

RESEARCH COMMUNICATION

Zebrafish Hairy/Enhancer of split protein links FGF signaling to cyclic gene expression in the periodic segmentation of somites

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Notch and fibroblast growth factor (FGF) signaling pathways have been implicated in the establishment of proper periodicity of vertebrate somites. Here, we show evidence that a *Hes6*-related *hairy/Enhancer of split*-related gene, *her13.2*, links FGF signaling to the Notch-regulated oscillation machinery in zebrafish. Expression of *her13.2* is induced by FGF-soaked beads and decreased by an FGF signaling inhibitor. *her13.2* is required for periodic repression of the Notch-regulated genes *her1* and *her7*, and for proper somite segmentation. Furthermore, *Her13.2* augments autorepression of *her1* in association with *Her1* protein. Therefore, FGF signaling appears to maintain the oscillation machinery by supplying a binding partner, *Her13.2*, for *Her1*.

Supplemental material is available at <http://www.genesdev.org>.

Received December 17, 2004; revised version accepted March 31, 2005.

Somites are the morphologically distinct segmental units that are transiently formed during early vertebrate development and subsequently give rise to metameric and fundamental structures such as the vertebrae of the axial skeleton, their associated muscles, and tendons. The somites are subdivided from the anterior end of the unsegmented paraxial mesoderm, called the presomitic

mesoderm (PSM), and sequentially generated in an anterior to posterior direction in a rhythmic fashion at regular spatiotemporal intervals. The molecular mechanism underlying the periodical formation of somites is coupled to an internal oscillator, referred to as the “segmentation clock”, which has been evidenced by the cyclic expression of genes in the PSM (Palmeirim et al. 1997; Maroto and Pourquie 2001). Most genes that exhibit a cyclic expression pattern in the PSM are involved in the Notch signaling pathway (Bessho and Kageyama 2003). Various *hairy/Enhancer of split (Espl)*-related basic helix–loop–helix (bHLH) genes (*hairy-1*, *hairy-2*, and *Hey2* in the chicken; *Hes1*, *Hes5*, *Hes7*, and *Hey2* in the mouse; *her1* and *her7* in the zebrafish) that are transcriptional targets of the Notch signaling are expressed in a dynamic pattern of stripes across the PSM in a posterior to anterior direction. In addition, *Lunatic fringe*, encoding a glycosyltransferase that modulates the Notch signaling in the chicken and mouse, and the Notch ligand *deltaC* in the zebrafish also show an oscillatory expression pattern in the PSM. These cyclic genes, as well as other components of the Notch signaling pathway, were shown to be required for the proper somite segmentation in mice (Hrabe de Angelis et al. 1997; Kusumi et al. 1998; Bessho et al. 2001) and zebrafish (Takke and Campos-Ortega 1999; Holley et al. 2000, 2002; Henry et al. 2002; Oates and Ho 2002) by mutant analysis or morpholino oligo (MO)-mediated gene-knockdown experiments. Furthermore, the cyclic expression of *Hes7*, *her1*, and *her7* requires their own activity, suggesting that a negative feedback loop involving these genes is a critical component of the oscillation machinery (Oates and Ho 2002; Bessho et al. 2003).

The oscillating and anteriorly propagating wave of gene expression, which is maintained in the posterior PSM, becomes fixed to cause segmentation in the anterior PSM. Activity gradients of signaling molecules, including fibroblast growth factor (FGF), Wnt, and retinoic acid are proposed to regulate the differentiation of PSM cells along the anteroposterior axis from a state permitting the oscillating gene expression to a state driving the segmentation program (Dubrulle et al. 2001; Sawada et al. 2001; Aulehla et al. 2003; Moreno and Kintner 2004). For instance, FGF signaling is the highest at the posterior end of the PSM with a gradual decrease toward the anterior, suggesting a role for FGF signaling in maintaining the characteristics of the posterior PSM cells (Dubrulle et al. 2001; Sawada et al. 2001). In fact, overexpression of *FGF8* in the entire PSM of chick embryos causes an increase in the expression of *Brachyury* in the posterior PSM and suppresses morphological segmentation (Dubrulle et al. 2001). Furthermore, transient activation or inhibition of FGF signaling results in the formation of smaller or larger somites, respectively. Thus, FGF signaling appears to maintain the posterior characteristics, which allows the cyclic gene expression, and the level of FGF activity regulates the transition of the posterior PSM cells into segmental tissues and determines the position of the segment boundaries. However, the molecular mechanism by which FGF signaling permits the cyclic gene expression in the posterior PSM has been totally unknown.

Here we focused on a zebrafish *hairy/Espl* gene,

[**Keywords:** Somite segmentation; FGF signaling; cyclic gene expression; *hairy/Espl*; negative feedback loop; zebrafish]

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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1291205>.

her13.2, which is expressed in the posterior PSM. Our results indicate that FGF signaling allows the cyclic gene expression by inducing *her13.2* in the posterior PSM.

Results and Discussion

To gain a better insight into the molecular mechanism underlying the segmentation process, we identified genes specifically expressed in the PSM by an in situ hybridization screening of mRNA expressed in the PSM to tailbud region of zebrafish embryos (18–21-somite stages). Among these genes, *her13.2*, coding for a bHLH protein closely related to mammalian Hes6 (Bae et al. 2000; Koyano-Nakagawa et al. 2000; Sieger et al. 2004), exhibited posterior-to-anterior decrease in expression, analogous to the FGF signaling gradient, in the posterior PSM and tailbud. Expression of *her13.2* was first observed in the blastoderm margin at the shield stage (Fig. 1A), and then became restricted to the posterior region at the tailbud stage, being excluded from the axial mesoderm (Fig. 1B). As segmentation proceeded, the transcripts became specifically localized to the posterior PSM and tailbud (Fig. 1C–F). Expression of *her13.2* was strong at the tailbud and gradually decreased along the PSM (Fig. 1D). In contrast to other *her* genes, e.g., *her1* and *her7*, which are known to exhibit oscillatory expression in the PSM and tailbud (Holley et al. 2000; Sawada et al. 2000; Henry et al. 2002; Oates and Ho 2002; Gajewski et al. 2003), *her13.2* is unique for its nonoscillatory expression.

Since the graded axial distribution of *her13.2* mRNA in the posterior PSM resembled that of FGF activity, we examined whether FGF signaling was involved in the expression of *her13.2*. With a brief treatment of embryos with SU5402, an FGF receptor kinase inhibitor (Mohammadi et al. 1997), expression of *her13.2* in the PSM was drastically reduced, whereas DMSO-treated control em-

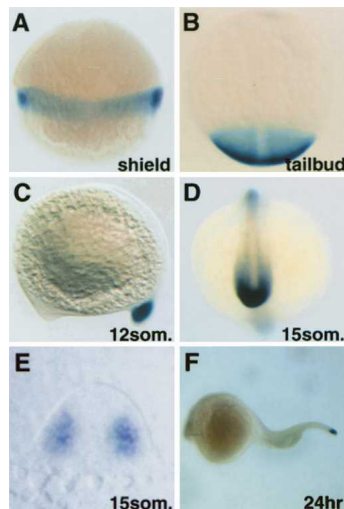


Figure 1. Zebrafish *her13.2* mRNA is expressed in the posterior PSM and tailbud. (A,C,F) Lateral view. (B,D) Dorsal view. Whole-mount in situ hybridization was carried out by using *her13.2* anti-sense probe and embryos at the developmental stages shown at the bottom right of each panel. *her13.2* expression is first seen at the blastoderm margin at the shield stage. During the segmentation period, the signal remains confined to the posterior PSM and tailbud. (E) Transverse section of a stained embryo at the level of posterior PSM. A signal is observed in the paraxial mesoderm.

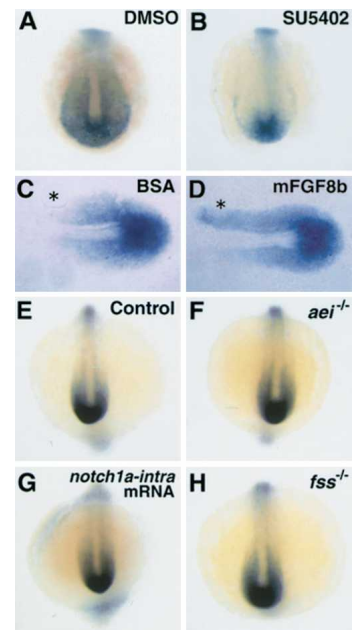


Figure 2. Expression of *her13.2* is regulated by FGF signaling. Each panel is shown from the dorsal aspect. (A,B) Transient inhibition of FGF signaling reduces the expression of *her13.2* in the posterior PSM. *her13.2* expression in embryos treated with DMSO ($n = 6$; 0% affected) and SU5402 at the concentration of 660 μM ($n = 6$; 100% affected). Reduced expression of *her13.2* was observed in embryos treated with SU5402 at any concentration that caused expansion of somites (150–660 μM ; data not shown). (C,D) Implantation of mouse recombinant FGF8b-soaked bead leads to the ectopic expression of *her13.2*. *her13.2* expression in flat-mounted embryos with BSA-soaked ($n = 6$; 0% affected) or mFGF8b-soaked ($n = 9$; 100% affected) bead. To minimize possible artifacts, beads were implanted lateral to the paraxial mesoderm. The anterior is left, and asterisks show the position of implanted beads. (E–H) Independence of *her13.2* expression from Notch signaling and *fss/tbx24*. Expression pattern of *her13.2* was comparable between sibling ($n = 16$; 0% affected) and *aei/deltaD*^{-/-} ($n = 11$; 0% affected) embryos. *her13.2* expression was not altered in embryos injected with mRNA encoding a constitutively active form of *notch1a* ($n = 20$; 0% affected), although ectopic expression of *her1* was observed in the entire PSM of these embryos ($n = 23$; 87% affected). *her13.2* expression was also not altered in *fss/tbx24*^{-/-} mutants ($n = 7$; 0% affected).

bryos maintained the normal expression of *her13.2* (Fig. 2A,B). Conversely, implantation of an mFGF8b-soaked bead into the anterior PSM caused anterior expansion of the expression domain of *her13.2*, whereas a bovine serum albumin (BSA)-soaked control bead did not affect *her13.2* expression (Fig. 2C,D). On the other hand, Notch signaling, which is known to activate *hairy/Espl*-related genes showing oscillatory expression in the PSM (Bessho and Kageyama 2003), did not affect the expression of *her13.2* in the PSM. Expression of *her13.2* in *aei/deltaD*^{-/-} embryos, as well as in embryos injected with mRNA encoding the intracellular domain of *notch1a*, which can activate the Notch signaling constitutively (Takke and Campos-Ortega 1999), appeared indistinguishable from that in normal embryos (Fig. 2E–G). Furthermore, expression of *her13.2* was not significantly altered in *fss/tbx24* mutant embryos (Fig. 2H). These results indicate that *her13.2* functions downstream of FGF signaling in the PSM, but is not under direct regulation by Notch signaling, or Tbx24 transcription factor, in contrast to other *hairy/Espl*-related genes.

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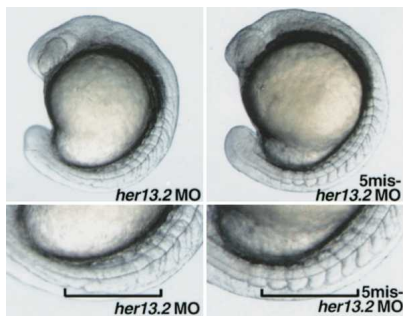


Figure 3. Altered somite patterning in *her13.2* MO-injected embryos. Lateral views of *her13.2* MO-injected and 5mis-*her13.2* MO-injected embryos at the 12-somite stage. In *her13.2* MO-injected embryos, the boundaries of somites are disrupted after the proper segmentation of the first eight somites, whereas no apparent defect is seen in 5mis-*her13.2* MO-injected embryos. Injected embryos were incubated at 23.5°C after the dome stage (4.5 hpf), because this segmental defect was more prominent after this stage.

To investigate the involvement of *her13.2* in somite development, we performed antisense MO-mediated gene-knockdown experiments. Injection of two distinct antisense MOs specific for *her13.2* resulted in disruption of the regular segmentation of somites posterior to the first seven to nine somites (Fig. 3). This phenotype closely resembled the phenotypes of previously identified segmentation mutants, such as *after eight*, *deadly*

seven, and *mind bomb* (van Eeden et al. 1996). In contrast, MOs carrying five base substitutions in the *her13.2*-specific MOs caused no such defect. Coinjection of variant *her13.2* mRNA lacking the *her13.2* MO-target sequence rescued embryos from this segmental defect in a dose-dependent manner (Supplementary Table 1). Thus, *her13.2* is required for the proper segmentation of somites in the posterior trunk. The segmental defect caused by *her13.2*-specific MOs was confirmed by analysis of the expression of a series of genes characteristically involved in the segmentation process. For instance, *deltaC*, *deltaD*, and *mesp-b*, which are normally expressed in a segmental pattern, were expressed in a scattered pattern in the anterior PSM of *her13.2* MO-injected embryos (Fig. 4A–F), as typically observed in embryos defective in the segmentation process (Jiang et al. 2000; Sawada et al. 2001; Holley et al. 2002). In contrast, the expression of *fss/tbx24*, which is normally expressed uniformly in the anterior and intermediate PSM (Nikaido et al. 2002), was not affected (Fig. 4G,H). Rostrocaudal polarity of somites was also perturbed in *her13.2* MO-injected embryos. Expression of *myod*, which is normally confined to the posterior region of somites (Weinberg et al. 1996), was expanded (Fig. 4I,J), whereas that of *paraxial protocadherin (papc)*, which is normally expressed in the PSM and anterior region of somites (Yamamoto et al. 1998), was scattered and reduced (Fig. 4K,L). These results indicate that *her13.2* function is essential for the process of somite segmentation.

In the process of segmentation, *hairy/Esp1*-related genes *her1* and *her7* in the zebrafish and *Hes7* in the mouse play essential roles in the segmentation machinery, because disruption of these genes results in defects in the segmentation and the oscillatory expression of the cyclic genes (Bessho et al. 2001; Henry et al. 2002; Holley

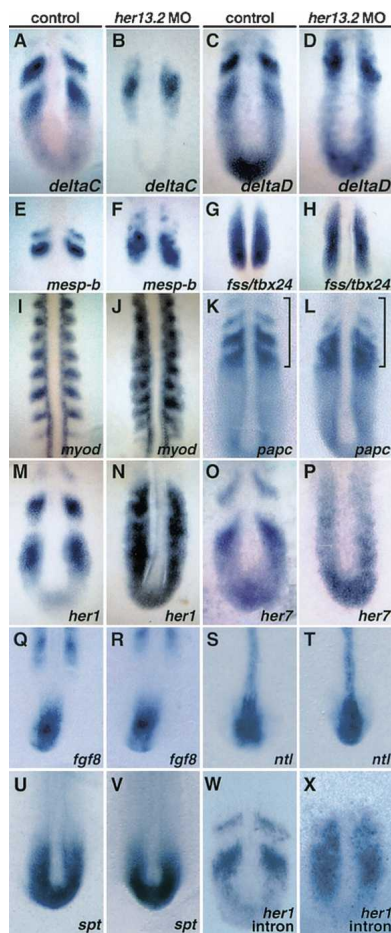


Figure 4. Ectopic expression of *her1* and *her7* in the PSM of *her13.2* MO-injected embryos. Expression analysis of *her13.2* MO-injected embryos by using probes indicated at the bottom right of each panel. Embryos were fixed at the 10–12-somite stages. Stained embryos were flat-mounted. (A–F) The segmental expression of *deltaC* ($n = 14$; 86% affected), *deltaD* ($n = 14$; 93% affected), and *mesp-b* ($n = 14$; 86% affected) has vanished. Instead, scattered expression is observed in the anterior PSM of *her13.2* MO-injected embryos. (G,H) *fss/tbx24*, normally expressed in the anterior and intermediate PSM, is not altered ($n = 15$; 0% affected). (I–L) The polarity of somites is posteriorized in *her13.2* morphants. The distribution of *myod*, expressed in the posterior region of somites, is expanded ($n = 14$; 93% affected). That of *papc*, expressed in the PSM and anterior part of somites, is scattered ($n = 15$; 87% affected) and emphasized by the bracket. (M–P) Cyclic expression patterns of *her1* and *her7*, categorized into three phases (Jiang et al. 2000; Oates and Ho 2002), are observed in the control embryos ($n = 16$; phase I, 5; phase II, 5; phase III, 6; and $n = 10$; phase I, 3; phase II, 3; phase III, 4, respectively). Expression pattern of *her1* in M represents phase I, and that of *her7* in O represents phase II. In *her13.2* morphants, however, expression of *her1* ($n = 23$; 87% affected) and *her7* ($n = 13$; 85% affected) is seen in the entire PSM, distinct from the three phases observed in the control embryos. (Q–V) *fgf8* ($n = 13$; 0% affected) and FGF-downstream genes such as *ntl* ($n = 11$; 0% affected) and *spt* ($n = 13$; 0% affected) are normally expressed in the posterior region of *her13.2* MO-injected embryos. (W,X) Transcriptionally active state of *her1* in the PSM of *her13.2* MO-injected embryos. Cyclic expression of *her1* primary transcripts is detected in control embryos by use of a *her1* intron probe ($n = 21$; phase I, 6; phase II, 7; phase III, 8). Expression pattern of nascent *her1* in W represents phase II. However, in *her13.2* MO-injected embryos, *her1* nascent mRNA is observed in the entire PSM ($n = 21$; 81% affected), as found with the *her1* exon probe (Fig. 4N).

et al. 2002; Oates and Ho 2002; Gajewski et al. 2003). These genes also exhibit periodical expression in the posterior PSM, the periodicity of which is generated by a negative feedback loop composed of repression of the genes by their own encoded proteins and break of this repression by ubiquitination-mediated degradation of the proteins (Oates and Ho 2002; Bessho et al. 2003; Hirata et al. 2004). Interestingly, in *her13.2* MO-injected embryos, the expression of *her1* and *her7* was observed in the entire region of the PSM (Fig. 4M–P), as observed in embryos deficient for *her1* and/or *her7* (Henry et al. 2002; Holley et al. 2002; Oates and Ho 2002; Gajewski et al. 2003), although the expression level was variable among cells in the PSM of the *her13.2*-deficient embryos. In contrast, the expression of *fgf8*, *no tail (ntl)*, and *spade tail (spt)*, which exhibit nonoscillating expression in the posterior PSM, was not significantly affected (Fig. 4Q–V). Thus, *her13.2* is required for the cyclic repression of *her1* and *her7* expression. On the other hand, expression of another cyclic gene, *deltaC*, was decreased by injecting *her13.2*-specific MO (Fig. 4A,B), indicating that *her13.2* acts differently on the expression of this gene. To determine whether the up-regulated expression of *her1* and *her7* genes was caused by decreased repression of their transcription or by stabilization of their mRNAs, we examined the distribution of *her1* nascent transcripts by using a *her1* intron probe. In normal embryos, the segmental expression of nascent *her1* transcripts was observed (Fig. 4W), as previously reported (Gajewski et al. 2003). On the other hand, in *her13.2* MO-injected embryos, the signal was widespread in the posterior PSM, as was the case when a *her1* exon probe was used (Fig. 4X). Thus, *her13.2* plays an essential role in cyclic repression of *her1* transcription in the posterior PSM.

We next addressed how Her13.2 is involved in repression of the *her1* gene transcription by conducting an in vitro luciferase reporter assay. The expression of the luciferase gene under the control of an 8.6-kb *her1* promoter is sufficient for the normal expression of the *her1* gene in the PSM (Gajewski et al. 2003). This promoter activity was repressed by Her1, but not affected by Her13.2, in 293T cells (Fig. 5A). Under this experimental condition, Her13.2 enhanced the self-repression activity of Her1 (Fig. 5B). Thus, Her13.2 can act as a transcriptional repressor with Her1, and enhancement of *her1* repression by Her13.2 may be required for the periodical repression of *her1* in the posterior PSM. To function as a transcriptional repressor, Her13.2 appears to require another bHLH protein as a binding partner, because Her13.2 has a uniquely short loop region within its HLH domain, causing an inability to bind to the target sequences (Bae et al. 2000). Thus, it is possible that Her13.2 may enhance the self-repression of *her1* through formation of Her1–Her13.2 heterodimer complex. In fact, an in vitro pulldown assay indicated that Her13.2 protein interacted physically with the Her1 protein (Fig. 5C). These results demonstrate that Her13.2 can strongly repress *her1* expression, presumably through complex formation with Her1.

Taken together, our findings indicate that FGF signaling induces the expression of *her13.2* in the posterior PSM, and, in turn, Her13.2 protein participates in the periodical repression of the Notch-regulated genes, *her1* and *her7*, presumably through formation of a transcriptional repressor complex with at least one of these Her proteins, Her1. FGF signaling has been shown to main-

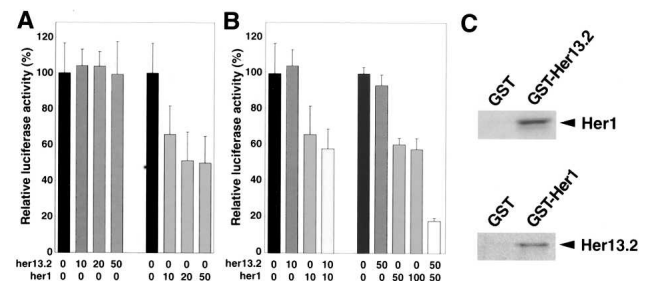


Figure 5. Her13.2 interacts with Her1 and synergistically represses the transcriptional activity of the *her1* promoter. (A,B) Her13.2 alone has little transcriptional effect on the *her1* promoter, but synergistically represses *her1* activity in the presence of Her1. Luciferase assay using 293T cells was conducted to examine the effects of introduction of *her13.2* and *her1* on the 8.6-kb fragment upstream of the translational start site of *her1* fused to the firefly luciferase gene. The amounts of pCS2+*her13.2* and pCS2+*her1* plasmids (in nanograms) used for transfection are shown under each bar. Total amount of DNA was adjusted with pCS2+ vector to be equal. All experiments were normalized for transfection efficiency by cotransfection with an expression vector for *Renilla* luciferase. The average normalized firefly luciferase without pCS2+*her13.2* and pCS2+*her1* 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. (C) Interaction between Her13.2 and Her1 in vitro. A pulldown assay was performed with in vitro labeled Her1 or Her13.2 proteins and purified Her13.2-GST or Her1-GST fusion proteins on glutathione Sepharose 4B, respectively. GST-Her13.2, not GST alone, associates with Her1 specifically, and vice versa. These specific interactions were confirmed in duplicate experiments.

tain posterior PSM cells in a state that allows the oscillatory expression of the cyclic genes, and also to be associated with expression of nonperiodic genes, e.g., *fgf8*, *ntl*, and *spt* (Dubrulle et al. 2001; Sawada et al. 2001), whose expression was not obviously altered in *her13.2* MO-injected embryos. Therefore, it is likely that FGF signaling is transmitted through several different molecular pathways in the posterior PSM, and that Her13.2 mediates specifically one of the roles of FGF signaling, the regulation of the cyclic genes. On the other hand, we cannot conclude at present whether *her13.2* expression is sufficient for this role of FGF signaling. Overexpression of *her13.2* did not give us an answer to this question, because the overexpression resulted in malformation of the presomitic mesoderm, which inhibited the precise analysis of the periodic gene expression (data not shown).

In addition to FGF signaling, Wnt signaling has been indicated to regulate the oscillation machinery (Aulehla et al. 2003). Recently, two groups reported that Wnt signaling directly regulates the expression of *Dll1*, a component of oscillation machinery in mouse embryos (Galceran et al. 2004; Hofmann et al. 2004). Thus, two molecular systems, which are individually activated by FGF or Wnt signaling, regulate the oscillation machinery. This may lead to speculation that these two regulatory systems coordinate in the maintenance of the oscillation machinery. However, it is uncertain whether these two regulatory systems function simultaneously in a particular species. In mouse, in contrast to zebrafish, the only structural counterpart of *her13.2*, *Hes6*, is not expressed in the PSM (Bae et al. 2000). Furthermore, in the zebrafish, despite a number of findings in the mouse, the involvement of Wnt signaling in the segmentation clock

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remains obscure. Thus, we can also speculate that different species may establish their own ways to transmit the signaling gradient to the segmentation clock. We expect that this issue will be solved by more extensive analysis in the future.

Materials and methods

Fish

All the experiments except mutant analysis were performed using the TL2 inbred line (Kishimoto et al. 2004). The mutant alleles used in this study are *after eight* (*aei^{tr233}*) and *fused somites* (*fss^{tl}*).

Construction of cDNA library, screening, and isolation of *her13.2*

A cDNA library was constructed with mRNA prepared from the PSM to tailbud region of zebrafish embryos at 18–21-somite stages. For collection of cDNA clones expressed in the PSM and tailbud region, a high-throughput in situ hybridization screening was performed in 96-well plates. The precise procedure for this screening will be described elsewhere. One of the cDNA clones obtained by this screening was a partial cDNA fragment of *her13.2*. Using this fragment as a probe, we obtained an ~1.2-kb cDNA from a 15–19 hours post-fertilization (hpf) cDNA library.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described (Nikaido et al. 1997). For the *her1* intron probe, the 1.4-kb genomic region corresponding to the *her1* intron was obtained by PCR with the following primers: 5'-GACCGATAATGGTAATTTGATGAGT-3' and 5'-TTGAGATGAAATGAAAAGGAAAAG-3', and hybridization was carried out as described (Gajewski et al. 2003).

Antisense MO and mRNA synthesis of *her13.2*

The sequences of MO used in this study are as follows: *her13.2* MO1, 5'-TGCAGTTCAGGACGCTTGAATGGG-3'; 5mis-*her13.2* MO1, 5'-TGCAcTTgAGGACcCTTgAtcGG-3'; *her13.2* MO2, 5'-GGCAGATGCTCGCGGTTCAGTTC-3'; 5mis-*her13.2* MO2, 5'-CGgAgTGGTC GaCGGTaCaCTTC-3'. The DNA fragment encoding the entire ORF of *her13.2* was inserted into pCS2⁺ vector. Capped *her13.2* mRNA was produced with an mMessage mMachine (Ambion).

SU5402 treatment and bead implantation

These experiments were performed essentially as described (Sawada et al. 2001).

Luciferase reporter assay

Reporter gene pGL3-her1 was constructed by inserting an 8.6-kb fragment upstream of the translational initiation site of zebrafish *her1* into the upstream of firefly luciferase gene in pGL3 Basic vector (Promega). About 1.0×10^4 293T cells were seeded into each well of a 24-well collagen-coated plate (Iwaki). pCS2⁺, pCS2⁺her1, and pCS2⁺her13.2 vector with pGL3-her1 (100 ng) and pRL-TK (0.2 ng) were transiently introduced into the cells using fugene6 (Roche). After incubation for 24 h, the cells were lysed, and the lysates were assayed with a Dual-Luciferase reporter assay system (Promega).

GST pull-down assay

To generate GST-Her13.2 and GST-Her1 proteins, the DNA fragment corresponding to the entire amino acid sequence of either *her13.2* or *her1* was inserted in-frame into pGEX4T-3 vector (Amersham). GST fusion proteins were purified on glutathione Sepharose 4B (Amersham). For synthesis of in vitro labeled proteins, 1 μg of either pCS2⁺her13.2 or pCS2⁺her1 containing the complete amino acid sequence of *her1* was added to a TNT Quick coupled Transcription/Translation Systems (Promega) in the presence of [³⁵S]-labeled methionine (Amersham). In 1 mL of 2% BSA in PBS(-), 5 μL of in vitro translation products was mixed and incubated with 5 μg of either GST, GST-Her13.2, or GST-Her1 for 60 min at 4°C. Following extensive washing, the bound proteins were separated in a 12% SDS-polyacrylamide gel.

Acknowledgments

We thank B. Appel for the 15–19 hpf cDNA library; J. Campos-Ortega for pCS2⁺her1 and pCS2⁺notch1a-intra plasmids; D. Tautz for *her1* pro-

moter; Y.J. Jiang for *aei* mutant and probe; M. Furutani-Seiki for *fss* mutant; H. Takeda, R. Kageyama, and M. Nikaido for probes; Y. Bessho for in situ hybridization protocol using intron probe; and A. Sawada for technical advice. We are grateful to Y. Kishimoto for discussions. This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, Culture, and Sports of Japan to S.T. and S.K., and by grants from the Japan Science and Technology Corporation and Mitsubishi Foundation to S.T. A.K. is a research fellow of the Japan Society for the Promotion of Science.

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Genes Dev. 2005, **19**:

Access the most recent version at doi:[10.1101/gad.1291205](https://doi.org/10.1101/gad.1291205)

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