

***spadetail*-Dependent Cell Compaction of the Dorsal Zebrafish Blastula**

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The dorsal marginal zone of the zebrafish blastula, equivalent to the amphibian Spemann organizer, is destined to become the tissues of the notochord and prechordal plate. Preceding gastrulation in the zebrafish, we find that these future mesendodermal cells acquire a cohesive cell behavior characterized by flattening and maximization of intercellular contacts, somewhat resembling cell compaction in mouse blastocysts. This behavior may suppress cell intermingling. Surprisingly, this blastula cell compaction requires normal function of *spadetail*, a gene known to be necessary for the dorsal convergent cell movement of paraxial mesoderm later in the gastrula. We propose that *spadetail*-dependent cell compaction subtly controls the early mixing and dispersal of dorsal cells that coalesce into the prospective organizer region. This early process may be necessary for the correct location of the boundary separating axial and paraxial cells. © 1998 Academic Press

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INTRODUCTION

In the zebrafish late blastula, epiboly begins the spread of the blastoderm over the giant yolk cell. Shortly following, cells at the blastoderm margin destined to become endoderm and mesoderm, begin to involute. Cells of the dorsal mesendoderm, after involuting, rapidly spread toward the animal pole and organize into the body axis; concomitantly, cells of the lateral and ventral mesendoderm migrate into paraxial positions. Cells of the dorsal mesendoderm form, for example, the notochord, hatching gland, skeletal head muscle, and pharyngeal endoderm, whereas cells of the lateral mesendoderm form somitic trunk muscle, stomach, and intestine (Kimmel *et al.*, 1990; Warga and Nüsslein-Volhard, submitted).

The function of the gene *spadetail* is necessary for cell movement toward the midline: *spadetail*-mutant lateral and ventral cells fail to converge dorsally and fail to become incorporated into trunk, resulting in an embryo with almost no somitic trunk muscle. Despite the lack of

convergent movements mutant cells continue an epibolic movement to the vegetal pole where they enter the tail bud. While these dislocated cells frequently form the mesenchyme of the “spade,” they sometimes give rise to structures which are normally derived from the dorsal region of the early gastrula (Ho and Kane, 1990; Kimmel *et al.*, 1989). Because *spadetail*-mutant embryos form a somewhat normal looking axis and head, it seems that *spadetail* is not required dorsally. Nevertheless, the expanded dorsal region of the fate map observed in mutant embryos (Kimmel *et al.*, 1989) suggests a broader role for *spadetail* in the sorting and regionalization of early embryonic fates.

While investigating the origin of mesendodermal structures in the blastula of wildtype and *spadetail* embryos, we observed that cells of the prospective mesendoderm on the future dorsal side of the zebrafish blastula display a unique cell compaction behavior. Surprisingly, *spadetail*, a gene with no obvious function on the dorsal side of the embryo, is essential for this dorsal-specific cell compaction. Such a behavior could be part of a mechanism which suppresses cell intermingling. In *spadetail* embryos, the anlage of the axial mesendoderm is enlarged at the expense of the paraxial mesendoderm. Dorsal cell compaction therefore may be the process initiating the formation of the axial-

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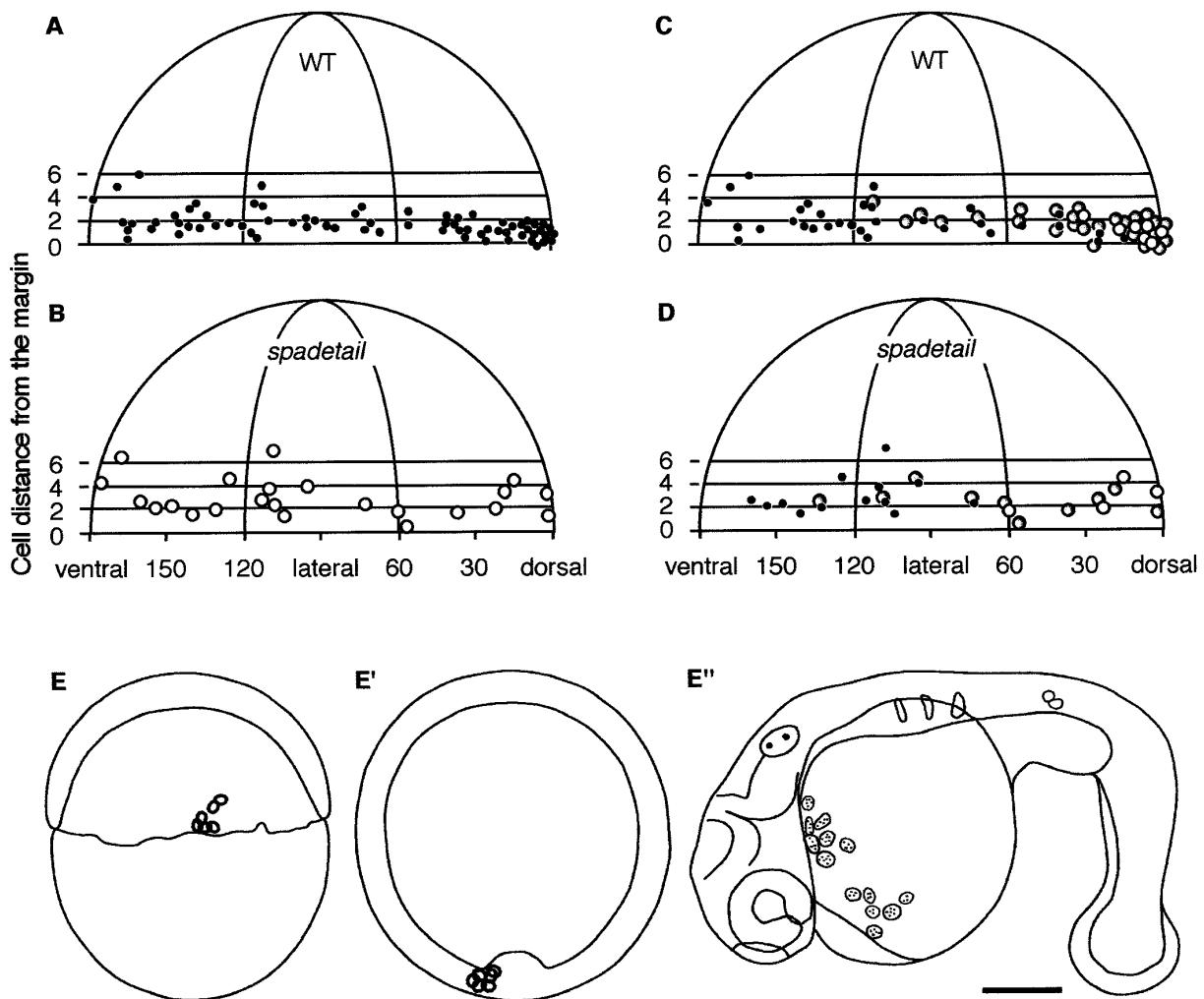


FIG. 1. Distribution of marginal clones at 40% epiboly is altered in *spadetail* mutants. The blastoderm is comprised of two cell populations: an outer epithelial monolayer (enveloping layer), and a multilayer of deeper cells which become the embryo proper (deep cells). The points on the blastula diagrams represent the central position of clonal by related deep cells. (A) Wild-type (filled circles; $n = 71$) and (B) *spadetail*-mutant embryos (open circles; $n = 23$). (C and D) The subset of the clones contributing to prechordal plate: hatching gland, pharyngeal endoderm, and head muscle (large gray circles); notochord (large open circles); and paraxial mesendoderm: somitic muscle, stomach, and intestine (small filled circles). (E) Dorsal marginal clones give rise to notochord and prechordal plate derivatives. Tracings are records of the same clone over time in a *spadetail* embryo. (E) 40% epiboly, side view; four cells of the clone cluster, whereas the remaining two cells have dispersed. (E') Shield stage, animal pole view; the dorsoventral position of the clone, centered just off the midline. (E'') 1 day, side view; the clone now differentiated into hatching gland over the yolk sac and five notochord cells in the trunk. Scale bar, 150 μ m.

paraxial boundary thought to be required for convergent extension in the gastrula.

MATERIALS AND METHODS

Embryos and Cell Labeling

Eggs were produced from fish heterozygous for *spadetail*^{b104}. At the 1K-cell stage, a single marginal cell was coinjected with a mixture of 5%

rhodamine-dextran (Warga and Kimmel, 1990) and 2% biotinylated-dextran (a fixable tracer, Molecular Probes) generating clones consisting of either enveloping layer cells and deep cells, or clones comprised wholly of deep cells. Enveloping layer cells were ignored in cell fate studies because these cells do not contribute to definitive embryonic tissues. At 40% epiboly, embryos were mounted in 3% methyl cellulose, and the labeled deep cells and blastoderm margin were recorded. At shield stage, the dorsoventral position of the clone was determined by examining the embryo from an animal pole view. Clones were followed up to 1–5 days to determine differentiated cell fates.

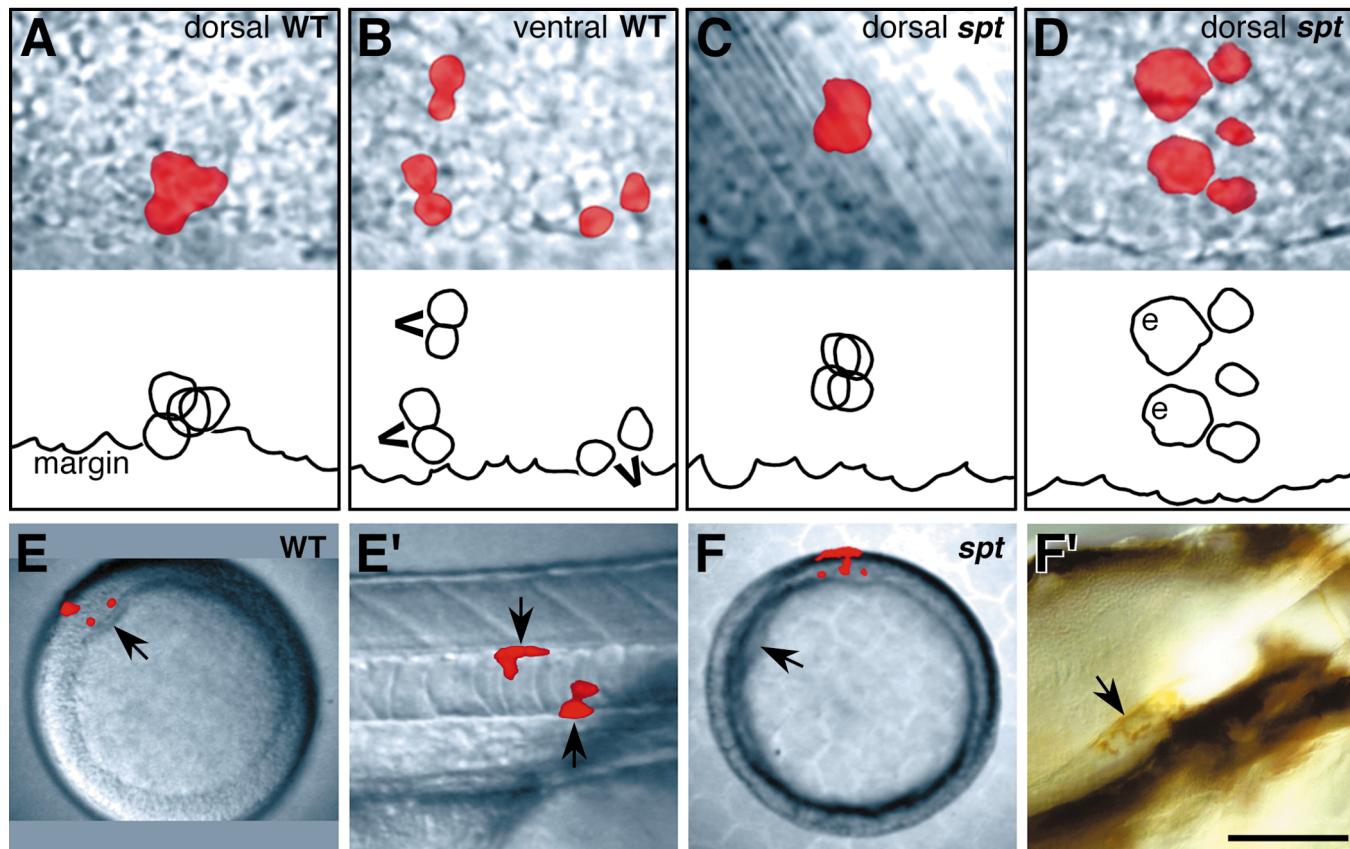


FIG. 2. Cell clustering and dorsoventral position of prospective axial mesendoderm is altered in *spadetail* mutants. (A–D) Labeled clones at 40% epiboly. The upper panel is the record, and the lower panel is a tracing of the individual deep cells, typically by this time there were 4 deep cells descended from the mid-blastula marginal cell ($3.7 \text{ cells} \pm 0.22$, wild type; $3.9 \text{ cells} \pm 0.39$, *spadetail* mutant); blastoderm margin is indicated; enveloping layer cells (e); recent cell divisions (v's). Dorsal clones in mutant embryos disperse significantly more than in normal embryos ($\chi^2 = 21.1$, $df = 4$; $P < 0.01$). (E and F) Labeled clones contributing to notochord in the trunk. (E and F) Animal pole views at shield stage; dorsal side (arrow). (E' and F') Side views. (E') 1 day wildtype embryo. The differentiated clone (arrows), located midtrunk, is notochord sheath, a derivative of the notochord (Melby *et al.*, 1996). (F') 4 day *spadetail*-mutant embryo, after whole-mount staining for the fixable tracer. The differentiated clone, now stained brown (arrow), is notochord sheath located in the anterior trunk. Scale bar: 50 μm (A–D), 100 μm (E' and F'), 250 μm (E and F).

Time-Lapse Video

Time-lapse recordings are described in Warga and Kimmel (1990) with the following modifications. We immobilized 20 embryos at the oblong stage in 0.075% agarose between microcoverslips. From sphere stage until shield stage, we recorded marginal deep cells by capturing 10 optical sections at 8- μm intervals every 20 seconds. At 40% epiboly, before determining the dorsoventral orientation of each embryo, we ranked the appearance of cells on an arbitrary scale from 1 to 6 (see Fig. 4). At gastrulation, we determined the dorsoventral orientation, and later the wildtype or mutant phenotype.

Immunostaining

Embryos were staged and fixed overnight at 4°C in 4% buffered paraformaldehyde. Whole-mount antibody staining and *in situ* were performed as in Schulte-Merker *et al.* (1992).

RESULTS AND DISCUSSION

To characterize region-specific differences in the zebrafish blastoderm, we labeled single marginal cells with lineage tracer dye in blastula stage embryos, mapped the resulting small clones of cells relative to the blastoderm margin at 40% epiboly, and later determined the dorsoventral location of each clone in the early gastrula after the dorsal side is visible. Without exception, progeny of these clones differentiated into mesodermal and endodermal derivatives (Warga and Nüsslein-Volhard, submitted). Since we labeled the clones blindly, before the dorsal side is apparent, we expected the labeled clones to be randomly located. However, the density of clones in the dorsal third of the blastula compared to that of the lateral or the ventral third was higher by a factor of 2 (34/18/19). Furthermore,

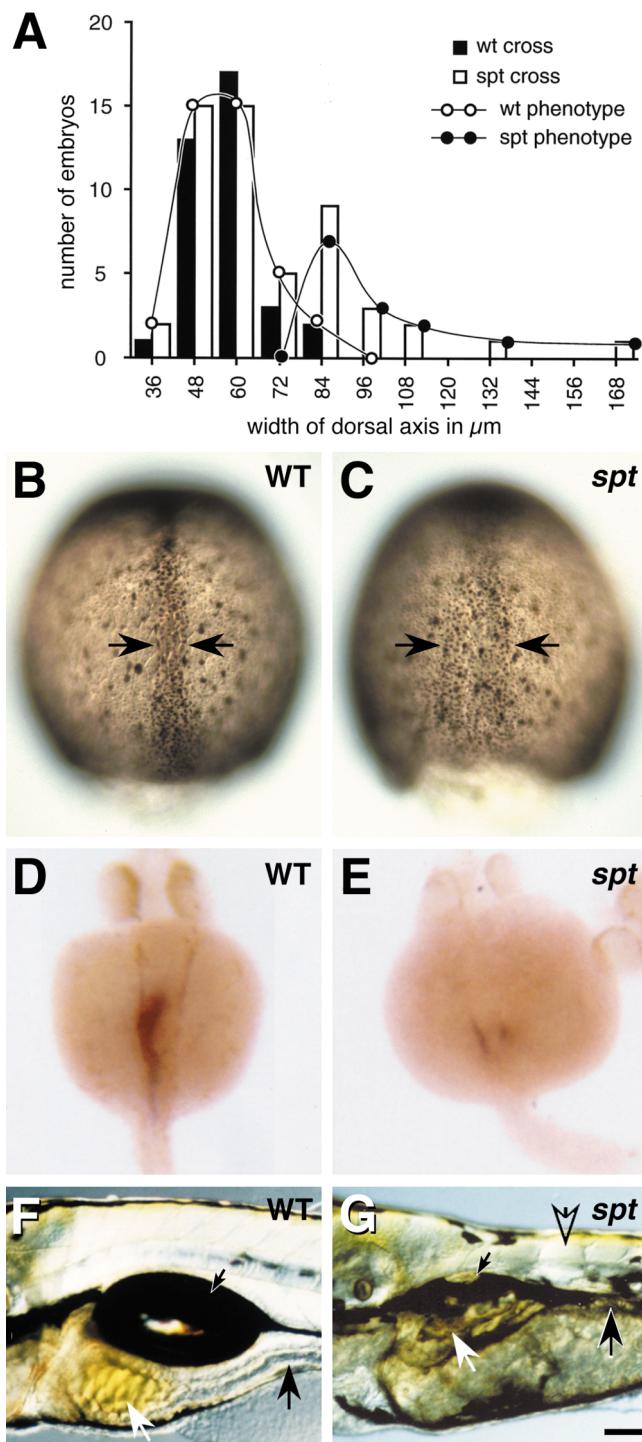


FIG. 3. There are more axial and less paraxial cells in *spadetail* mutants. (A) The bimodal distribution in the width of the dorsal axis for embryos derived from a cross of *spadetail* heterozygotes (open bars), and the bell curve for embryos derived from a cross of wildtype homozygotes (solid bars). Overlap is apparent between the larger peak in the *spadetail* cross and the wildtype peak, suggesting that embryos within this region of the graph are phenotypically

dorsal clones were centered closer to the margin than lateral or ventral clones (Fig. 1A). Thus, in between labeling and 40% epiboly, a displacement of marginal cells toward dorsal and a displacement of dorsal midline cells toward the margin must have occurred prior to gastrulation. Furthermore, blastula cells in dorsal clones were tightly clustered (Fig. 2A) and in lateral or ventral clones were more scattered (Fig. 2B), demonstrating that cell dispersion is not equivalent from dorsal to ventral.

In *spadetail*-mutant embryos, clones did not get displaced toward dorsal (Fig. 1B). Additionally, cells in dorsal clones only sometimes clustered (Figs. 2C and 2D). In mutant embryos, clonally related cells separated by approximately a cell diameter (0.9 ± 0.5 cell diameters, $n = 8$), whereas in normal embryos clonally related cells remained together (0.1 ± 0.2 cell diameters, $n = 34$). Whether cells clustered or not, dorsal clones gave rise to prechordal plate as well as notochord, in both wildtype and mutant (Fig. 1E). However, in *spadetail* embryos, prechordal plate and notochord progenitors were not restricted to the dorsal midline as in wildtype (compare Fig. 1C to 1D). For example, trunk notochord, which is derived exclusively from dorsal cells in wildtype embryos (Melby *et al.*, 1996), was derived from more lateral cells in some mutant embryos (Figs. 2E and 2F). By comparison, paraxial mesendoderm progenitors, which are derived from the broad dorsolateral to ventral region of the margin in wildtype embryos, were derived from only the more ventrolateral cells in *spadetail* embryos (compare Fig. 1C to 1D), a smaller region than normal.

These mapping data suggest that in *spadetail* embryos the number of axial progenitors could be increased at the expense of paraxial progenitors. Using anti-Fkd2 immunostaining, which visualizes the axial mesendoderm (Warga and Nüsslein-Volhard, submitted), we stained embryos derived from parents heterozygous for *spadetail*. At 80% epiboly, approximately 1/4 of the embryos had markedly wider dorsal axis not observed in embryos derived from homozygous wildtype fish (Figs. 3A–3C). A fraction of these embryos (13%), inferred mutant, had many more notochord

wildtype. The smaller peak in the *spadetail* cross would represent mutant embryos. Black lines with open or closed circles are the inferred distribution based on the expected mutant frequency. Axis were measured on a dissection scope using an ocular micrometer. (B and C) Dorsal axis (arrows) visualized using anti-Fkd2 immunostaining at 80% epiboly. (B) Representative wildtype embryo. (C) Presumed mutant embryo. (D and E) The embryonic gut in 1-day embryos visualized using *fkd2* RNA expression (Odenthal and Nüsslein-Volhard, 1998). (F and G) The differentiated gut in 5 day embryos, the stomach (white arrow), intestine (black arrow), and swimbladder (small black arrow), all derived from the paraxial region (Warga and Nüsslein-Volhard, submitted) are present, but diminutive in the mutant. Note that muscle is now apparent, but less abundant in the mutant trunk as revealed by birefringence (open arrow). Scale bar: 60 μ m (B and C), 100 μ m (D–G).

cells (332 versus 190 in wildtype; $n = 6$) visualized using anti-Ntl immunostaining, a notochord-specific marker (Schulte-Merker *et al.*, 1992). Embryos mutant for *spadetail* have almost no trunk paraxial muscle. Additionally, using expression of *fkd2* RNA in the differentiating gut at pharyngula stage, we found that mutant embryos develop almost no trunk endodermal tissue (Figs. 3D and 3E). However, during later development, trunk endoderm in mutant embryos is detectable, as is the case for paraxial muscle (Figs. 3F and 3G). Thus consistent with our fate mapping data, we demonstrate there is more axial tissue and less paraxial tissue in *spadetail* mutant embryos.

Many of the defects we observed in *spadetail* embryos could result from the altered clustering of dorsal clones. Using time-lapse video we observed the behavior of cells along the margin of the blastoderm. Prior to epiboly, marginal cells of wildtype embryos are round and loosely associated. Following the onset of epiboly, dorsal marginal cells within a region of 60° arc flatten and maximize intercellular contact becoming completely adjoined (Fig. 4A). Throughout this time period, lateral and ventral cells neither flatten nor compact (Figs 4C and 4D). Embryos mutant for *spadetail* show abnormal cell compaction (7 observations; a total of 84 embryos were recorded, 29 of which were mutant). In two *spadetail*-mutant embryos, no dorsal cell compaction occurred. In the remaining five, compacted patches (Fig. 4B; white arrow) were interspersed by extracellular space (black arrow), and loosely associated cells. Ranking the appearance of cells along the blastoderm margin at 40% epiboly (Fig. 4E), dorsal cells are significantly more compacted than lateral cells ($P < 0.001$) or than mutant dorsal cells ($P < 0.001$).

Blastula cell morphology was further characterized by labeling with an antibody against β -catenin protein to visualize the cell membrane (Schneider *et al.*, 1996). This showed that at 30% epiboly, cells in normal embryos possess numerous contact sites irrespective of dorsoventral position (Fig. 4F). Consistent with the above observations, cells in *spadetail* mutants, regardless of position, exhibited aberrant and fewer contact sites (Fig. 4G; black arrow). These observations suggest that the *spadetail* gene product is required for cell adhesion, consistent with a function for *spadetail* in dorsal cell compaction and in later cell movement toward the midline.

Our studies reveal a control of cell contact following the onset of epiboly. Dorsal compaction appears to be the earliest morphogenetic movement of the embryo, coinciding with the expression of genes necessary for organizer function (Stachel *et al.*, 1993; Schulte-Merker *et al.*, 1997; Odenthal and Nüsslein-Volhard, 1998). During early epiboly, the blastoderm dramatically thins as central medial cells radially intercalate outward among the more superficial cells (Warga and Kimmel, 1990). This movement forces neighboring cells to scatter (Held *et al.*, 1994). If radial intercalation was even throughout the blastoderm, then we would expect the scatter of cells to be even throughout the blastoderm. However, we observed that there is a lack of

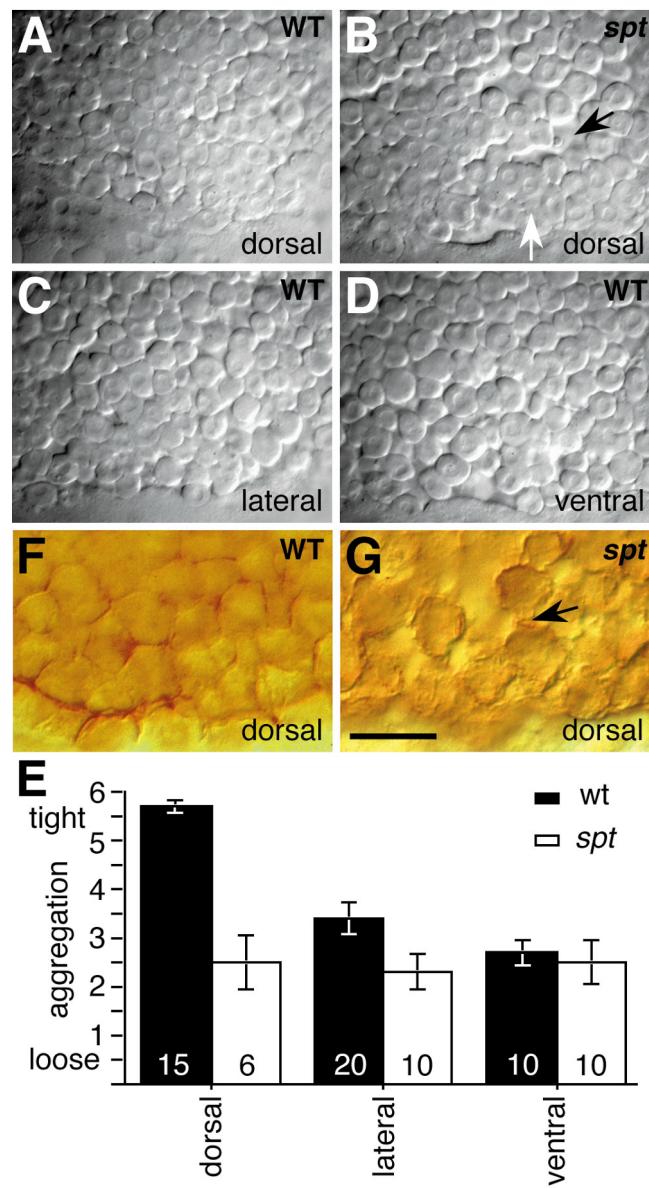


FIG. 4. The behavior of blastula marginal cells is changed in *spadetail* mutants. (A–D) Marginal cells at 40% epiboly, after progression of cell compaction. (E) Assessment of appearance of marginal deep cells just below the surface of the enveloping layer between 30 and 40% epiboly, respective of position in wildtype (solid bar) and *spadetail* mutants (open bar); lines represent standard error. For reference we ranked A as 6, tightly associated cells, and B and C as 3, tightly and loosely associated cells, and D as 2, loosely associated cells. Cell compaction was statistically compared between wildtype dorsal and lateral cells (Wilcoxon rank sum = 150, $n = 15$, $m = 20$) and between wild-type dorsal and mutant dorsal cells (Wilcoxon rank sum = 120, $n = 15$, $m = 6$). (F and G) Cell membranes visualized using anti- β -catenin immunostaining at 30% epiboly. Scale bar: 50 μ m (A–D), 25 μ m (F and G).

cell dispersion on the dorsal side of the embryo. Perhaps during early epiboly, differential adhesion of the dorsal margin forces a subtle displacement of marginal cells toward the dorsal side of the embryo, driven by an asymmetric radial intercalation of central medial cells toward the ventral side of the blastoderm.

In embryos mutant for *spadetail*, control of cell contact is aberrant. Dorsal marginal cells scatter as do ventral cells, and dorsal displacement of cells does not occur. Regulation of cell contact may be a component of the mechanism used to consolidate cells into a developmental field. Such regulation could result in isolating axial cells having organizer function from adjacent paraxial cells and initiate formation of the axial-paraxial boundary which, for example, acts in *Xenopus* as an organizing center for cellular behaviors that direct convergence and extension of cells to the midline during gastrulation (Domingo and Keller, 1995). By this argument, *spadetail* function may be essential for the regulation of the relative size of the axial and paraxial fields. Supporting this notion we find that the number of cells contributing to notochord in *spadetail*-mutant embryos is greater, whereas the number of cells contributing to paraxial mesendoderm is diminished. Remarkably, *spadetail* function is nonessential in determining axial cell fate: the notochord in *spadetail* mutants, although enlarged, appears to form approximately normally and the head seems completely normal. Given the early effects in the mutant blastoderm, it appears that later *spadetail* function on the dorsal side is not required in contrast to the lateral and ventral mesendoderm where severe pattern deficiencies are observed in *spadetail*-mutant embryos. Our results indicate that *spadetail* function may be necessary for keeping cells with organizer function from dislocating into the paraxial domain where they could divert trunk progenitors toward an axial fate, perhaps disrupting a phenomenon similar to the "community effect" required during *Xenopus* gastrulation for establishing a homogeneous population of muscle progenitors (Gurdon *et al.*, 1993). Further investigation will reveal to what extent failure of *spadetail*-mutant lateral and ventral cells to converge dorsally and subsequent loss of the trunk territory can be attributed to the earlier defects in the future organizer region.

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