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Generation of segment polarity in the paraxial mesoderm of the zebrafish through a T-box-dependent inductive event

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Abstract

The first morphological sign of vertebrate postcranial body segmentation is the sequential production from posterior paraxial mesoderm of blocks of cells termed somites. Each of these embryonic structures is polarized along the anterior/posterior axis, a subdivision first distinguished by marker gene expression restricted to rostral or caudal territories of forming somites. To better understand the generation of segment polarity in vertebrates, we have studied the zebrafish mutant *fused somites* (*fss*), because its paraxial mesoderm lacks segment polarity. Previously examined markers of caudal half-segment identity are widely expressed, whereas markers of rostral identity are either missing or dramatically down-regulated, suggesting that the paraxial mesoderm of the *fss* mutant embryo is profoundly caudalized. These findings gave rise to a model for the formation of segment polarity in the zebrafish in which caudal is the default identity for paraxial mesoderm, upon which is patterned rostral identity in an *fss*-dependent manner. In contrast to this scheme, the caudal marker gene *ephrinA1* was recently shown to be down-regulated in *fss* embryos. We now show that *notch5*, another caudal identity marker and a component of the Delta/Notch signaling system, is not expressed in the paraxial mesoderm of early segmentation stage *fss* embryos. We use cell transplantation to create genetic mosaics between *fss* and wild-type embryos in order to assay the requirement for *fss* function in *notch5* expression. In contrast to the expression of rostral markers, which have a cell-autonomous requirement for *fss*, expression of *notch5* is induced in *fss* cells at short range by nearby wild-type cells, indicating a cell-non-autonomous requirement for *fss* function in this process. These new data suggest that segment polarity is created in a three-step process in which cells that have assumed a rostral identity must subsequently communicate with their partially caudalized neighbors in order to induce the fully caudalized state.

Keywords

Fused somites; tbx24; T-box; Segment polarity; Somitogenesis; Paraxial mesoderm; Induction; Community effect

Introduction

Somitogenesis is the serial production, from anterior to posterior along the embryonic axis, of epithelial blocks of mesodermal cells, termed somites, from the morphologically unsegmented presomitic mesoderm (PSM) in the growing vertebrate embryo (reviewed in Pourquie, 2001). Somites are bilaterally symmetrical, and differentiate into the muscle, skin, and axial skeleton.

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Positioning of boundaries, or furrows, between each successive somite is thought to be controlled in part by a biochemical oscillator active in cells of the tailbud and posterior PSM. This appears to consist of a feedback loop involving genes and proteins of the Delta/Notch signaling system and its Her repressor gene targets (reviewed in Rida et al., 2004; Weinmaster and Kintner, 2003). In addition, the Wnt and Fgf signaling systems may modulate the action of the oscillator (Aulehla et al., 2003; Dubrulle et al., 2001; Sawada et al., 2001). Genes that display the distinctive dynamic, wavelike expression domains characteristic of the segmentation oscillator are known as cyclic genes (Pourquie and Tam, 2001), and the posterior region of the PSM and tailbud that exhibits these periodic changes will be here termed the oscillatory zone.

Each of the somites is clearly polarized along its rostral/caudal axis, as evidenced by differential permissiveness to neural crest and sensory nerve axon outgrowth in rostral and caudal halves (reviewed in Pourquie, 2001). In addition, skeletal elements derived from sclerotomal cells of the somite project from the vertebral body, or centra, in a specific, polarized manner. Even prior to somite boundary formation, the prospective somitic cells within the anterior end of the PSM exhibit striped expression of many genes, indicating that the prepatterning of these cells into a polarized array prefigures the morphological aspects of segment polarity. The region in the anterior PSM in which these stable, polarized stripes of gene expression are first seen will here be termed the segment polarity zone. Despite the importance of this segmental polarity to the functional form of the animal, the mechanism of its generation remains unresolved.

One strategy to understand the generation of segment polarity is to isolate and characterize mutants that fail in one or more aspects of this process. The zebrafish recessive viable mutant *fused somites* (*fss*) does not form embryonic somites, and the striped expression of marker genes in the segment polarity zone of *fss* mutant embryos is lost (van Eeden et al., 1996). Genes usually found restricted to the caudal halves of prospective and formed somites, such as *myoD*, *snail1* (van Eeden et al., 1996), *ephrinB2* (Durbin et al., 2000), and *deltaC* (Jiang et al., 2000), are instead expressed ubiquitously throughout the paraxial mesoderm of *fss*, whereas rostral marker genes such as *fgf8*, *ephA4*, *deltaD*, *mespa*, *mespb*, *papc*, and *lfng* (Durbin et al., 2000; Jiang et al., 2000; Prince et al., 2001; Sawada et al., 2000) are either absent or dramatically down-regulated. These molecular marker data indicate that the paraxial mesoderm of *fss* embryos is profoundly caudalized (Durbin et al., 2000), suggesting a simple 2-step model for the generation of segment polarity. In the first step, paraxial mesoderm is formed during gastrulation with a default caudal identity. In the second step, the *fss* gene is required to generate regions of rostral identity, in some manner, from a tissue that otherwise has a uniform caudal state. However, the recent finding that the caudal segment polarity marker *ephrinA1* is down-regulated in *fss/tbx24* PM (Barrios et al., 2003) suggests that this 2-step model may be incomplete.

The *fused somites* gene has recently been cloned and encodes a novel transcription factor of the T-box family, *tbx24*, which is expressed throughout the anterior oscillatory and segment polarity zones and the two most recently formed somites of the zebrafish embryo (Nikaido et al., 2002). Importantly, *tbx24* is ubiquitously expressed in the PSM, leaving open the question of how *fss/tbx24* might act to generate segment polarity, and conflicting evidence exists regarding whether *fss/tbx24* is required cell-autonomously for expression of rostral identity (Barrios et al., 2003; Durbin et al., 2000). One possibility is that a cryptic segmental pattern exists at the protein level, either by differential abundance, sub-cellular localization, or by interaction with some other localized factor. Alternatively, the *fss/tbx24* phenotype may result from a simple defect in the maturation of PSM cells (Holley and Takeda, 2002; Nikaido et al., 2002).

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The perturbation of segment polarity in *fss/tbx24* embryos could simply reflect an earlier disorganization of segmental prepatterning as a whole, but this appears unlikely to be the case. The hemal and neural arches of the axial skeleton, which normally project only from the rostral half of each vertebral centrum, grow also from the caudal half and are severely distorted in *fss/tbx24* (van Eeden et al., 1996). However, the vertebral bodies themselves appear normally segmented, implying that underlying segmental information is still generated in the *fss/tbx24* embryo. Consistent with this notion, examination of the wavelike expression domains of the cyclic genes *her1*, *her7*, and *deltaC* (*dlc*) in the oscillatory zone of *fss/tbx24* embryos reveals an essentially normal sequence of stripes, suggesting that the segmentation oscillator is still functional (Gajewski et al., 2003; Holley et al., 2000; Jiang et al., 2000). In the anterior PSM of *fss/tbx24* embryos, however, instead of increasing in level and arresting at the site of the future somite furrow as expected in wild-type embryos, the wavelike expression domains of *her1*, *her7*, and *dlc* grow weaker and disappear (Gajewski et al., 2003; Holley et al., 2000; Jiang et al., 2000; van Eeden et al., 1998). Thus, the somitogenic defect in *fss/tbx24* does not appear to be at the level of basic spatial subdivision, but rather at some later point between the oscillator and morphogenesis. Since embryological experiments in chick have shown the importance of juxtaposing somite halves with different polar identities for morphological boundary formation (Aoyama and Asamoto, 1988; Sato et al., 2002; Stern and Keynes, 1987), the lack of polarity was postulated to be the underlying cause of the failure to form epithelial boundaries seen in the *fss/tbx24* mutant embryo (Durbin et al., 2000).

A link between segment polarity and somite morphogenesis is provided by the Eph and ephrin families of cell-contact repulsion receptors and ligands, which are expressed in rostrally and caudally polarized stripes in the wild-type PSM (Barrios et al., 2003; Durbin et al., 1998, 2000; Xu et al., 1994). This juxtaposition of fields of receptor and ligand-bearing cells is lost in *fss/tbx24* (Durbin et al., 2000). Elegant transplantation studies indicate that the direct cause of the failure of somite furrow formation in *fss/tbx24* embryos is likely defective Eph/ephrin signaling (Barrios et al., 2003; Durbin et al., 2000). Indeed, this signaling process can drive the mesenchymal to epithelial transition of somite boundary formation in *fss/tbx24* host tissue without generating segment polarity, suggesting that Eph/ephrin signaling directly mediates the morphogenetic changes of somitogenesis without affecting cell fate in the PSM (Barrios et al., 2003). Thus, current evidence suggests that the segmentation defect in *fss/tbx24* mutant embryos stems from a failure to establish segment polarity; downstream of the segmentation oscillator, and upstream of the Eph/ephrin signaling system.

In this paper, we start by confirming that the paraxial mesoderm of *fss/tbx24* mutants is not truly caudalized, as was previously thought, because, like *ephrinA1* (Barrios et al., 2003), the caudal half-segment marker gene *notch5* (Westin and Lardelli, 1997) is severely down-regulated in the segment polarity zone and somites. We have used cell transplantation together with confocal microscopy and fluorescent gene probes to better characterize the *fss/tbx24* phenotype. We show by analysis of mosaic embryos that *fss/tbx24* is responsible for acquisition of rostral half-segment identity in a cell autonomous manner, and that *notch5* expression can be induced in neighboring *fss/tbx24* host cells by wild-type grafts. Thus, our results are the first evidence of inductive patterning during the generation of segment polarity in the zebrafish, and lead us to propose an additional step in segment polarity in the zebrafish, in which a ground state of partial caudal identity in the PSM must be further patterned by rostral cells in order for a complete caudal cell state, and thus complete segment polarity, to be established.

Materials and methods

Maintenance of fish and mutant strains

Zebrafish were maintained according to standard conditions (http://www.zfin.org) on a 14-h light, 10-h dark cycle. Embryos were collected by natural spawning, raised at 28.5°C and staged

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according to Kimmel et al. (1995). Mutant allele used was *fused somites/tbx24* (*fss*^{te314a}), first described by van Eeden et al. (1996).

Cell transplantation

Single and double blastoderm cell transplantations were carried out according to Ho and Kane (1990). Briefly, donor embryos were labeled at the one- or two-cell stage with 5% fluorescein-labeled 40 kDa fixable dextran (Molecular Probes, Oregon), and grown to sphere stage, whereupon a forged micropipette was used to remove cells from a donor embryo, and place varying numbers of cells at the margin of an unlabelled host embryo. The resulting chimeric embryo was grown until segmentation stages and the location of labeled donor cells within the paraxial mesoderm, as well as the development of morphologically distinct boundaries was monitored under a fluorescent dissection microscope (Leica, New York). Embryos with features of interest were mounted in 3% methylcellulose and examined at higher magnification with a Zeiss Axioscop. Images were captured using a Nikon D1 digital SLR, and stored as Adobe Photoshop files for manipulation and analysis. Embryos were fixed in 4% paraformaldehyde and used in subsequent in situ hybridization steps.

In situ hybridization and microscopy

In situ hybridization was according to Prince et al. (1998) with modifications according to Oates et al. (2000). Probes to *mespb*, *deltaC*, *deltaD*, *papc*, *notch5*, and *fgf8* have been previously described (Dornseifer et al., 1997; Furthauer et al., 1997; Oates and Ho, 2002; Westin and Lardelli, 1997; Yamamoto et al., 1998). After color development, some embryos were counterstained with 1 μg/mL Hoechst 43222 for 30 min, then washed 2× in PBT/10 mM EDTA before equilibrating in PBS/80% glycerol/10 mM EDTA. Embryos were either photographed on a Leica dissecting microscope with a Nikon D1 digital camera in whole mount, or, after deyolking, and flat mounting, photographed on a Zeiss Axiophot with a Nikon D1 digital camera. After transplantation and in situ hybridization, all embryos were examined and the position of transplanted cells and gene expression were recorded by confocal microscopy on a Zeiss Axiovert 100 M LSM510. Images were imported into Adobe Photoshop and adjusted for contrast in parallel before building into figures.

Results

Segment polarity defects in presomitic mesoderm of fused somites/tbx24 mutant embryos

Previous reports have shown that all genetic markers of rostral segmental polarity that have been examined are absent or severely down-regulated in presomitic mesoderm (PSM) and somites of *fss/tbx24* mutant embryos, whereas markers of caudal polarity are ubiquitously expressed (Durbin et al., 2000; Sawada et al., 2000; van Eeden et al., 1996). For example, previously examined rostral markers *papc*, *mespb*, and *fgf8*, which will be used below to assay transplantation experiments, are severely or completely down-regulated in *fss/tbx24* mutants (Figs. 1A, B, C), whereas caudal marker *myoD* is expressed widely throughout the paraxial mesoderm (PM) (Fig. 1D). We have extended these results to include 3 additional markers of rostral (*artl*, *tbx18*, and *robo2*) and 4 of caudal identity (*fgfr4*, *uncx4*, *slit2b*, and *fkh6*) that are consistent with a caudalized state in *fss/tbx24* (data not shown). Barrios et al. (2003) previously saw down-regulation of the caudal marker *ephrinA1* in *fss/tbx24* PM. Strikingly, we now find that *notch5* (Westin and Lardelli, 1997), which in wild-type embryos is expressed in the caudal halves of the formed somites and in presumptive segments of the segment polarity zone (Fig. 1E, arrowheads), is also absent from the PM of *fss/tbx24* mutant embryos during early segmentation (Fig. 1F). Thus, the PM of *fss/tbx24* mutants is not completely caudalized, as was previously thought (Durbin et al., 2000), indicating that *fss/tbx24* function is required for some aspects of caudal, as well as rostral identity. To determine the role of *fss/tbx24* in the generation of rostral and caudal identities, we used cell transplantation to create genetic mosaics

between wild-type and *fss/tbx24* cells in an attempt to recreate the generation of segment polarity lacking in the mutant.

Morphological furrow formation in fused somites/tbx24 mutant hosts is rescued by high density of wild-type cells

Using pre-gastrula cell transplantation (Ho and Kane, 1990), we found that transplantation of varying numbers (10–100) of wild-type donor cells into the blastoderm margin of a wild-type host gave rise to regions of low and high cell density within the PSM and PM, and did not disrupt somitogenesis (Fig. 2A). Under these conditions in 15/32 *fss/tbx24* hosts, wild-type cells at high densities tended to compact together in the PSM, and were able to cause local formation of furrows, both within the wild-type donor clones, and between the edge of a wild-type clone and neighboring *fss/tbx24* cells (arrowheads, Fig. 2B). In doing so, the wild-type cells formed varying length rows of somite-like blocks, each with a sharp rostral border (arrowheads, Fig. 2C). The internal organization of 5 of these donor cell blocks, or clusters in the host embryos was investigated with confocal microscopy. Figs. 2D and D' show neighboring confocal sections through two such groups. The lateral surfaces of the clusters were completely donor derived (Fig. 2D), as were the internal rostral borders (arrowheads, Fig. 2D'), whereas the cells in the caudal half of the cluster interdigitated with their *fss/tbx24* host neighbors (asterisks). This arrangement indicates that the clusters were polarized across their rostral–caudal axes. Wild-type donor cells that were scattered at low density were not associated with furrow formation and remained indistinguishable from their *fss/tbx24* host neighbors by morphology, both during the time of somitogenesis (arrows, Fig. 2D), and later after differentiation into muscle fibers (Fig. 2F). Control wild-type donor cells always aligned with endogenous boundaries in wild-type hosts (Fig. 1E). Thus, transplantation of wild-type cells into *fss/tbx24* hosts recapitulates the overt morphological features of wild-type somitogenesis, and we next examined whether aspects of segment polarity were associated with these wild-type donor cells, or the surrounding *fss/tbx24* host cells.

Wild-type cells autonomously express rostral polarity markers in fused somites/tbx24 mutant hosts

To determine whether *fss/tbx24* function in wild-type donor cells was sufficient for adoption of rostral segment polarity fate in *fss/tbx24* mutant host embryos, we assayed the expression of a number of rostral marker genes after transplanting fluorescently labeled wild-type cells into *fss/tbx24* host embryos. A series of preliminary experiments using non-fluorescent detection of gene expression (NBT/BCIP, DAB) and DIC microscopy yielded data of insufficient accuracy to unambiguously determine which cells expressed a given gene (data not shown). However, using confocal microscopy to detect fluorescent labeling of both donor cells and expression of target genes enabled this distinction to be made at cellular resolution (Table 1). We first examined the ability of wild-type cells located in the *fss/tbx24* mutant PSM to express the *mespb* gene. *mespb* is normally a marker of rostral segment polarity in the two presumptive segments in the segment polarity zone, and in the most recently formed somite (Fig. 1A) (Durbin et al., 2000;Sawada et al., 2000). Wild-type cells placed into wild-type hosts expressed *mespb* normally after transplantation (Fig. 3A). However, in *fss/tbx24* hosts that possessed wild-type grafts at the same A/P level as the segment polarity zone, and so occupying the normal territory of *mespb* expression, we found examples of autonomous *mespb* expression only in the wild-type donor cells, and never in the mutant host cells (Fig. 3B, Table 1). Thus, *fss/tbx24* function appears to be sufficient for autonomous *mespb* expression in the wild-type donor cells, but not sufficient to induce expression in neighboring *fss/tbx24* cells.

Expression of the cell adhesion gene *papc* is normally found in adaxial cells, in the rostral portions of two presumptive somites in the segment polarity zone, and along the rostral border of the most recently formed somite, but is absent from the segment polarity zone and more

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mature PM of *fss/tbx24* mutants (Fig. 1B; Yamamoto et al., 1998). Because *fss/tbx24* mutants retain *papc* expression in the adaxial cells, it is straightforward to determine the approximate A/P position of wild-type donor cells. We found that wild-type cells expressed *papc* readily in the segment polarity zone of the *fss/tbx24* host, often in a striped pattern (arrows, Figs. 3C, D, Table 1). These stripes varied in their mediolateral width depending on the extent of the wild-type donor-derived clone and appeared remarkably well spaced along the A/P axis. Importantly, *papc* expression was restricted to the wild-type donor cells (Fig. 3C', $n = 21$ hosts), indicating that *fss/tbx24* is required cell-autonomously for *papc* expression. Indeed, even a single, isolated wild-type cell was capable of expressing *papc* (data not shown). However, in two cases, expression of *papc* was observed in a single *fss/tbx24* host cell neighboring large wild-type grafts (Figs. 3D, D', arrow in D').

We also examined the expression of *fgf8* in this assay (Figs. 3E–F', Table 1), which in wild-type embryos is expressed in two broad stripes in the segment polarity zone, and subsequently in the rostral half of every somite (Fig. 1C; Furthauer et al., 1997). We observed instances of strictly donor cell autonomous expression in the posterior of the axis (Figs. 3E, E'), as well as infrequent expression of *fgf8* in neighboring *fss/tbx24* host cells (Figs. 3F, F'). Combined, the above results indicate that *fss/tbx24* function is sufficient for expression of markers of rostral identity in the same cell, consistent with a cell-autonomous requirement. However, the striped *papc* expression patterns also indicate that some form of segment polarity can be generated within the larger clones of wild-type donor cells in an *fss/tbx24* environment. Although nearly all cells expressing rostral markers in these experiments were wild type in origin, the existence of a few *fss/tbx24* host cells able to express *papc* and *fgf8* indicates that intercellular communication can overcome the *fss/tbx24* block in some circumstances.

Wild-type cells at high density induce neighboring fused somites/tbx24 cells to express missing caudal polarity gene

Given that the *notch5* caudal polarity marker gene is not expressed in the *fss/tbx24* PM (Fig. 1F), we next examined whether wild-type cells transplanted into a *fss/tbx24* host could assume a complete caudal segment polarity state that included *notch5* expression, and whether they could induce this state in neighboring *fss/tbx24* host cells. In mutant embryos containing a low density of donor cells in the PM, we did not detect expression of *notch5* in either donor or host cells (Fig. 4A). However, in *fss/tbx24* embryos in which wild-type donor cell density was high, we observed strong *notch5* expression in both host and donor cells, often in a series of stripes associated with the donor cells (Figs. 4B, C, Table 1). Examination of these embryos revealed that within the donor cell clusters themselves, *notch5* expression was high in the caudal region and excluded from the rostral half (asterisks, Fig. 4C'). This indicates that wild-type cell clusters establish correct rostro-caudal polarity, recapitulating another aspect of normal somitogenesis. Strikingly, *notch5* was also expressed in surrounding mutant host cells at a distance of up to 3 cell diameters from the wild-type donors (Figs. 4B arrows, C, C' arrowheads), indicating that wild-type cells can induce their mutant *fss/tbx24* neighbors to express a marker of caudal segment polarity normally missing in this genetic background. The induced expression of *notch5* in *fss/tbx24* cells did not always have a clearly striped pattern (e.g., Fig. 4C'), most likely because of close proximity (3 cell diameters) to wild-type cells and an inability of *fss/tbx24* cells to actively repress caudal markers (see Discussion). This result demonstrates that PSM cells do not themselves require *fss/tbx24* for direct expression of *notch5*, but rather for production of a signal that induces the expression of *notch5* in neighboring cells.

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Discussion

In this report, we address the role of the T-box gene *fused somites/tbx24* in the generation of segment polarity in the paraxial mesoderm of the zebrafish embryo. Using cell transplantation between wild-type and mutant embryos combined with cellular-resolution analysis of gene expression, we present evidence that *fss/tbx24* is required cell-autonomously for the expression of rostral segment identity. Further, we have uncovered a novel inductive event producing complete caudal half-segment identity, which requires *fss/tbx24* in the sending cells. This induction likely takes place temporally downstream of the Delta/Notch-dependent somitogenesis oscillator, and upstream of the Eph/ephrin-mediated production of epithelialized somite boundaries.

Autonomy of fused somites/tbx24 action

Since in *fss/tbx24* mutant embryos there is a profound loss of rostral segment identity, a primary question has been whether *fss/tbx24* is responsible for this state in a cell-autonomous manner. Transplantation experiments by Durbin et al. (2000) led to the idea that the *fss/tbx24* gene was acting non-cell-autonomously with respect to rostral segment polarity, since wild-type cells in *fss/tbx24* hosts did not express the rostral marker *fgf8*. Consistent with this, *fss/tbx24* cells in wild-type hosts expressed *fgf8* when located in the rostral epithelial boundary of a somite, suggesting that the wild-type host environment rescues the defect caused by loss of *fss/tbx24* function. In contrast, Barrios et al. (2003) have recently shown that rostral markers *papc* and *dld* are expressed within wild-type grafts in *fss/tbx24* hosts, suggesting that in fact *fss/tbx24* acts cell autonomously with respect to generation of rostral half-segment identity.

Our results using *papc* and *dld* (data not shown) expression as markers of rostral identity are in good accordance with those of Barrios, and in addition, we find that cell-autonomous *fss/tbx24* function is sufficient for *mespb* and *fgf8* expression. The differing results using *fgf8* (Durbin et al., 2000) may be a simple consequence of our host embryos containing higher donor cell densities than previously examined. This explanation fits well with our observation of somite-like wild-type donor cell clusters with complete rostral boundaries and morphological and molecular internal polarity occurring only in *fss/tbx24* hosts with high local densities of wild-type donor cells. Such structures were not seen after transplantation of wild-type cells into *fss/tbx24* host embryos by Barrios et al. (2003), who found that over-expression of *EphA4* or *ephrinB2* was required to induce morphological boundary formation. These differences may highlight a role for a community effect in segmentation and/or somitogenesis (Buckingham, 2003; Gurdon, 1988). Indeed, the existence of a community effect modifying gene expression in the zebrafish PSM has been previously suggested by Holley et al. (2000), who showed that the autonomous dependence of *her1* expression on *fss/tbx24* function in wild-type cells transplanted into *fss/tbx24* mutant hosts could be overcome in the reciprocal transplant when mutant donor cells were surrounded by wild-type host cells.

Genetic variation between wild-type donor strains seems unlikely to have a role in the differences between our transplants and previous studies, since we observed cluster formation from donor clones from several different wild-type laboratory and commercial lines. As we have not characterized the cell polarity or epithelial character of these cell clusters, their exact relationship to the Eph/ephrin-induced somite-like structures seen by Barrios et al. (2003) is not clear. We emphasize that even though somite-like clusters of cells were readily generated, their formation was not a prerequisite for cell-autonomous expression of rostral markers, or for the induction of *notch5* in cells neighboring the graft. This observation is consistent with the findings of Barrios et al. (2003), who showed that Eph/ephrin-induced boundary morphogenesis and segment polarity can be uncoupled.

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The role of mesp genes in segment polarity

The two members of the zebrafish *Mesp* family of bHLH genes are expressed in two or three thin stripes in anterior PSM, where they become restricted to the rostral-most cells of the prospective somites, suggesting that they may play an important role in establishing segment polarity (Durbin et al., 2000; Sawada et al., 2000). Mutation in the mouse *Mesp2* gene results in a loss of rostral half-segment identity (Saga et al., 1997), essentially the same phenotype as produced by the *fss/tbx24* mutation, in which expression of both *mespa* and *mespb* is dramatically reduced (Durbin et al., 2000; Sawada et al., 2000). Given that *mespb* is sufficient to cause widespread activation of rostral segment polarity markers (such as *notch6*, *fgfrf1*, and *papc*), at the expense of caudal markers (such as *myoD* and *notch5*)in over-expression experiments (Sawada et al., 2000), it has been proposed that the *fss/tbx24* phenotype is due in large part to the failure to express *mespb* (Sawada et al., 2000). The relationship between *mespb* function and furrow formation is not yet clear, however, as over-expression of *mespb* does not rescue this aspect of the *fss/tbx24* phenotype (Holley and Takeda, 2002; AO, unpublished). The cell-autonomous expression of *mespb* in grafted wild-type cells is likely an important feature of our assay system, suggesting that *mespb* is in fact a direct target of the Fss/Tbx24 transcription factor. Thus, donor wild-type cells in the PSM of an *fss/tbx24* host expressing *mespb* may be positioned at the top of a regulatory cascade that leads to the adoption of the rostral identity, and repression of the caudal state.

Inductive activities of wild-type cells

Our demonstration that *fss/tbx24* mutant embryos are missing *notch5* PSM expression, and therefore are not fully caudalized, raises the question of the role of *fss/tbx24* in the generation of caudal identity. The caudal marker *ephrinA1* was recently shown to be absent from the PM of *fss/tbx24* mutant embryos (Barrios et al., 2003), indicating that *notch5* is not the only missing caudal marker gene, and that the deficiency in caudal identity could be more severe than previously appreciated. Whether *ephrinA1* is responsive to the community effect-derived signal responsible for *notch5* induction or whether it is controlled by some other activity is currently under investigation. At present, we do not know the function of *notch5* itself in segment polarity, although over-expression of an activated form of the *notch5* receptor (ICD) disrupts somitogenesis (AO, unpublished), suggesting that it may have an important role. Since *fss/tbx24* is expressed in the segment polarity zone in both rostral and caudal halves of prospective somites (Nikaido et al., 2002), the failure to express *notch5* in the caudal half could be in principle a result of the lack of a direct activation by *fss/tbx24* in these cells. We show, however, that the *fss/tbx24* gene is not required in PSM cells for *notch5* expression, and that it is sufficient for *fss/tbx24* to be present in a nearby cell. This non-autonomy of *fss/tbx24* function indicates the existence of an *fss/tbx24*-dependent signal or interaction that is capable of inducing *notch5* expression. Mutant *fss/tbx24* PSM cells must therefore express the receptor and signal transduction proteins for this signal. These results imply that in the wild-type zebrafish, generation of complete segment polarity involves at least one inductive step.

The pattern of induced *notch5* expression in the *fss/tbx24* host was not always segmentally arranged, although autonomous *notch5* expression in donor cell clusters often was (for example, Fig. 4). In these cases, a continuous band of *notch5*-expressing cells was found immediately medial or lateral to the wild-type donor cells. This is most likely a consequence of the three-cell diameter inductive range of the signal, and the mediolateral position of the wild-type donor clone. In wild-type embryos, *notch5*-inductive signals would be released from cells within a domain of rostral identity that spans the mediolateral extent of the PSM. Therefore, target cells of partial caudal identity would not normally be available laterally. The striped pattern of *notch5* expression exhibited in wild-type embryos and wild-type cell clusters would be generated despite the inductive signal because rostral cells would themselves inhibit *notch5* expression through a *mesp*-dependent mechanism (Sawada et al., 2000). It is important

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to note that the presence and details of the *notch5*-inductive events were only apparent when a cellular-level resolution was obtained using a combination of fluorescent gene expression detection and confocal microscopy.

Nature of the inductive signal

The spatial distribution of induced *notch5* expression may reveal some properties of the signaling process itself. Since *notch5* can be induced at a distance of three cell diameters, one hypothesis is that an inducing molecule released in the segment polarity zone is active only over short ranges. Instability or binding to extracellular matrix components might restrict the range of a diffusible molecule. A cell-contact-dependent signal could also act in a relay, and thus spread the induction of *notch5* to a distance of three cell diameters. Alternatively, if a cell–cell signal was delivered in the oscillatory zone, potential cell mixing occurring during the transit of cells through the PSM could scatter *notch5*-expressing cells that had been in direct contact with the wild-type donor clusters at some earlier point, giving the appearance of a three-cell range. A large number of signaling molecules from different families are expressed in the PSM of zebrafish, including members of the Fgf, Delta, and Notch families. In chick and mouse embryos, inductive activity of Delta/Notch signaling is thought to mediate some aspects of segment polarity (Sato et al., 2002; Takahashi et al., 2000, 2003). In zebrafish, loss of *fgf8* function in the *acerebellar* mutant gives a somitogenic phenotype, but without strong segment polarity defects (Reifers et al., 1998), although functional redundancy with other *fgf* genes (Reifers et al., 2000) may mask an effect. Clearly, direct functional tests must be made in zebrafish before conclusions can be drawn.

3-step model for sequential generation of segment polarity

The current hypothesis for the generation of segment polarity in zebrafish PM can be termed the “two-step” model. In the first step, PM is produced through gastrulation with a default and complete caudal state (Durbin et al., 2000). The next step requires action of the *fss/tbx24* gene, which produces regions of rostral identity from within the field of caudal cells (Durbin et al., 2000; Sawada et al., 2000). The spatial patterning information for this step may derive from the site of arrest of the segmentation oscillator in the segment polarity zone (Henry et al., 2002; Holley et al., 2000, [2002]; Oates and Ho, 2002; Sawada et al., 2001). Subsequently, morphological inter-somitic furrows are developed from the juxtaposition of cells with rostral and caudal identity using Eph/ephrin signaling (Barrios et al., 2003; Durbin et al., 1998, 2000). Our findings now indicate that this model needs revision. In the first step, the default state of PM produced by gastrulation is an incomplete or partial caudal identity, as shown by the absence of *notch5* expression from the PM of *fss/tbx24* mutant embryos. In the second step, the production of rostral identity is effected by *fss/tbx24* in an almost entirely cell-autonomous manner. We now add a third step in which *notch5* is induced in the caudal region of the forming somite by *fss/tbx24*-expressing cells of the neighboring rostral half-segment, thus completing segment polarization.

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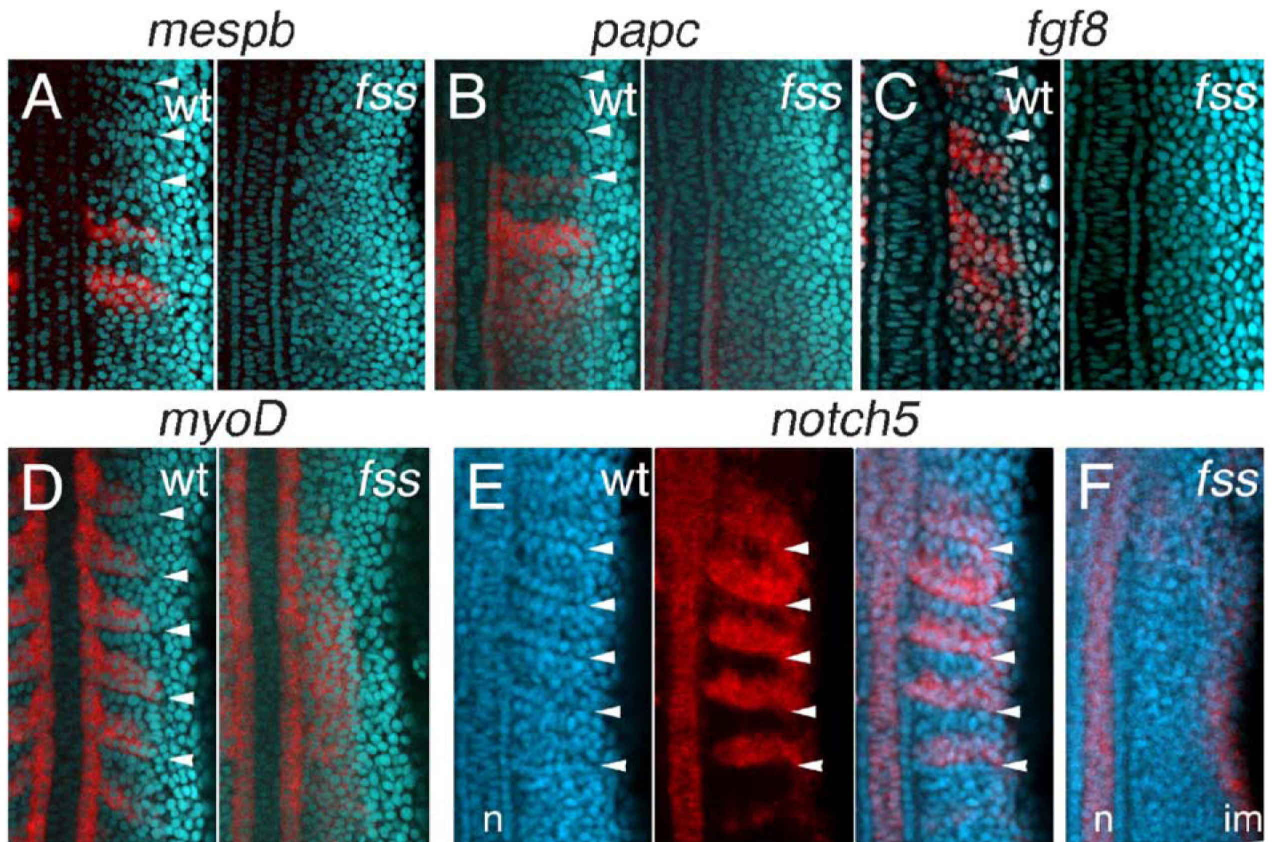

Fig. 1.

Gene expression defects in the paraxial mesoderm of *fused somites/tbx24* mutant embryos. Expression of (A) *mespb*, (B) *papc*, (C) *fgf8*, and (D) *myoD* mRNA in paraxial mesoderm and tailbud of wild-type (left panel) and *fss/tbx24* mutant (right panel) embryos at 5–6 (A, B) and 10 (C, D) somite stages. Embryos are deyolked and flat mounted with anterior up. Arrowheads indicate somitic boundaries. (E–F) Comparison of *notch5* expression in wild type and *fss/tbx24* backgrounds at the 5 somite stage. (E) Expression of *notch5* mRNA in caudal half of PM segments. From left to right, panels show cell nuclei, *notch5* expression, and a merge of the two. (F) Paraxial *notch5* expression is absent from *fss/tbx24* mutant embryos, although retained in the notochord and intermediate mesoderm.

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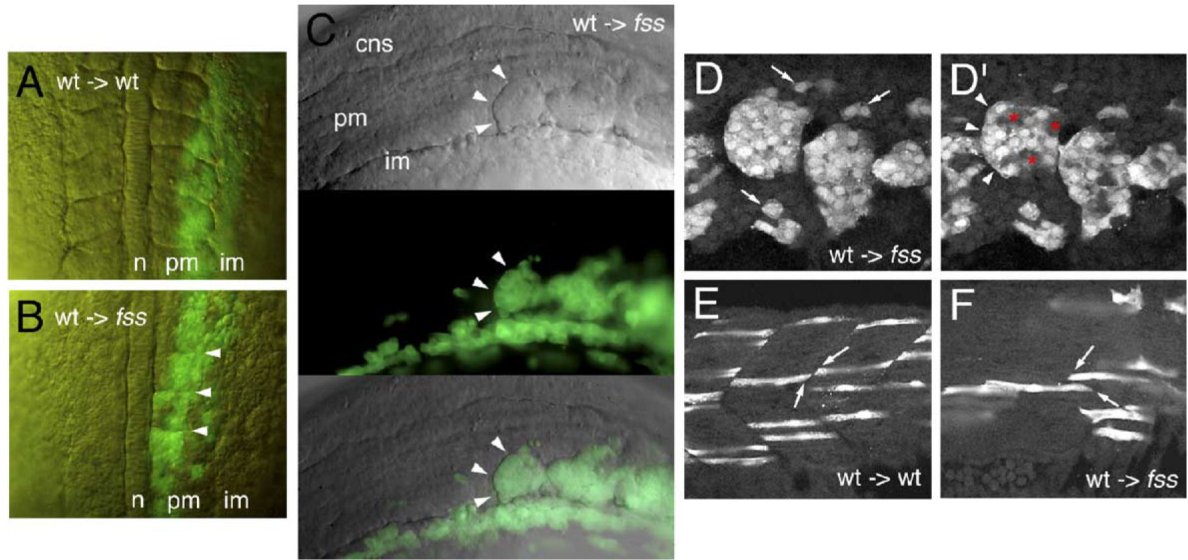

Fig. 2.

Rescue of morphological boundary formation by wild-type cells in *fused somites/tbx24* mutant hosts. Formation of morphological boundaries in PM of *fss/tbx24* host embryos after transplantation of wild-type cells (green), shown in live embryos: dorsal view in panels A–B, anterior up; lateral views in panels C–F, anterior left. (A) Normal segmentation in 6-somite stage wild-type embryo after transplantation of wild-type cells into PM. (B) Morphological boundary formation (arrowheads) in sibling *fss/tbx24* host associated with wild-type donor cell clusters. (C) Appearance of somite-like wild-type donor cell clusters at A/P level of somite 6 in paraxial mesoderm of *fss/tbx24* host at 12-somite stage, showing strong boundary morphology (arrowheads). Top panel is DIC image, middle panel is fluorescent image of green transplanted wild-type cells and bottom panel is a merge. (D–F) Confocal sections through PM of wild-type (E) and *fss/tbx24* (D, D', F) embryos at 24 hpf. (D, D') Arrangement of wild-type cells at high-density forming compact cell clusters in *fss/tbx24* host embryos. (D) Section through lateral surface of cluster. Arrangement of wild-type cells at low density is indicated with arrows. (D') More medial section through center of cluster, showing distinctive rostral morphological boundary (arrowheads), and interdigitation of wild-type with *fss/tbx24* cells on caudal side of cluster (asterisks). (E) Ends of wild-type muscle fibers at low density align to segmental boundaries in the trunk of wild-type hosts (arrows). (F) Ends of wild-type muscle fibers at low density do not align in the trunk PM of *fss/tbx24* host embryos (arrows). cns = central nervous system, pm = paraxial mesoderm, im = intermediate mesoderm, n = notochord.

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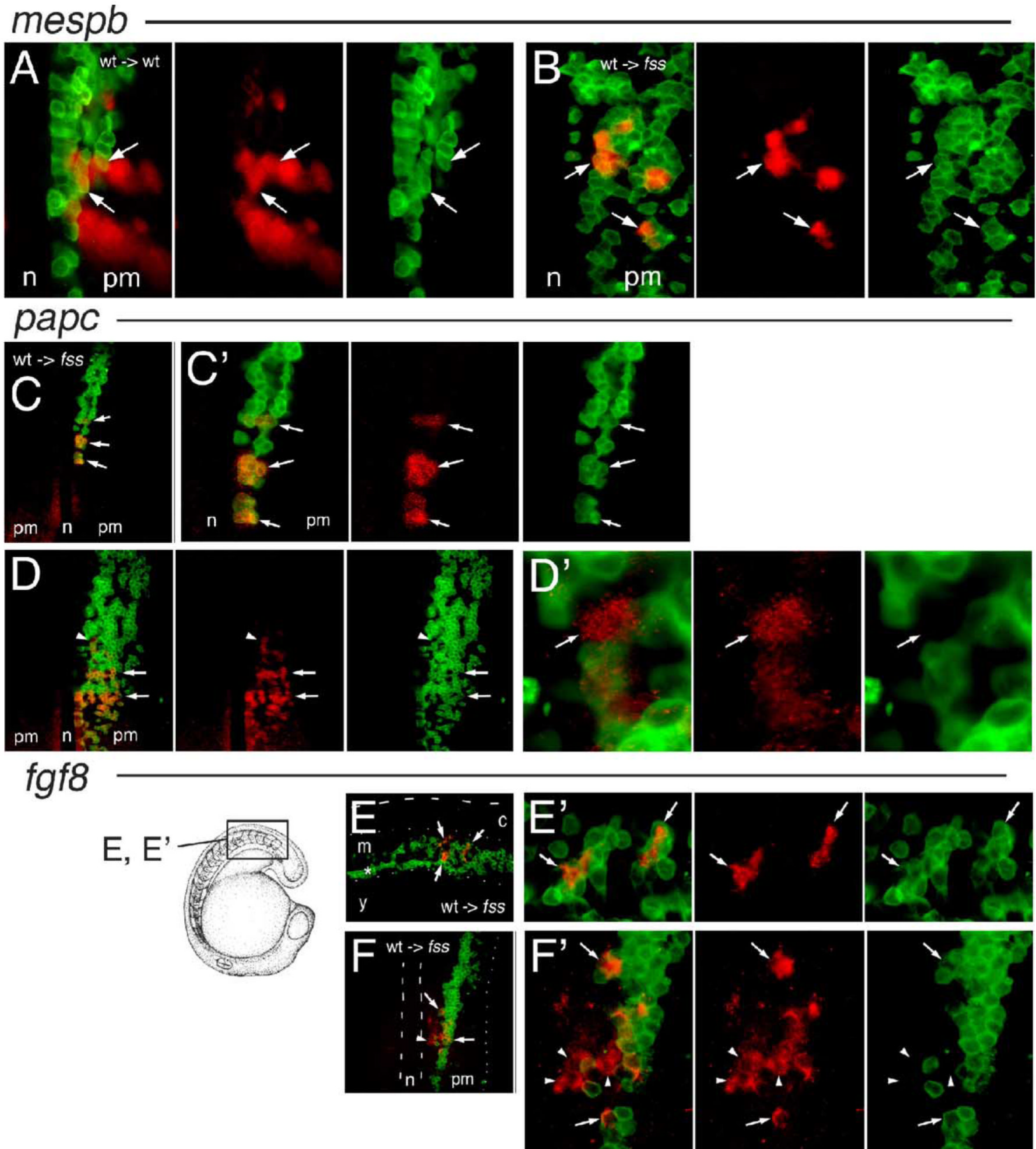

Fig. 3.

Expression of the rostral segment polarity marker genes *mespb*, *papc*, and *fgf8* by wild-type cells in *fused somites/tbx24* hosts. (A–B) Expression of *mespb* mRNA (red) in confocal sections of the right-hand side of the PM at the A/P level of the segment polarity zone in 8-somite stage embryos containing transplanted wild-type cells (green). (A) Normal *mespb* expression in wild-type embryos, arrows indicate transplanted cells expressing *mespb*. (B) Cell-autonomous *mespb* expression in wild-type donor cells in *fss/tbx24* host PSM (arrows). (C–D') Expression of *papc* mRNA (red) in confocal sections of the paraxial mesoderm at an A/P level spanning the segment polarity zone in 8-somite stage *fss/tbx24* mutant host embryos containing transplanted wild-type cells (green). (C) *papc* expression associated with small wild-type donor

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cell clusters (arrows), in contrast to absence of *papc* on contralateral side. (C') Higher magnification of C showing *papc* expression only in wild-type donor cells (arrows). (D) Striped expression of *papc* in large, high-density clone of wild-type cells (arrows) and location of *papc* expression in host cell (arrowhead). (D') Higher magnification of region indicated by arrowhead in D showing *papc* expression in *fss/tbx24* host cell (arrow). (E–F') Expression of *fgf8* mRNA (red) in confocal sections of the paraxial mesoderm at an A/P level spanning the segment polarity zone in an 18-somite (lateral view E, E') and 6-somite stage (dorsal view F, F') *fss/tbx24* mutant host embryos containing transplanted wild-type cells (green). Location of E, E' shown in diagrammatic form. (E) *fgf8* expression in wild-type cells (arrows), dashed line indicates the dorsal extent of the embryo, the dotted lines delimit the paraxial mesoderm, and the asterisk marks the intermediate mesoderm. (E') Higher magnification of *fgf8* expressing region in panel C, arrows mark *fgf8*-positive wild-type donor cells. (F) Expression of *fgf8* associated with wild-type donor cells in paraxial mesoderm, notochord delineated with dashed line and position of the lateral edge of embryo with a dotted line. (F') Higher magnification of F, showing *fgf8* expression in wild-type donor (arrows) and *fss/tbx24* mutant host cells (arrowheads). c = central nervous system, m = paraxial mesoderm, y = yolk, n = notochord, pm = paraxial mesoderm.

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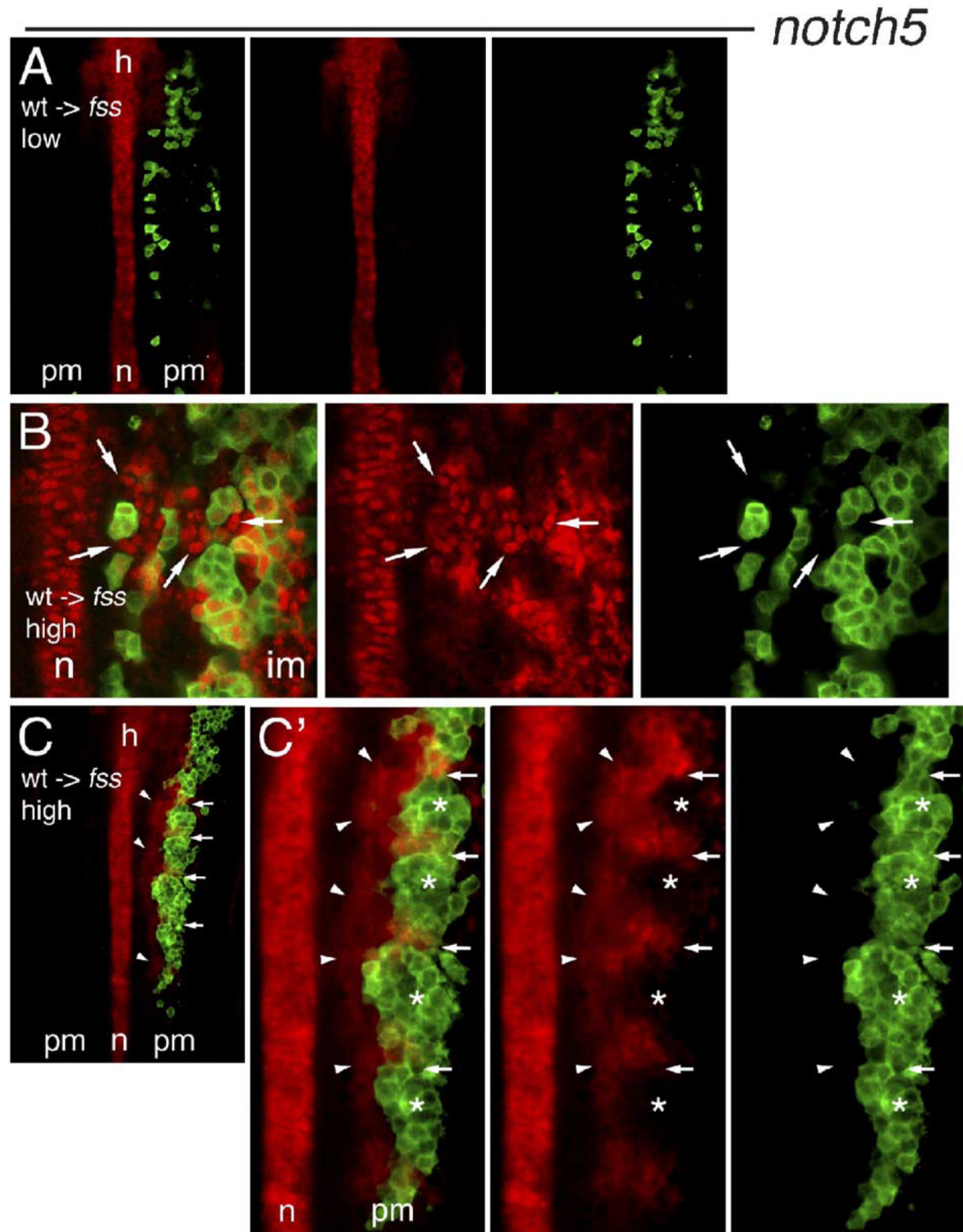

Fig. 4.

Induction of *notch5* expression in *fss/tbx24* host cells by wild-type neighbors. Expression of *notch5* mRNA (red) in confocal sections of the PM of 5–6 somite stage *fss/tbx24* host embryos containing transplanted cells (green) from wild-type donors. Embryos are flat mounted, anterior up. (A) *notch5* is not expressed in the PM of *fss/tbx24* host embryo with low density of wild-type donor cells. (B) High-magnification view of PM of *fss/tbx24* host embryo containing high density of wild-type donor cells. Autonomous *notch5* expression is seen in wild-type cells, and *notch5* induction in numerous *fss/tbx24* host cells (arrows) up to three cell diameters from the wild-type cell clones. (C) Periodic stripes of *notch5* expression in *fss/tbx24* host embryo containing 5 somite-like clusters of wild-type donor cells in the right-hand

PM. Contralateral side does not express *notch5*. Arrows indicate the boundaries between clusters, arrowheads mark *fss/tbx24* host PM cells expressing *notch5*.(C') High magnification of the region of *notch5* expression. Arrows and arrowheads as in panel C, asterisks mark the rostral half of cell clusters. Note high-level *notch5* expression in the caudal part of each somite-like cluster. n = notochord, h = hindbrain, pm = paraxial mesoderm, im = intermediate mesoderm.

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Table 1

Segmentation gene expression in wild-type–*fss/tbx24* mosaic experiments

Marker	*n* embryos[a]	n fss/tbx24[b]	Location of donor cells[c]		Location of expression[d]	
			n **somitic PM**	***n*** **PSM**	***n*** **donor (%)**	***n*** **host (%)**
papc	45	25	17[e]	-	0	0
			-	21	21 (100)	2 (9)
mespb	51	27	16[e]	-	0	0
			-	10	3 (30)	0
fgf8	56	28	15	-	0	0
			-	9	4 (44)	1 (11)
notch5	128	72	47	-	24 (51)	23 (49)
			-	19	3 (16)	1 (5)

[a]Number of host embryos of all genotypes that received transplants.

[b]Number of *fss/tbx24* host embryos.

[c]Number of embryos with donor wild-type cells in either more mature (the equivalent of somitic level) PM, or in PSM. Both situations can occur in a given embryo.

[d]Location of gene expression with respect to wild-type donor or *fss/tbx24* host cells. Percentage given is relative to number of embryos with wild-type donor cells in indicated area of PM (*n* somitic PM or *n* PSM).

[e]Gene is not normally expressed in this region.