Control of *her1* expression during zebrafish somitogenesis by a *Delta*-dependent oscillator and an independent wave-front activity

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Somitogenesis has been linked both to a molecular clock that controls the oscillation of gene expression in the presomitic mesoderm (PSM) and to *Notch* pathway signaling. The oscillator, or clock, is thought to create a prepattern of stripes of gene expression that regulates the activity of the *Notch* pathway that subsequently directs somite border formation. Here, we report that the zebrafish gene *after eight* (*aei*) that is required for both somitogenesis and neurogenesis encodes the Notch ligand DeltaD. Additional analysis revealed that stripes of *her1* expression oscillate within the PSM and that *aei/DeltaD* signaling is required for this oscillation. *aei/DeltaD* expression does not oscillate, indicating that the activity of the *Notch* pathway upstream of *her1* may function within the oscillator itself. Moreover, we found that *her1* stripes are expressed in the anlage of consecutive somites, indicating that its expression pattern is not pair-rule. Analysis of *her1* expression in *aei/DeltaD*, *fused somites* (*fss*), and *aei;fss* embryos uncovered a wave-front activity that is capable of continually inducing *her1* expression de novo in the anterior PSM in the absence of the oscillation of *her1*. The wave-front activity, in reference to the clock and wave-front model, is defined as such because it interacts with the oscillator-derived pattern in the anterior PSM and is required for somite morphogenesis. This wave-front activity is blocked in embryos mutant for *fss* but not *aei/DeltaD*. Thus, our analysis indicates that the smooth sequence of formation, refinement, and fading of *her1* stripes in the PSM is governed by two separate activities.

[*Key Words*: Somitogenesis; *Delta*; *her1*; oscillator; wave front; zebrafish]

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Somites are reiterated, epithelial structures within the paraxial mesoderm of the vertebrate embryo that give rise to the vertebrae and muscle of the trunk and tail. They are derived from the unsegmented, mesenchymal, presomitic mesoderm (PSM) flanking the notochord and form in an anterior to posterior sequence as clusters of cells undergo furrow formation and epithelialization. The somites are patterned by the adjacent notochord, neural tube, lateral plate mesoderm, and surface ectoderm giving rise to the appropriate, innervated muscle types and vertebrae. In turn, the somites influence the pattern of the neural tube along the anterior–posterior axis and provide the appropriate signals to guide ventrally migrating neural crest cells (Stern et al. 1991; Itasaki et al. 1996; for review, see Bronner-Fraser 1999).

Embryological experiments had suggested that during somitogenesis, segmentation of the paraxial mesoderm takes place in the PSM before morphological signs of segments are evident (Elsdale et al. 1976; Kimmel et al. 1991). Furthermore, it has been shown in the chick that anterior–posterior polarity of the somite is established early and is maintained independently of its orientation with respect to the environment (Aoyama and Asamoto 1988). Additional grafting experiments suggest that somite borders form only when anterior and posterior somite compartments are juxtapposed to each other (Stern and Keynes 1987). More recently, identification of genes expressed in a striped pattern within the PSM and subsequent gene knockout experiments in the mouse have given clear evidence for a molecular prepattern that is linked to somite formation (summarized in del Barco Barrantes et al. 1999).

The clock and wave front and the Meinhardt models describe mechanisms that could regulate somite formation (Cooke and Zeeman 1976; Meinhardt 1982, 1986; Cooke 1998). Both models predicted the existence of an oscillator within the cells of the PSM that produces a spatial pattern that governs somite formation. The existence of an oscillator was revealed by the analysis of *c-hairy1*, a chick homolog of the *Drosophila* pair-rule gene *hairy*, that is expressed in a single stripe that progresses through the cells of the PSM in a posterior to anterior direction. A single stripe traverses the entire PSM in 180 min (two somite cycles) and fades in the anterior PSM as the next somite border is formed (Palmeirim et al. 1997; Forsberg et al. 1998). This wave of

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expression repeats each somite cycle and does not appear to be directed by cell displacement or by an intercellular signal. It was postulated that these waves of expression are created by an oscillator within each cell that coordinates expression of this gene (Palmeirim et al. 1997). In the clock and wave-front model, the oscillator imposes a stepwise, and thus segmental, progression to the wave front by controlling competence of the cells to respond to (the morphogenic signals of) the wave front that moves from anterior to posterior. In the original version of the model, the wave front is an independent entity, and the stepwise interaction between the wave front and the clock is what leads to regulated somite furrow formation (Cooke and Zeeman 1976; Cooke 1998). A modification of this model was made such that the wave front is a direct output of the clock. In this version, cells would "count" the number of cycles that they had been through; upon reaching the appropriate count, they would form a somite furrow that then must be integrated with the existing spatial pattern (the last formed somite border) (Cooke 1998). The Meinhardt model proposes that the wave front and the oscillator are derived from a common underlying mechanism. In this model, cells in the PSM oscillate between signals responsible for anterior and posterior specification of half-somites. A single cell cannot express both the anterior and the posterior signals. However, an anterior-expressing cell will instruct its neighbors to express posterior signals, and posterior-expressing cells instruct its neighbors to express anterior signals. In computer simulations, these interactions create oscillations that spread in a wave-like fashion from posterior to anterior and refine into bands that come to rest at the appropriate distance because they are stabilized by anterior–posterior signals (the wave front) from the last formed somite. Upon stabilization, the somite furrow would form (Meinhardt 1982, 1986). Although molecular evidence for the clock or oscillator exists, none has been found for either an oscillator-dependent or -independent wave front.

The oscillator has been linked to the *Notch* pathway in several ways. *lfng* expression oscillates in both the mouse and chick PSM (Forsberg et al. 1998; McGrew et al. 1998; Aulehla and Johnson 1999). This oscillation, in contrast to *c-hairy* oscillation, is dependent on protein synthesis, initially suggesting that the *Notch* pathway acts downstream of the clock (Palmeirim et al. 1997; McGrew et al. 1998). Mouse knockouts or mutants of several components of the *Notch* pathway, including *Notch1*, *Delta-like 1* (*Dll1*), *Delta-like 3* (*Dll3*), *RBP-Jκ* (*suppressor of hairless*), *presenilin*, and *lunatic fringe* (*lfng*), produce embryos with defects in segmentation and/or anterior–posterior patterning of the somites (Conlon et al. 1995; Oka et al. 1995; Hrabé Angelis et al. 1997; Wong et al. 1997; Evrard et al. 1998; Kusumi et al. 1998; Zhang and Gridley 1998). Further analysis indicated that *Dll1*, *Notch1*, and *RBP-Jκ* are required for proper *lfng* expression in the mouse, suggesting that the *Notch* pathway is required for at least some readouts of the clock (del Barco Barrantes et al. 1999).

Somitogenesis in the zebrafish embryo commences at 10.5 hr post-fertilization with a somite pair being created approximately every 30 min until 26–30 somite pairs are formed (for review, see Holley and Nüsslein-Volhard 1999). In the zebrafish, the *Notch* homologs *Notch1a*, *Notch1b*, *Notch5*, and *Notch6* have complex expression patterns that include the PSM and/or the developing somites (Bierkamp and Campos-Ortega 1993; Westin and Lardelli 1997). The *Notch* ligand homologs, *DeltaD* and *DeltaC*, are expressed in the tailbud and in two stripes in the anterior PSM. After somite formation, *DeltaD* is expressed in the anterior and *DeltaC* is expressed in the posterior of each somite (Dornseifer et al. 1997; Haddon et al. 1998). Similar to data from *Xenopus* experiments, misexpression of *DeltaD* or *DeltaC* via mRNA injection leads to defects in somite formation (Dornseifer et al. 1997; Jen et al. 1997, 1999; Takke and Campos-Ortega 1999).

her1, a *hairy* homolog in the zebrafish, is expressed in the tailbud and in two or three stripes in the more anterior PSM. The most anterior stripe fades just before morphological somites can be distinguished, and, at the same time, new stripes of expression emerge from the tailbud. Cell labeling experiments suggested that the stripes of *her1* expression correspond to the odd number somites beginning with the fifth somite, whereas the intervening nonexpressing stripes correspond to the even number somites (Müller et al. 1996).

In the zebrafish, the *fss*-type mutants are defective in both the segmentation and anterior–posterior patterning of the somites. *fss* is required for the formation of all somites, whereas in *beamter* (*bea*) embryos, the first three to four somites form but the remainder do not. In *after eight* (*aei*), *deadly seven* (*des*), and *white tail* (*wit*) embryos, the first seven to nine somites form but the more posterior somites do not. In each of these mutants, in the unsegmented regions, the segmental expression of genes such as *MyoD* is lost, and expression is seen throughout the somitic mesoderm (Jiang et al. 1996; van Eeden et al. 1996). Moreover, each of these mutants display defects in *her1* expression within the PSM (van Eeden et al. 1998).

Here, we show that the *fss*-type gene *aei* encodes the zebrafish *DeltaD* protein. In addition to the previously described somite phenotype, we found that *aei*/*DeltaD* embryos exhibit a neuronal hyperplasia. We undertook an extensive analysis of *her1* expression and found that, like *c-hairy* and *lfng* in the chick and in the mouse or chick, respectively, its expression oscillates within the PSM. Moreover, we found that *her1* stripes are expressed in the anlage of consecutive somites, indicating that *her1* is not expressed in a pair-rule pattern. *aei*/*DeltaD* activity is required for the oscillation of *her1* expression, whereas *aei*/*DeltaD* expression itself does not oscillate, indicating that the *Notch* pathway functions upstream of *her1* mRNA oscillation, possibly within the clock itself. Analysis of *her1* expression in *aei*, *fss*, and *aei*/*fss* embryos uncovered a wave-front activity that is blocked in embryos mutant for *fss* but not *aei*/*DeltaD*. This analysis indicates that *her1* expression has two phases. The first phase of expression within the posterior and

intermediate PSM involves periodic oscillations of stripes of *her1* expression and requires *aei*/*DeltaD* signaling. This establishes a molecular prepattern of *her1* stripes within the PSM that appears to be stabilized during the second phase of expression by an anterior wave-front activity acting through *fss*. This wave-front activity then appears to be required to transform the molecular prepattern into regularly spaced and patterned somites. Finally, cell transplantation experiments demonstrate that *fss* is required cell-autonomously to propagate this molecular and morphogenic wave-front activity.

Results

aei *is* DeltaD

Radiation hybrid mapping placed *DeltaD* within a 5- to 10-cM region to the right of z6104 on linkage group 13, whereas *aei* was mapped within the interval between z6104 and z687 (Fig. 1A; Geisler et al. 1999). No additional markers were available within this interval to map *DeltaD* more accurately with respect to *aei*. Thus, *DeltaD* was cloned from aei^{AR33} (previously aei^{tr233}) via RT–PCR and sequenced in the hope of uncovering a polymorphism that could be used for mapping via PCR. A T → A substitution was found that created a stop codon within the fifth EGF repeat of *DeltaD* (Fig. 1B). PCR primers were designed such that the 3′ base of one primer matched the wild-type sequence but not the mutant sequence (allele-specific PCR). To further destabilize primer annealing, the penultimate base of the primer was altered such that it did not match either sequence. The second primer matched both wild-type and mutant sequences perfectly. As an internal control, primers specific to *sonic hedgehog* (*shh*) that maps to linkage group 7 were included in each reaction. PCR was performed on DNA preparations from individual mutant aei^{AR33} and wild-type sibling embryos. Whereas the sibling embryos gave both the *shh* and *DeltaD* PCR products, 0 out of 175 aei^{AR33} embryos gave the *DeltaD* product (Fig. 1C). Genetically, this maps *DeltaD* within 0.3 cM of *aei*. Sequencing of a second allele, aei^{AG49} (previously aei^{tg249}) revealed a stop codon 63 amino acids from the amino terminus, well before the Delta:Serrate:Lag-2 (DSL) domain that is thought to mediate ligand–receptor interactions, producing what should be a null allele (Fig. 1B; for review, see Artavanis-Tsakonas et al. 1999). The analysis of Islet-1-expressing neurons in the trunk of 13 somite stage *aei* embryos revealed a hyperplasia of Rohon-Beard neurons (Fig. 2A–C). In each *aei* allele, the number of Islet-1-expressing neurons is roughly doubled. This neuronal hyperplasia phenotype is analogous to the classic neurogenic phenotype observed for *Notch* pathway mu-

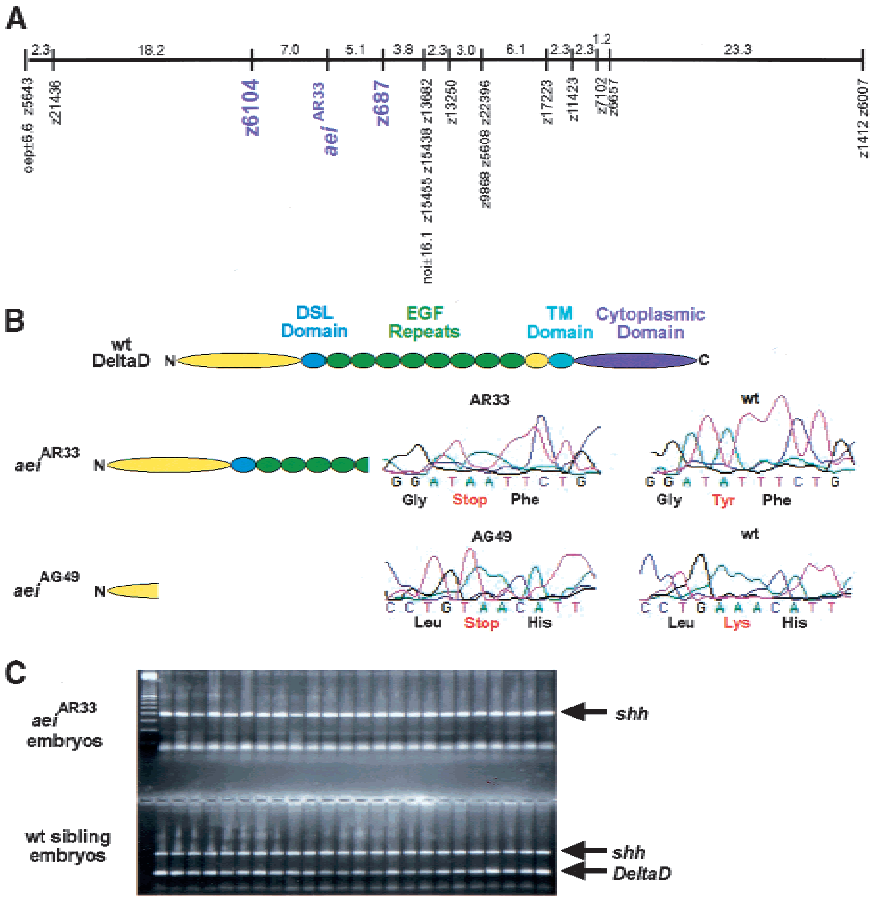

Figure 1. *aei* is *DeltaD*. (*A*) The genetic map of linkage group 13 is shown with *aei* and the markers to which it was linked (purple). Numbers indicate genetic distance in cM. (*B*) Schematics are shown of a wild-type *DeltaD* protein and of the two predicted protein products produced in aei^{AR33} and aei^{AG49}. Sequencing trace profiles of the region altered in each mutant allele are presented along with the corresponding wild-type sequences. (*C*) The products of mapping PCR reactions performed on 24 aei^{AR33} embryos and 24 wild-type embryos are displayed. Although all embryos produce the *shh* control product, only the sibling embryos produce the *DeltaD* product.

tants in *Drosophila*. Additionally, the fact that the two alleles exhibit the same severity of phenotype suggests that despite having the DSL domain, aei^{AR33} is also a null allele.

aei/DeltaD *regulates its own expression in the PSM*

During somitogenesis, *aei/DeltaD* is expressed in the tailbud and in two stripes in the anterior PSM. The anterior-most stripe refines and persists after furrow formation within the anterior of each somite (Fig. 2D; Dornseifer et al. 1997). In each of the *fss*-type mutants, the striped expression in the anterior PSM is lost and *aei/DeltaD* is expressed in all cells of this region (Fig. 2E–I). Thus, in each of the mutants in which somite formation is affected, *aei/DeltaD* expression is perturbed. The expression of *DeltaD* in *fss* embryos is sometimes different from that observed in the other mutants (Fig. 2F). This is perhaps significant and is discussed later within the context of additional data. Finally, Figure 2E indicates that *aei/DeltaD* activity is required for proper regulation of *aei/DeltaD* expression.

Examination of her1 *expression*

her1 is misexpressed in each of the *fss*-type mutants, but unlike *aei/DeltaD* expression that is affected in basically the same way in each of the *fss*-type mutants, the *her1* expression pattern differentiates the mutants into three groups (van Eeden et al. 1998). *fss* embryos form stripes of *her1* expression but always lack the anterior-most stripe. In *bea*, no *her1* stripes are formed, and *her1* is expressed uniformly throughout the PSM. In *aei*, *des*, and *wit*, *her1* stripes do not form, and only a disorganized expression domain is seen in the anterior PSM. To better comprehend the significance of these phenotypic differences, a better understanding of *her1* expression in wild-type embryos was required. We staged embryos at the 7-, 8-, 12-, and 15-somite stages and examined *her1* expression using *MyoD* as a reference. *MyoD* is the most reliable, robust, segmentally expressed in situ marker for the zebrafish somitic mesoderm. *MyoD* labels the posterior of each somite, and whereas the anterior border of the *MyoD* expression domain changes as the somite matures, the posterior border of expression remains at the posterior border of each somite. In Figures 3D and 5A, a distinct row of cells can be seen along the posterior of most of the *MyoD* stripes. These cells are morphologically distinct in that they form the epithelium of the somite and express *MyoD* less strongly than the cells to their immediate anterior (Weinberg et al. 1996). Measurements were made in reference to the most posterior cells in each *MyoD* stripe (the posterior border of the somite) and the anterior border of each *her1* stripe.

During somitogenesis, *MyoD* is expressed in the adaxial cells that form longitudinal stripes on each side of the notochord and in lateral stripes in the posterior of each somite. Formation of these lateral stripes precedes somite border formation by 1–2 somites, that is, an embryo with 12 somites may have 13–14 *MyoD* stripes (Weinberg et al. 1996). Thus, whereas *MyoD* expression arises just before somite boundary formation, *her1* expression fades before boundary formation. In 11% of the examined embryos, a *MyoD* stripe arose before the *her1* stripe just anterior to it had faded. Some embryos from each of the data sets in Figures 3 and 4 (40 of 341 total) exhibited *her1* stripes immediately posterior to consecutive stripes of *MyoD* expression indicating that *her1* is expressed in the anlage of consecutive somites (Fig. 3A–C). Moreover, the distance between the posterior-most *MyoD* stripe and the anterior-most *her1* stripe was never the size of two somites. These two stripes were either directly adjacent to each other (in most cases) or were separated by a distance of roughly one somite (Fig. 3D, III). These results suggest that *her1* is not expressed in a pair-rule pattern and are in contradiction with previous studies (Müller et al. 1996).

Analysis of the graphs suggests that the distances between the more posterior *her1* stripes (Fig. 3D, IV) are

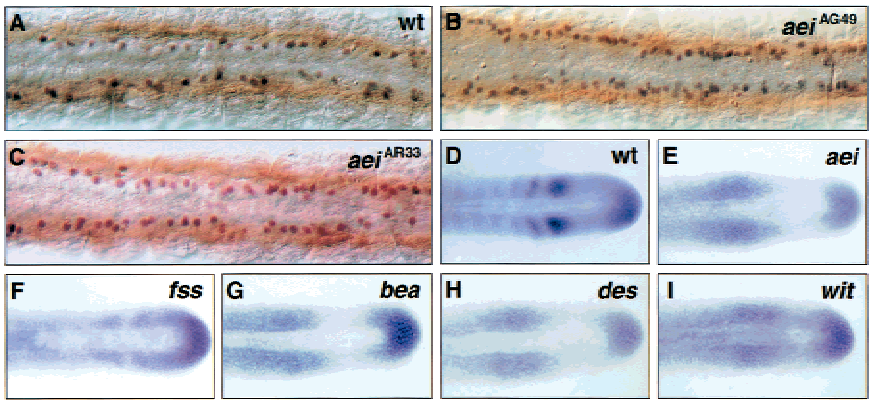

Figure 2. *aei/DeltaD* is required for both neurogenesis and patterning of *aei/DeltaD* expression in the PSM. (*A–C*) Examination of Islet-1-expressing neurons (black/blue) in the trunk of *aei* embryos revealed a neuronal hyperplasia. Using myosin (brown) as a reference for somite position, Islet-1-expressing neurons anterior to the posterior border of somite 5 were counted in wild-type (*A*) and *aei* embryos (*B*,*C*). Wild-type embryos ($n = 20$) averaged 33 Islet-1-expressing neurons in this region, whereas aei^{AG49} ($n = 19$) and aei^{AR33} ($n = 20$) averaged 58 and 61 such neurons, respectively. All embryos are at about the 13-somite stage. Photos show dorsal views of the trunk region. (*D–I*) Expression of *aei/DeltaD* is mispatterned in each of the *fss*-type mutants. Note, in *fss* embryos (*F*), 10%–15% show some evidence of *aei/DeltaD* stripe formation. Most embryos, however, exhibit expression patterns as seen in *aei/DeltaD* and the other *fss*-type mutants. All embryos are at about the 15-somite stage. Photos show dorsal views of the tailbud and posterior trunk. In all panels, anterior is to the *left* and posterior to the *right*.

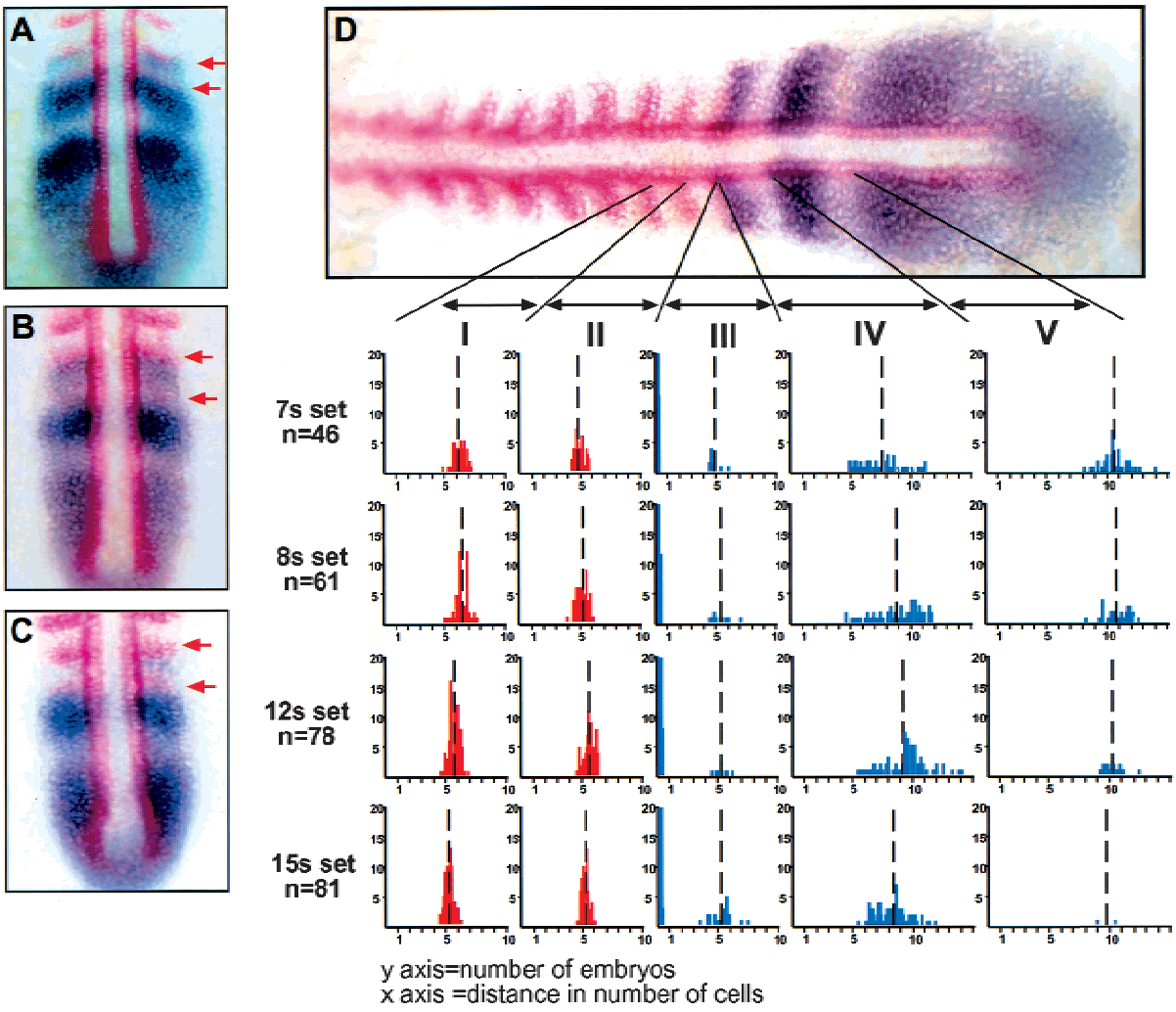

Figure 3. *her1* is expressed in the anlage of consecutive somites. Embryos (7-, 8-, 12-, and 15-somite stage) were double-stained for *her1* (blue) and *MyoD* (red). Some embryos from each data set were found to have *her1* stripes immediately posterior to consecutive *MyoD* stripes: (*A*) 7 somites; (*B*) 12 somites; (*C*) 15 somites. The last two *MyoD* stripes are indicated by red arrows. (*D*) A summary of all of the measurement data is shown. The distance between the posterior of the third to last and penultimate *MyoD* stripes (interval I), between the posterior of the penultimate and the last *MyoD* stripes (interval II), between the posterior of the last *MyoD* stripe and the anterior of the first *her1* stripe (interval III), between the anterior of the first and the anterior of the second *her1* stripe (interval IV), and between the anterior of the second and the anterior of the third *her1* stripe (interval V) were made. Vertical broken lines represent mean values. Note that in most cases, including the embryo shown in *D*, the last *MyoD* stripe and the first *her1* stripe are directly juxtaposed giving a value of 0 for that measurement. This is indicated by the large number of data points at 0 in interval III. Adobe Photoshop 5.0 was used to analyze the expression domains of the digitally photographed in situ images. Distances between expression domains were measured in pixels, and the number of pixels per cell (seven) was calibrated using the in situ as well as DAPI stained embryos.

more variable than the distances between *MyoD* stripes (Fig. 3D, I and II). For instance, the distance between the anterior of the first and second *her1* stripe varied continuously from one to two somites in length (Fig. 3D, IV). The distances between the anterior of the second and third *her1* stripes also appear more variable than the distances between *MyoD* stripes. However, the sample size for this measurement is significantly smaller given that not all embryos at the seven- or eight-somite stage have three *her1* stripes, and, as somitogenesis proceeds and the tailbud becomes more compact, usually only one or two *her1* stripes are observed (cf. graphs in Fig. 3D, V).

Could the variation in spacing between posterior *her1* stripes be indicative of a directionally refining expression pattern? Given that the spacing between consecutive *MyoD* stripes is one somite in length, that the distance between the posterior-most *MyoD* stripe and the anterior-most *her1* stripe is either 0 or one somite in length, and that *her1* is expressed in the anlage of consecutive somites, the distances between the first and second and second and third *her1* stripes must decrease during time as the PSM matures. Concomitantly, the width of each *her1* stripe along the anterior–posterior axis also should diminish. For instance, Figure 3, B and C, shows

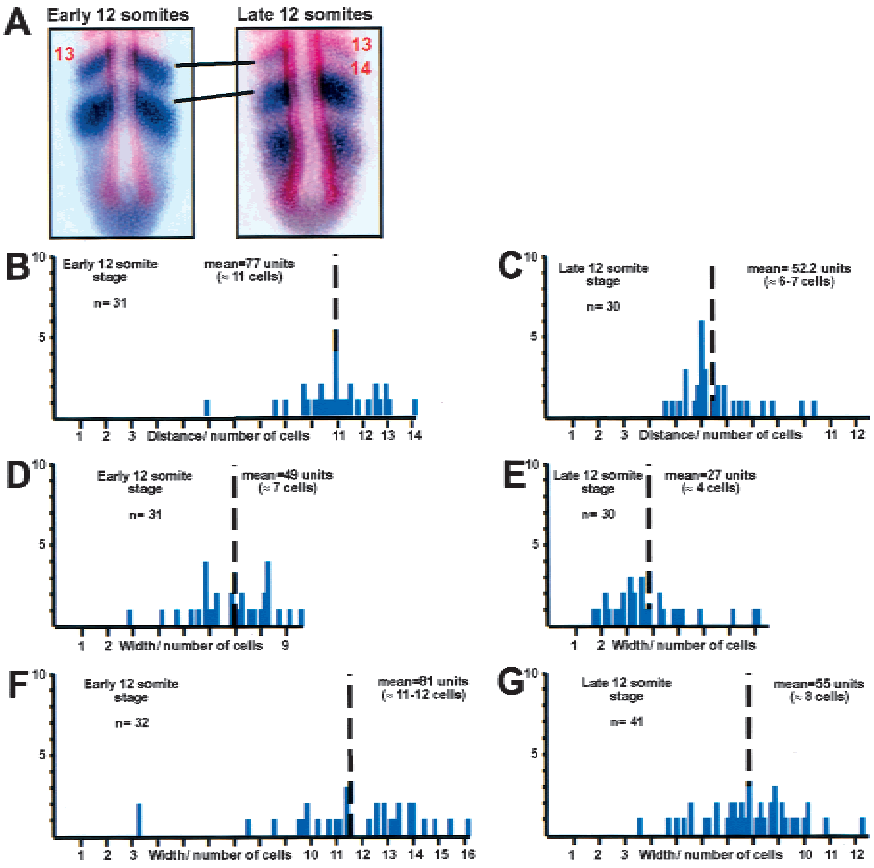

Figure 4. The stripes of *her1* expression progress anteriorly during somitogenesis. (*A*) Early 12-somite embryos and late 12-somite embryos were stained for *her1* (blue) and *MyoD* (red). Measurements were made between the anterior of the *her1* stripe immediately posterior to the thirteenth *MyoD* stripe and the anterior of the next, posterior *her1* stripe. In many of the late 12-somite embryos (as pictured), the fourteenth *MyoD* stripe already had formed, but the *her1* stripe immediately posterior to the thirteenth *MyoD* stripe had not faded. (*B–G*) Plots of individual measurements are shown with the *y*-axis representing the number of embryos and the *x*-axis representing distance from anterior (*left*) to posterior (*right*). The distance between the two *her1* stripes decreases with time (cf. *B* to *C*). Likewise, the width of the two *her1* stripes along the anterior–posterior axis decreases with time at the same rate (cf. *D* to *E* and *F* to *G*). Mean values are indicated by the broken vertical line. Means were compared by two-sample *t*-test. The difference between the means in *B* and *C* is 29 pixels with a 95% C.I. from 19.5 to 30.7 pixels. The difference between the means in *D* and *E* is 21.6 pixels with a 95% C.I. from 15.9 to 27.4 pixels. The difference between the means in *F* and *G* is 25.7 pixels with a 95% C.I. from 17.8 to 33.7 pixels. Thus, the differences between the means in each comparison is statistically significant.

embryos in which the last *MyoD* stripe directly abuts a *her1* stripe that is between 6 and 12 cells in width. This *her1* stripe must diminish in width as the PSM matures. Although the interface between the *MyoD* stripe and this *her1* stripe refines as indicated by the variability observed in the 79% of embryos that have this interface, this border is maintained because the only measurable distance between these stripes is one somite in length: The anterior border does not gradually fade away (posteriorly) from the *MyoD* stripe. Moreover, the variability seen in interval II of Figure 3D that has a range of three cells means that the *MyoD* stripe does not move posteriorly to maintain the interface with the *her1* stripe as it fades from the anterior: This would require that the range of variability in interval II be >12 cells. Thus, the data in Figure 3 indicate that the fading of each *her1* stripe along the anterior–posterior axis must occur directionally from the posterior.

The question remains as to whether the variabilities seen in intervals IV and V of Figure 3D are due to the distances between the anterior borders of the *her1* stripes decreasing as the PSM matures. To test this hypothesis, a more precise staging experiment was performed. Embryos were examined under a dissecting microscope as they developed and fixed at either the early or late 12-somite stage. Embryos that just had begun to form the thirteenth furrow were classified as early 12 somites, whereas those with the thirteenth furrow well formed on both sides were called late 12 somites. These embryos were stained for both *her1* and *MyoD*, and the distances between the anterior of the *her1* stripe immediately posterior to the thirteenth *MyoD* stripe and the anterior of the next, posterior *her1* stripe were measured (Fig. 4A). This distance averaged almost two somites in length in the early 12-somite embryos (Fig. 4B), whereas in the late 12-somite embryos, the average distance was closer to 1 somite in length (Fig. 4C). Thus, the distance between these two *her1* stripes decreases with time. Analysis of the width of these two *her1* stripes along the anterior–posterior axis indicates that both the anterior (Fig. 4D,E) and posterior (Fig. 4F,G) *her1* stripes fade from the posterior at equal rates as time progresses. The differences between the means compared are all statistically significant. Time-lapse analysis of somitogenesis indicates that these decreases in distances are due to neither cell migration nor cell compaction toward the anterior (supplemental information). This latter point is demonstrated again by nuclear staining (DAPI) (data not shown). Finally, this decrease in distance is not due to patterned programmed cell death within the tailbud as demonstrated by TUNEL (data not shown).

Cumulatively, these observations suggest that *her1*

expression oscillates within the zebrafish PSM such that stripes of *her1* expression progress anteriorly through the cells of the PSM as somitogenesis proceeds. The anterior border of a *her1* stripe would have to progress anteriorly at a rate of six to eight cells per somite cycle (30 min), until it is a one-somite distance posterior of where the *her1* stripe anterior to it had stopped and faded.

aei/DeltaD *signaling is required for* her1 *oscillation*

As described previously, each of the *fss*-type genes is required for proper regulation of *her1* expression (van Eeden et al. 1998). The observation that *her1* oscillates within the PSM now allows a better understanding of how somitogenesis is affected in the *fss*-type mutants. As shown in Figure 5B, *aei/DeltaD* is required for *her1* stripe formation. Although stripes of *her1* expression are sometimes seen in *aei/DeltaD* embryos before the five- to six-somite stage, we have not observed *her1* stripes in *aei/DeltaD* embryos past this stage (van Eeden et al. 1998). In *aei* embryos, *her1* is expressed constantly in the anterior PSM just posterior to the *MyoD* expression domain. There is little variability to this expression pattern, and no stripes of expression are ever seen separating from the tailbud. Thus, there is no oscillation of *her1* expression. This means that cells in the posterior PSM do not express *her1* at all but do so once they mature and are just posterior to the *MyoD* expression domain. This anterior expression is de novo, independent of the oscillation of *her1*, and must be constantly induced or propagated to always flank the *MyoD* expression domain posteriorly.

If *her1* expression oscillates and *aei/DeltaD* signaling is required for this oscillation, then the question arises as to whether *aei/DeltaD* expression also oscillates. If *aei/DeltaD* expression does oscillate, then this would indicate that it, along with *her1*, is a readout of the clock. If, however, *aei/DeltaD* does not oscillate, then this would suggest that *aei/DeltaD* signaling functions within the clock itself. *aei/DeltaD* is expressed in one or two stripes in the anterior PSM. The anterior-most stripe is always immediately posterior to a *MyoD* stripe. Measurements of the distance between the anterior borders of the two *aei/DeltaD* stripes indicate that the anterior border of the posterior stripe is not more than approximately eight cells (<1.5 somites) away from the posterior-most *MyoD* stripe ($n = 64$). Thus, although a *aei/DeltaD* stripe may progress anteriorly over roughly two cell diameters to be within a one-somite distance from the stripe anterior to it, this refinement in expression of *aei/DeltaD* cannot account for the oscillation of *her1* that cycles within the more posterior region of the PSM. The oscillation of *her1* is probably dependent on the protein product of *aei/DeltaD* that is derived from mRNA that is transcribed within the posterior tailbud. These observations indicate that *aei/DeltaD* signaling, but not oscillation of *aei/DeltaD* expression, is required for the oscillation of *her1* expression. This is supported by the fact that although *aei/DeltaD* stripes do not form completely in *fss* embryos (Fig. 2F), *her1* expression nevertheless oscillates (Fig. 5C). Moreover, misexpression of *aei/DeltaD* via mRNA injection perturbs somite border formation but not *her1* transcription (Takke and Campos-Ortega 1999), indicating that regulated expression of *aei/DeltaD* is not required for *her1* oscillation but is required for regular somite border formation.

Although *aei/DeltaD* stripes may progress in an anterior direction to a limited extent, examination of *aei/DeltaD* expression relative to *MyoD* and *her1* expression reveals more about the formation of *aei/DeltaD* stripes. Formation of each *aei/DeltaD* stripe appears to result from a refining of a diffuse band of expression immediately posterior to a mature *aei/DeltaD* stripe (Fig. 5E). This band of expression refines both anteriorly and posteriorly (Fig. 5F,G). While the borders of the *aei/DeltaD* stripes refine, the level of expression concomitantly in-

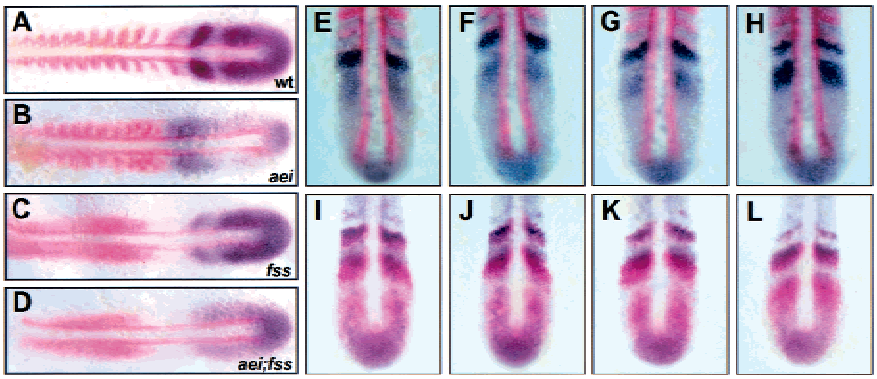

Figure 5. *aei/DeltaD* signaling is required for oscillation of *her1* expression. (*A–D*) Anterior is *left* and posterior is *right*. Expression of *her1* (blue) and *MyoD* (red) in wild-type (*A*), *aei* (*B*), *fss* (*C*), and *aei;fss* (*D*) embryos. (*E–H*) A hypothetical time course illustrating *aei/DeltaD* (blue) stripe formation relative to *MyoD* expression (red) is depicted. Anterior is *top* and posterior is *bottom*. In *E*, one *aei/DeltaD* stripe is seen immediately posterior to the most posterior *MyoD* stripe. Posterior to this *aei/DeltaD* stripe is a broad, weaker domain of *DeltaD* expression. This weaker domain of expression appears to refine such that a region of nonexpression begins to form immediately posterior to the strong *aei/DeltaD* stripe (*F*,*G*). As this intervening region not expressing *aei/DeltaD* becomes more refined, the posterior stripe of expression increases in intensity (*H*). (*I–L*) A hypothetical time course comparing *aei/DeltaD* expression (blue) with *her1* expression (red) is depicted. *aei/DeltaD* expression goes through the same process of refinement as shown in *E–H*, whereas the *her1* stripes proceed anteriorly. Although in *J* and *K* the anterior limit of the posterior *her1* and *aei/DeltaD* stripes are not aligned as the anterior limits of the more anterior stripes are, ultimately, these more posterior stripes do align (*L*). Measurements of the distance between the anterior border of the two *aei/DeltaD* stripes suggest that the posterior *aei/DeltaD* stripe may progress anteriorly over one to two cell diameters.

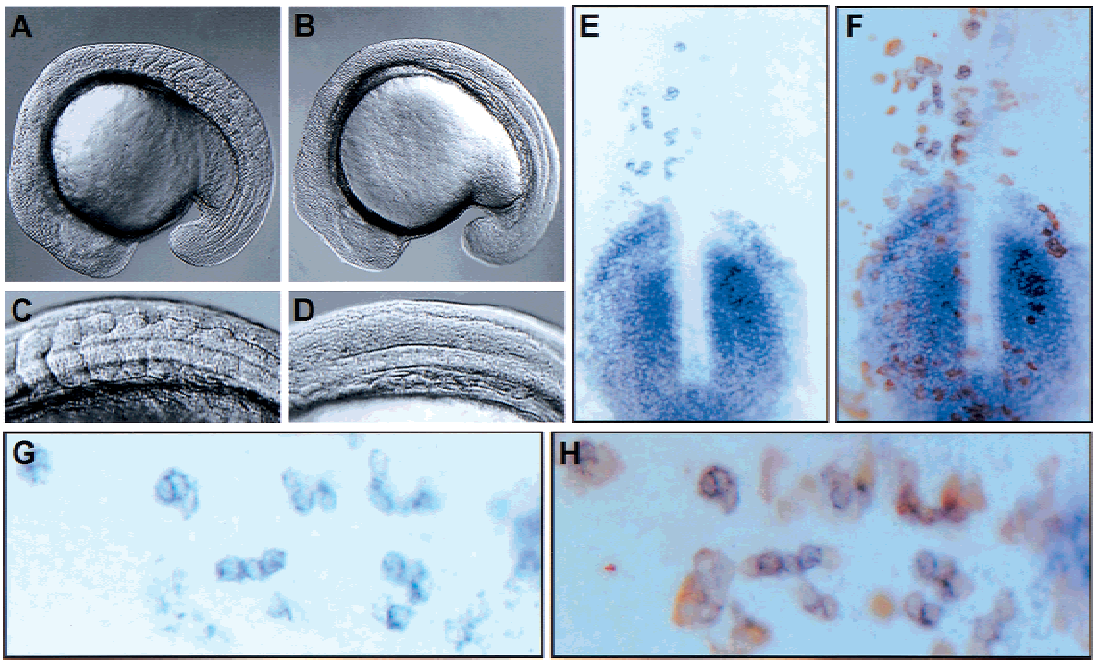

Figure 6. *fss* activity is required cell autonomously to propagate a molecular and morphogenic wave-front activity. (*A–D*) Approximately 17-somite stage embryos are shown. *C* and *D* are higher magnification views of the embryos in *A* and *B*, respectively. *aei/DeltaD* embryos (*A*,*C*) have a qualitatively different phenotype than *fss* embryos (*B*,*D*). The "unsegmented" posterior somitic mesoderm in *aei/DeltaD* embryos is convoluted with irregular somite borders (*A*,*C*), whereas no border morphogenesis is observed in *fss* embryos (*B*,*D*). (*E–H*) *her1* expression (blue) in a *fss* embryo that has received wild-type cells (brown) via transplantation is shown. *G* and *H* are higher magnification views of *E* and *F*, respectively, with anterior to the *left*. Embryos were first stained for *her1* expression, dissected, mounted, and photographed. Then, the dissected tailbuds were stained for the biotin–dextran-labeled donor cells. All *her1*-expressing cells in the anterior domain are wild-type donor cells (cf. *G* to *H*). This result was observed in a total of 61 embryos from five independent experiments.

creases (Fig. 5H). This refinement occurs while stripes of *her1* expression progress anteriorly until the anterior border of the *her1* stripe aligns with the crisp anterior border of an *aei/DeltaD* stripe (Fig. 5I–L). The two stripes then continue to refine and fade together (see the anterior-most stripes in Fig. 5I–L). This analysis also shows that consecutive *her1* stripes overlap with consecutive *aei/DeltaD* stripes that refine and are ultimately expressed in the anterior half of every somite.

fss *is required for propagation of a wave-front activity*

As reported previously, *fss*, which has the strongest morphological phenotype of the *fss*-type mutants, has the most subtle effect on *her1* expression (van Eeden et al. 1998). In *fss* embryos, *her1* stripes emerge from the tailbud region, but the anterior-most stripe is always absent (Fig. 5C). There are no stripes of *MyoD* expression that could be used as a reliable reference point in *fss* embryos; so we are not able to do the same measuring experiments as were performed in wild-type embryos. However, by analogy with the wild-type embryos, we interpret the formation of stripes and variability (observed by eye) of *her1* expression in *fss* embryos as indicating that oscillation of *her1* expression occurs. Thus, the activity of *fss* is required for the maintenance or refinement of the *her1* pattern established by the oscillator. In *aei;fss* double mutant embryos, no *her1* stripes are formed as in *aei/DeltaD* embryos, and the anterior expression that is constant in *aei/DeltaD* embryos is lost (Fig. 5D). Therefore, *fss* is required for this anterior activity that in the absence of *her1* oscillation is sufficient to induce or propagate *her1* expression in the anterior PSM. This anterior activity interacts with the oscillator-derived pattern to produce the smooth changes seen in *her1* expression. This *fss*-dependent activity has some characteristic of the "wave front" in that it interacts with the oscillator-derived pattern in the anterior PSM and is required for the formation of the somites. However, this observed activity is not identical to the wave front; so we refer to it as a "wave-front activity" to make this distinction. *aei/DeltaD*, on the other hand, is required for the oscillation of *her1* and does not affect propagation of the wave-front activity. This difference also is seen in the morphology of the somitic mesoderm in the two mutants. In *aei/DeltaD*, the posterior somites fail to form, but unpatterned or irregular furrows are seen in the posterior somitic mesoderm (Fig. 6A,C). In contrast, the somitic mesoderm in *fss* embryos remains smooth with no evidence of furrow formation (Fig. 6B,D). In time-lapse movies of wild-type somitogenesis, a "morphogenic wave front" can be seen as cells in the PSM undergo the furrow formation and cell shape changes that

constitute epithelial somite formation in a single anterior to posterior wave. Accordingly, in time-lapse analysis, this morphogenic wave front is preserved in *aei/DeltaD* embryos (albeit unpatterned), but none is observed in *fss* embryos (supplemental information; http://www.eb.tuebingen.mpg.de/abt.3/research_interests/somitogenesis.html).

fss *function is required cell automomously*

her1 expression, therefore, has two phases: the initial oscillating phase that requires *aei/DeltaD* signaling and a second phase in the anterior, mature PSM that is under control of a wave-front activity acting through *fss*. The characteristics of the wave-front activity remain unknown. To address how *fss* functions and, thus, to try to characterize the wave-front activity, cell transplantation experiments were performed. Wild-type cells within the anterior PSM, where *her1* is normally not expressed in *fss* embryos, are able to express *her1* but not able to induce expression in host cells (61 embryos from five experiments; Fig. 6E–H). This indicates that *fss* functions cell autonomously. However, this cell-autonomous requirement is not absolute because in reciprocal transplantations, *fss* cells in a wild-type anterior PSM environment will express *her1* (49 embryos from three experiments; data not shown). Thus, a community effect is able to overcome the cell-autonomous requirement for *fss*. This same phenomenon is observed with *floating head* that encodes a homeobox gene (Talbot et al. 1995). Although this gene encodes for a transcription factor that is required for notochord development, small clones of *floating head* mutant cells in a wild-type environment can become notochord (Amacher and Kimmel 1998). These experiments show that mutations in *fss* do not directly block the propagation of an anteriorly derived extracellular signal that controls *her1* expression and subsequent somite morphogenesis.

Although this data indicates that *fss* function is required cell autonomously, it also suggests that *fss* has cell nonautonomous effects. Transplanted wild-type cells are capable of expressing *her1* in the *fss* host. However, the images in Figure 6, E–H, also show that these wild-type cells do not turn off *her1* as they should. If the transplanted wild-type cells were able to regulate *her1* expression in a completely wild-type manner, then a "mosaic stripe" pattern should be observed. Although *fss* appears to be required cell autonomously, it is likely that these cells are required to communicate with their neighbors to consummate the patterning events initiated by the oscillator. The observation that *aei/DeltaD* is sometimes differently expressed in *fss* than the other mutants may reflect the distinct role *fss* plays in this process. For example, in *aei/DeltaD* embryos, the oscillator readout is perturbed, and thus, no prepattern is established, and *aei/DeltaD* is expressed consistently in a broad disorganized domain. In *fss*, the oscillator is functioning and the prepattern is established but not maintained or refined. Thus, in some *fss* embryos *aei/DeltaD* stripe formation, which appears to be dependent on *her1* (Takke and Campos-Ortega 1999), is initiated but not completed because the wave-front activity that is required to interact with the oscillator pattern at this point is compromised. The reason that only ~15% of *fss* embryos appear as in Figure 2F may be because the oscillator-derived pattern is present in the anterior PSM during only a small interval within the somite cycle. In summary, *fss* appears to function cell autonomously to promote *her1* expression and somite formation by propagation of an anterior wave-front activity. However, because of intercellular interactions involving the cells requiring *fss* activity, *fss* has indirect cell-nonautonomous effects.

Discussion

Using *MyoD* as a reference, we performed a detailed study of *her1* expression within the PSM. We provide evidence that *her1* expression oscillates within the cells of the PSM such that stripes of expression emerge from the tailbud and progress through the PSM in a posterior to anterior direction. The anterior border of a stripe progresses six to eight cell diameters each somite cycle (~30 min), and a given cell will go through multiple cycles of expression. As somitogenesis proceeds and the tailbud decreases in size, cells of the PSM would go through fewer cycles of *her1* expression: A cell in the posterior PSM at the 7- or 8-somite stage would go through eight or so cycles, whereas a cell at the same position in a 12- or 15-somite stage embryo would go through six to seven or five cycles, respectively.

Here, we have demonstrated that the *fss*-type gene *aei* is the *Notch* ligand *DeltaD*. Additionally, we show that *aei/DeltaD* signaling is required for *her1* oscillation throughout the PSM but that *aei/DeltaD* stripes appear to form and refine only within the anterior PSM. During gastrulation, both *aei/DeltaD* and *her1* are expressed in the marginal zone, and it is the continuation of this expression that is seen in the posterior tailbud (Müller et al. 1996; Dornseifer et al. 1997). The transient expression of *aei/DeltaD* in the posterior tailbud (and/or earlier expression) is the likely source of *aei/DeltaD* protein whose signaling activity is required for the oscillation of *her1* expression. The fact that *her1* oscillates in *fss* embryos in the absence of *aei/DeltaD* stripe formation again supports the conclusion that oscillation of *aei/DeltaD* signaling but not transcription is required for *her1* oscillation. Accordingly, ubiquitous misexpression of *aei/DeltaD* or an activated form of *Notch* via mRNA injection perturbs somite formation, but only the activated *Notch* affects *her1* stripe formation (Takke and Campos-Ortega 1999), again indicating that regulated *Notch* activity but not regulated *DeltaD* expression is required for the oscillation of *her1* expression. Cumulatively, these findings indicate that *Notch* signaling is required within the oscillator or at least for some readouts of the oscillator.

By examining *her1/MyoD* expression in *aei*, *fss*, and *aei/fss* mutant embryos, a wave-front activity was uncovered that requires *fss* but not *aei/DeltaD* activity. Accordingly, in time-lapse analysis, the morphogenic wave front is preserved in *aei/DeltaD* but not *fss* em-

bryos. This wave-front activity is capable of continually inducing *her1* expression in the anterior PSM just posterior to the *MyoD* expression domain independently of *her1* oscillation. This de novo expression, however, is unpatterned in that *her1* is expressed in a broad domain in the anterior PSM. Thus, although neither *aei/DeltaD* signaling nor *her1* oscillation appears to be required for the anterior induction of *her1* by the wave front, they are required for restraining the inductive activity. The oscillator thus appears to provide pattern, whereas the wave-front activity appears to provide a spatial–temporal signal that stabilizes and translates this pattern into regularly patterned somites.

Recent research had suggested that the oscillator acts to regulate *Notch* signaling that then directs somite border formation, possibly via *Eph:Ephrin* interactions (for review, see Durbin et al. 1998; Pourquié 1999). This relationship was largely inferred from the observation that although *c-hairy* oscillation was independent of protein synthesis, oscillation of the *Notch* pathway gene *lfng* was dependent on translation (Palmeirim et al. 1997; Forsberg et al. 1998; McGrew et al. 1998; Aulehla and Johnson 1999). However, the observation that *lfng* expression within the PSM is affected in *Dll1* and *RBP-Jκ* mutant mice even though neither of these genes shows such a dynamic expression pattern suggested that *Notch* signaling may act upstream of oscillating gene expression in the PSM. Here, we provide clear evidence that *Notch* pathway signaling is required for *her1* oscillation. This regulation of *her1* activity then appears to be required for the patterned expression of *aei/DeltaD* in the anterior PSM as *aei/DeltaD* stripe formation is perturbed in mutant embryos in which *her1* oscillation is lost (*aei/DeltaD*, *bea*, *des*, and *wit* mutant embryos). The failure to maintain and refine *her1* expression in *fss* embryos also correlates with defect in *aei/DeltaD* stripe formation. Moreover, misexpression of *her1* via mRNA injection perturbs *aei/DeltaD* stripe formation and somite formation (Takke and Campos-Ortega 1999). Thus, genetic data indicate that *aei/DeltaD* acts upstream of *her1* to regulate oscillation of *her1* expression, and the combination of genetic and misexpression data shows that *her1* functions upstream of *aei/DeltaD* stripe formation that is then required for proper somite border formation. The relative changes in expression of these two genes within the PSM during somite formation are depicted in Figure 7.

In both the mouse and in the zebrafish, many of the mutants in which somite segmentation is perturbed show an anterior to posterior polarity in phenotype in that the anterior regions are less severely affected than the posterior regions. In the zebrafish, *aei/DeltaD*, *des*, and *wit* form the anterior-most seven to nine somites but not the more posterior somites. Interestingly, the development of these anterior somites exhibits several differences from the development of the more posterior somites. The first six somites form more rapidly (three pairs per hour) than the posterior somites (two pairs per hour) (Westerfield 1995). These anterior somites also display a more synchronous development. For example, in

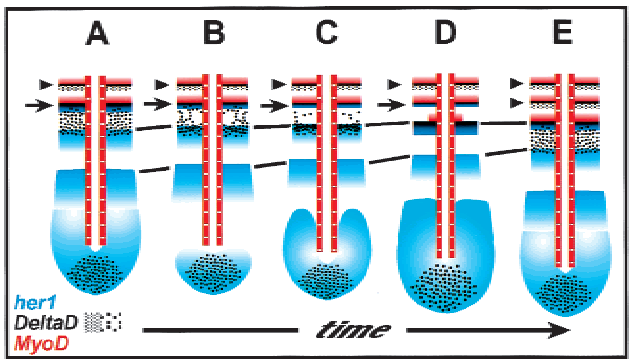

Figure 7. A model for the concomitant changes in *MyoD*, *her1*, and *aei/DeltaD* gene expression during zebrafish somite formation. Anterior is *top* and posterior is *bottom*. (*A–E*) A time series during one cycle of somite formation is represented. Somite furrows are indicated by arrowheads. *MyoD* is expressed in the posterior of each somite, whereas *aei/DeltaD* is weakly expressed in the anterior of each somite. The anterior-most stripe of *her1* overlaps with a strong stripe of *aei/DeltaD* (arrows). These two stripes refine and fade together (*A–D*) leaving only a faint stripe of *aei/DeltaD* expression (*E*). Around this time, the next, posterior stripe of *MyoD* emerges (*D*), and somite furrow formation occurs (second arrowhead in *E*). During this time period, the more posterior stripes of *her1* expression progress anteriorly (*A–E*) (equivalent stripes are indicated by connecting lines). Meanwhile, the weak domain of *aei/DeltaD* expression immediately posterior to the strong stripe of expression (arrow) in the anterior PSM (*A*) begins to refine (*B,C*). Ultimately, this process results in a strong stripe of expression that aligns anteriorly with a stripe of *her1* expression (*D*). Posterior to these overlapping stripes, a broad, weak domain of expression of *aei/DeltaD* forms (*E*) as was originally present in *A*. In the more posterior PSM and tailbud, an additional stripe of *her1* expression emerges (*B–E*). The expression of *aei/DeltaD* in the posterior tailbud remains relatively constant (*A–E*).

the first five somites, the adaxial cells undergo shape change and rearrangement simultaneously, whereas within the posterior somites the adaxial cells rearrange in an anterior to posterior progression as each somite matures (van Eeden et al. 1996). The refinement of *snail* expression, the initiation of *engrailed* expression, and the formation of the lateral *MyoD* stripes occur simultaneously in the anterior five to seven somites but sequentially in the posterior somites (Kimmel et al. 1991; Hammerschmidt and Nüsslein-Volhard 1993; Weinberg et al. 1996). The first *her1* stripe was originally fate mapped to the anlage of the fifth somite (Müller et al. 1996). However, our data indicates that *her1* oscillates through the PSM, which brings the fate mapping data into question. It is possible that if Müller et al. (1996) very carefully staged and positioned their embryos, they could have labeled the first and second *her1* stripes and also labeled the anlage of the fifth and seventh somite. In other words, by carefully staging the embryos, probably within at most a 10-min interval, and labeling in the same region that happened to correspond to the anlage of somite 5 and 7, they also could have caught *her1* expression just as the two stripes were passing through this region. Because the *her1* stripes would continue to move

anteriorly, they ultimately could stop and fade in the anterior of the anlage of somite 3 or 4, for example. Thus, it is not clear when the oscillator output of *her1* stripes begins to function during somitogenesis. Within this context, it is interesting to note that *aei*/*DeltaD* activity is not required for anterior somite formation, indicating that segmentation of these somites occurs independently of *aei*/*DeltaD*-dependent oscillations. This raises the possibility that anterior somite formation occurs independently of any oscillator. Conversely, *fss* activity is necessary for formation and anterior–posterior patterning of these somites, indicating that the wave-front activity or some additional function of *fss* is required.

How do the results presented here affect our understanding of the conservation of segmentation mechanisms in higher animals? Much excitement arose from the initial finding that *her1* was expressed in a pair-rule pattern as in the *Drosophila* embryo (Müller et al. 1996). In conjunction with the finding that the *amphioxus engrailed* gene (*AmphiEn*) is expressed in the posterior of the eight anterior-most somites, many suggested a possible conservation of segmentation mechanisms between protostomes and deuterostomes (Holland et al. 1997). Although there is precedent for such conservation in the roles that the *hox* genes play in defining segment identity and that the *dpp;Bmp/sog;chordin* patterning system plays in establishing embryonic dorsal–ventral polarity, it seems less likely now that such conservation exists for segmentation mechanisms because here we find that *her1* is not expressed in a pair-rule pattern. It seems more likely that the conservation of *hairy* function during segmentation reflects a conserved use of the *Notch* pathway during somitogenesis. That *Notch* function and a molecular oscillator are required for somitogenesis in all vertebrates seems likely at this point. However, differences in how this regulatory network is organized may exist. For example, which *Notch* pathway genes are direct outputs of the oscillator may vary. Although *lfng* expression oscillates in both the chick and mouse, the one known *lfng* homolog in zebrafish does not oscillate (Forsberg et al. 1998; McGrew et al. 1998; Aulehla and Johnson 1999; S. Holley and T. Vogt, unpubl.). One intriguing possibility concerning conservation of segmentation mechanisms between protostomes and deuterostomes concerns the development of the anterior somites. In *amphioxus* where *AmphiEn* is expressed in a metameric pattern, the anterior somites form via a mechanism distinct from the posterior somites (Holland et al. 1997). Given that anterior somitogenesis appears distinct in both mouse and zebrafish, it is possible that the as yet unidentified genetic mechanism that controls this process exhibits some homology to protostome segmentation mechanisms.

Materials and methods

Fish work

Fish were raised as described by Westerfield (1995). Eggs were derived from natural crosses. *aei;fss* double mutant embryos were derived from a cross between double heterozygous parents. Morphologically, the *fss* phenotype is dominant to *aei*. One quarter of the morphologically *fss* embryos displayed the composite (*fss* + *aei*) *her1* expression phenotype. These were classified as double mutant embryos.

Mapping

Radiation hybrid mapping was performed as described by Geisler et al. (1999). For the allele-specific PCR, 5-day-old embryos were chilled on ice for 30 min. Single embryos were placed in 96-well microtiter plates in 25 µl of 70 mg/ml proteinase K in TE and incubated at 70°C for 4 hr and at 75°C for 30 min. Each preparation was then diluted with 50 µl ddH_2O, and 5 µl was used for each PCR reaction. For the PCR reactions, primers AR33 MAP-3 (5′-gttgcactgatcttgaaaacacctacagct-3′), AR33 MAP-2 (5′-ccagcataacccatcgggcactggcagaca-3′), *shh* 7 (5′-ccggagatccgcgcctcgac-3′), and *shh* 10 (5′-ctgtgtcatgagcctgtccgctc-3′) were used at a final concentration of 0.225 µM. Additional final PCR conditions were 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 0.5 units of *Taq* polymerase. PCR cycling conditions were as follows: 94°C for 2 min; 35 cycles of 94°C for 30 sec, 67°C for 30 sec, 73°C for 1 min; and 73°C for 5 min. Reactions were analyzed by electrophoresis in 2% agarose gels. Three 96-well microtiter dishes containing 72 mutant and 24 sibling embryos were assayed twice, and only samples that produced the same result were counted. Sixty-one of seventy-two (84.7%) sibling embryos gave both the *shh* and *DeltaD* product. Two hundred and six aei^{AR33} embryos gave the *shh* product but not the *DeltaD* product.

Cloning and sequencing

Total RNA was isolated from mutant embryos using TriStar reagent (Angewandte Gentechnologie Systeme GmbH) according to kit protocol. Poly(A) RNA was subsequently isolated using Qiagen Oligotex. RT–PCR was performed using the SuperScript kit (GIBCO BRL). Three independently derived PCR products were either cloned into Bluescript or into PCR2.1 using the TA-cloning kit (Invitrogen). All clones were sequenced using the Thermo Sequenase fluorescent labeling primer cycle sequencing kit (Amersham), run on an A.L.F. sequencer (Pharmacia) and analyzed with the Lasergene software package.

In situ and antibody stainings

In situ hybridizations were performed according to standard protocols. Briefly, embryos were hybridized simultaneously with digoxigenin- and fluorescein-labeled RNA probes. Antibody incubation and NBT/BCIP staining for the digoxigenin-labeled probe were performed, followed by fixation, 2 × 15-min incubations in 100 mM glycine (pH 2.2), through a methanol series to 100% methanol for 1 hr, through a methanol series to PBST, washing, preincubation, and incubation with the anti-fluorescein antibody. The second staining reaction used the alkaline phosphatase substrate Fast Red (Roche). Embryos were cleared in 75% glycerol in PBST.

For antibody stainings, embryos were fixed in 4% PFA, blocked, incubated with the α-Islet-1 antibody (39.4D5 monoclonal, 1:500 dilution; Developmental Studies Hybridoma Bank), washed, incubated with 2% biotinylated anti-mouse (Vector), washed, and stained according to standard procedures. The first staining reaction, using the Elite ABC peroxidase kit (Vector), was performed in the presence of 0.03% $CoCl_2$ to give the black/gray stain. The second incubation with the α-myosin antibody (A4.1025 monoclonal, 1:50 dilution; Developmental

Studies Hybridoma Bank) was performed as above, and the second staining reaction was performed as above but without $CoCl_2$ to give the brown stain. Embryos were then fixed, washed, and cleared.

Cell transplantations

Cell transplantations were performed essentially as described by Westerfield (1995). Donor embryos were labeled with a fresh mixture of 1.25% biotin–dextran, MW10,000 and 1.25% tetramethyl-rhodamine–dextran MW10,000 (Molecular Probes). Roughly 20 cells were transplanted to host embryos. After in situ hybridizations were performed, donor cells were visualized using the Elite ABC peroxidase kit (Vector). Briefly, after the standard NBT/BCIP staining reaction, embryos were fixed for 20 min in 4% PFA, washed, put through a methanol series, incubated in methanol for 1 hr, through a methanol series to PBST. Tailbuds then were dissected, flat-mounted, and photographed. The tailbuds were recovered and incubated in 2% Blocking Reagent (Roche) for 1 hr. Meanwhile, 1 µl each of solutions A and B from the Elite ABC kit were mixed in 1 ml of blocking solution and preincubated for 45 min to 1 hr. The AB mixture was added to the embryos and incubated for 15 min. Embryos were washed three times for 30 min in PBST and preincubated in DAB (20 µg/ml) for 15 min. The staining reaction was initiated by adding 2 µl 0.3% H_2O_2/ml. Embryos were then fixed, washed, and cleared in 75% glycerol.

Photomicroscopy and graphics work

All embryos were digitally photographed (400 dpi) and analyzed using Adobe Photoshop 5.0. Figures were compiled using Freehand 8.0.

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