 7 injections of the purified proteins, sera (#1 and #2) were recovered from the rabbits; and their reactivity and specificity were assessed by Western blotting (Figure 2A). Whole mount immunostaining was conducted using one of the antisera (#1) at a dilution of 1:200 in 2%BSA-PBS containing 0.1% Triton-x100, with 48 hrs incubation at 4^{o}C. Detection was carried out using Alexa Fluor-555 anti-rabbit antibody (Invitrogen) at 1:1000 dilution in 2%BSA-PBS containing 0.1% Triton-x100. For quantification of the expression levels of *tbx6* mRNA and Tbx6 protein in the PSM, signal intensity was measured by ImageJ software (National Institute of Health) and background was subtracted. Obtained intensity values were normalized to a range between 0 and 1. Double immunostaining was performed using anti-Tbx6 and anti-pERK as described previously (Matsui et al. 2011).

Antisense MO injection

The sequences of morpholinos used in this study were the following: *her1* MO 5'-GACTTGCCATTTTTGGAGTAACCAT-3' and *her7* MO 5'-TTTCAGTCTGTGCCAGGATTTTCA-3' (Henry et al., 2002); *ripply1* MO1, 5'-CATCGTCACTGTGTTTTTCGTTTTG-3' and 5mis-*ripply1* MO1, 5'-CtTCcTCAgTGTcTTTTTCcTTTTG-3' (Kawamura et al., 2005); *ripply2* MO1, 5'- TCGTGAAAGTGATGTTCTCCATAGT-3' (Moreno et al*.,* 2008); 5mis-*ripply2* MO1, 5'-AGTCATCTTCTGCATAGTCTCGATG-3' and *ripply2* MO2, AGTGATGTTCTCCATAGTGTCCATG. Neither of the *ripply2*

morpholinos gave a phenotypic change when injected alone. I continued the experiments with the *ripply2* MO2. One ng of *ripply1* morpholino; 2 ng of *ripply2* morpholino and 1:2 of *ripply1*:*ripply2* MOs were injected. Embryos were injected at the 1-cell stage and fixed at 8 somite stage with 4%PFA in PBS, for overnight at 4°C. *her1* and *her7* morpholinos were each diluted to 4mg/ml working solution and co-injected at a ratio of 1:1. The morphants exhibit weak boundaries as described (Henry et al., 2002). The morpholinos were diluted in sterile milliQ water and supplemented with 0.1% Phenol red (SIGMA) in 0.1M KCl (Nacalai Tesque) for injection.

mRNA injection and preparation of cell lysates for SDS PAGE

Capped mRNAs were transcribed from linearized pCS2+zripply1-Myc, pCS2MT+zripply1-6Myc, pCS2MT+zripply1mutFPVQ-6Myc and pCS2+zTbx6-Flag, pCS2+mRipply2-Myc, pCS2+mTbx6-Flag, pCS2+GFP and pCS2+mBrachyury-Flag by using an mMessage mMachine Sp6 kit (Ambion). The zebrafish Tbx6-Flag was synthesized by PCR. mRNAs were injected, at the desired concentrations, at the 1-cell stage and the eggs were harvested after 6 hrs of incubation at 28.5°C. After careful dechorionation, the intact eggs were collected into 1.5-ml tubes (20 eggs /tube) on ice. Then the eggs were triturated with a 200-μl micro pipette having a broken tip. Next, 2x SDS PAGE buffer (2 μl/embryo) was added to the pellet and the cells were vortexed. In some experiments, 200 μl of protease inhibitor cocktail (Nacalai Tesque) was added

before the trituration, then the eggs were centrifuged twice at 1000rpm at 4°C for 1 min each and the supernatant was carefully removed. After the tubes had been immersed in liquid nitrogen, the samples were either stored at -80°C or continued by boiling for 10 min at 95°C before loading into the PAGE gel (Westerfield, 2000). Western blotting was performed according to a standard procedure with anti-zebrafish Tbx6 rabbit polyclonal antibody (antisera #2), anti-Myc mouse monoclonal antibody clone 4A6 (Upstate, 05-724), anti-Myc rabbit polyclonal antibody (Abcam, ab9106), anti-GFP rabbit polyclonal antibody (MBL, 598) and anti-Flag rabbit polyclonal antibody (Sigma, F7425).

Immunoprecipitation

Whole cell lysates were prepared from 293T or COS7 cells transfected with pCS2+zTbx6-Flag, pCS2MT+zripply1-6Myc, pCS2+zripply1mutFPVQ-6Myc, pCS2+mTbx6-Flag, pCS2+mRipply2-Myc, pCS2+mRipply2mutFPIQ-Myc, or pCS2+ expression vectors accordingly. The lysates were pre-cleared by passing over Protein G resin bed (GE Healthcare) for 2hrs at 4°C to eliminate any unspecific binding. The solution was then incubated with anti-FLAG M2 resin (SIGMA) for 3hrs at 4°C to allow antibody-antigen complexes to form. The precipitated complex was washed several times, and the proteins were collected in 2x SDS sample buffer and separated by SDS PAGE. Western blotting was conducted and the proteins were detected by anti rabbit polyclonal antibody against zebrafish tbx6 (#2) or rabbit polyclonal anti-Myc (Abcam, ab9106) antibodies accordingly.

Chemical treatment of zebrafish embryos

DAPT, N-[N-(3,5-difluorophenyacetyl)-L-alanyl]-S-phenyl glycine t-butylester, widely used as a Notch pathway inhibitor was administered as described earlier (Özbudak and Lewis, 2008). Chorions were removed from eggs at around the 75% epiboly stage, and the embryos were incubated with 100 μM DAPT and then fixed at 10 somite stage. SU5402 was used as described previously (Sawada et al., 2001). Embryos were dechorionated and treated with 0.4 mM SU5402 at 2 somite stage for 8 min. After thorough washing, they were incubated at 28°C and then fixed at 6 somite stage for overnight at 4°C with 4% PFA. Some of the embryos were fixed just after SU5402 treatment and analyzed. Statistical analysis was performed by the following procedure. Distance was measured from the chordo neural hinge (CNH) to the anterior border of the posteriormost band of the *ripply1* mRNA expression for both the control and the SU5402 treated embryos expressing *ripply1* mRNA by ImageJ. Standard deviation for each measurement was calculated and a student's t test was performed for two samples with unequal variances. A $p < 0.05$ value was obtained.

Gal4-UAS system to generate *hsp*-Gal4/UAS-*mesp-ba*

The *hsp*:Gal4 line was kindly gifted by Dr. Kawakami (Asakawa and Kawakami 2008). The UAS:*mesp-ba* line was generated (Yabe, unpublished data). To construct the plasmid for UAS:*mesp-ba* transgenesis, the coding region of

mesp-ba was amplified by PCR and cloned into the EcoRI-XhoI site of the pT2-UAS-mcs vector (gifted by Dr. Kawakami). To generate UAS:*mesp-ba* transgenic fish, 25pg of pT2-UAS-mcs containing *mesp-ba* was co-injected with 50pg of *Tol2* mRNA into the fertilized eggs. The F0 fish were mated with *hsp*-Gal4 fish to assess the germline transmission by the Gal4 dependent induction of *mesp-ba* expression. The heat shock treatment was carried out by soaking the embryos in egg water (3% salt in RO water and 0.01mg/L methylene blue), for 1hr at 37^{o}C (Shoji, 2008). The embryos were further incubated at 28.5^{o}C for 3 hrs and fixed with 4%PFA for overnight at 4^{o}C.

Results

Periodical change in the anterior border of the Tbx6 domain in zebrafish embryos

In zebrafish, mutants deficient of *tbx6/fss* (previously called *tbx24*) show defects in segmentation boundary formation (Nikaido et al., 2002). As a first step toward understanding the regulation of zebrafish Tbx6 protein expression during somitogenesis, antibody against zebrafish Tbx6 was generated suitable for immunohistochemistry. Antibody specificity was confirmed by western blotting analysis (Figure 2A) and its localization was observed in the PSM in zebrafish embryos at around the 8-somite stage. As predicted from its mRNA pattern, the zebrafish Tbx6 protein was broadly expressed in the anterior PSM without the posterior PSM and the tail bud (Figure 2B, 2D). However, similar to its counterpart in the mouse, the anterior border of the Tbx6 protein domain was posterior to that of its mRNA domain, whereas the posterior border was almost identical between these 2 domains (Figure 2E-2G). This result suggests that the anterior border of the Tbx6 protein was regulated post-transcriptionally as in the case of the mouse (Oginuma et al, 2008).

However, unlike mouse Tbx6 proteins, an additional distinct band of zebrafish Tbx6 protein was detected anterior to this broad domain in 35% of stained embryos (Figure 2I). I refer this distinct band as the “upper band“ and the broad protein domain as the “core domain” hereinafter. Of note, the length of the

core domain along the A-P axis changed within the length of 1 segment (Figure 2H-2J). To examine whether the patterns of Tbx6 proteins correlated with the phases of oscillation, I examined the expression pattern of *her1,* a zebrafish gene related to *hairy* and *enhancer of split* (Sawada et.al., 2000; Muller et.al., 1996), in the same embryos and identified the phases of the oscillation cycle (Figures 3A-3C; (Pourquie and Tam, 2001). In the PSM of zebrafish embryos, *her1* is expressed in several distinct domains along the anterior-posterior axis. During a segmentation cycle, the most posterior expression is initially observed in broad area of the posterior PSM (phase I) then this expression becomes more discrete and gradually shifts to the anterior direction (phase II and III). The comparative analysis revealed that a long "core domain", without the "upper band", of Tbx6 protein was observed in phase III (Figure 3A'). At this phase, the anterior limit of the Tbx6 protein domain coincided with B-II, the boundary between presumptive somite S-II and S-III. The "upper band" emerged from late phase III to early phase I (Figure 3B'), then this upper band diminished (Figure 3C') and the core domain, whose anterior limit coincided with B-III, gradually extended to the posterior direction by 1 segment length during phase II and III. This means that elimination of Tbx6 proteins takes place in a two-step fashion; it first started at the anterior part of the "core domain" except the most anterior part of it, the "upper band"; then in the next step, proteins persisting in the "upper band" subsequently disappeared. Taking into consideration that spatial pattern of *tbx6* mRNA remained continuous without showing any "upper band" during a single

segmentation cycle, this dynamic change in Tbx6 proteins shows the importance of post-transcriptional regulation in the spatial patterning of the Tbx6 domain.

To examine relationship between Tbx6 protein pattern and the prospective segmentation border, I next compared the spatial pattern of zebrafish Tbx6 proteins with that of mRNA of *mesp* genes. The zebrafish possesses at least 4 *mesp* genes; 2 recently identified ones, *mesp-ab* and *mesp-bb* (Cutty et.al., 2012), in addition to *mesp-aa* and *mesp-ba,* previously referred to as *mesp-a and mesp-b,* respectively. These 4 *mesp* genes are expressed in the anterior PSM in a similar fashion. For instance, the anterior expression border of these 4 *mesp* genes coincides with the prospective segmentation boundaries in the anterior PSM (Sawada et.al., 2000; Cutty et.al., 2012). The onset of *mesp-ab* and *mesp-ba* expression, which occurred at the level of S-II, was observed at the most anterior region of the "core domain" of the Tbx6 protein (Figures 4A-4C and Figure 4E-4G). Thus, as in the case of mouse embryos, the anterior border of the Tbx6 "core domain" basically coincided with the prospective segmentation boundary even in the zebrafish, suggesting that the mechanism governing Tbx6 protein-mediated segmentation may be conserved between mouse and zebrafish.

If this is true, the anterior border of the Tbx6 domain should be perturbed in embryos in which formation of the intersomitic boundary is defective. In the zebrafish, *her1* and *her7* encoding transcriptional repressors crucial for establishment of the segmentation clock are required for proper formation of somite boundaries. The anterior border of the Tbx6 protein domain was not

clearly defined in embryos injected with antisense morpholino oligo specific both for *her1* and *her7* (Figure 2K). In addition, Notch-defective embryos show impaired segmentation due to de-synchronization of oscillation among PSM cells, resulting in a change in the expression patterns of several *mesp* genes to "salt-and-pepper" ones (Jiang et al., 2000). We also observed that the anterior border of the Tbx6 proteins was actually disturbed in embryos treated with DAPT, N-[N-(3,5-difluorophenyacetyl)-L-alanyl]-S-phenyl glycine t-butylester, which inhibits γ-secretase and widely used as a Notch pathway inhibitor, supporting the correlation between the anterior border of the Tbx6 domain and the prospective segmentation boundary (Figure 2L and Figure 4D).

ripply1 and *ripply2* are required for proper positioning of Tbx6 domain in zebrafish embryos

Recent studies in mouse demonstrated that *Ripply1* and *Ripply2* are required for elimination of Tbx6 (Takahashi et al., 2010). So, I next compared the expression of zebrafish *ripply1* and *ripply2* with the Tbx6 domain (Figure 4H-4J, 4K-4M). The newest and most posterior expression of the *ripply* genes occurred at the S-II level in the anterior part of the "core domain" of the Tbx6 domain (Figure 4H", 4K"). After these earliest signs of *ripply1* and *ripply2* mRNA expressions, Tbx6 protein started to reduce in the anterior part of the core domain (Figure 4I", 4L") while the "upper band" continued to express (Figure 4I', 4L'). Since the region where Tbx6 proteins became reduced, well coincided with the area where

ripply1 and *ripply2* had been expressed in the core domain, these Ripplys appear to function to reduce expression of the Tbx6 protein. The posterior-most bands of *ripply1* and *ripply2* become strongly expressed above the "core domain" of Tbx6 while the "upper band" disappeared (Figure 4J'- 4J", 4M'- 4M").

To validate our theory that *ripply1* and *ripply2* actually play a role in reducing the Tbx6 protein level in zebrafish embryos, I examined the spatial pattern of Tbx6 proteins in *ripply1* and/or *ripply2*-deficient embryos (Figure 5). Injection of antisense morpholino oligos specific for zebrafish *ripply1* and *ripply2* caused severe expansion of the Tbx6 domain in zebrafish embryos (Figure 5D). This expansion was certainly, or at least to some extent, a result of posttranscriptional dysregulation, since the *tbx6* mRNA domain was not so severely, but only slightly expanded as compared to the protein domain in *ripply1/ripply2* double-deficient embryos (Figure 5E, 5F). On the other hand, *ripply1* single morphants exhibited less severe expansion of the Tbx6 domain (Figure 5B); whereas this domain looked normal in *ripply2* single morphants (Figure 5C), indicating a redundant role between these 2 ripplys in the regulation of Tbx6 protein expression. Consistent with these results, *ripply1/ripply2* double-deficient embryos, as well as *ripply1* single morphants, exhibited no segmentation boundaries; whereas *ripply2* single morphants seemed normal in the morphology of their somites (Kawamura et al., 2005; data not shown). Therefore, both the ripplys are required for the reduction in the Tbx6 protein level, as observed in the mouse, and for proper formation of the anterior border of the Tbx6 domain in zebrafish embryos.

Ripply can decrease Tbx6 protein level in zebrafish eggs

Next, I asked the molecular mechanism by which the anterior border of the Tbx6 domain was established in the PSM. In the mouse, *Mesp2* is one of the key molecules involved in this establishment, because a newly formed border of the Tbx6 domain is established nearby the caudal border of the *Mesp2* expression domain (Oginuma et al, 2008). Furthermore, the anterior border of the Tbx6 domain is anteriorly expanded in *Mesp2*-deficient mouse embryos. These results indicate the requirement of *Mesp2* in the proper positioning of the Tbx6 domain (Oginuma et al, 2008). Similarly, *Ripply1* and *Ripply2* are also required for this positioning, since *Ripply1* and *Ripply2* double-deficient embryos also exhibited anterior expansion of the Tbx6 domain (Takahashi et al., 2010). Because expression of *Ripply1* and *Ripply2* is lost in the PSM in *Mesp2* mutant embryos (Takahashi et al., 2010), it seems likely that the loss of Ripplys' expression is a more direct cause for anterior expansion of the Tbx6 domain in *Mesp2*-deficient embryos. Furthermore, *Ripply1/Ripply2* double-mutant embryos exhibited expanded anterior border of the Tbx6 domain although Mesp2 expression was also increased. Thus, *Mesp2* expression, which is required for the anterior positioning of the Tbx6 domain, itself was not sufficient for elimination of Tbx6 proteins. Rather, *Ripply1* and *Ripply2* appear to play a role downstream or parallel to *Mesp2* in the anterior positioning of the Tbx6 domain.

Therefore, I next examined whether Ripply could actually reduce the Tbx6 protein level. First, the COS7 cell line was used for this analysis, but the assay

failed to detect a Ripply-dependent reduction in the level of mouse Tbx6 proteins (data not shown). Next, I used the zebrafish egg as an assay system to examine whether a reduction in the level could be detected by injecting mouse or zebrafish *tbx6* mRNA along with *ripply* mRNA into zebrafish eggs (Figure 6). The amount of zebrafish Tbx6 protein was severely decreased by injection of zebrafish *ripply1* mRNA, indicating that Ripply possessed strong activity to reduce the Tbx6 protein level (Figure 6A). Similarly, mouse *Ripply2* mRNA also decreased the mouse Tbx6 protein level (Figure 6B), as did zebrafish *ripply1* mRNA (Figure 6C). Thus, the ability of Ripplys to reduce Tbx6 protein level is conserved between mouse and zebrafish. Of note, these effects by Ripplys were canceled when a FPVQ in zebrafish Ripply1 or its corresponding amino acid stretch FPIQ in mouse Ripply2, both of which are amino-acid sequences essential for physical association with Tbx6 (Kawamura et al., 2008, Figure 6D-6E, 6G-6H), was deleted. Thus, Ripply reduced the Tbx6 protein level probably through a direct protein-to-protein interaction. In addition to that of Tbx6, the protein level of another T-box factor, mouse Brachyury, was decreased by Ripply2 (Figure 6F), indicating that Ripply can reduce the level of several T-box proteins.

mesp regulates *ripply1* expression at least in the anterior PSM

Previous studies in mouse have demonstrated that Mesp2 is required for expression of Ripply2 (Morimoto et al., 2007) and Ripply1 (Takahashi et al., 2010). Both the expression of *Ripply1* and *Ripply2* was lost in *Mesp2* mutants

indicating that they are similarly regulated by Mesp2 and may function in coordinated manner in the somite segmentation. However, it is still unclear whether Mesp is sufficient for Ripply regulation. To address this question, zebrafish *mesp-ba,* the homologue of mouse *Mesp2,* was over-expressed by generating *hsp*-Gal4/UAS-*mesp-ba* transgenic line and expression of *ripply was* observed. The embryos showed severed posterior body defects (Figure 7C) compared to the control (Figure 7A). The *mesp-ba* was ubiquitously expressed in the whole embryo (Figure 7D) while the control embryos showed normal *mesp-ba* expression (Figure 7B). The *ripply1* expression was uniformly expressed throughout the somites at the anterior PSM but no significant upregulation was visible at the posterior PSM (Figure 7H) as compared to its normal expression (Figure 7E). The expression of *ripply2* on the other hand, was unchanged in these induced embryos (data not shown). This indicates that similar to mouse, zebrafish *rippy1* is regulated by mesp at least in the anterior PSM. However, mesp is not sufficient to regulate ripply1 at the posterior PSM. Some other factor, for instance FGF, etc may be involved in the regulation of *ripply* in the posterior PSM. While in contrast to mouse, ripply2 may be differently regulated by some other factor.

Downregulation of Tbx6 anterior domain in *hsp*-Gal4/UAS-*mesp-ba* embryos is compensated by *ripply1* knockdown

To observe if this induction of *mesp-ba* and upregulation of *ripply1* has any

affect on Tbx6, I also checked the expression patterns of *tbx6* mRNA and Tbx6 protein in the hsp-Gal4/UAS-*mesp-ba* embryos. The anterior domain of Tbx6 protein (Figure 7J) and *tbx6* mRNA (Figure 7I) were significantly downregulated, however the posterior domain of both were unchanged when compared to the control embryos (Figure 7G, 7F) respectively. Previous studies have also shown that ripply proteins suppress Tbx-mediated transcription of *mesp* genes by recruiting the Groucho/TLE co-repressor (Kawamura et al., 2008, Kondow et al., 2007) indicating that this downregulation of Tbx6 at the anterior PSM may be a result of *ripply1* upregulation. In order to confirm that ripply is actually required for Tbx6 elimination caused by mesp, I knockdown *ripply1* in the *hsp*-Gal4/UAS-*mesp-ba* embryos and checked the expression of Tbx6 protein. As expected, the anterior Tbx6 domain, which was eliminated in the *hsp*-Gal4/UAS-*mesp-ba* embryos (Figure 7M), was rescued on injecting *ripply1* MO (Figure 7N). No significant difference was observed between the wild type embryos injected with *ripply1* morpholino (Figure 7L) and the *hsp*-Gal4/UAS-*mesp-ba* embryos injected with *ripply1* morpholino (Figure 7N).

High Tbx6 / low FGF signaling zone requisite for *ripply* expression in zebrafish embryos

Because *ripply1* and *ripply2* were necessary and sufficient for reducing the level of Tbx6 proteins, an understanding of the regulation of their expression would be important for also understanding the mechanism of the boundary

formation of somites. Tbx6 is a positive regulator in this regulation because the expression of *ripply1* and *ripply2* is lost in *tbx6/fss* mutant zebrafish embryos (Kawamura et al. 2005 and data not shown). In contrast, since the Tbx6 domain is posteriorly shifted in mouse embryos defective in the FGF receptor 1 (Oginuma et al. 2008), it seems plausible to consider that FGF signaling may negatively regulate *ripplys'* expression in the PSM. To test this possibility, I examined Tbx6 domain and *ripply1* expression in zebrafish embryos treated with SU5402, a chemical inhibitor of FGF signaling. As predicted, SU5402 treatment caused a posterior shift in the Tbx6 domain in zebrafish embryos (Figure 8C and 8D), although the expression of *tbx6* mRNA was not obviously changed by this treatment (Figure 8A and 8B). Furthermore, SU5402 treatment also caused a posterior shift in *ripply1* expression (Figure 8E-8G). Thus, this treatment hastened the onset of *ripply1*, indicating FGF signaling was required for suppression of *ripply* expression in the posterior PSM.

Recently, the anterior border of FGF/pERK activity was shown to shift posteriorly in a stepwise manner during a single segmentation cycle in zebrafish embryos (Akiyama et al. 2014). Because this border corresponds to future somite boundary, it was proposed that the positioning of prospective somite boundary is already defined at this border of FGF signaling. If this is the case, it should be interesting to understand the process in which this stepwise shift of FGF signaling border leads to the stepwise shift of the Tbx6 domain, especially in terms of regulation of *ripply* expression. Thus, I next examined the spatio-temporal

activation of FGF/pERK and compared it with the position of the Tbx6 domain (Figure 8H-8J) and *ripply* expression as well. The anterior border of FGF signaling, monitored with anti-phosphorylated-ERK antibody (Akiyama et al., 2014), was positioned posterior to that of the Tbx6 domain in all of embryos examined. As far as my observation, the gap between these 2 borders changed almost within 1 to 2 segment lengths during a segmentation cycle. Comparing these results with the expression of *ripply1* and *ripply2* shown in Figure 4H-4J, 4K-4M, I concluded that the initial or most posterior expression of *ripply1* and *ripply2* was observed in this gap region (Figure 4H-4J, 4K-4M, Figure 8H-8J, Figure 9), indicating that expression of the *ripply* genes was primarily established within the region where the level of Tbx6 was high and that of FGF signaling was low. Thus, a state with high Tbx6 protein and low FGF signaling is likely to be requisite for *ripply* expression; and periodical activation of *ripply* genes in the high Tbx6 / low FGF signaling zone appears to have caused elimination of Tbx6 proteins in this zone and subsequent positioning of the intersomitic boundary.

Discussion

Anterior border of Tbx6 is important for segmentation boundary formation even in zebrafish

In mouse, the Tbx6 anterior border has been demonstrated to be important for segmentation boundary formation by regulating Mesp2 (Yasuhiko et al., 2006). Similarly, zebrafish *tbx6/fss* mutants do not form segmentation boundaries (van Eeden et al., 1996) and zebrafish *mesps* are not expressed in these mutants (Nikaido et al., 2002, Oates et al., 2005, data not shown). In order to understand the mechanism of segmentation boundary formation in zebrafish and to test if Tbx6-mediated somite boundary formation is conserved in vertebrates, I used zebrafish Tbx6 antibody to show that the function of Tbx6 is whether or not conserved in zebrafish and mouse. Similar to mouse, the anterior border of zebrafish Tbx6 also coincides with the presumptive boundary as observed by co-staining with *mesp* genes (Figure 4A-4G, data not shown), and the Tbx6 domain also undergoes phases of oscillation similar to *her1* expression (Figure 3). This indicates that the anterior border of Tbx6 domain is important for somite boundary positioning even in zebrafish. I also observed that the expression pattern of *ripply* is also corresponsive with this Tbx6 anterior domain at different phases of oscillation indicating that ripply also determines the positioning of Tbx6 in zebrafish.

Mechanism of Ripply-mediated reduction in Tbx6 protein level

Creation of a discrete border of Tbx6 proteins in the anterior PSM was first reported in the mouse and was found to be essential for activation of *Mesp2* expression (Yasuhiko et al., 2006). The Tbx6 transcription factor directly binds to the Mesp2 enhancer and analysis of enhancer-specific knockout mouse showed diminished *Mesp2* expression and segmentation defect similar to that of *Mesp2*-null mouse (Yasuhiko et al., 2008). The anterior domain of the *Mesp2* expression domain also accords with the anterior border of Tbx6 domain (Oginuma et al., 2008). Previous studies have demonstrated the periodical expression of Mesp2 transcription factor in the anterior PSM and that it is required both for segmental border formation and establishment of the rostro-caudal patterning within a somite (Morimoto et al., 2005, Takahashi et al., 2000). Since the expression of *Mesp2* requires Tbx6, this border accordingly defines the expression domain of *Mesp2*, which specifies the rostral side of a somite (Yasuhiko et al., 2006). Therefore, the creation of the anterior border of the Tbx6 domain has been considered to be a crucial process in the positioning of the segmentation boundaries of somites. Here, using zebrafish eggs as an assay system, I showed that both mouse and zebrafish Ripply could act in reducing the Tbx6 protein level to define the segmentation boundary. I also showed that physical interaction between Tbx6 and Ripply appears to be required for this reduction, because a mutant form of Ripply that could not interact with Tbx6 was not able to cause this reduction.

Interestingly, the reduction in the Tbx6 protein level in the PSM appeared to be regulated in a ubiquitin-proteasome-dependent manner, because mouse embryos treated with chemical inhibitor of proteasome, MG132, exhibit anterior expansion of the Tbx6 domain (Oginuma et al., 2008). Thus, it seems highly plausible that a ubiquitin-proteasome machinery is involved in the Ripply-mediated reduction of Tbx6 protein level. Given that Ripply family proteins are relatively small, consisting of about 100 amino acids (Kawamura et al., 2005), and do not possess similarity to any component of ubiquitin-proteasome machineries known to us, it is likely that some other component directly or indirectly involved in the ubiquitin-proteasome machinery may associate with the Tbx6-Ripply protein complex. At present, it is uncertain if such a molecule is actually involved in the determination of the Tbx6 domain; but further extensive analysis, for instance, screening and identification of Ripply-associated molecules, should make it clear.

Mechanism of boundary positioning and rostro-caudal patterning in zebrafish somitogenesis

Previously, 2 different functions of Ripply were proposed with respect to the regulation of Tbx6 during somite segmentation. One of them is a reduction in the Tbx6 protein level (Takahashi et al., 2010); and the other, suppression of the transcriptional activity of Tbx6 by recruiting the co-repressor Groucho/TLE to Tbx6 (Kawamura et al. 2005, Kawamura et al., 2008, Kondow et al., 2006,

Kondow et al., 2007, Hitachi et al., 2009). In the mouse, our group previously showed that the level of the Tbx6 protein, but not that of its mRNA, is specifically affected in *Ripply1/Ripply2* double mutants (Takahashi et al., 2010). A mathematical modeling based on this finding strongly suggests that Ripply's role in Tbx6 expression can be more suitably explained by its function in protein reduction rather than that in transcriptional regulation. Rather, our previous studies with culture cells showed a function of zebrafish ripply in transcriptional regulation of Tbx6. On the other hand, it had been unclear until now whether Ripply may play the same role in the somite segmentation in another animal such as the zebrafish.

In this study, by generating anti-zebrafish Tbx6 antibody, I showed that a presumptive somite boundary was created at the anterior border of the Tbx6 domain. Moreover, analysis with *ripply1* and *ripply2* double-deficient embryos indicated that Tbx6 protein level was specifically reduced. Hence Tbx6 was negatively regulated by both *ripply1* and *ripply2* Further analysis in *hsp*-Gal4/UAS-*mesp-ba* showed that the downregulation of Tbx6 in *hsp*-Gal4/UAS-*mesp-ba* embryos was an outcome of the upregulation of *ripply1,* and hence knockdown of *ripply1* in these embryos could recover the Tbx6 domain. These results strongly support the idea that reducing Tbx6 protein expression may be the major function of Ripply even in the zebrafish, although we cannot exclude the other possibility that Ripply-mediated transcriptional regulation may also play a role.

Given that Ripply is a regulator that defines the anterior border of the Tbx6 domain in both the mouse and the zebrafish, one of the critical processes in the positioning of the somite boundary should be the regulation of Ripply expression. In the mouse, the expression of Ripply1 and Ripply2 in the PSM is dependent on Mesp2, because expression of these Ripplys is lost in Mesp2 null-mutant embryos (Takahashi et al., 2010). Here, I showed that in zebrafish, *mesp-ba* can activate *ripply1* expression at least in the anterior PSM but some other factor may be required to regulate it at the posterior PSM (Figure 7H) while on the other hand, *ripply2* was not significantly altered (data not shown). This indicates that unlike mouse, *ripply1* and *ripply2* are differently regulated in zebrafish.

At present, it is uncertain whether this regulation between Ripply and Mesp is conserved even in zebrafish. Especially, since the period of segmentation is shorter in zebrafish somitogenesis (20 to 30 min) than in the mouse one (120 min), zebrafish may require a more speedy interaction for the generation of each boundary.

Another important point for understanding the mechanism of the boundary positioning is how the temporal information created by the oscillation affects the timing of *ripply* expression and Tbx6 border formation. In the mouse, the combination of the oscillatory changes in both Notch and FGF signalings determines the onset of *Mesp2* expression in the anterior PSM (Niwa et al., 2011). Since the activation of *Ripply1* and *Ripply2* expression and subsequent definition of the Tbx6 protein border is dependent on this *Mesp2* expression in the mouse,

the Mesp2/Ripply/Tbx6-mediated machinery converts the oscillation into the boundary positioning (Takahashi et al., 2010). In the zebrafish, in addition to the uncertainty of involvement of *mesp* genes, Notch signaling does not seem to be required for induction of *ripply* expression, but is needed for proper patterning of it, since *ripply1* and *ripply2* are still expressed in Notch-defective embryos in spite of impaired pattern of expression (Kawamura et al. 2005). On the other hand, we showed that FGF signaling negatively regulates expression of *ripply1* and *ripply2* in the zebrafish, similarly as in the mouse. Expression of *ripply1* and *ripply2* was induced in the high Tbx6 protein / low FGF signaling zone. Because the anterior border of FGF activity is changed in a step-wise fashion in the zebrafish (Akiyama 2014), retreat of the FGF border may regulate the onset of *ripply1* and *ripply2* expression. On the other hand, given that *ripply1* and *ripply2* expression shift from caudal to rostral part of a somite (Figure 9), it seems plausible that some oscillatory molecule, but not Notch signaling itself, regulates the expression of *ripply1* and *ripply2* in this zone in the zebrafish. Further extensive studies should reveal the similarity and/or diversity in the mechanism underlying the positioning of intersomitic boundary between zebrafish and mouse, and identify the core and conserved process resulting in the boundary positioning.

Finally, I would like to note that the pattern of Tbx6 proteins, which is observed in this study, may provide a clue for understanding the mechanism of the rostro-caudal patterning within a somite. In addition to the lack of somite boundaries, *tbx6/fss* zebrafish mutants display caudalization of the somites.

However, this caudalization phenotype has not yet been well explained because *tbx6* mRNA is widely expressed in the anterior PSM. Interestingly, we also found that Tbx6 proteins remain for a while at the rostral side of a presumptive somite, forming the “upper band.” Given that *mesp-ba* expression is dependent on Tbx6 even in zebrafish, the persistent Tbx6 proteins seem to be important for rostralization, because it is likely that their presence results in rostral-specific enhancement of *mesp-ba* expression.

Figure legends

Figure 1. Somite boundary formation in zebrafish and mouse.

(A) Zebrafish embryo at 18 somite stage with indications of the PSM and somites. A schematic diagram demonstrating the somite boundary formation in zebrafish and mouse as indicated. The several molecular factors and signaling pathways involved in the program are shown in different colors in the diagram and are indicated accordingly. In zebrafish, the presumptive somite boundary defined by expression of the earliest/newest *mesp* band is observed at the S-II region while in mouse, it is observed at the S-I region. The regulations and molecular interactions involved in segmental border formation in mouse are shown in different phases. In the phase I, Notch active domain on reaching the anterior PSM, activates the expression of *Mesp2* at the Tbx6 positive and pERK negative region. In phase II, Mesp2 protein activates *Ripply2.* In phase III, Ripply suppressed Tbx6 expression at the anterior PSM in the Mesp2 expressing domain. pERK signaling is also seen to oscillate in the PSM. (B) Molecular interactions between Notch, Tbx6, Mesp2 and Ripply in mouse somite boundary formation. The Notch signaling and Tbx6 positively regulate *Mesp2.* Mesp2 activates *Ripply1/2* and Ripply1/2 are responsible for downregulation of Tbx6 mediated *Mesp* transcription. Mesp2 also suppresses Notch activity (Morimoto et.al., 2005).

Figure 2. Periodic expression of Tbx6 protein and post

transcriptional regulation of its anterior border.

(A) The specificity of newly generated antibody against zebrafish Tbx6 was confirmed by western blotting analysis. Lysates of 293T cells expressing either zebrafish Tbx6 tagged with FLAG peptide at C terminus or vector only, were loaded on the SDS-PAGE gel. Detection of Tbx6 was achieved by both antisera #1 and #2 of the antibody against zebrafish Tbx6 and also anti-FLAG antibody. Asterisks indicate the band size corresponding to Tbx6. (B-D) Immunostaining with anti-Tbx6 antibody (C) and *in situ* hybridization with *tbx6* probe (B) were performed using zebrafish embryos at the 8 somite stage (*n*=15). Merged image (D) combined (B) and (C) is also shown. Zebrafish *tbx6* mRNA is expressed broadly throughout the anterior PSM. At the same time, the Tbx6 protein is also expressed throughout the anterior PSM, however, its anterior border is restricted far posterior to the anterior border of the mRNA. The position of each segmental unit is also indicated from SIII to S-II. (E-G) Quantitative analysis of *tbx6* mRNA and protein expression in the PSM. Intensity of *tbx6* mRNA signals in a boxed area in the PSM (E; the boxed area shown in (B) is indicated by 90° rotation) and protein signals in the corresponding area (F; the boxed area shown in (C) is indicated by 90° rotation) was scanned and indicated by green and magenta lines, respectively, in (G). While *tbx6* mRNA is gradually decreased in the anterior region (shown by dark green line), Tbx6 protein level is abruptly decreased (shown by red line). Anterior is left and posterior is right. (H-J) Indication of 3 typical patterns of embryos stained with anti-Tbx6 antibody. Embryos were

observed at 8 somite stage Comparative analysis with *her1* mRNA expression shown in Figure 3 indicates that the anterior border of the Tbx6 protein follows a phase of periodic change. After a long core domain of Tbx6 proteins is generated (H), an anterior part of the Tbx6 protein domain was eliminated, resulting in appearance of the "upper band", which is indicated by an arrowhead (I), then this upper band disappeared, resulting in a short Tbx6 domain (J). Out of a total of 154 embryos examined, around 40% of them showed (H), 35% showed (I), 25% showed (J) type of expression pattern. (K) A 10 somite stage embryo injected with both *her1* and *her7* specific antisense morpholino oligo was stained with anti-Tbx6 antibody. The defects were observed in 97.5% of the injected embryos (*n*=40). (L) A 10 somite stage embryo treated with DAPT, a Notch inhibitor, was stained with anti-Tbx6 antibody. The defects were observed in all of the embryos treated with DAPT (*n*=22). Pattern of Tbx6 proteins was disturbed in anterior area indicated by a bracket (K, L). The yellow dotted lines indicate S-II (H, I) and S-II and S-III (J) regions.

Figure 3. Comparative analysis of the anterior border of the Tbx6 domain with expression of *her1*

Spatial pattern of the Tbx6 protein (A'-C'; magenta) is compared with those of *her1* mRNA expression (A-C; green) at 3 different phases of segmentation cycle at around the 8 somite stage. Merged images are also indicated (A"-C"). According to the general nomenclature (Pourquie and Tam, 2001), the phases shown in A, B and C appear to correspond to the phase III, I and II, respectively.

(B-B") Anterior Tbx6 starts to disappear with some remains (the "upper band": arrowhead) (B'). (C-C") The upper band of Tbx6 disappears and the next Tbx6 anterior border shifts posteriorly. (A-A") The core domain extends posteriorly. Out of a total of 42 embryos examined, around 35% of them showed A type, 41% showed B type, 24% showed C type of expression. The dotted lines indicate S-II (A", B") and S-II, S-III (C") regions.

Figure 4. Comparative analysis of the anterior border of the Tbx6 domain with *mesp* and *ripply* gene expressions

Spatial pattern of the (magenta) Tbx6 protein is compared with those (green) of *mesp-ab* mRNA (A-D), *mesp-ba* mRNA (E-G) and *ripply1* mRNA (H-J), *ripply2* mRNA (K-M) at 3 different phases of segmentation cycle (the phases in embryos shown in A, B or C are identical to those shown in E, F or G and H, I or J; and K, L or M, respectively) at around the 8 somite stage. Tbx6 pattern was also compared with *mesp-ab* mRNA in embryos treated with DAPT (D). Merged images are indicated (A"-G"). Out of a total of 59 embryos examined, around 46.5% of them showed A and E phase, 27% showed B and F phase, 26.5% showed C and G phase types of expression. Note that the anterior limit of newly expressed, or most posterior band, of *mesp-ab* and *mesp-ba* bands coincided with the anterior border of the Tbx6 core domain (A-A"; E-E"). Then these expression bands coincided with the "upper band" of Tbx6 protein, when elimination of the anterior Tbx6 domain has started (B-B"; F-F"). On the other hand, new/

posteriormost *ripply1* and *ripply2* expression emerged within the anterior part of Tbx6 domain (H-H"; K-K") and the Tbx6 domain starts to reduce in areas where *ripply1* and *ripply2* were expressed (I-I"; L-L"). (C-C") Expression patterns of *mesp-ab* and (G-G") *mesp-ab* are sharper and more distinct towards anterior compartment of the newly formed somite, while (J-J") *ripply1* and (M-M") *ripply2* expression patterns were expanded into almost full somite length. In (D), the defects were observed in all of the embryos treated with DAPT (*n*=22). Patterns of Tbx6 proteins and *mesp-ab* mRNA were disturbed in anterior area indicated by a bracket. The dotted lines indicate S-II (A", B", E", F", H", I", K", L") and, S-II and S-III (C", G", J", M") regions. White arrowheads indicate the upper band of Tbx6 protein.

Figure 5. Proper positioning of Tbx6 domain depends on ripply.

(A-D) Patterns of Tbx6 protein at the 8 somite stage in control (A), *ripply1* morphant (B), *ripply2* morphant (C) and *ripply1/ripply2* double morphant (D). Comparison of Tbx6 protein (E', F') with the respective mRNA (E, F) patterns in control (E, E') and *ripply1/ripply2* double morphant (F, F'). *ripply1* morphants show graded expansion of Tbx6 protein anteriorly (B) but *ripply2* morphants (C) show no significant difference from control embryos (A). Double knockdown of *ripply1* and *ripply2* show strong expansion of Tbx6 protein anteriorly (D). In the double morphants, *tbx6* mRNA is also anteriorly expanded to some level, but not so significantly as Tbx6 protein (F, F'). A total of 20 injected embryos were

observed for each injection. While *ripply2* morphant appeared indistinguishable from control embryos in Tbx6 protein pattern, 100% of the *ripply1* morphants and 100% of the *ripply1* and *ripply2* double morphants displayed anterior expansion of Tbx6 protein shown in (B) and (D), respectively. The dotted lines indicate S-II (A, E") region.

Figure 6. Ripply can reduce Tbx6 protein level.

(A) Western blotting with proteins recovered from embryos injected with Flag tagged zebrafish *tbx6* mRNA *(ztbx6-Flag)* and Myc tagged zebrafish *ripply1* mRNA *(zripply1-Myc)*. Three hundred pg of *ztbx6-Flag* with or without 300pg of *zripply1-Myc* were injected into zebrafish eggs at 1 cell stage. (B, C) Similar experiments shown in (A) were performed with 150pg of Flag tagged mouse *Tbx6* mRNA (*mTbx6-Flag*) and 50pg of Myc tagged mouse *Ripply2* mRNA *(mRipply2-Myc)* (B) or 120pg of *zripply1-Myc* (C). Mouse Tbx6 proteins were reduced when *mTbx6-Flag* was co-injected with *mRipply2-Myc* (B) and also *zripply1-Myc* (C). (D) Similar experiments shown in (A) were performed with 300pg of *ztbx6-Flag*, and 300pg of wild-type or FPVQ-mutated form of *zripply1-Myc*. In FPVQ-mutated form, this 4 amino acid stretch was replaced with four alanines as previously described (Kawamura et al. 2008). (E) Similar experiments shown in (A) were performed with 150pg of *mTbx6-Flag* and 50pg of wild-type or FPIQ-mutated form of *mRipply2-Myc*. The FPIQ stretch in the mouse Ripply2 exists at the corresponding position to the FPVQ in the zebrafish

Ripply1. In FPVQ-mutated form, this 4 amino acid stretch was replaced with four alanines. When co-injected with the mutated forms of zebrafish *ripply1* or mouse *Ripply2*, the reduction of Tbx6 proteins was canceled. (F) Similar experiments shown in (A) were performed with 50pg of mouse Flag-tagged *Brachyury (T)* mRNA and 50pg of *mRipply2-Myc*. Mouse *Ripply2* also reduced mouse Brachyury protein level. As internal controls to validate the consistency between injection experiments, 100pg (B, C, E, F) or 200pg (A, D) of *GFP* mRNA was also injected and its expression was examined. (G, H) Co-immunoprecipitation assay using 293T cells (G) or Cos 7 cells (H) were performed to show that FPIQ/FPVQ site is important for physical interaction between Tbx6 and Ripply for Tbx6 protein reduction. The mutated forms of Ripply failed to co-precipitate with the either zebrafish (G) or mouse (H) Tbx6.

Figure 7. Overexpression of *mesp-ba* induces Tbx6 protein reduction via upregulation of *ripply;* Mesp is not sufficient to regulate Ripply in the posterior PSM in zebrafish;

Over expression of zebrafish *mesp-ba* using the *hsp*-Gal4/UAS-*mesp-ba* transgenic fish line resulted in ubiquitous expression of *mesp-ba* (D) when compared to (B). The embryos showed severed posterior body defects (C) while the control embryos grow normally at 24hpf (A). Anterior expression of *ripply1* was upregulated ubiquitously from the anterior PSM but not towards the posterior PSM (H). Both the mRNA (I) and protein (J) levels of Tbx6 showed defects in the

anterior regions while the posterior domains look normal. The control embryos show normal expression patterns of *mesp-ba* (B), *ripply1* (E), *tbx6* (F) and Tbx6 (G). (K-N) Morpholinos were injected to wild type (K-L) or *hsp*-Gal4/UAS-*mesp-ba* transgenic (M-N) embryos. The anterior reduction of Tbx6 protein expression in (M) was rescued by *ripply1* knockdown (N). White arrowheads indicate the anterior limit of Tbx6 protein domain.

Figure 8. FGF signaling is required for *ripply* suppression at the posterior PSM.

(A-F) Effect of SU5402, a chemical inhibitor against FGF signaling, on *tbx6* mRNA (A, B), Tbx6 protein (C, D), and *ripply1* mRNA (E, F) patterns in embryos at the 8 somite stage. Control embryos treated with DMSO (A, C, E) and embryos treated with SU5402 (B, D, F) are shown. While *tbx6* mRNA expression was unchanged (A, B), the anterior border of the Tbx6 domain was shifted posteriorly in SU5402 treated embryos when compared to control embryos at the same phase of the segmentation cycle (C, D). Note that both of these embryos are at the stage when Tbx6 proteins just started to be eliminated in the anterior domain. A total of 15 number set of embryos were observed each for A and B, and all of the treated embryos did not show any change in *tbx6* mRNA expression pattern when compared to control embryos. Another 32 number set of embryos were treated with SU5402 and examined for Tbx6 protein where, about 87% of the embryos showed posterior shift of anterior domain of Tbx6 protein when

compared to control embryos. (E, F) *ripply1* expression is initiated earlier (yellow arrowheads) in SU5402 treated embryos (F) when compared to control ones (E) at the same phase of the segmentation cycle. Asterisk indicates the position of the chordo neural hinge (CNH). (G) The distance of the anterior border of the posteriormost expression of *ripply1* from the chordo neural hinge in SU5402 treated embryos was significantly shorter than the control embryos; * $p<0.05$ (*n*=17 for control embryos and *n*=19 for SU5402 treated embryos). Error bars indicate standard deviation. (H-J) Spatial distribution of FGF/pERK signaling was examined during a segmentation cycle in comparison with Tbx6 protein domain at the 8 somite stage. The upper band of Tbx6 is indicated by a white arrowhead. The dotted lines indicate S-II (C, H", I") and, S-II and S-III (J") regions.

Figure 9. Schematic diagram of spatial patterns of Tbx6 and p-ERK domains with *ripply1*, *ripply2* and *mesp-ba* expressions during a single segmentation cycle.

The newest/posteriormost band of zebrafish *mesp-ba* expression is activated in the anterior border of the Tbx6 domain at the anterior PSM, and gets restricted to the rostral compartment of the somite. The *mesp-ba* expression is seen as a two or three band patterns depending on the phase. *mesp-ba* then activates *ripply1* in the Tbx6 high / FGF low signaling region while *ripply2* is also activated at the same time by some other factor(s). These activated ripplys appear to suppress Tbx6 protein at the anterior region of the Tbx6 domain resulting in formation of a new

anterior border of the Tbx6 core domain, and the “upper band”. Then, *ripply* expression domain expands anteriroly to a somite length causing complete elimination of the “upper band” of Tbx6 protein. At the same time, the anteriormost band of *mesp-ba,* which is restricted to the rostral compartment of S0 region, disappears and a new posterior band starts to appear at the newly generated anterior domain of Tbx6 at S-II.

Figure 10. Comparison of molecular mechanism of somite boundary formation between zebrafish and mouse

(A) Mouse segmentation is initiated when Notch active domain reaches the anterior PSM and activates *Mesp2* at Tbx6 high and FGF/pERK low region. Mesp2 then activates *Ripply1/2* and then Ripply1/2 suppressed Tbx6 at the protein level, shifting the anterior border of Tbx6 posteriorly by a somite length. This new anterior border of Tbx6 defines the next presumptive border. FGF/pERK domain is dynamic and represents a rippled wavefront from posterior to anterior PSM. (B) Zebrafish segmentation also starts at S-II at the anterior PSM, with activation of *mesp* in a Tbx6 dependent manner. Notch is required for proper synchronization of the oscillation but not for *mesp* regulation. The main regulator of *mesp* is unknown. Mesp can activate *ripply1* at least in the anterior PSM but not in the posterior PSM, while *ripply2* is regulated independently by some other factor(s). However, both *ripply1* and *ripply2* are required for proper termination of Tbx6 at the anterior border, and similar to mouse, the next position of Tbx6 protein

determines the next presumptive somite boundary. FGF/pERK shows a steep gradient from the posterior PSM/tail bud to the intermediate PSM, and its anterior limit between S-IV and S-V represents the future somite boundary.

Figure 1A

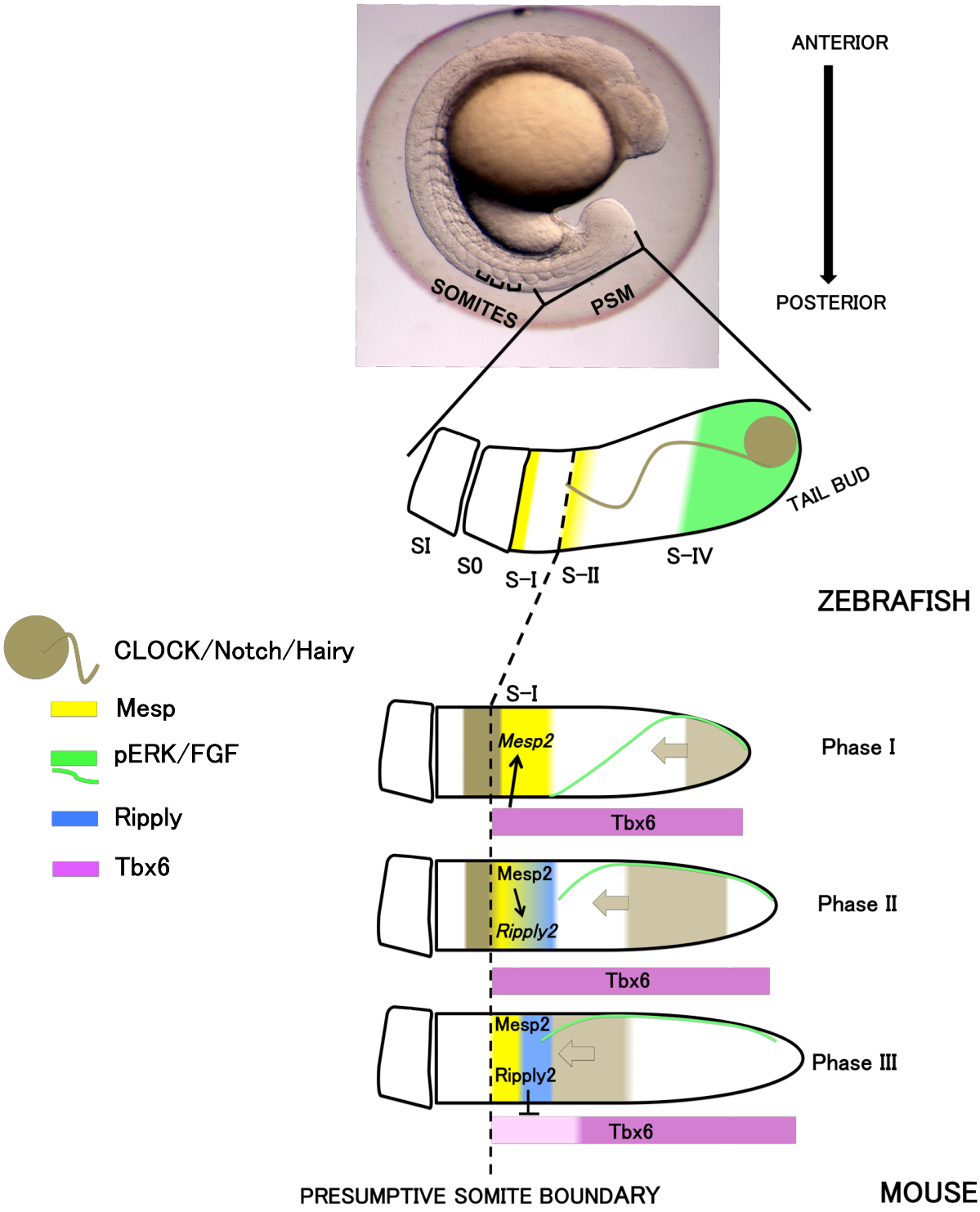
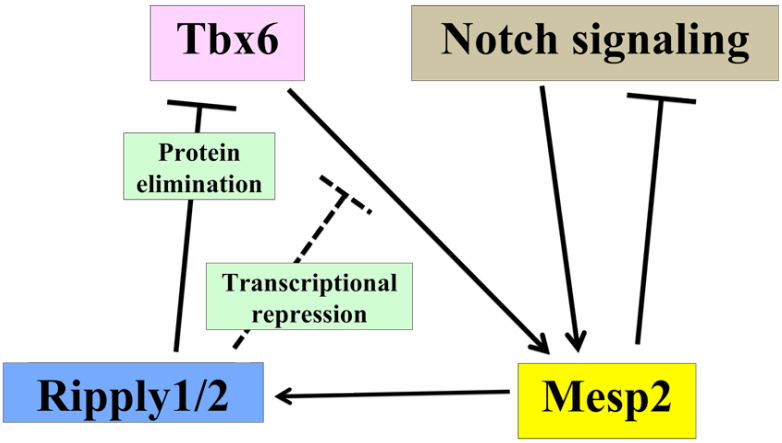

ANTERIOR
POSTERIOR
SOMITES
PSM
TAIL BUD
SI
S0
S-I
S-II
S-IV
ZEBRAFISH
CLOCK/Notch/Hairy
Mesp
pERK/FGF
Ripply
Tbx6
S-I
Mesp2
Tbx6
Phase I
Mesp2
Ripply2
Tbx6
Phase II
Mesp2
Ripply2
Tbx6
Phase III
PRESUMPTIVE SOMITE BOUNDARY
MOUSE

Figure 1B



Mouse

Figure 2A

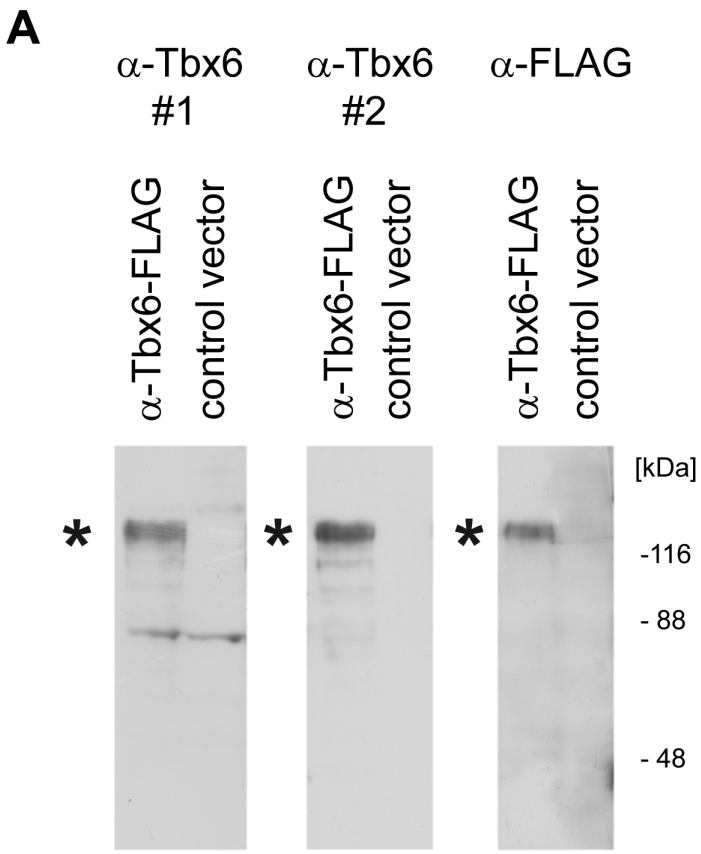

A
α-Tbx6 #1
α-Tbx6 #2
α-FLAG
α-Tbx6-FLAG
control vector
α-Tbx6-FLAG
control vector
α-Tbx6-FLAG
control vector
[kDa]
-116
- 88
- 48
*
*
*

Figure 2B-2L

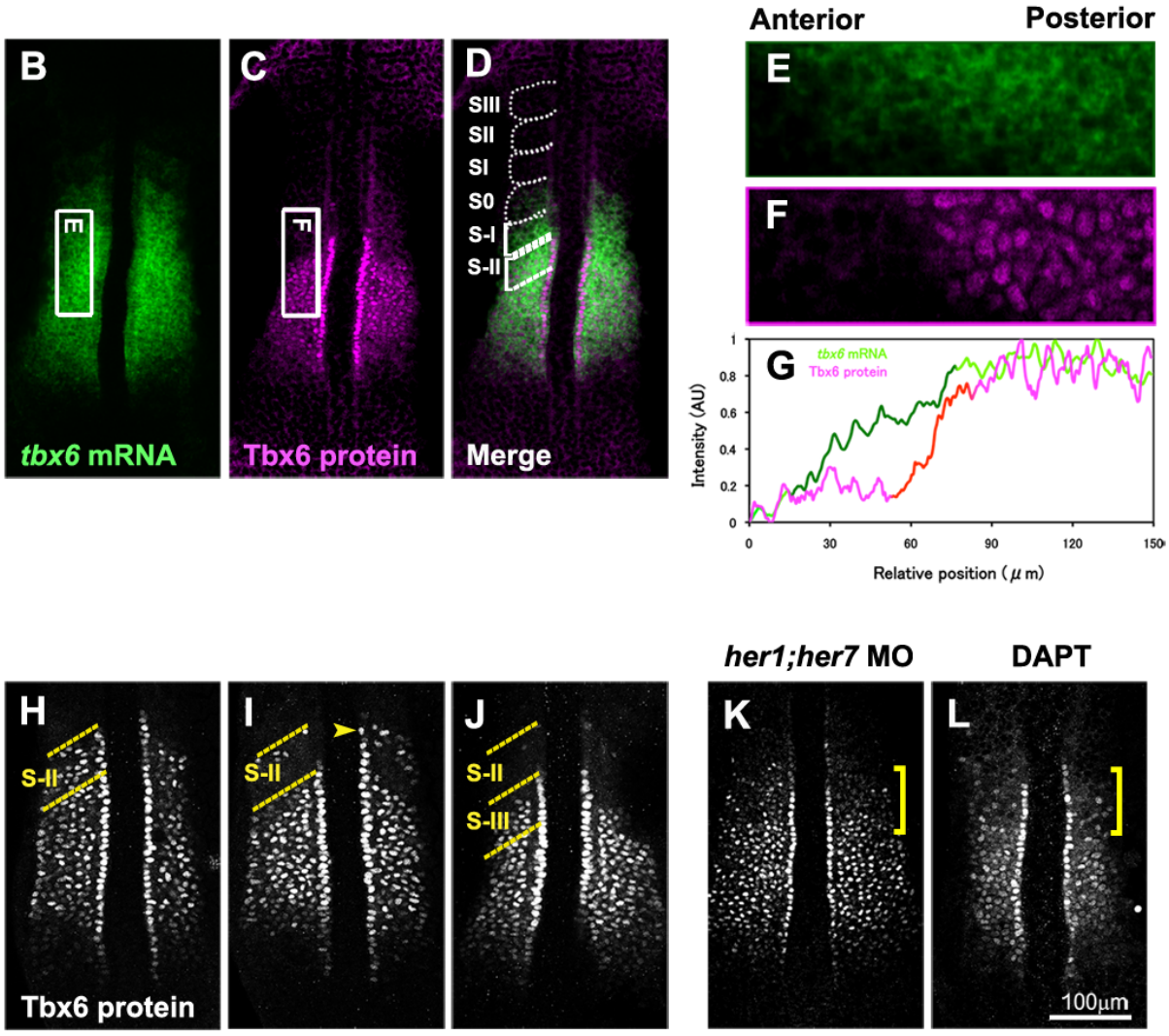

B
C
D
SIII
SII
SI
S0
S-I
S-II
tbx6 mRNA
Tbx6 protein
Merge
Anterior
Posterior
E
F
G
tbx6 mRNA
Tbx6 protein
Intensity (AU)
Relative position (μm)
H
I
J
S-II
S-III
Tbx6 protein
her1;her7 MO
DAPT
K
L
100μm

Figure 3

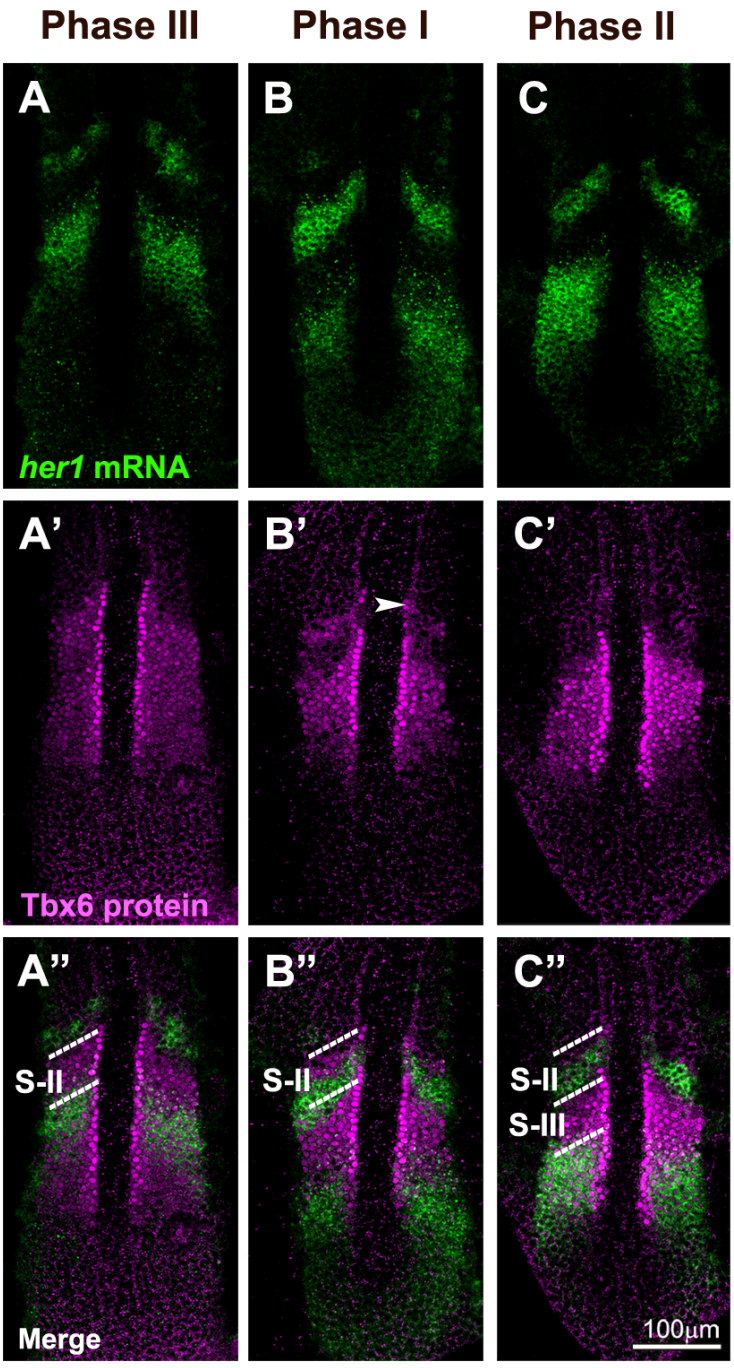

Phase III
Phase I
Phase II
A
B
C
her1 mRNA
A'
B'
C'
Tbx6 protein
A''
B''
C''
S-II
S-II
S-II
S-III
Merge
100μm

Figure 4A-4D

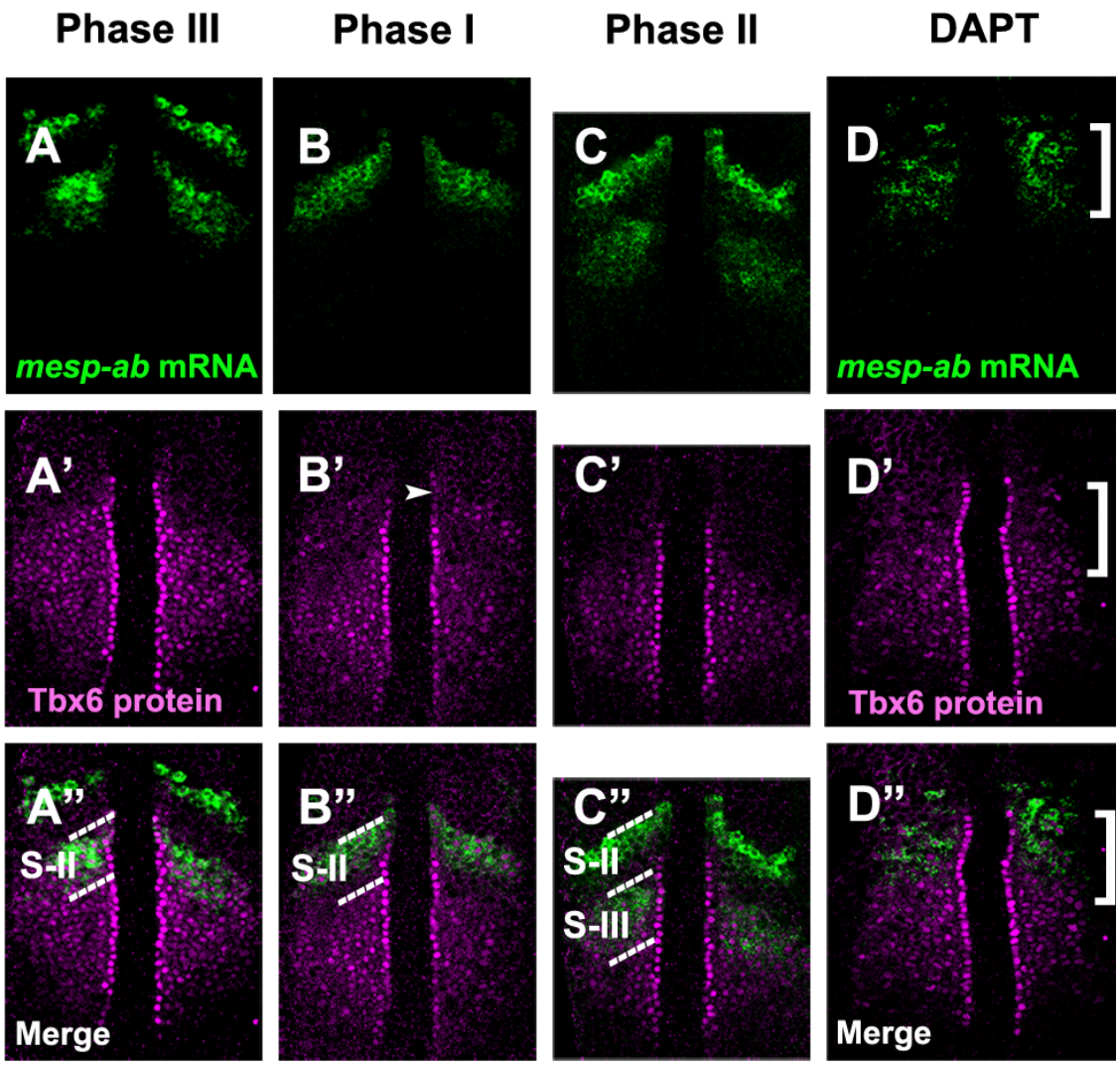

Phase III
Phase I
Phase II
DAPT
A
B
C
D
mesp-ab mRNA
mesp-ab mRNA
A'
B'
C'
D'
Tbx6 protein
Tbx6 protein
A''
B''
C''
D''
S-II
S-II
S-II
S-III
Merge
Merge

Figure 4E-4G

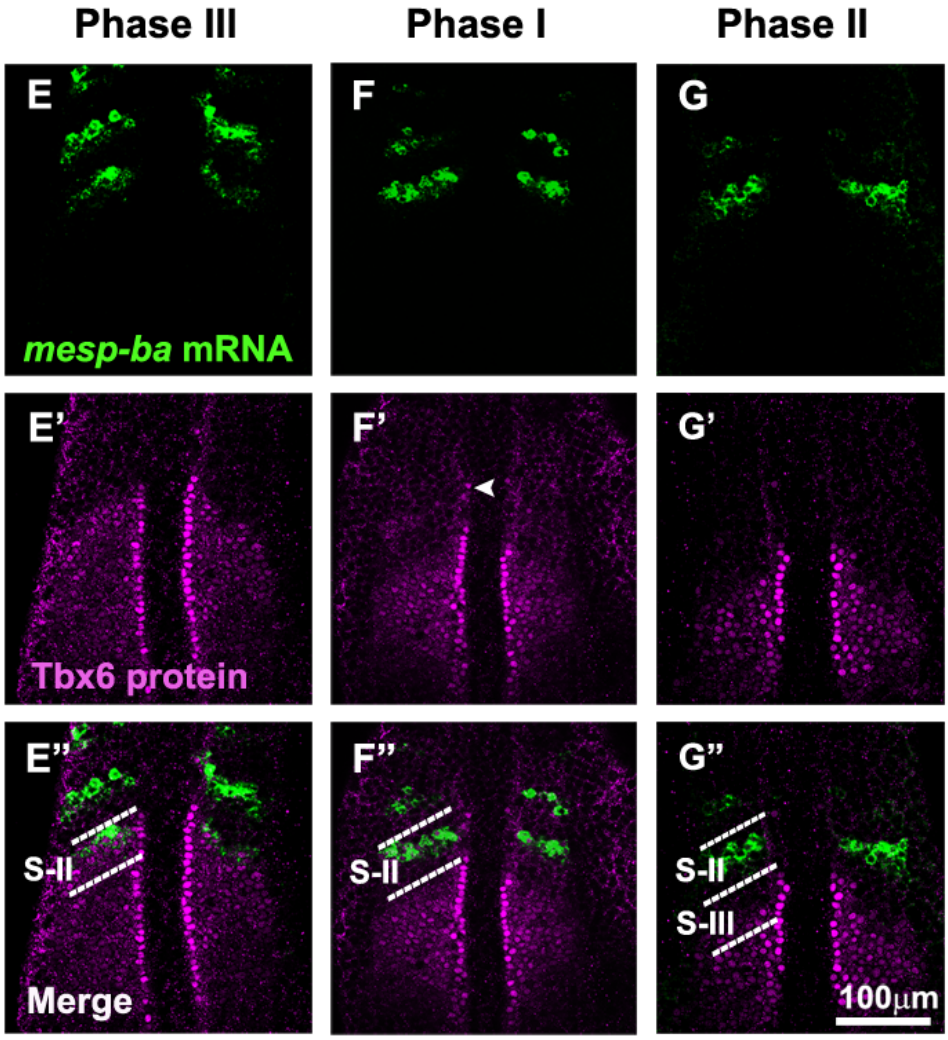

Phase III
Phase I
Phase II
E
F
G
mesp-ba mRNA
E'
F'
G'
Tbx6 protein
E''
F''
G''
S-II
S-II
S-II
S-III
Merge
100μm

Figure 4H-4J

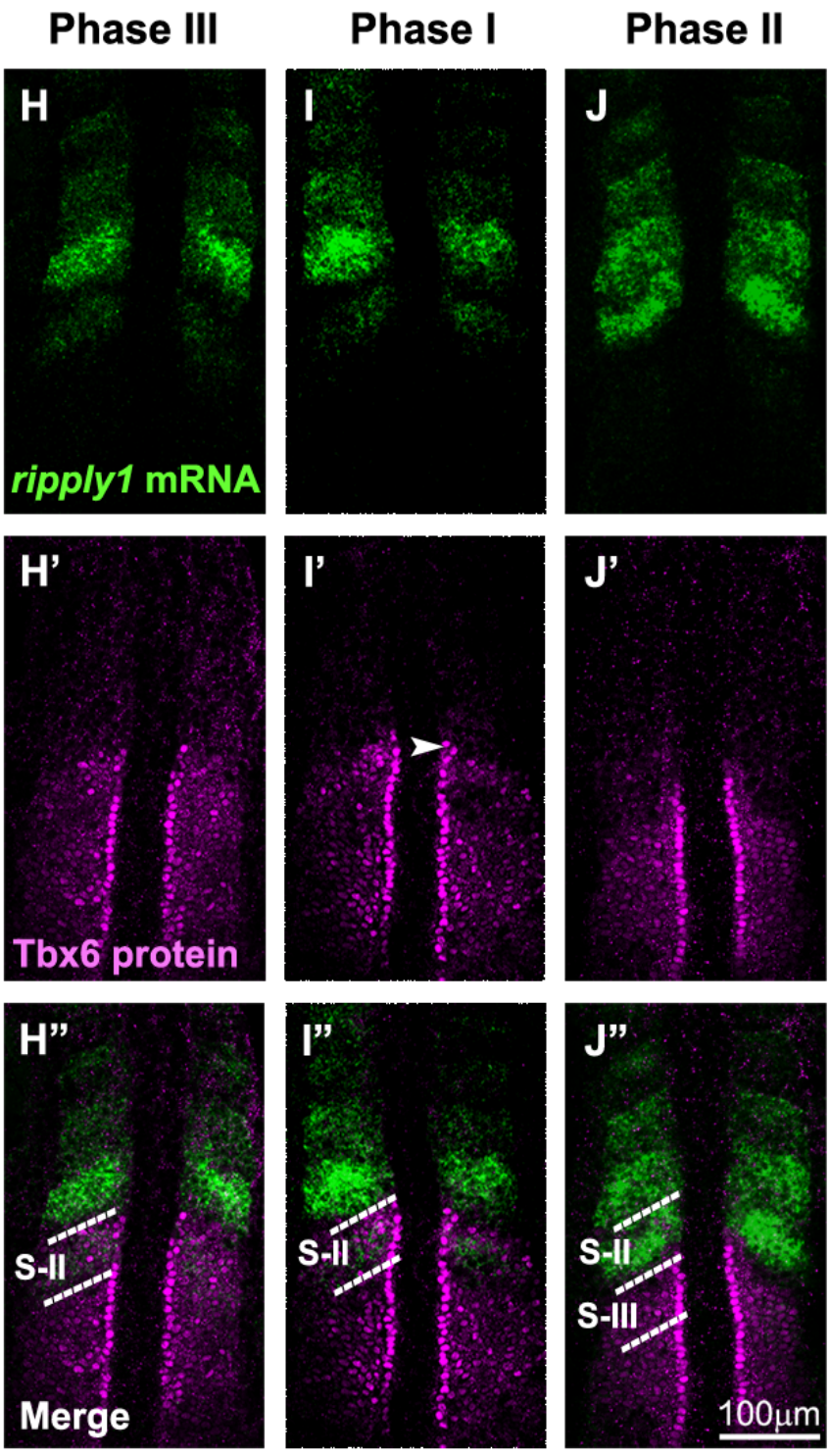

Phase III
Phase I
Phase II
H
I
J
ripply1 mRNA
H'
I'
J'
Tbx6 protein
H''
I''
J''
S-II
S-II
S-II
S-III
Merge
100μm

Figure 4K-4M

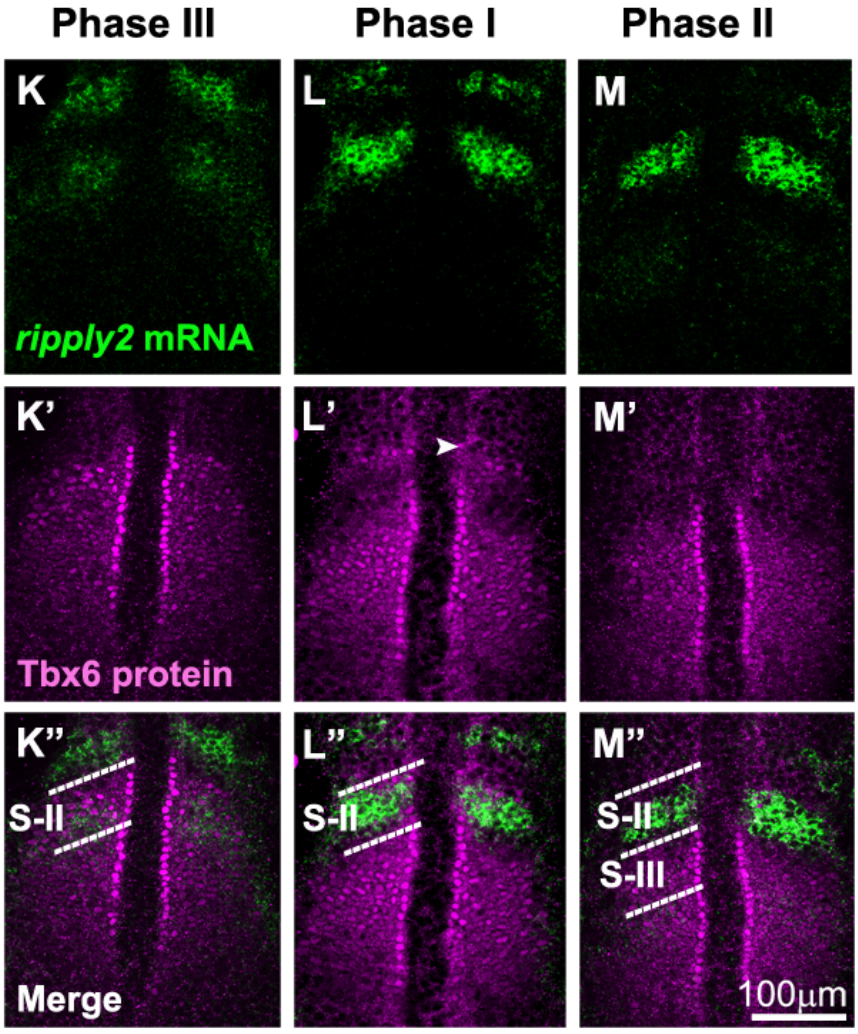

Phase III
Phase I
Phase II
K
L
M
ripply2 mRNA
K'
L'
M'
Tbx6 protein
K''
L''
M''
S-II
S-II
S-II
S-III
Merge
100μm

Figure 5

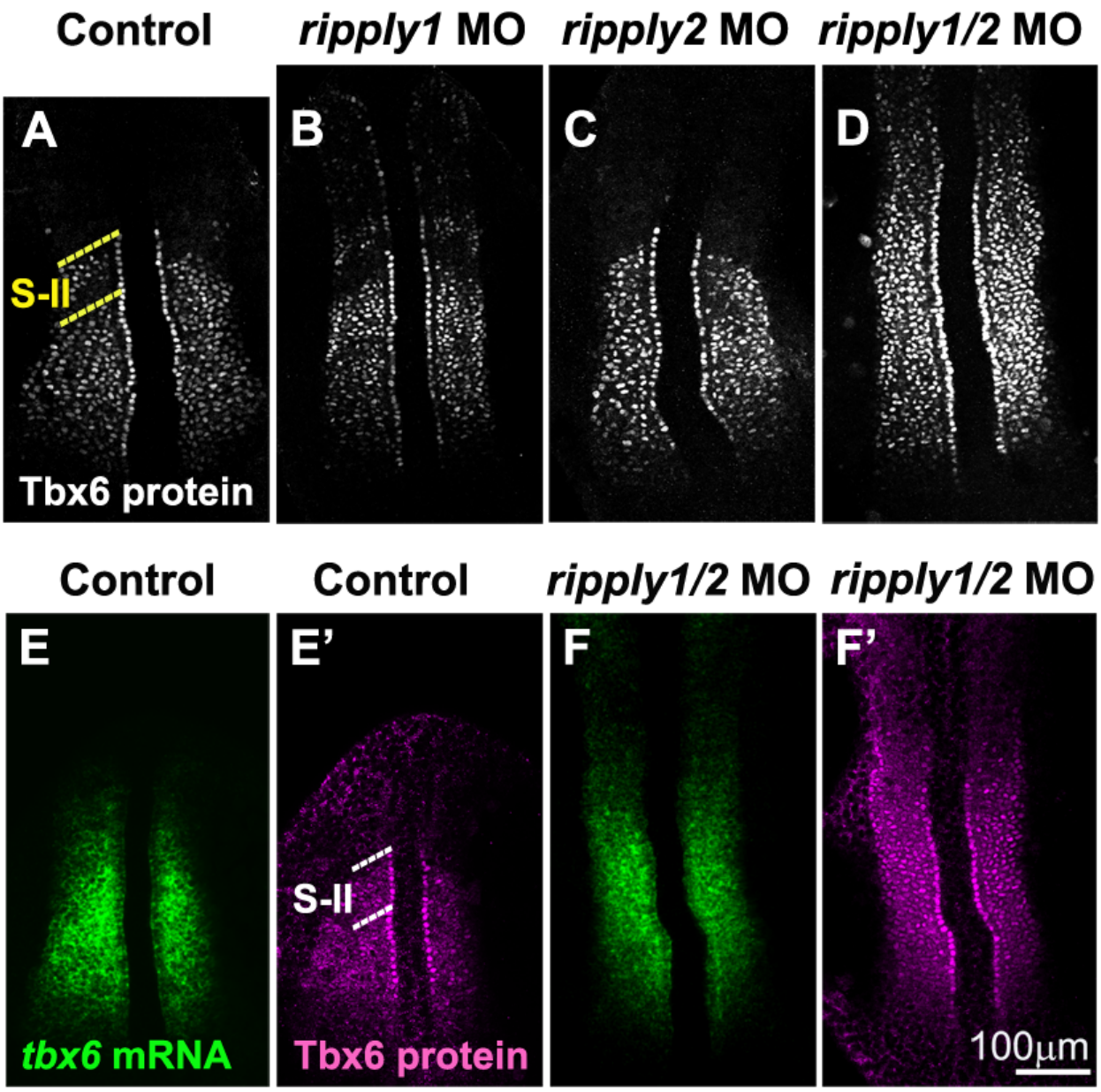

Control
ripply1 MO
ripply2 MO
ripply1/2 MO
A
B
C
D
S-II
Tbx6 protein
Control
Control
ripply1/2 MO
ripply1/2 MO
E
E'
F
F'
S-II
tbx6 mRNA
Tbx6 protein
100μm

Figure 6A-6F

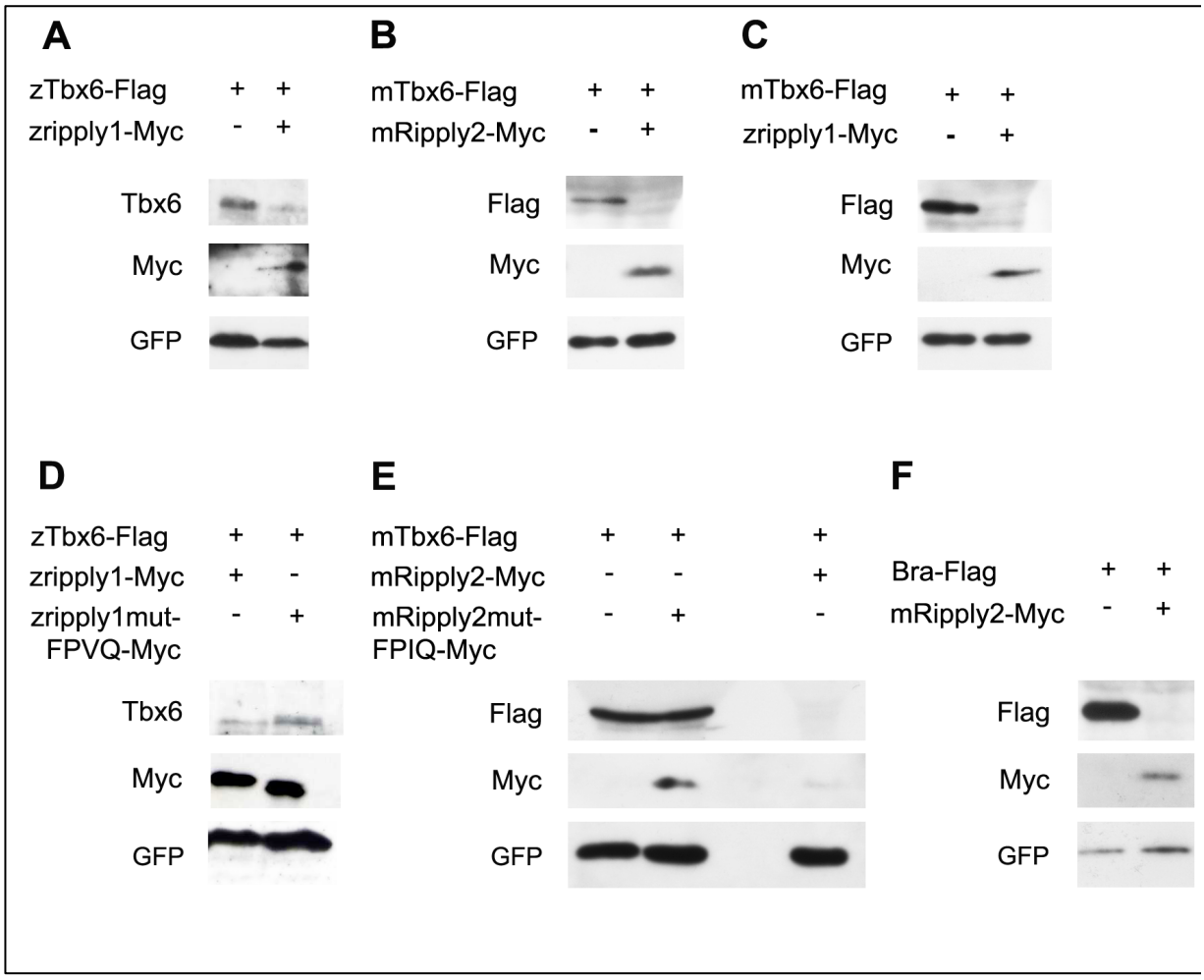

A
zTbx6-Flag + +
zripply1-Myc - +
Tbx6
Myc
GFP
B
mTbx6-Flag + +
mRipply2-Myc - +
Flag
Myc
GFP
C
mTbx6-Flag + +
zripply1-Myc - +
Flag
Myc
GFP
D
zTbx6-Flag + +
zripply1-Myc + -
zripply1mut-FPVQ-Myc - +
Tbx6
Myc
GFP
E
mTbx6-Flag + + +
mRipply2-Myc - - +
mRipply2mut-FPIQ-Myc - + -
Flag
Myc
GFP
F
Bra-Flag + +
mRipply2-Myc - +
Flag
Myc
GFP

Figure 6G-6H

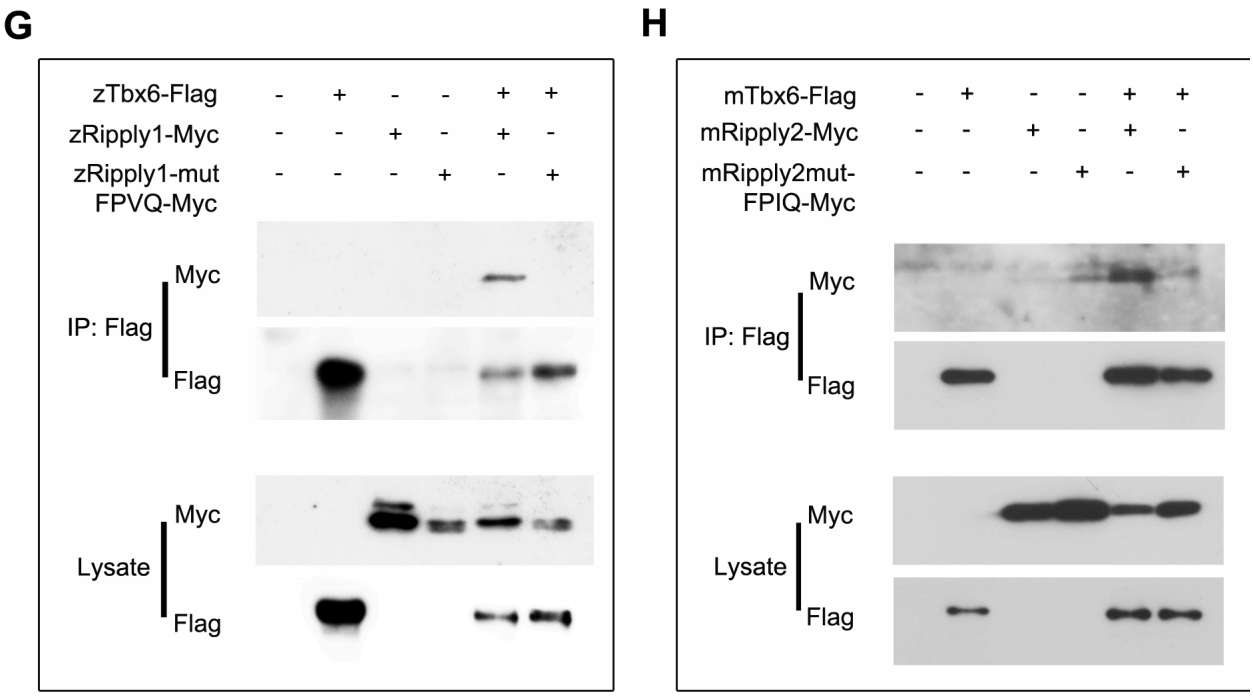

G
zTbx6-Flag - + - - + +
zRipply1-Myc - - + - + -
zRipply1-mut FPVQ-Myc - - - + - +
IP: Flag
Myc
Flag
Lysate
Myc
Flag
H
mTbx6-Flag - + - - + +
mRipply2-Myc - - + - + -
mRipply2mut-FPIQ-Myc - - - + - +
IP: Flag
Myc
Flag
Lysate
Myc
Flag

Figure 7A-7J

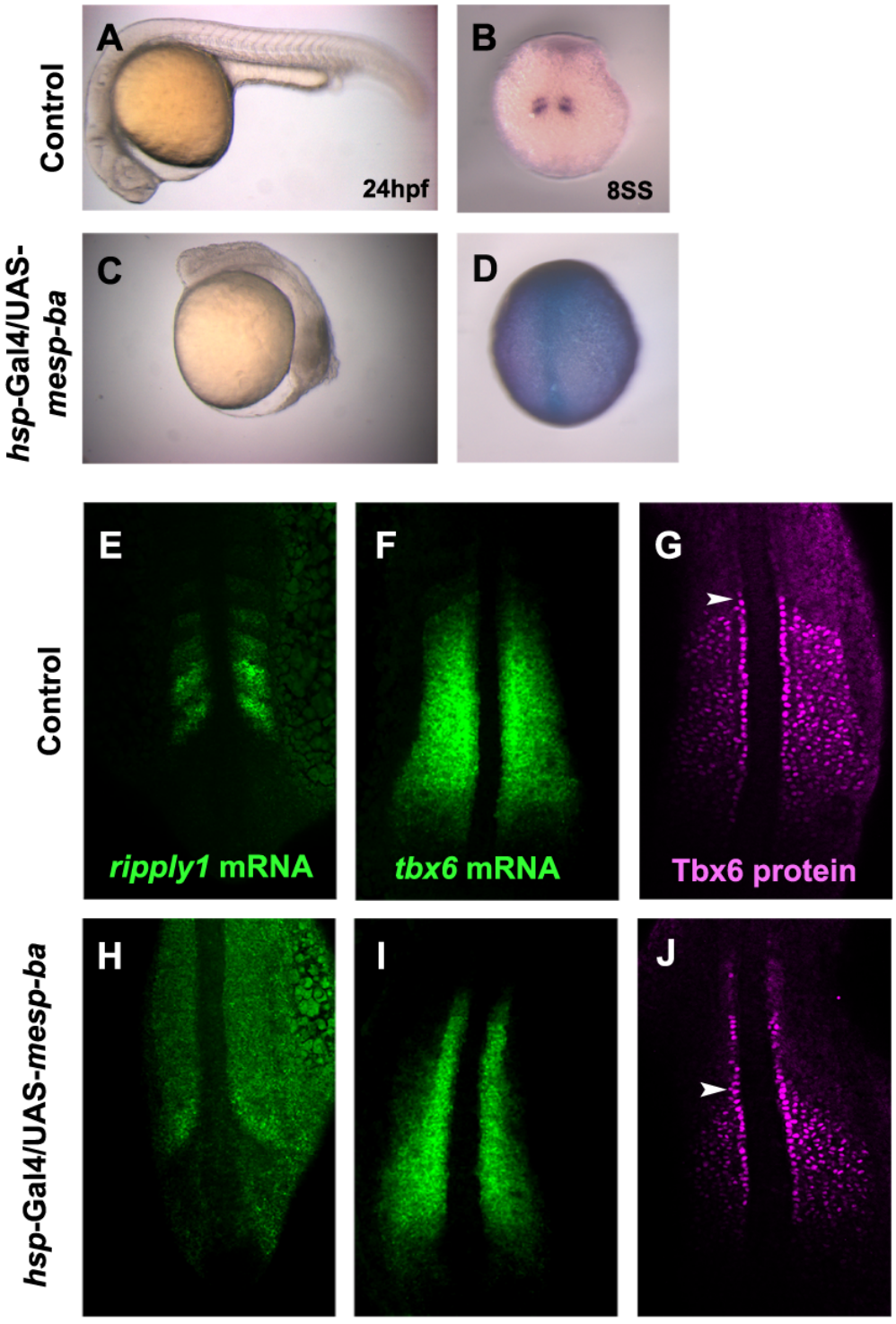

Control
A
24hpf
B
8SS
hsp-Gal4/UAS-mesp-ba
C
D
Control
E
ripply1 mRNA
F
tbx6 mRNA
G
Tbx6 protein
hsp-Gal4/UAS-mesp-ba
H
I
J

Figure 7K-7N

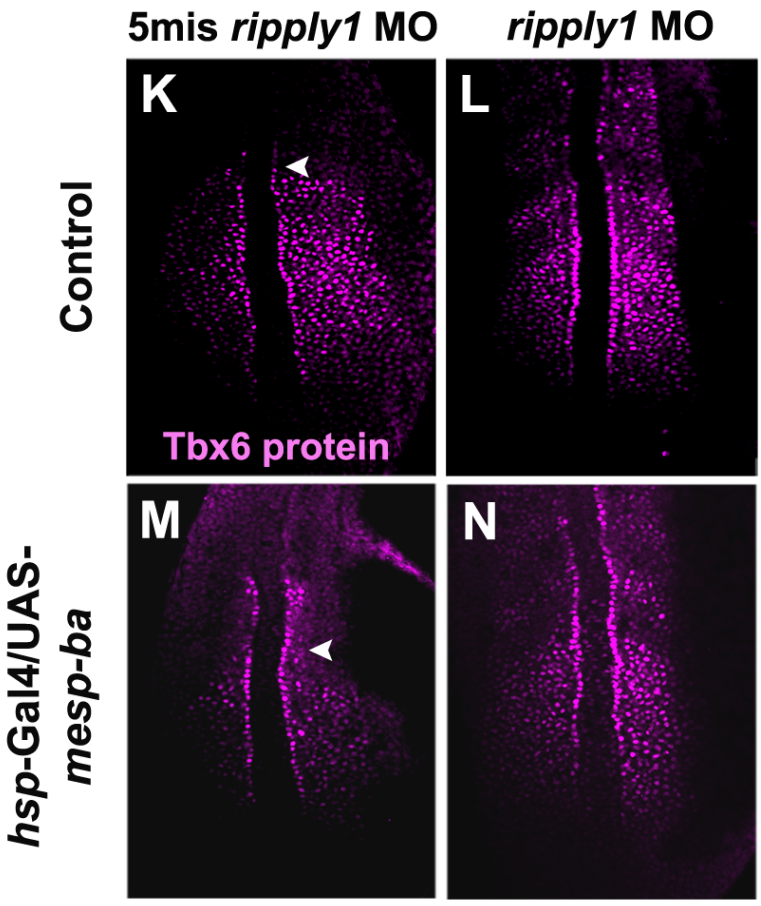

5mis *ripply1* MO
ripply1 MO
K
L
M
N
Control
hsp-Gal4/UAS-*mesp-ba*
Tbx6 protein

Figure 8A-8G

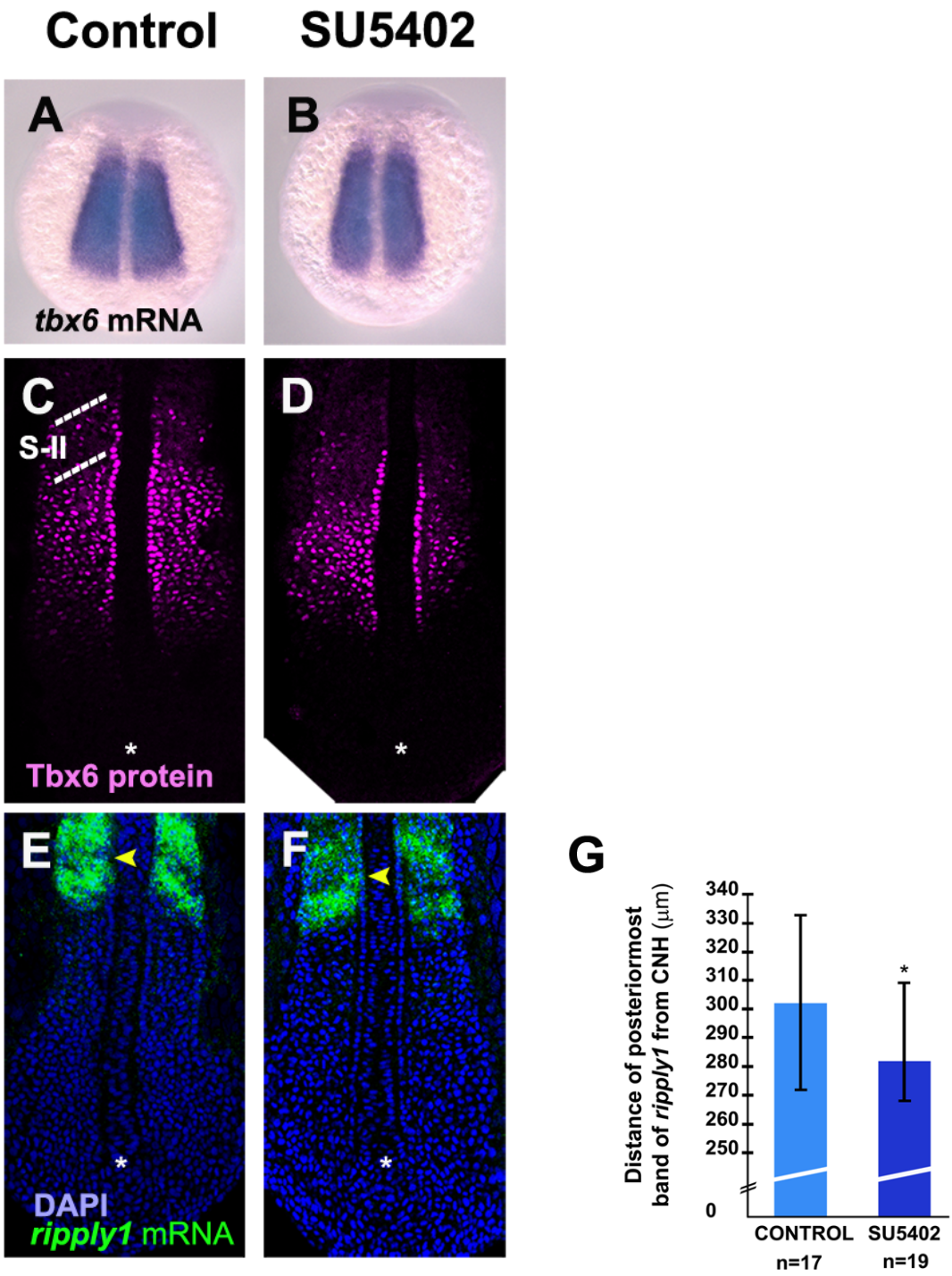

Control
SU5402
A
B
tbx6 mRNA
C
S-II
D
*
Tbx6 protein
E
F
DAPI
ripply1 mRNA
G
Distance of posteriormost band of ripply1 from CNH (μm)
340
330
320
310
300
290
280
270
260
250
0
CONTROL
n=17
SU5402
n=19

Figure 8H-8J

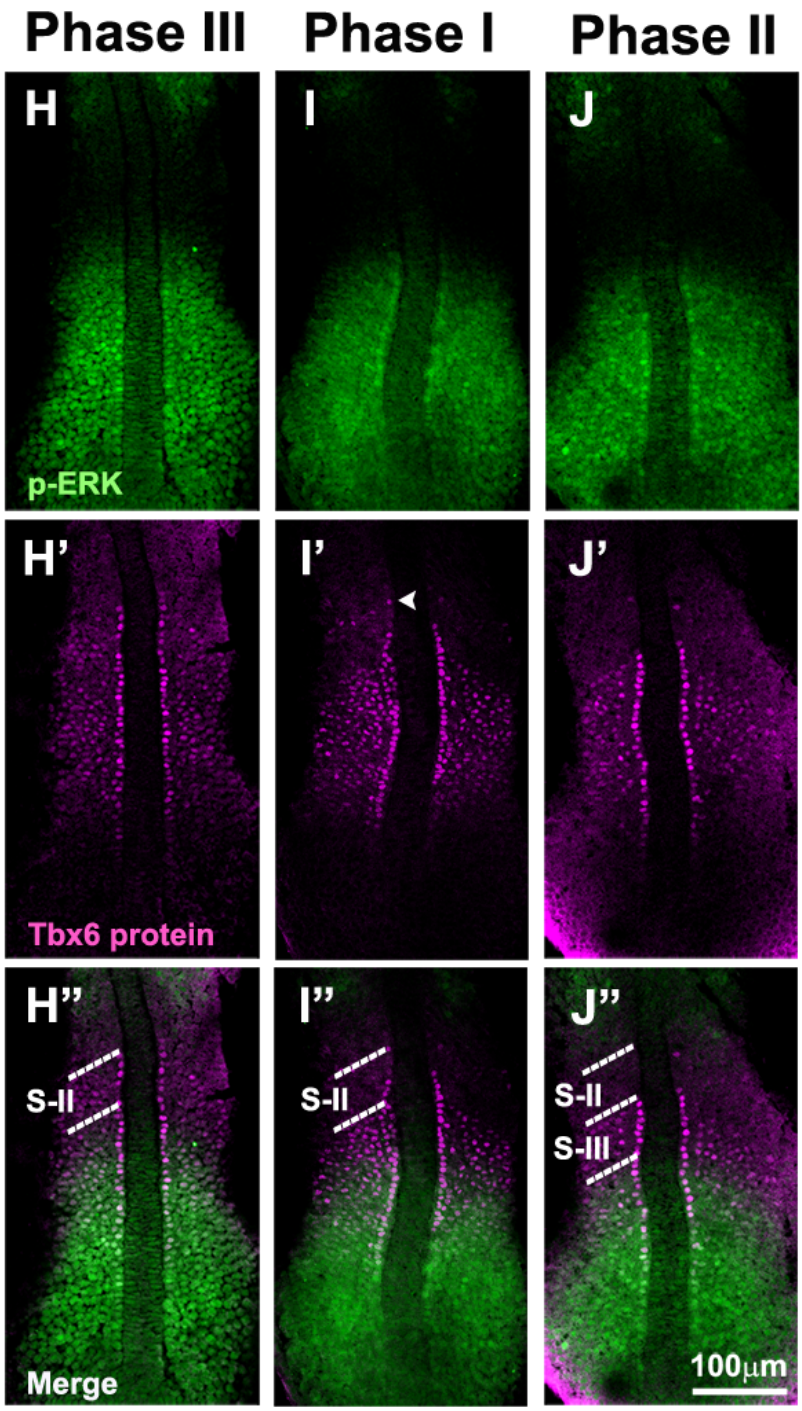

Phase III
Phase I
Phase II
H
I
J
p-ERK
H'
I'
J'
Tbx6 protein
H''
I''
J''
S-II
S-II
S-II
S-III
Merge
100μm

Figure 9

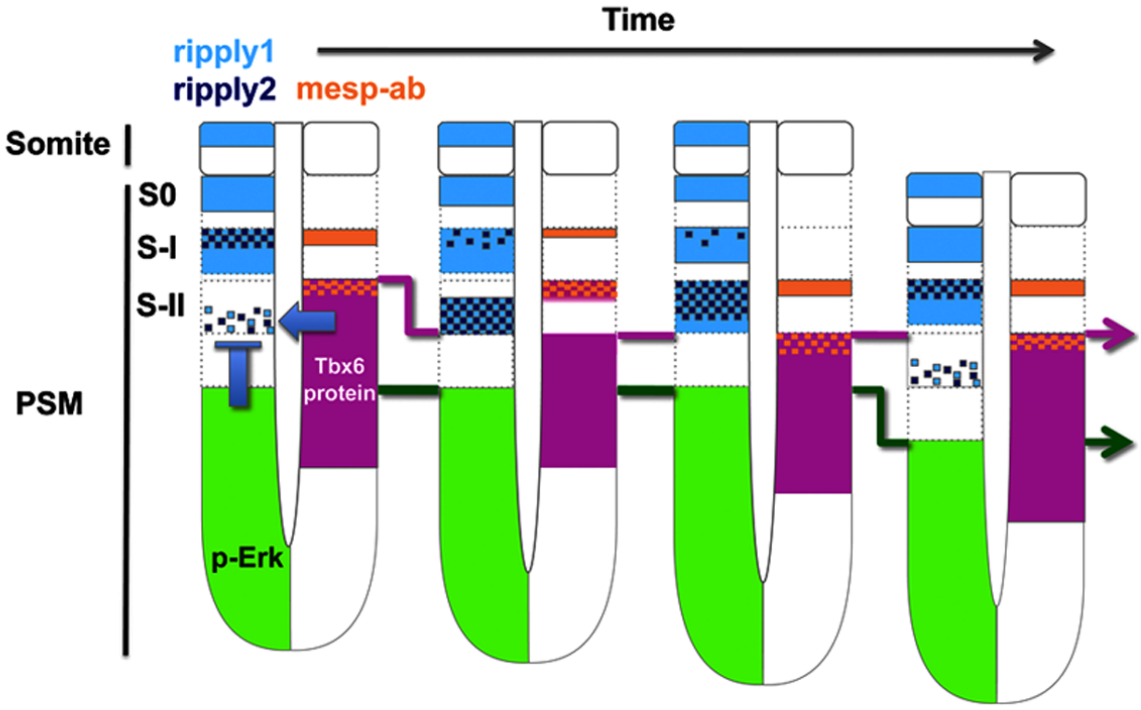
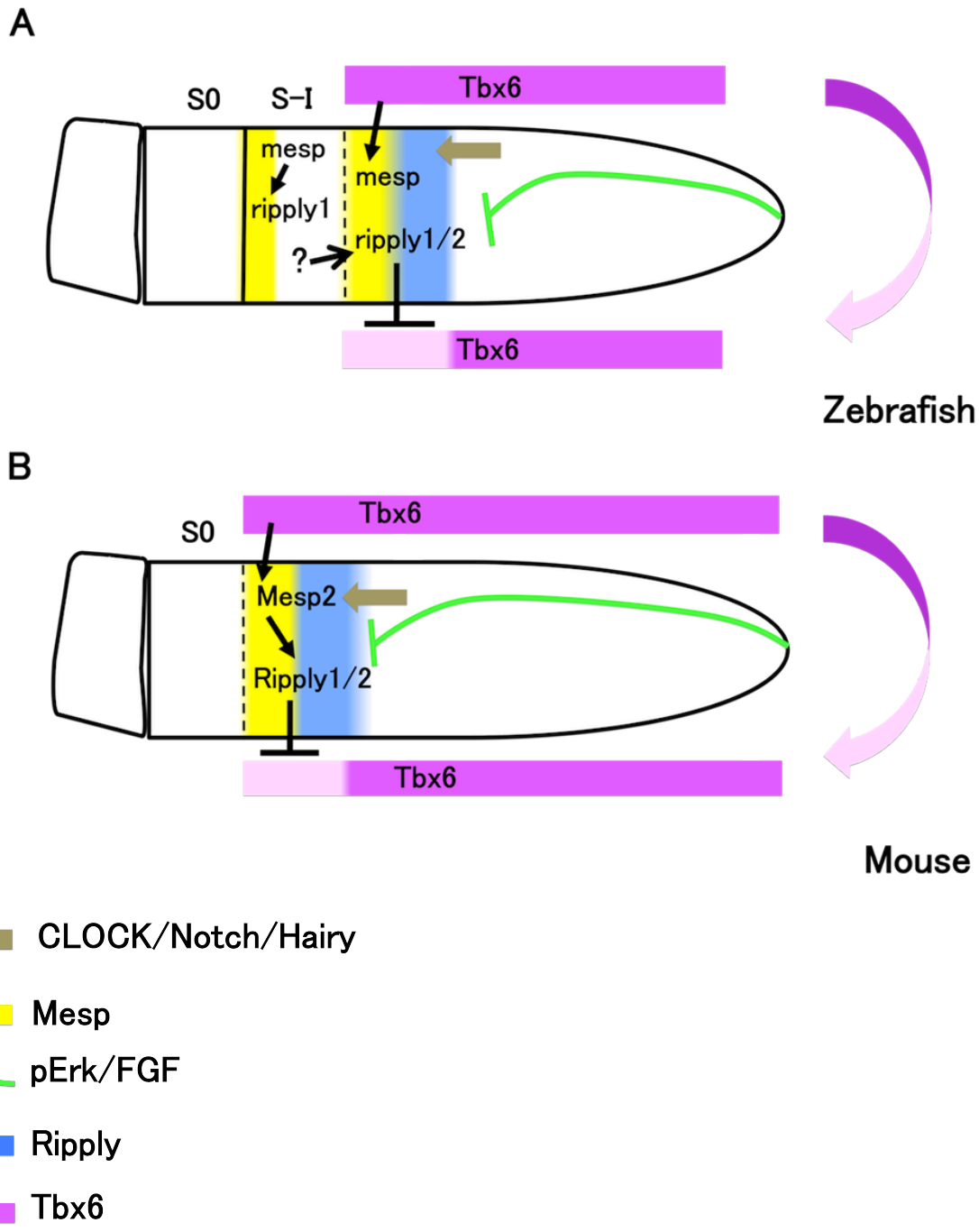

Time
ripply1
ripply2
mesp-ab
Somite
S0
S-I
S-II
PSM
Tbx6
protein
p-Erk

Figure 10


A
S0
S-I
Tbx6
mesp
ripply1
?
mesp
ripply1/2
Tbx6
Zebrafish
B
S0
Tbx6
Mesp2
Ripply1/2
Tbx6
Mouse
CLOCK/Notch/Hairy
Mesp
pErk/FGF
Ripply
Tbx6

Reference

Akiyama R, Masuda M, Tsuge S, Bessho Y, Matsui T (2014) An anterior limit of FGF/Erk signal activity marks the earliest future somite boundary in zebrafish. Development 141: 1104-1109.

Asakawa K, Kawakami K (2008) Targeted gene expression by the Gal4-UAS system in Zebrafish. Develop Growth Differ 50: 391-399.

Aulehla A, Pourquie O (2010) Signaling gradients during paraxial mesoderm development. Cold Spring Harb Perspect Biol 2: a000869.

Aulehla A, Wehrle C, Brand-Saberi B, Kemler R, Gossler A, et al. (2003) Wnt3a plays a major role in the segmentation clock controlling somitogenesis. Dev Cell 4: 395-406.

Aulehla A, Wiegraebe W, Baubet V, Wahl MB, Deng C, et al. (2008) A beta-catenin gradient links the clock and wavefront systems in mouse embryo segmentation. Nat Cell Biol 10: 186-193.

Bajard L, Morelli LG, Ares S, Pecreaux J, Julicher F, et al. (2014) Wnt-regulated dynamics of positional information in zebrafish somitogenesis. Development 141: 1381-1391.

Baker RE, Schnell S, Maini PK (2008) Mathematical models for somite formation. Current Topics in Developmental Biology 81:183-203.

Chapman DL, Agulnik I, Hancock S, Silver LM, Papaloannou VE (1996) Tbx6, a mouse T-Box gene implicated in paraxial mesoderm formation at gastrulation. Dev Biol 180:534-542.

Cooke J, Zeeman EC (1976) A clock and Wavefront model for control of the number of repeated structures during animal morphogenesis. J Theor Biol 58:455-476.

Cutty SJ, Fior R, Henriques PM, Saude L, Wardle FC (2012) Identification and expression analysis of two novel members of the Mesp family in zebrafish. Int J Dev Biol 56: 285-294.

Dubrulle J, McGrew MJ, Pourquie O (2001) FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. Cell 106: 219-232.

Gibb S, Zagorska A, Melton K, Tenin G, Vacca I, et al. (2009) Interfering with Wnt signalling alters the periodicity of the segmentation clock. Dev Biol 330: 21-31.

Henry CA, Urban MK, Dill KK, Merlie JP, Page MF, et al. (2002) Two linked hairy/Enhancer of split-related zebrafish genes, her1 and her7, function together to refine alternating somite boundaries. Development 129: 3693-3704.

Hitachi K, Danno H, Tazumi S, Aihara Y, Uchiyama H, et al. (2009) The Xenopus Bowline/Ripply family proteins negatively regulate the transcriptional activity of T-box transcription factors. Int J Dev Biol 53: 631-639.

Jiang YJ, Aerne BL, Smithers L, Haddon C, Ish-Horowicz D, et al. (2000) Notch signalling and the synchronization of the somite segmentation clock. Nature 408: 475-479.

Julich D, Hwee Lim C, Round J, Nicolaije C, Schroeder J, et al. (2005) beamter/deltaC and the role of Notch ligands in the zebrafish somite segmentation, hindbrain neurogenesis and hypochord differentiation. Dev Biol 286: 391-404.

Kageyama R, Niwa Y, Isomura A, Gonzalez A, Harima Y (2012) Oscillatory gene expression and somitogenesis. Wiley Interdiscip Rev Dev Biol 1: 629-641.

Kawamura A, Koshida S, Hijikata H, Ohbayashi A, Kondoh H, et al. (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, 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.

Kishimoto Y, Koshida S, Furutani-Seiki M, Kondoh H (2004) Zebrafish maternal-effect mutations causing cytokinesis defect without affecting mitosis or equatorial vasa deposition. Mech Dev 121: 79-89.

Kitabayashi AN, Takahashi Y, Kitajima S, Inoue T, Takeda H et al. (2002) Hypomorphic *Mesp* allele distinguishes establishment of rostrocaudal polarity and segment border formation in somitogenesis. Development 129:2473-2481.

Kondow A, Hitachi K, Ikegame T, 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, 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.

Maroto M, Bone RA, Dale JK (2012) Somitogenesis. Development 139: 2453-2456.

Matsui T, Thitamadee S, Murata T, Kakinuma H, Nabetani T, Hirabayashi Y, Hirate Y, Okamoto H, Bessho Y (2011) Canopy1, a positive feedback regulator of FGF signaling, controls progenitor cell clustering during Kupffer`s vesicle organogenesis. Proc. Natl. Acad. Sci. USA 108 (24): 9881-6.

Moreno TA, Jappelli R, Izpisua Belmonte JC, Kintner C (2008) Retinoic acid regulation of the Mesp-Ripply feedback loop during vertebrate segmental patterning. Dev Biol 315: 317-330.

Morimoto M, Takahashi Y, Endo M, Saga Y (2005) The Mesp2 transcription factor establishes segmental borders by suppressing Notch activity. Nature 435: 354-359.

Nikaido M, Kawakami A, Sawada A, Furutani-Seiki M, Takeda H, et al. (2002) Tbx24, encoding a T-box protein, is mutated in the zebrafish somite-segmentation mutant fused somites. Nat Genet 31: 195-199.

Nikaido M, Tada M, Saji T, Ueno N (1997) Conservation of BMP signaling in zebrafish mesoderm patterning. Mech Dev 61: 75-88.

Niwa Y, Shimojo H, Isomura A, Gonzalez A, Miyachi H, et al. (2011) Different types of oscillations in Notch and Fgf signaling regulate the spatiotemporal periodicity of somitogenesis. Genes Dev 25: 1115-1120.

Oates AC, Rohde LA, Ho RK (2005) Generation of segment polarity in the paraxial mesoderm of the Zebrafish through a T-box dependent inductive event. Dev Biol 283:204-214.

Oates AC, Morelli LG, Ares S (2012) Patterning embryos with oscillations: structure, function and dynamics of the vertebrate segmentation clock. Development 139: 625-639.

Oginuma M, Niwa Y, Chapman DL, Saga Y (2008) Mesp2 and Tbx6 cooperatively create periodic patterns coupled with the clock machinery during mouse somitogenesis. Development 135: 2555-2562.

Ozbudak EM, Lewis J (2008) Notch signalling synchronizes the zebrafish segmentation clock but is not needed to create somite boundaries. PLoS Genet 4: e15.

Pourquie O (2011) Vertebrate segmentation: from cyclic gene networks to scoliosis. Cell 145: 650-663.

Saga Y (2012) The mechanism of somite formation in mice. Curr Opin Genet Dev 22: 331-338.

Sawada A, Fritz A, Jiang YJ, Yamamoto A, Yamasu K, et al. (2000) Zebrafish Mesp family genes, mesp-a and mesp-b are segmentally expressed in the presomitic mesoderm, and Mesp-b confers the anterior identity to the developing somites. Development 127: 1691-1702.

Sawada A, Shinya M, Jiang YJ, Kawakami A, Kuroiwa A, et al. (2001) Fgf/MAPK signalling is a crucial positional cue in somite boundary formation. Development 128: 4873-4880.

Shoji W, Maeda MK (2008) Application of heat shock promoter in transgenic zebrafish. Develop Growth Differ 50:401-406.

Takahashi J, Ohbayashi A, Oginuma M, Saito D, Mochizuki A, et al. (2010) Analysis of Ripply1/2-deficient mouse embryos reveals a mechanism underlying the rostro-caudal patterning within a somite. Dev Biol 342: 134-145.

van Eeden FJ, Granato M, Schach U, Brand M, Furutani-Seiki M, et al. (1996) Mutations affecting somite formation and patterning in the zebrafish, Danio rerio. Development 123: 153-164.

White PH, Farkas DR, McFadden EE, Chapman DL (2003) Defective somite patterning in mouse embryos with reduced levels of Tbx6. Development 130: 1681-1690.

White PH, Chapman DL (2005) *Dll1* is a downstream target of Tbx6 in the paraxial mesoderm. Genesis 42:193-202.

Westerfield M (2000) Molecular methods In: The zebrafish book: A guide for the laboratory use of zebrafish (Danio rerio). Eugene: University of Oregon Press. pp. 289-294.

Yasuhiko Y, Haraguchi S, Kitajima S, Takahashi Y, Kanno J, et al. (2006) Tbx6-mediated Notch signaling controls somite-specific Mesp2 expression. Proc Natl Acad Sci USA 103: 3651-3656.

Yasuhiko Y, Kitajima S, Takahashi Y, Oginuma M, Kagiwada H, et al., (2008) Functional importance of evolutionally conserved Tbx6 binding sites in the presomitic mesoderm-specific enhancer of Mesp2. Development 135:3511-3519.

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