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her1 and the *notch* pathway function within the oscillator mechanism that regulates zebrafish somitogenesis

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SUMMARY

Somite formation is thought to be regulated by an unknown oscillator mechanism that causes the cells of the presomitic mesoderm to activate and then repress the transcription of specific genes in a cyclical fashion. These oscillations create stripes/waves of gene expression that repeatedly pass through the presomitic mesoderm in a posterior-to-anterior direction. In both the mouse and the zebrafish, it has been shown that the *notch* pathway is required to create the stripes/waves of gene expression. However, it is not clear if the *notch* pathway comprises part of the oscillator mechanism or if the *notch* pathway simply coordinates the activity of the oscillator among neighboring cells. In the zebrafish, oscillations in the expression of a *hairy*-related transcription factor, *her1* and the *notch* ligand *deltaC* precede somite formation. Our study focuses on how the oscillations in the expression of these two genes is affected in the mutants *aei*/*deltaD* and *des*/*notch1*, in 'morpholino knockdowns' of *deltaC* and *her1* and in double 'mutant' combinations. This analysis indicates that these oscillations in gene expression are created by a genetic circuit comprised of the *notch* pathway and the *notch* target gene *her1*. We also show that a later function of the *notch* pathway can create a segmental pattern even in the absence of prior oscillations in *her1* and *deltaC* expression.

Supplementary data available at http://www.eb.tuebingen.mpg.de/papers/holley_dev_2002.html

Key words: Zebrafish, *deadly seven*, *notch1*, *her1*, Somite, Segmentation, Oscillator, Morpholino

INTRODUCTION

Somites are segments within the vertebrate embryo that are the precursors of the vertebrae and muscle of the trunk and tail. These segments form sequentially from anterior to posterior, concomitant with the posterior extension of the embryo. Embryological studies first suggested the existence of a prepattern within the morphologically unsegmented presomitic mesoderm (PSM) that presages segment border formation (Aoyama and Asamoto, 1988). Subsequently, this prepattern was linked to an oscillator mechanism (Cooke, 1998; Cooke and Zeeman, 1975; Meinhardt, 1982; Meinhardt, 1986; Palmeirim et al., 1997) that causes cells of the PSM to go through repeated cycles of expression and repression of homologues of the *notch* pathway target gene *hairy* (Palmeirim et al., 1997; Holley et al., 2000; Jouve et al., 2000; Sawada et al., 2000) and homologues of the *notch* pathway genes *lunatic fringe* (*lfng*) (Forsberg et al., 1998; McGrew et al., 1998; Aulehla and Johnson, 1999) and *delta* (Jiang et al., 2000). These studies show that the cells within the PSM undergo multiple cycles of expression and repression of these genes, in a manner that is coordinated intercellularly to create stripes of mRNA expression that travel through the cells of the PSM in a posterior-to-anterior direction. This oscillation halts in the anterior PSM as the pattern is stabilized and interpreted to give rise to regular somite borders. While earlier studies had demonstrated the involvement of the *notch* signaling pathway in somitogenesis (Conlon et al., 1995; Dornseifer et al., 1997; Evrard et al., 1998; Hrabé Angelis et al., 1997; Jen et al., 1997; Jen et al., 1999; Kusumi et al., 1998; McGrew et al., 1998; Oka et al., 1995; Takke and Campos-Ortega, 1999; Wong et al., 1997; Zhang and Gridley, 1998), the requirement for the *notch* pathway in creating the oscillations in gene expression was not appreciated until more recently (del Barco Barrantes et al., 1999; Holley et al., 2000; Jouve et al., 2000). Nevertheless, both the specific function(s) of the *notch* pathway in this process and the identity of the oscillator remain unknown.

after eight (*aei*; *dld* – Zebrafish Information Network), *deadly seven* (*des*), *fused somites* (*fss*), *beamter* (*bea*) and *white tail*/*mindbomb* (*wit*) are the five genes that are necessary for normal somite formation isolated in our zebrafish genetic screen (van Eeden et al., 1996; Jiang et al., 1996). We have shown previously that *aei* codes for the *notch* ligand *deltaD* (Dornseifer et al., 1997; Holley et al., 2000). Moreover, we have shown that *aei*/*deltaD* is required to create the oscillating pattern of *her1*, but that its mRNA expression does not oscillate (Holley et al., 2000). However, none of the genes shown to be necessary to produce the oscillating pattern of mRNA expression actually oscillate themselves. Thus, it is not clear if these genes [*aei*/*deltaD* in the zebrafish and *Delta-like1* (*Dll1*)

in the mouse] constitute core components of the oscillator or if they simply are necessary to produce the oscillator readout. Furthermore, the analysis of the oscillating genes *hairy* and *lfng* in the chick, and *Hes1* and *Lfng* in the mouse suggest that neither of these genes functions within the oscillator mechanism (Palmeirim et al., 1997; McGrew et al., 1998; Forsberg et al., 1998; Aulehla and Johnson, 1999; Jouve et al., 2000). Thus, it is likewise not clear if any of the known oscillating genes are central components of the oscillator.

We show that *des* encodes for *notch1* (Bierkamp and Campos-Ortega, 1993). Like *aei*/*deltaD, des*/*notch1* expression does not oscillate, but its protein is required for the oscillation of both *her1* and *deltaC* expression. Using 'morpholino' oligonucleotides (mo), we performed a series of gene 'knockdown' experiments to ascertain the functions of the oscillating genes *her1* and *deltaC* during somitogenesis. We find that both genes are required to create the oscillating pattern of *her1* and *deltaC* expression. Further analysis of double-mutant and double-'knockdown' embryos indicates that the epistatic relationship between the *notch* pathway and *her1* changes along the anterior-posterior axis of the PSM. This demonstrates that these *notch* pathway genes have at least two functions during somitogenesis and that these genes operate within a *notch* pathway→*her1*→*notch* pathway regulatory circuit (Takke and Campos-Ortega, 1999). Because this circuit is comprised of genes that are necessary to create the oscillations in gene expression, these data suggest a model in which both the *notch* pathway and *her1* comprise part of the oscillator that regulates zebrafish somitogenesis.

MATERIALS AND METHODS

Fish work

Fish were raised as described elsewhere (Haffter et al., 1996). Embryos were derived from natural crosses at 28°C.

Mapping

Radiation hybrid mapping was performed as previously described (Geisler et al., 1999). For mapping of *des*, PCR reactions for specific SSLPs were performed as for the radiation hybrid mapping but at half the volume per reaction.

Allele sequencing

For each allele of *des*, PCR products derived from three independent reverse transcriptase (RT) reactions were sequenced using the ABI system and analyzed using the Lasergene software package. Total RNA was isolated from mutant embryos using TriStar reagent (Angewandte Gentechnologie Systeme GmbH) according to kit protocol. RT-PCR was performed using the SuperScript kit (GIBCO BRL). From each RT reaction, the *notch1* mRNA was amplified in nine overlapping 1 kb fragments. Current allele designations relate to the originals (van Eeden et al., 1996) as follows: des^{AXO1B}, des^{tx201}; des^{H35B}, des^{th35b}; des^{P37A}, des^{tp37}; des^{M145B}, des^{tm145}.

Morpholino injections

Morpholinos (Gene-Tools, http://www.gene-tools.com) were injected at the one-cell stage at a concentration of 50 μM ($deltaC^{mo1}$, 5′-agccatctttgccttcttgtctgct-3′), 50 μM ($deltaC^{moC}$, 5′-agtcatctttggctt-cttgtgttct-3′), 250 μM ($deltaC^{mo2}$, 5′-cgatagcagactgtgagagtagtcc-3′), 100 μM ($deltaD^{mo}$ 5′-aaacagctatcattagtcgtcccat-3′), 100 μM ($notch1^{mo}$, 5′-ttcaccaagaaacggttcataactc-3′), 1 mM ($her1^{mo1}$, 5′-cgacttgccatttttggagtaacca-3′), 1 mM ($her1^{moC}$, 5′-cgatttgacatttttgg-actaatca-3′) and 100 μM ($her1^{mo2}$, 5′-tggctgaaaatcggaagaagacgat-3′) in 1×Danieau (Nasevicius and Ekker, 2000).

In situ hybridization

In situ hybridization experiments were performed as previously described (Holley et al., 2000).

RESULTS

deadly seven is *notch1*

Examination of both the morphology of the somitic mesoderm and gene expression within the PSM has failed to identify any clear difference between *des* and *aei*/*deltaD*, suggesting that *des* also encodes for a *notch* pathway gene. Genetic linkage between *des* and *notch1* was found using a zebrafish microsatellite map to position *des* and an anchored radiation hybrid map to position *notch1* (Fig. 1C) (Geisler et al., 1999; Knapik et al., 1996; Knapik et al., 1998; Postlethwait et al., 1998; Shimoda et al., 1999). We then sequenced the *notch1*-coding region from four alleles of *des* and found premature stop codons in two alleles and amino acid substitutions in the other two alleles (Fig. 1D).

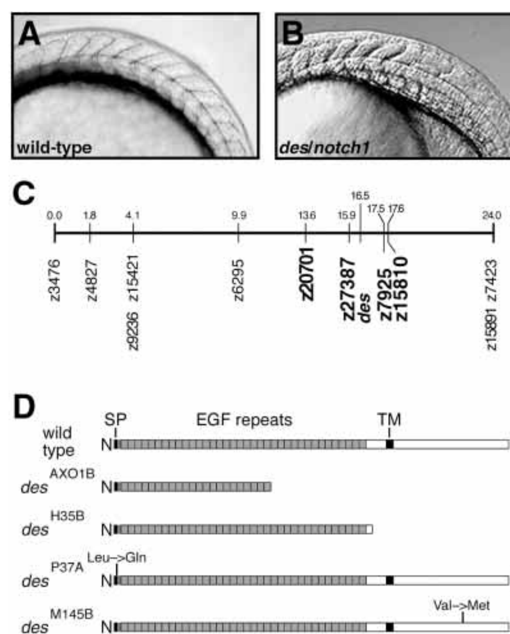

Fig. 1. *des* is *notch1*. Morphological phenotypes of (A) wild-type and (B) des^{P37A} embryos at about the 15-somite stage. *des* embryos form the first seven to nine somites but not the posterior somites. Anterior is leftwards. (C) *des* was mapped to linkage group 21 between z20701 and z7925, while *notch1* was mapped between z27387 and z7925 (z15810). Genetic distance from the top of linkage group 21 (left) is given in cM. (D) Four independent alleles of *des* were sequenced. des^{AXO1B} has a 7 bp insertion (5′-TGTGCAG-3′) between bases 2738 and 2739, creating a frame-shift and premature stop, seven codons to the C terminus. des^{H35B} has a T to A transition at base 4552, converting a Cys to a stop. des^{P37A} has a T to A transition at base 186, creating a Leu to Gln substitution within the hydrophobic domain of the signal peptide (SP). des^{M145B} has a G to A transition at base 6683, causing a Val to Met substitution. There are no obvious differences between these alleles in the severity of the somite phenotype. Nucleotide and amino acid sequences refer to the published wild-type sequences (Bierkamp and Campos-Ortega, 1993). TM, transmembrane domain.

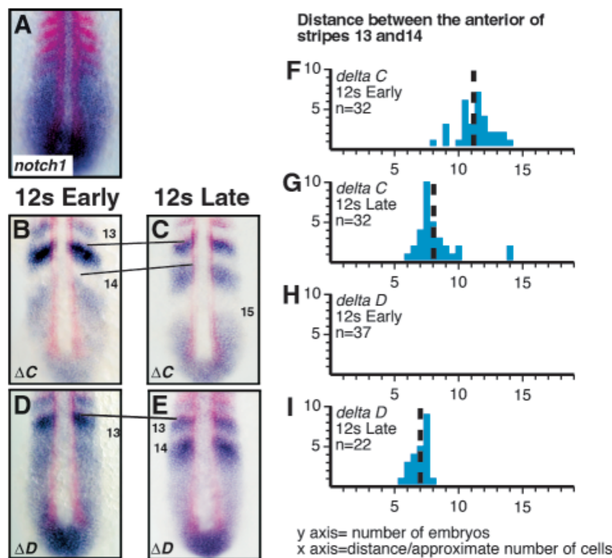

Fig. 2. Comparison of the oscillating pattern of *deltaC* expression with the non-oscillating expression of *deltaD* and *notch1*. (A) In wild-type embryos, *notch1* is always expressed uniformly throughout the PSM, indicating that its expression does not oscillate. *myoD* expression is shown in red. (B-I) Wild-type embryos were staged and analyzed as previously described (Holley et al., 2000).
(B-E) Representative early and late 12-somite stage embryos stained for *deltaC* (ΔC) (B,C) or *deltaD* (ΔD) (D,E) in blue and counterstained for *myoD* in red. In each panel, anterior is upwards. (F-I) Graphs depicting the distances that separate anterior of *deltaC* stripes 13 and 14 at the early (F) and late (G) 12 somite stage. The distances separating *deltaD* stripes 13 and 14 at the early and late 12 somite stages are depicted in H and I, respectively. Note that in H, no data points exist because the more posterior *deltaD* stripe has not formed yet. Measurements were made in pixels and later converted to number of cells (8 pixels/cell). Mean values are represented by broken lines. The mean values for F and G were compared using a two-sample *t*-test. The difference between the means in F and G is 25.25 pixels (3.1 cells) with a 95% C.I. from 18.7 to 31.7 pixels indicating that the differences between the data in F and G are significant. This indicates that, as for *her1*, the distance between consecutive *deltaC* stripes decreases as the somite cycle progresses.

Neither *des/notch1* nor *aei/deltaD* expression oscillates

des/notch1 mRNA is expressed uniformly throughout the PSM (Fig. 2A) (Bierkamp and Campos-Ortega, 1993). Thus, *des/Notch1* expression does not oscillate, but its protein is necessary for the oscillation of both *her1* and *deltaC* expression (Fig. 3F,L) (van Eeden et al., 1998; Holley et al., 2000; Jiang et al., 2000). While our previous analysis suggested that *deltaD* expression did not oscillate, several recent papers state that *deltaD* expression oscillates, although there are no data in the literature to support this conclusion. In light of these discrepancies, we compared the expression of *deltaD* with the oscillating expression of *deltaC* (Jiang et al., 2000) using the same protocol that we had previously used to show that *her1* expression oscillates (Holley et al., 2000). We staged embryos morphologically at the early and late 12 somite stage and performed double in situ analysis. Embryos at both the early and late 12 somite stages were probed for either *myoD* and *deltaC* expression (Fig. 2B,C) or *myoD* and *deltaD* expression (Fig. 2D,E). These embryos were flat mounted and digitally photographed. Using Adobe Photoshop, we measured the distances between the anterior of the *deltaC* or *deltaD* stripe immediately posterior to the 13th *MyoD* stripe ('13' in Fig. 2B-E) and the next posterior stripe ('14' in Fig. 2B-E). This converts the in situ data into numerical data (distance in pixels) that can be analyzed statistically (Fig. 2F-I). These studies indicate that the distance between the anterior borders of consecutive *deltaC* stripes decreases with the progression of the somite cycle: at the early 12-somite stage, this distance averages about 11 cells (Fig. 2F), while 20-25 minutes later at the late 12-somite stage, this distance averages seven to eight cells (Fig. 2G). This is the behavior that we have seen with *her1* expression, and, accordingly, double in situ analysis of *her1* and *deltaC* expression indicates that their oscillating expression patterns in the PSM superimpose (not shown). Our previous timelapse analysis revealed no anterior cell compaction or cell migration within the PSM before somite formation, indicating that the stripes of expression are moving through the cells of the PSM. Similar to *her1* expression, the anterior most *deltaC* stripe is immediately posterior to the 13th *myoD* stripe (Fig. 2C) and (Holley et al., 2000). As *myoD* is expressed in the posterior half-somite, this *deltaC* stripe must correspond to the anlage of the next anterior half-somite. Subsequently however, *deltaC* expression is found in the posterior half of each somite (Jiang et al., 2000), meaning that *deltaC* expression reverses polarity during the course of morphological somite formation.

In contrast to *deltaC*, we found no evidence that *aei/deltaD* expression oscillates. The more posterior *deltaD* stripe was never seen in the early 12-somite stage embryos but was formed in the late 12-somite stage embryos (Fig. 2D,E). This indicates that each *deltaD* stripe forms relatively late in each somite cycle. Additionally, the distance between the two *deltaD* stripes does not vary significantly (the data points in Fig. 2I are tightly clustered) indicating that, once formed, the stripes do not move. In the posterior tailbud, *deltaD* expression exhibits much less variation in expression than that observed in *her1* and *deltaC* expression, and no clear distinction in this posterior expression can be made when comparing early and late 12-somite stage embryos (not shown). We therefore think that the slight posterior variation in *deltaD* expression is random and not due to the activity of the oscillator. In summary, while *deltaD* expression undergoes cyclical changes each somite cycle, the formation of the *deltaD* stripes is a process that is specific to the anterior PSM and is distinct from the oscillating expression of *her1* and *deltaC*. This distinction is also seen in the anterior PSM of *fss* embryos, where *deltaD* expression persists, but *her1* and *deltaC* expression is lost (van Eeden et al., 1998; Holley et al., 2000; Jiang et al., 2000).

The oscillations in gene expression are dependent upon both *her1* and *deltaC*

Because we do not have zebrafish mutants that correspond to either of the oscillating genes, *her1* and *deltaC*, we have used a reverse genetic approach to ascertain the function of these genes in generating the oscillating pattern. Morpholino oligonucleotides specifically inhibit the translation of their target mRNAs (Nasevicius and Ekker, 2000), and here we show that injection of morpholinos specific to either *deltaD* or *notch1*, can recapitulate the phenotype of *aei* and *des*, respectively, with over 90% penetrance (Fig. 3A,B,F,L).

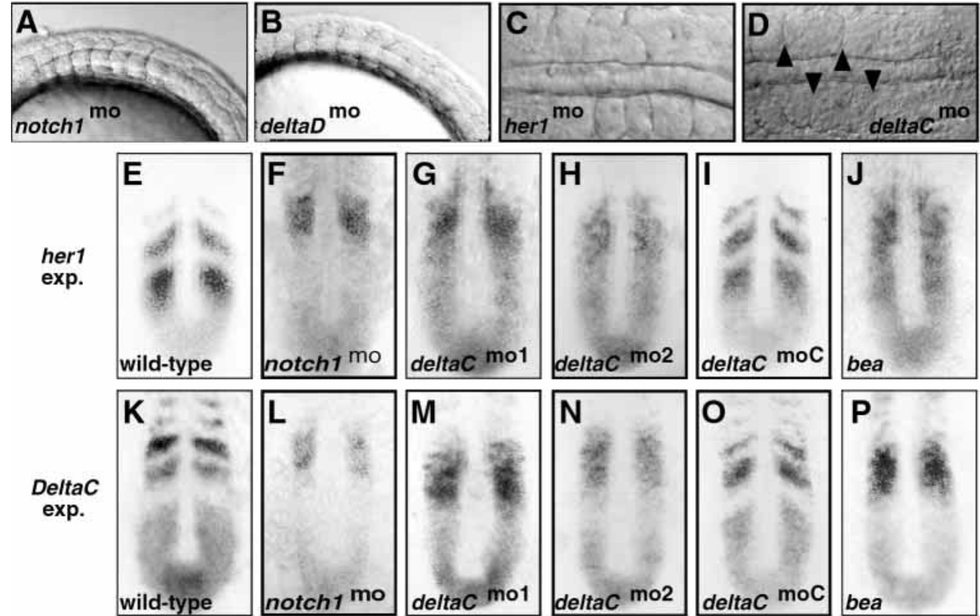

Fig. 3. Injection of morpholinos specific to *notch1*, *deltaD*, *her1* or *deltaC* perturbs somite formation. Embryos injected with (A) *notch1*^{mo} (four experiments; n=201; 97% affected) or (B) *deltaD*^{mo} (four experiments; n=127; 99% affected) form the anterior seven to nine somites but fail to make regular posterior segments. (C,D) Dorsal views of *her1*^{mo1} (4 experiments n=200; 91% affected) or *deltaC*^{mo1} (6 experiments; n=545; 78% affected) injected embryos, respectively. In contrast to *aei*/*deltaD* and *des*/*notch1*, *her1* and *deltaC* are necessary for the formation of both the anterior and posterior somites. Arrowheads in D indicate the misplaced somite borders. (E-P) The expression patterns of *her1* and *deltaC* seen in wild-type, *bea* and morpholino-injected embryos. These embryos are between the 8 and 12 somite stages. Anterior is upwards. (E,K) Wild-type expression patterns of *her1* and *deltaC*, respectively. Injection of *Notch1*^{mo} causes defects in *her1* expression (F) (two experiments; n=133; 100% affected) and *deltaC* expression (L) (two experiments; n=38; 100% affected) identical to that observed in *des* embryos. Injection of *deltaD*^{mo} recapitulates the pattern of gene expression that is observed in *aei*/*deltaD* embryos (not shown). Injection of *deltaC*^{mo1} disrupts *her1* expression (G) (four experiments; n=109; 98% affected) and *deltaC* expression (M) (6 experiments; n=168; 100% affected). Injection of a second *deltaC* morpholino, *deltaC*^{mo2}, that does not overlap the sequence of *deltaC*^{mo1}, produces the same defect in the expression of both *her1* (H) (four experiments; n=145; 100% affected) and *deltaC* (N) (four experiments; n=187; 100% affected). Conversely, a control morpholino identical to *deltaC*^{mo1}, except for four nucleotide substitutions, *deltaC*^{moC}, has no effect on the expression of either *her1* (I) (three experiments; n=62; 0% affected) or *deltaC* (O) (3 experiments; n=56; 0% affected). (J,P) Expression of *her1* and *deltaC*, respectively, in *bea*^{M98B} embryos. *bea*^{M98B} embryos were collected from a mating of homozygous *bea*^{M98B} adults. *deltaD* expression is the same in all of the mutants and knockdown embryos, with the exception of ~15% of *fss* embryos, as previously noted (not shown) (Holley et al., 2000). For F-H and L-N, percentages are in reference to n, the number of pre-sorted morphologically affected embryos examined. The specificity of the individual morpholinos is illustrated by the fact that: (1) both the *deltaD*^{mo} and *notch1*^{mo} phenocopy their known mutant phenotypes; and (2) *deltaC*^{mo1} and *deltaC*^{mo2} produce the identical phenotype, while *deltaC*^{moC} produces no phenotype.

Injection of morpholinos specific to either *her1* or *deltaC* leads to irregular somite border formation (Fig. 3C,D), and examination of gene expression indicates that both genes are necessary to generate the oscillating pattern of *her1* and *deltaC* expression (Fig. 3G,H,M,N; Fig. 4B,D). The expression patterns that are observed in *deltaC*^{mo} embryos are somewhat similar to the patterns observed in the existing mutants (Fig. 3). However, the expression patterns seen in *her1*^{mo} embryos are unique. In *her1*^{mo} embryos, *her1* is expressed throughout the PSM and shows no variation in levels of expression between neighboring cells (Fig. 4B; see http://www.eb.tuebingen.mpg.de/papers/holley_dev_2002.html). This pattern reveals no evidence of oscillations in gene expression, indicating that Her1 protein is required to generate the oscillations in expression of *her1* mRNA. *deltaC* is expressed weakly in the posterior and intermediate PSM of *her1*^{mo} embryos and more strongly in the anterior PSM. Again, there is no heterogeneity in the levels of expression of *deltaC* among neighboring cells in these embryos, except for the refinement seen in the anteriormost PSM (Fig. 4D, Fig. 5I; see http://www.eb.tuebingen.mpg.de/papers/holley_dev_2002.html). Therefore, *her1* function also is necessary to generate the oscillations of *deltaC* expression.

Multiple requirements for *notch* signaling during somitogenesis

Gain-of-function experiments have suggested the existence of a *notch* pathway→*her1*→*notch* pathway regulatory loop within the zebrafish PSM (Takke and Campos-Ortega, 1999). Our loss-of-function analysis of mutant and morpholino-injected embryos shows that the *notch* pathway (*aei*/*deltaD*, *des*/*notch1* and *deltaC*) acts upstream of *her1* to promote *her1* expression, and that *her1* feeds back on the *notch* pathway to regulate *deltaC* expression. An additional series of epistasis experiments independently demonstrate the existence of this regulatory loop by showing that the *notch* pathway functions both upstream and downstream of *her1* in the anterior PSM.

The first set of epistasis experiments uses the *fss* phenotype as a reference. *fss* is unique among the known zebrafish genes in that it functions not in creating the oscillating pattern but in maintaining this pattern in the anterior PSM. In *fss* embryos, one or two *her1* (and *deltaC*) stripes are present, but the anteriormost stripe is always missing (Fig. 5C) (van Eeden et al., 1998; Holley et al., 2000). Analysis of *fss*;*des*/*notch1* and *fss*;*aei*/*deltaD* double mutants indicated that the ‘salt and pepper’ expression of *her1* in the anterior PSM of *aei*/*deltaD* and *des*/*notch1* embryos is dependent entirely upon *fss* function (Fig. 5B-D) (van Eeden et al., 1998; Holley et al., 2000). This indicates that *fss* activity is required in the anterior PSM in the absence of *des*/*notch1* and *aei*/*deltaD*. Thus, in the anterior PSM, *fss* functions downstream of *des*/*notch1* and *aei*/*deltaD*. Ectopic expression of *her1* in the anterior PSM is also observed in *deltaC*^{mo} embryos, *her1*^{mo} embryos and *bea* embryos (Fig.

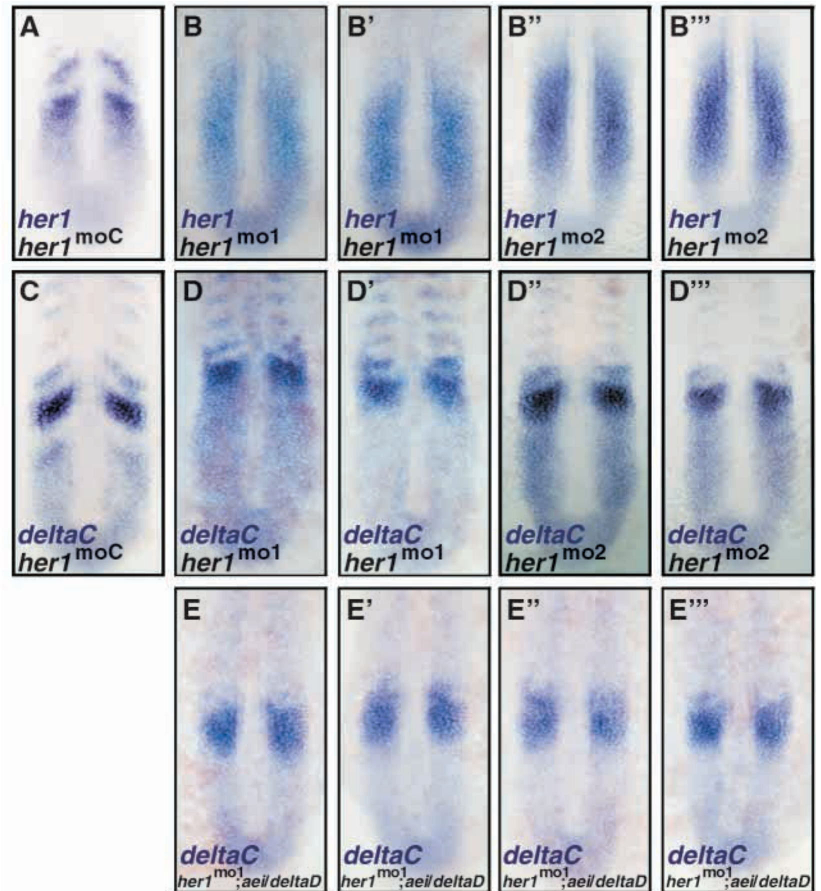

Fig. 4. Loss of *her1* function eliminates all evidence of the oscillations in gene expression. These embryos are between the 8 and 12 somite stages. In all panels, anterior is upwards. (A) The wild-type *her1* expression pattern is observed in all embryos injected with a control morpholino, *her1*$^{\mathrm{moC}}$, that is identical to *her1*$^{\mathrm{mo1}}$, except for four nucleotide substitutions (four experiments; *n*=182; 0% affected). (B,B') injection of *her1*$^{\mathrm{mo1}}$ into wild-type embryos leads to a de-repression of *her1* expression (three experiments; *n*=76; 100% affected). (B'',B''') injection of a second *her1* morpholino, *her1*$^{\mathrm{mo2}}$, which does not overlap the sequence of *her1*$^{\mathrm{mo1}}$, produces the identical defect in *her1* expression (three experiments; *n*=128; 100% affected). Notice that there is no heterogeneity in the levels of expression between neighboring cells. (C) Wild-type expression pattern of *deltaC* is seen in embryos injected with *her1*$^{\mathrm{moC}}$ (three experiments; *n*=162; 0% affected). (D,D') in embryos injected with *her1*$^{\mathrm{mo1}}$, *deltaC* expression is reduced throughout the posterior and intermediate PSM (three experiments; *n*=77; 100% affected). In the anterior PSM, *deltaC* is expressed in a smooth domain that undergoes a refinement in the anteriormost PSM. This refinement appears to originate from the anterior and creates stripes of *deltaC* expression that can be later seen in the somitic mesoderm. (D'',D''') injection of *her1*$^{\mathrm{mo2}}$ produces the identical defect (three experiments; *n*=122; 99% affected). (E-E''') The refinement of *deltaC* expression is lost in *her1*$^{\mathrm{mo1}}$;*aei*/*deltaD*$^{\mathrm{AR33}}$ embryos. Additionally, the stripes of *deltaC* expression in the somitic mesoderm are lost.

3G,H,J; Fig. 4B). We have found that this anterior expression is lost in *fss*;*deltaC*$^{\mathrm{mo}}$ embryos and *fss*;*bea* embryos but not *fss*;*her1*$^{\mathrm{mo}}$ embryos (Fig. 5E-G). Therefore, while *fss* functions downstream of both *deltaC* and *bea*, *her1* is the only gene found so far that functions downstream of *fss* in the anterior PSM.

The second set of epistasis experiments makes use of a unique feature of the *deltaC* expression pattern in *her1*$^{\mathrm{mo}}$ embryos: the strong domain of *deltaC* expression in the anterior PSM is refined, resulting in stripes of *deltaC* expression that persist in the somitic mesoderm (Fig. 4D; Fig. 5I). These stripes resemble the stripes of *deltaC* expression seen in wild-type embryos (Fig. 3K, Fig. 4C, Fig. 5H). We have used this refinement of *deltaC* expression in *her1*$^{\mathrm{mo}}$ embryos as an assay to test for additional functions for *fss* and the *notch* pathway in the anteriormost PSM, downstream of *her1*.

her1 is epistatic to *fss* with regard to *deltaC* expression in the anterior PSM [i.e. *deltaC*, like *her1*, is expressed in the anterior PSM of *her1* $^{\mathrm{mo}}$;*fss* embryos (Fig. 5J) but not *fss* embryos (Jiang et al., 2000)]. However, the refining of the *deltaC* expression domain observed in *her1*$^{\mathrm{mo}}$ embryos is lost (compare Fig. 4D and Fig. 5I with Fig. 5J). Thus, while *her1* acts downstream of *fss* with regard to the maintenance of *deltaC* expression in the anterior PSM, *fss* functions downstream of *her1* with regard to the later refining of *deltaC* expression in the anteriormost PSM. Analysis of double mutants between *her1*$^{\mathrm{mo}}$ and either *aei*/*deltaD, des*/*Notch1, deltaC*$^{\mathrm{mo}}$ or *bea*, indicate that each of these latter genes functions downstream of *her1* in the anteriormost PSM to create the refining pattern of *deltaC* (Fig. 5K-N). In these double mutant embryos, this refining pattern is converted into a weak 'salt and pepper' pattern, and the stripes of *deltaC* expression in the somitic mesoderm are eliminated (Fig. 4E; Fig. 5J-M).

DISCUSSION

her1 and the *notch* pathway may function within the oscillator

Both *aei*/*deltaD* and *des*/*Notch1* are necessary to promote the expression of the oscillating genes *her1* and *deltaC*. Meanwhile, *her1* regulates *deltaC* expression and functions, directly or indirectly, in a negative feedback loop to repress its own transcription. Thus, the *notch* pathway functions upstream of *her1* to promote the transcription of *her1* mRNA, and *her1* functions upstream of the *Notch* pathway to create the oscillating pattern of *deltaC* transcription. This identifies a rudimentary genetic loop (*notch* pathway→*her1*→*notch* pathway) that functions within the PSM [Fig. 6A (I)]. Further, we show that *fss* functions downstream of the *notch* pathway but upstream of *her1* in the anterior PSM [Fig. 6A (II)], and that the *notch* pathway and *fss* function downstream of *her1* slightly later in the anteriormost PSM [Fig. 6A (III)]. Therefore, the regulatory circuit consisting of *her1* and the *notch* pathway exists throughout the PSM. Because this genetic circuit comprises genes that are required to create the oscillations in gene expression, these findings suggest that *her1*

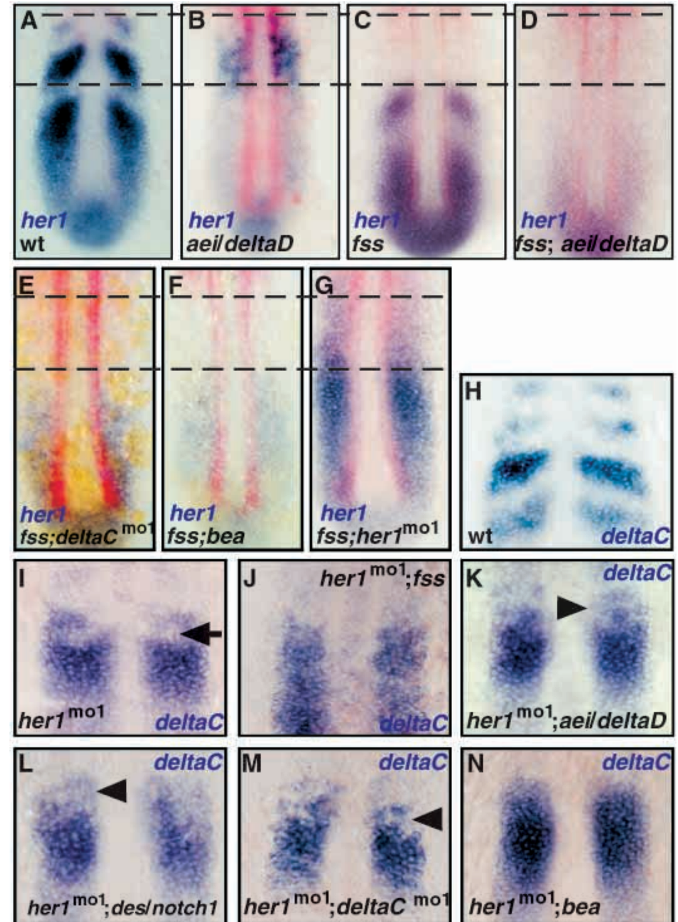

Fig. 5. The epistatic relationship between *her1* and the *notch* pathway changes along the anteroposterior axis of the PSM. Anterior is upwards. (A-G) *her1* expression is in blue and that of *myoD* is in red. (A) In wild-type embryos, stripes of *her1* expression are seen throughout the PSM. This embryo is a sibling of the mutant *aei*/*deltaD*^{AR33} embryo shown in B. In *aei*/*deltaD* embryos, no stripes of *her1* expression are observed, and *her1* is always exclusively expressed in a 'salt and pepper' pattern in the anterior PSM (between the broken lines). (C) *her1* stripes form in *fss*^{AE114} embryos, but expression in the anterior PSM is always lost. (D) A *fss*^{AE114};*aei*/*deltaD*^{AR33} double mutant sibling of the embryo shown in C. In *fss*;*aei*/*deltaD* double mutant embryos, no stripes of *her1* expression are formed (as in *aei*/*deltaD* embryos) and there is no expression of *her1* in the anterior PSM (as in *fss* embryos). (E) Injection of *deltaC*^{mo1} into *fss*^{AE114} embryos produces a pattern of *her1* expression similar to that observed in *fss*;*des*/*notch1* and *fss*;*aei*/*deltaD* double mutant embryos: no stripes of expression are formed and no expression is seen in the anterior PSM (four experiments; n=123; 99% affected). (F) *fss*^{AE114};*bea*^{M98B} double mutant embryos also lack both stripes of *her1* expression and expression in the anterior PSM. These embryos were derived from a cross between double homozygous parents. (G) Injection of *her1*^{mo1} into *fss*^{AE114} embryos produced a *her1* expression pattern identical to that of *her1*^{mo} embryos (three experiments; n=158; 99% affected). A parallel analysis of *deltaC* expression yielded similar results (not shown). (H-N) All panels show *deltaC* expression. (H) *deltaC* expression in wild-type embryos. (I) *her1*^{mo1} embryos (four experiments 72% of 97 embryos) exhibit a refining stripe within the *deltaC* expression domain in the anteriormost PSM, arrow. In *her1*^{mo1};*fss*^{AE114} embryos (J) (two experiments 0% of 184 embryos), *her1*^{mo1};*aei*/*deltaD*^{AR33} embryos (K) (four experiments 0.5% of 202 embryos), *her1*^{mo1};*notch1*^{mo1} embryos (L) (three experiments 0.7% of 153 embryos), *her1*^{mo1}; *deltaC*^{mo1} (M) (three experiments 0.6% of 169 embryos) and *her1*^{mo1}; *bea*^{M98B} (N) (two experiments 1% of 86 embryos), this refining stripe is lost. Sometimes in these double mutant embryos the pattern (stripe) of repression is converted into a 'salt and pepper' pattern, arrowheads in K-M.

and the *notch* pathway have cyclical functions at the center of the somitogenesis oscillator.

The genetic analysis of *her1* and the *notch* pathway suggest a model in which these genes somehow generate the oscillations in gene expression. The initiation of the oscillations may be coupled to the commitment to become paraxial mesoderm. The expression of each of these genes (*her1*, *deltaC*, *aei*/*deltaD* and *des*/*notch1*) is initiated at the tip of the tailbud as cells subduct to form the paraxial mesoderm (Bierkamp and Campos-Ortega, 1993; Müller et al., 1996; Dornseifer et al., 1997; Jiang et al., 2000; Kanki and Ho, 1996). The subsequent activities of these proteins could then initiate the interactions that create the oscillations in gene expression (Fig. 6B). *deltaC*, *aei*/*deltaD* and *des*/*notch1* signaling would activate the transcription of *her1* and *deltaC*. The subsequent increase in Her1 protein would then act to block the transcription of *her1*. As the *hairy* proteins typically function as transcriptional repressors (Fisher et al., 1996; Paroush et al., 1994), an increase in Her1 should result in an increase in repressive activity, and the gradual degradation of this protein would produce a gradual decrease in this repressive activity. Therefore, the anterior progression/activation of a stripe of gene expression could be driven by the gradual loss of a repressive activity generated during the previous somite cycle. The positive regulation via *notch* could also display a cyclical variation, but ultimately the re-initiation of *her1* and *deltaC* transcription would not occur until the level of Her1 drops below a specific threshold. In essence, this model suggests that the anterior progression of a stripe of gene expression is, at least in part, driven by the degradation of an existing, repressive activity (Her1), as opposed to the de novo synthesis of an activating component.

The loss-of-function phenotype of these genes now can be explained within the context of this model. After the expression of each of these genes is initiated at the posterior of the tailbud, the resulting proteins would initiate the oscillations. If Her1 is absent, then *her1* expression is never downregulated in the PSM (Fig. 4B). If *aei*/*deltaD* or *des*/*notch1* function is lost, then Her1 derived from the initial burst of *her1* expression in the tailbud will repress the transcription of its own mRNA, and the loss of *notch* signaling would then lead to a failure to re-initiate *her1* transcription (Fig. 5B). The phenotypes seen in the anterior PSM in the *notch* pathway mutants (the 'salt and pepper' pattern) are likely to be the result of an anterior-specific activity.

This model is in agreement with misexpression studies in *Xenopus*, suggesting that periodic changes in *notch* signaling activity occur in the PSM (Jen et al., 1999). Our model also can explain the observation that the anterior progression of a wave of chick *hairy* expression is unperturbed when the PSM is physically separated into anterior and posterior halves (Palmeirim et al., 1997). The 'gradient' of the repressive activity of chick *hairy* could provide an instructive memory within the cells of the PSM, and the remaining cell-cell

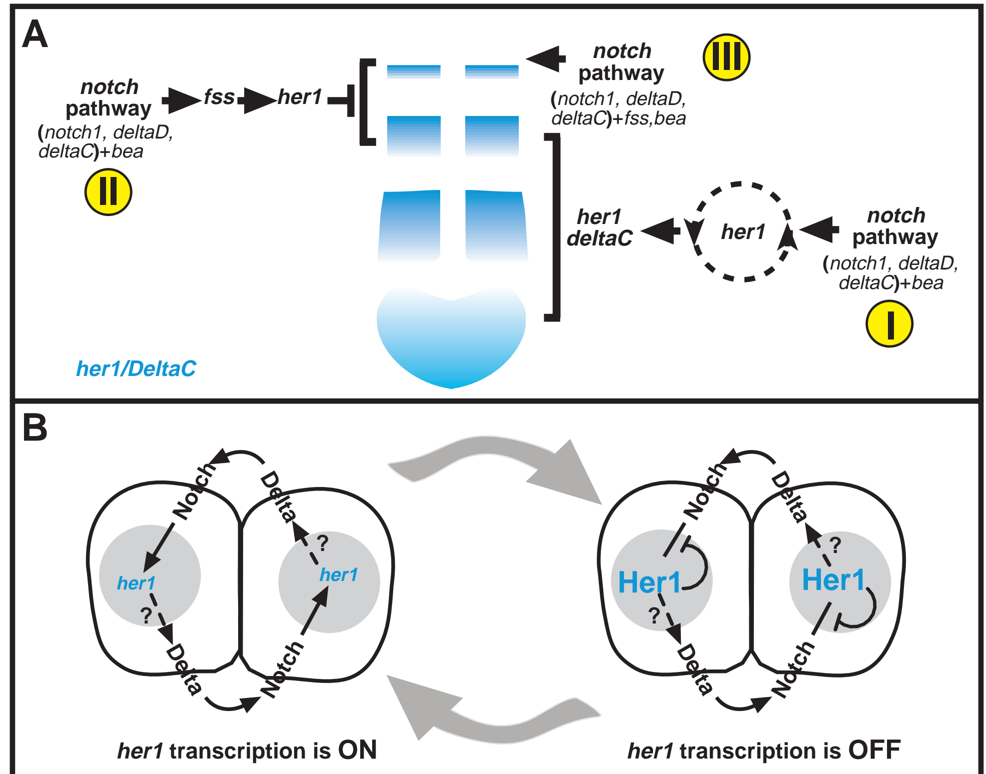

Fig. 6. A summary of the genetic analysis of the functions of *her1* and the *notch* pathway during somitogenesis. (A) Anterior is upwards. (I) the *notch* pathway and *bea* are required to generate the oscillating expression of *deltaC* and *her1* in the posterior and intermediate PSM. *her1* probably functions within the oscillator and feeds back on the *notch* pathway to create the oscillating pattern of both *deltaC* and *her1*. (II) *fss* functions downstream of the *notch* pathway but upstream of *her1* in the anterior PSM. (III) Slightly later, the *notch* pathway, *bea* and *fss* function in the anteriormost PSM/somitic mesoderm. (B) A model in which the *notch* pathway→*her1*→*notch* pathway circuit creates the oscillations in gene expression. While the actual oscillator is probably more complicated, this model reflects the present data. Two states are represented: one in which *her1* transcription is on (left) and one in which *her1* transcription is off (right). Activation of Notch via interaction with Delta expressed on the surface of neighboring cells causes the activation of *her1* transcription (left). The subsequent increase in Her1 would then act in a negative feedback loop to repress its own transcription (right). In time, the amount of Her1 would drop below a threshold and would allow *her1* transcription to be activated again (left). Non-autonomous effects of the oscillations may be mediated by DeltaD, DeltaC or perhaps an unidentified ligand. The data addressing this aspect of the oscillations are not easily interpreted (represented by the '?' and the broken arrow).

contacts would provide the required Notch-Delta signaling interactions needed to re-initiate chick *hairy* expression. This type of regulation would not require the oscillating signal to always be propagated from the posterior by an intercellular relay.

notch-dependent or *notch*-independent oscillations?

In wild-type embryos, neighboring cells oscillate together (they turn on *her1* expression together and turn off *her1* expression together). This coordination creates the stripes of *her1* expression. It has been proposed that the function of the *notch* pathway during somitogenesis is to synchronize, not to generate, the oscillations of gene expression (Jiang et al., 2000). According to this model, perturbation of *notch* pathway signaling will cause the cells to lose coordination, and the cells will continue to oscillate independently of their neighbors. These de-synchronized oscillations would not create stripes of gene expression. Instead, a 'salt and pepper' pattern is created in which there is random heterogeneity in levels of gene expression among neighboring cells. The important difference between the de-synchronization model and the model presented in this paper is that the former proposes that the *notch* pathway does not create the oscillations in gene expression and that in the absence of *notch* signaling, the oscillations in gene expression persist. The model presented here proposes that the *notch* pathway generates the oscillations in gene expression and that in the absence of *notch* pathway signaling, oscillations in gene expression no longer occur.

The phenotype of the *her1*^{mo} embryos supports the model in which *her1* and the *notch* pathway create the oscillations in gene expression and is inconsistent with the de-synchronization model. *her1* is expressed throughout the PSM in *her1*^{mo} embryos and there is no significant variation in this expression between sibling embryos, i.e. there is no evidence of coordinated oscillations. Moreover, *her1*^{mo} embryos show no variation in the levels of *her1* expression among neighboring cells, i.e. there is no evidence of de-synchronized oscillations (Fig. 4B; see http://www.eb.tuebingen.mpg.de/papers/holley_dev_2002.html). The expression of *deltaC* in *her1*^{mo} embryos is more similar to the expression pattern seen in the *notch* pathway mutants: *deltaC* is expressed weakly in the posterior PSM and in a strong domain always found in the anterior PSM. However, this anterior expression domain of *deltaC* in *her1*^{mo} embryos is uniform and not in a 'salt and pepper' pattern, i.e. there is no evidence of asynchronous oscillations (Fig. 4D, Fig. 5I). These phenotypes indicate that the oscillations in *her1* and *deltaC* expression do not occur if *her1* function is absent.

The de-synchronization model originally suggested that the 'salt and pepper' patterns of *her1* and *deltaC* expression seen in *aei/deltaD* embryos are indicative of continued but de-synchronized cellular oscillations in gene expression (Jiang et al., 2000). However, this model does not account for the absence of stripes of gene expression within the posterior PSM of *aei/deltaD* embryos because the 'salt and pepper' pattern is restricted to the anterior PSM. *aei/deltaD* embryos do not have cells within the posterior PSM that express *her1* at levels equivalent to the high levels of expression seen within the

posterior stripes in wild-type sibling embryos (compare Fig. 5A with 5B). Therefore, there is nothing to indicate that these cells in the posterior and intermediate PSM are oscillating in the absence of *aei*/*deltaD* function (Holley et al., 2000). Furthermore, the de-synchronization model does not explain why there is an abrupt or coordinated onset of the 'salt and pepper' pattern within the *middle* of the domain in which the oscillations normally are observed. If the oscillations in *her1* and *deltaC* expression persisted in *aei*/*deltaD* embryos, then virtually all of these embryos should exhibit a strong 'salt and pepper' pattern gradually arising within the more *posterior* PSM, as observed for *her1* expression in *bea* embryos (Fig. 3J).

In *fss* embryos, the oscillations in gene expression occur, but the anteriormost stripe is always missing, indicating that *fss* is not required to generate the oscillating pattern but is required to maintain this pattern in the anterior PSM (Fig. 5C) (van Eeden et al., 1998; Holley et al., 2000; Jiang et al., 2000). Here, the de-synchronization model would make a simple prediction: removal of *notch* pathway activity in the *fss* background via *fss*;*aei*/*deltaD* and *fss*;*des*/*notch1* double mutant combinations should create a de-synchronized version of the oscillating pattern observed in *fss* embryos, i.e. a 'salt and pepper' pattern instead of stripes. However, only weak posterior expression is observed in these embryos and there is no variation in levels of expression among neighboring cells (Fig. 5D; see http://www.eb.tuebingen.mpg.de/papers/holley_dev_2002.html) (van Eeden et al., 1998; Holley et al., 2000). The cells turn on *her1* expression posteriorly and together, gradually lose their expression as they mature and become relatively more anterior. Thus, the loss of *Notch* pathway function results in an elimination, not de-synchronization, of oscillations in gene expression.

These analyses indicate that all evidence of the oscillations in *her1* and *deltaC* expression is absent in backgrounds in which either *her1* or *aei*/*deltaD* function is missing. This suggests that the generation of the oscillations and the coordination of the oscillations between cells are one and the same, and that the two processes cannot be separated. Nevertheless, one cannot exclude the possibility that the oscillations persist in these mutants in some way that is not observed and that the oscillations in gene expression are a subset of a more general, unseen oscillation.

The anterior presomitic mesoderm

A 'salt and pepper' expression pattern could be created by a number of patterning processes gone awry and is not indicative inherently of oscillations. In fact, non-oscillating genes such as *deltaD* and *mesp-b* also can exhibit a patchy 'salt and pepper' pattern in the anterior PSM of the *notch* pathway mutants (not shown) (Durbin et al., 2000; Sawada et al., 2000). More importantly, we know that the anterior PSM is distinct from the posterior PSM, and the analysis of *fss*;*aei*/*deltaD* and *fss*;*des*/*Notch1* embryos indicates that the strong anterior 'salt and pepper' expression domain of *her1* and *deltaC* in *aei*/*deltaD* and *des*/*notch1* embryos is dependent entirely upon *fss* (van Eeden et al., 1998; Holley et al., 2000) and, therefore, is dependent upon an activity specific to the anterior PSM (Holley et al., 2000). This explains why the 'salt and pepper' pattern is found only in the anterior PSM of *aei*/*deltaD* embryos, and also led us to propose previously that this anterior expression was induced de novo by a separate, anterior 'wave-front activity.' The wave-front would move from anterior to posterior along the body axis as the embryo extends posteriorly. This wave-front activity requires the function of the *fss* gene that normally functions in the anterior PSM to maintain or stabilize the oscillating pattern emanating from the posterior tailbud. In the absence of oscillations, this wave-front activity can induce or facilitate the expression of the oscillating genes in the anterior PSM, leading to the abrupt onset of the 'salt and pepper' pattern in the anterior PSM of the *aei*/*deltaD* embryos (Holley et al., 2000). Recent studies performed in the chick suggest that the wave-front could correlate with a drop in the level of FGF signaling, which is highest in the posterior PSM (Dubrulle et al., 2001).

The analysis of *deltaC* expression in *her1*^{mo} embryos uncovers an additional *Notch*-dependent patterning activity in the anterior PSM. This activity can create a segmental pattern of gene expression in the absence of any evidence of oscillations in *her1* and *deltaC* expression: a smooth domain of *deltaC* expression is refined anteriorly to create stripes of expression that persist in the somitic mesoderm. This refinement requires the activity of *fss*, *aei*/*deltaD*, *des*/*notch1*, *deltaC* and *bea*, indicating that each of these genes has an additional function in the anterior-most PSM, downstream of *her1*. This is consistent with the fact that *aei*/*deltaD*, *deltaC* and *des*/*notch1* are each transcribed within the PSM and later in the somitic mesoderm. In fact, this refining pattern is likely to be revealed only within the *her1*^{mo} embryos because *her1* is the only one of these cloned genes whose expression is restricted to the PSM (Bierkamp and Campos-Ortega, 1993; Dornseifer et al., 1997; Jiang et al., 2000; Müller et al., 1996). Ultimately, this indicates that the phenotypes observed in *aei*/*deltaD* and *des*/*notch1* embryos are composites of defects that occur both upstream and downstream of *her1* (oscillator) function. It has been shown that *notch* pathway signaling is involved in establishing the anteroposterior pattern within each somite (Conlon et al., 1995; Oka et al., 1995; Evrard et al., 1998; Hrabé Angelis et al., 1997; Kusumi et al., 1998; Wong et al., 1997; Zhang and Gridley, 1998; Takahashi et al., 2000). The late activity of the *notch* pathway described here probably represents this same anteroposterior patterning function. What is remarkable is that this late function can create a segmental pattern in the absence of prior oscillations in *her1* and *deltaC* expression.

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