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Defects of the body plan of mutant embryos lacking *Lim1*, *Otx2* or *Hnf3β* activity

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ABSTRACT The orientation of the anterior-posterior (A-P) axis was examined in gastrula-stage *Hnf3β*, *Otx2* and *Lim1* null mutant embryos that display defective axis development. *In situ* hybridization analysis of the expression pattern of genes associated with the posterior germ layer tissues and the primitive streak (*T, Wnt3* and *Fgf8*) and anterior endoderm (*Cer1* and *Sox17*) revealed that the A-P axis of mutant embryos remains aligned with the proximo-distal plane of the gastrula. Further analysis revealed that cells which express *Chrd* activity are either absent in *Hnf3β* mutant embryos or localised in heterotopic sites in *Lim1* and *Otx2* null mutants. *Lim1*-expressing cells are present in the *Hnf3β* mutant embryo albeit in heterotopic sites. In all three mutants, *Gs*c-expressing cells are missing from the anterior mesendoderm. These findings suggest that although some cells with organizer activity may be present in the mutant embryo, they are not properly localised and fail to contribute to the axial mesoderm of the head. By contrast, in *T/T* mutant embryos that display normal head fold development, the expression domains of organizer, primitive streak and anterior endoderm genes are regionalised correctly in the gastrula.

KEY WORDS: *axis alignment, gastrulation, mouse, mutant.*

Introduction

In the Zebrafish, *Xenopus* and chick embryo, the orientation and polarity of the embryonic axes can be traced back to asymmetrical features of the embryo before the onset of gastrulation (Yamanaka *et al.*, 1998; Harland and Gerhard, 1997; Bachvarova *et al.*, 1998; Izpisua-Belmonte *et al.*, 1993). Recently, embryological studies in the mouse blastocyst have revealed that the polar body formed at fertilization remains tethered to the trophectodermal descendant of a blastomere during pre-implantation development. In the blastocyst, the polar body is localized asymmetrically in regards to the inner cell mass (ICM), and its position marks the animal-vegetal plane of the blastocyst that lies orthogonally to the embryonic-abembryonic axis (Gardner, 1997). Fate mapping studies have demonstrated that ICM cells located at the animal pole (towards the polar body) contribute descendants to the visceral endoderm (VE) that resides in a more distal location in the 5.5-day embryo than those derived from ICM cells at the vegetal pole away from the polar body (Weber *et al.*, 1999). By the early-gastrula stage, the visceral endodermal cells derived from the two poles of the ICM are distributed unevenly to the prospective anterior and posterior sides of the embryo (Weber *et al.*, 1999). Although a relationship between the animal-vegetal axis of the blastocyst and any of the three embryonic axes (i.e. anterior-posterior [A-P], left-right [L-R] and dorso-ventral [D-V]) has yet to be established, these findings are consistent with the concept that, during pre-gastrulation development, the A-P axis is re-aligned from an initial proximo-distal plane to the transverse plane of the egg cylinder (Thomas *et al.*, 1998; reviewed by Beddington and Robertson, 1998, 1999). The re-orientation of the A-P axis is revealed by the coincidental shift of the *Hex*-expression domain in the VE and the displacement of endodermal cells from the distal to anterior region of the embryo between 5.5 and 6.5 dpc (Thomas *et al.*, 1998). Concomitant with the changes of gene expression in the VE, the expression domains of two genes (*T* and *Wnt3*) in the proximal epiblast are transformed from a symmetrical ring to a lopsided configuration in anticipation of the formation of the primitive streak on the posterior side of the egg cylinder (Thomas and Beddington, 1996; Liu *et al.*, 1999). Whether the shift in these expression domains in the proximal epiblast and the visceral endoderm is brought about by differential regulation of transcriptional activity or concerted tissue movement in the germ layers, or both is presently unresolved.

Abbreviations used in this paper: A, anterior; AVE, anterior visceral endoderm; D, dorsal; Dist, distal; dpc, days post coitum; ICM, inner cell mass; L, left; P, posterior; Prox, proximal; R, right; V, ventral; VE, visceral endoderm.

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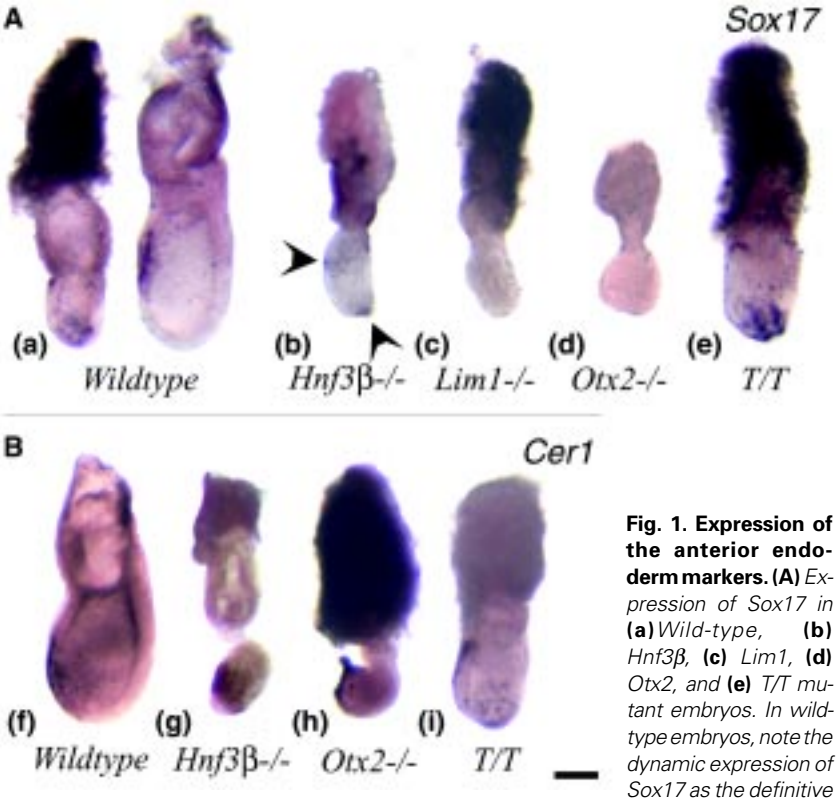

Fig. 1. Expression of the anterior endoderm markers. (A) *Expression of Sox17 in* **(a)** *Wild-type,* **(b)** *Hnf3β,* **(c)** *Lim1,* **(d)** *Otx2, and* **(e)** *T/T mutant embryos. In wild-type embryos, note the dynamic expression of Sox17 as the definitive endoderm is displaced from the distal posterior region to the anterior. Arrowheads in* **(b)** *mark the sites of Sox17 expression in the endoderm of the distal and anterior regions of the Hnf3β mutant embryo.* **(B)** *Expression of Cer1 in* **(f)** *Wild-type,* **(g)** *Hnf3β,* **(h)** *Otx2, and* **(i)** *T/T mutant embryos. Note the widespread Cer1 expression in the anterior and distal region in the Hnf3β mutant (***g***). Anterior is to the left and distal is down. Scale bar, 200 μm.*

The endodermal cells originating from different parts of the ICM are not only spatially segregated in the visceral endoderm, they are also organized in coherent clonal populations. By contrast, descendants of ICM cells in the epiblast intermingle extensively. The breakdown of the clonal coherence may be caused by the segregation of clonally related cells during the epithelialization of the epiblast and the separation of the mitotic descendants of epiblast cells as they alter cell-cell and cell-basal lamina contacts during cell proliferation (Gardner and Cockroft, 1998). This difference in the coherence of cell populations between the epiblast and the endoderm raises the possibility that, if an earlier asymmetry in the blastocyst is critical to the specification of the embryonic polarity that emerges in the postimplantation embryo, the regionalised information would have to be perpetuated by the tissue with the least disruption in the spatial relationship of clonally derived cell populations. This would implicate the primitive endoderm and not the epiblast as the key tissue responsible for driving the patterning of the embryo.

During gastrulation, visceral endoderm cells continue to be displaced towards the anterior and proximal sides of the embryo by the newly formed definitive endoderm (Lawson *et al.*, 1986; Lawson and Pedersen, 1987; Tam and Beddington, 1992). Concomitantly, the proximal epiblast which is fated to be extraembryonic mesoderm and amnionic ectoderm is displaced posteriorly towards the primitive streak (Lawson *et al.,* 1991; Kinder *et al.*, 1999), and more distal epiblast moves to this position (Quinlan *et al.*, 1995). Migration of more distal epiblast to a more proximal location may occur in concert with the migration of the mesoderm and definitive endoderm (Parameswaran and Tam, 1995, Tam *et al.*, 1997; reviewed by Tam and Behringer, 1997), though such coordinate movement of the two germ layers has not yet been shown.

In the mouse, the loss of *Lim1* or *Otx2* function results in the truncation of the anterior body axis (Shawlot and Behringer, 1995; Ang *et al.*, 1996; Acampora *et al*; 1995; Matsuo *et al.*, 1995). Analysis of the expression of molecular markers has revealed defects in the patterning of the visceral endoderm in the *Lim1* mutant embryo, where the *Cer1*-expressing endoderm (presumptive AVE) is localized ectopically in the distal region of the gastrula (Shawlot *et al.*, 1998). The localization of the *Cer1*-expressing endoderm in the *Otx2* mutant embryo varies from distal to anterior sites (Biben *et al.*, 1998). In the *Cripto* null mutant which shows no neural axis morphogenesis, *Cer1-, Hesx1-* and *Hex*-expressing endodermal cells remain in a distal position and are not found in the AVE (Ding *et al.*, 1998). These findings suggest that the distal endoderm may behave like the AVE for the specification of certain anterior neural characteristics (Thomas and Beddington, 1996). However, despite the induction of BF1 activity in the distal epiblast, no head morphogenesis or development of the A-P embryonic axis is found in *Cripto* null mutant embryos which fail to gastrulate (Ding *et al.*, 1998). In the *Wnt3* null mutant, the presence of *Lim1-* and *Cer1*-expressing cells in the anterior endoderm is similarly insufficient for initiation of anterior development (Liu *et al.*, 1999). Induction of head development therefore needs more than a functional AVE and has been shown to require the synergistic interaction of organizer-derived tissues and the anterior endoderm and epiblast tissue of the early

TABLE 1

EXPRESSION PATTERN OF ANTERIOR ENDODERM MARKERS (*Sox17* AND *Cer1*) IN 7.5-DAY *Hnf3ß-/-, Otx2-/-, Lim1-/-* AND *T/T* MUTANT EMBRYOS

Gene	Mutant	Expression pattern (N)
Sox17	*Hnf3β-/-*	Distal endoderm (4/5) Anterior endoderm (2/5)
	Lim1-/-	No expression (6/6)
	Otx2-/-	No expression (3/4) Localized to a clump of loose endoderm-like tissue at the distal tip (1/4)
	T/T	Distal and anterior endoderm (6/7) Anterior endoderm only (1/7, late gastrula)
Cer1	*Hnf3β-/-*	Distal endoderm (7/8) Proximal tissues (4/8) Anterior endoderm (3/8)
	Lim1-/-	*Weak or no expression (12/14) Shawlot et al., 1998* *Distal endoderm (2/14) Shawlot et al., 1998*
	Otx2-/-	Anterior and distal germ layer tissues (3/3) and Biben et al., 1998.
	T/T	Anterior and distal endoderm (5/5)

(N), number of specimens showing the specific expression over total number examined. Results of other studies are in italics.

mouse gastrula (Tam and Steiner, 1999). In the *Hnf3β* mutant embryo, a rudimentary neural axis with proper A-P molecular subdivisions is formed despite the absence of any recognisable organiser or axial mesendoderm. However, tissues resembling the AVE are found in the mutant gastrula-stage embryo (Klingensmith *et al.*, 1999), suggesting that there may be sufficient A-P organising activity for the initial patterning of embryonic tissues. The analysis of the developmental potential of chimaeras that comprise mutant and wild-type embryonic cells reveals that, in addition to the normal AVE function, *Lim1, Otx2* or *Hnf3ß* activity is also required in other germ layer tissues for anterior development (Shawlot *et al.*, 1999; Dufort *et al.*, 1998; Rhinn *et al.*, 1998, 1999).

To elucidate if some common morphogenetic defects may underlie the disruption of the body plan of *Hnf3ß, Lim1* and *Otx2* mutant embryos, we have conducted a comparative *in situ* hybridization study of genes that are expressed in the anterior mesendoderm, primitive streak and the organizer which maybe significant in axis formation. Our analysis focused on the expression of markers that may reveal the orientation of the A-P axis and the presence of the organizer-derived tissues in gastrula-stage mutant embryos. In parallel, we have examined the expression of the same set of genes in the *T/T* mutant embryo which forms head structures but fails to develop the trunk (Chesley *et al.*, 1935).

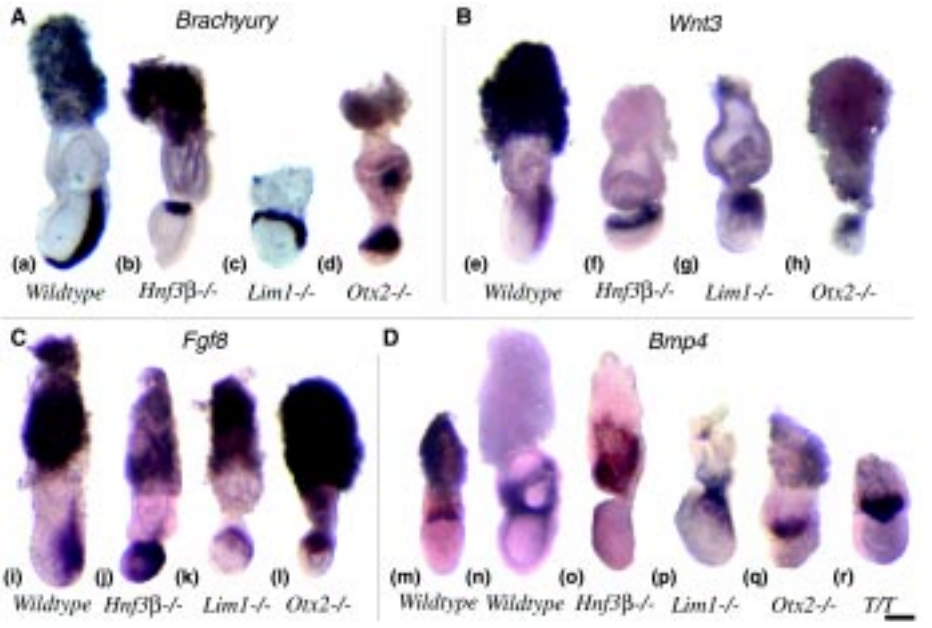

Fig. 2. **Expression of molecular markers of the primitive streak and extraembryonic tissues.** *Expression of* **(A)** *T,* **(B)** *Wnt3,* **(C)** *Fgf8 and* **(D)** *Bmp4 in Wild-type, Hnf3β, Otx2, Lim1 and T/T mutant embryos. Note the expression of T, Wnt3 and Fgf8 in the proximal region of the mutant embryos, and* **(p)** *the ectopic expression of Bmp4 in the embryonic mesoderm in the Lim1 mutant. Anterior is to the left and distal is down. Scale bar, 200 μm.*

Results

Ectopic localization of the anterior endoderm

The expression of *Cer1* and *Sox17* was examined in wild-type and mutant embryos at 7.5 days post coitum (dpc). In wild-type embryos, *Cer1* and *Sox17* are first expressed in the AVE in the early-streak stage embryo (Belo *et al.*, 1997; Biben *et al.*, 1998; Shawlot *et al.*, 1998; J. Gad and K. Bolton, per. comm.). During gastrulation, the expression of both genes shows a dynamic pattern that appears to reflect the formation and movement of the definitive endoderm to the prospective foregut (Figs. 1 a,f). In *Hnf3ß* mutant embryos *Sox17* expression was frequently found in the distal endoderm but sometimes also in the anterior endoderm (Fig. 1b, arrowheads). No *Lim1* mutant embryos showed any *Sox17* expression (Fig. 1c). Similarly most *Otx2* mutant embryos showed no *Sox17* expression (Fig. 1d) except one in our series which displayed expression in a loose cluster of endoderm-like cells at the distal tip of the egg cylinder (data not shown, Table 1). In *T/T* mutant embryos, *Sox17*- expressing cells were found in the anterior and distal endoderm (Fig. 1e), but mutant embryos appeared developmentally retarded compared to the wild-type littermates.

Previous studies have shown that *Cer1* is expressed correctly in the anterior endoderm of the 7.5-day *Hnf3ß* mutant embryo (Klingensmith *et al.*, 1999). In the eight *Hnf3ß* mutant embryos studied, *Cer1* expression in the endoderm was found to be variable from being in the distal region to the proximal-anterior region (Fig. 1g, Table 1). In *Otx2* mutant embryos *Cer1* was expressed in an anterior and distal domain, as previously described (Fig. 1h, Biben *et al.*, 1998), while in a few *Lim1* mutants, *Cer1* expression is restricted to the distal endoderm (Table 1; Shawlot *et al.*, 1998). In *T/T* mutant embryos *Cer1*-expressing cells were found in the anterior and distal endoderm (Fig. 1i).

Because of the retarded development of mutant embryos, it is difficult to determine whether the *Sox17*- and *Cer1*-expressing population in the endoderm represents the AVE, or the definitive endoderm or both. If the expressing cells are representative of the AVE, the detection of distal localization in some *Hnf3*β mutant embryos may indicate the failure of the re-orientation of the A-P embryonic axis. However, some *Hnf3*β mutant embryos seemed to display the anterior expression pattern of both genes that is characteristic of the younger embryo. Alternatively, if the expressing cells are precursors of the definitive endoderm, their distal location could reflect retarded migration or abnormal placement of the prospective foregut endoderm. The abnormal localization of *Sox17*-and *Cer1*-expressing endoderm may be correlated with the absence of foregut in the *Hnf3*β mutant embryo (Ang *et al.*, 1994). However, the lack of *Sox17* expression in the *Otx2* and *Lim1* mutant embryos is difficult to reconcile with the formation of foregut-like structures in these embryos (Shawlot and Behringer, 1995; Ang *et al.*, 1996). *Cer1* however is expressed in all mutant embryos, but is either distally localised (*Lim1* or *Otx2*) or scattered in *Hnf3*β null mutant embryos. Taking together the findings of *Cer1* and *Sox17* expression and disregarding the nature of mutant endodermal population that may express these genes, it seems that the A-P axis may be aligned incorrectly in *Hnf3*β, *Lim1*

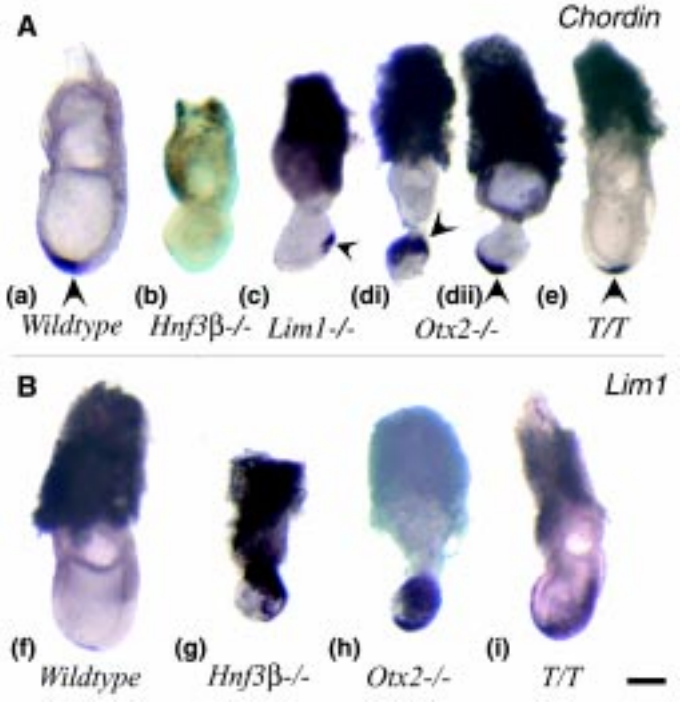

Fig. 3. Expression of *Chrd* and *Lim1* in mutant embryos. (A) *Expression of Chrd (arrowheads) in Wild-type, Hnf3β, Otx2, Lim1 and T/T mutant embryos.* **(d)** *Two specimens of Otx2 mutant embryo showing* **(i)** *proximal and* **(ii)** *distal and the anterior midline expression of Chrd.* **(B)** *Expression of Lim1 in Wild-type, Hnf3β, Otx2 and T/T mutant embryos. Anterior is to the left and distal is down. Scale bar, 200 μm.*

and *Otx2* mutants and that the anterior pole of the axis has remained in the distal region of the embryo.

Localization of the primitive streak

The localization of the primitive streak was examined in mutant embryos by the expression of *T, Wnt3* and *Fgf8*. In the wild-type late-streak (7.5 dpc) embryo, *T* expression marks the full extent of the primitive streak on the posterior aspect of the gastrula and the axial mesendoderm anterior to node (Fig. 2a). As previously reported for the *Hnf3ß, Lim1* and *Otx2* mutant embryos (Table 2), the primitive streak did not extend the full length of the posterior side of the embryo. In many mutant embryos, *T* expression was localized to the proximal epiblast cells of the gastrula (Figs. 2 b-d; Table 2). This is reminiscent of the proximal localization of *T* activity in the wild-type pre-gastrula embryo (Thomas and Beddington, 1996), suggesting the germ layer tissues are formed at an abnormal site in the mutant embryo.

Similar to the *T* gene, *Wnt3* is expressed uniformly in the proximal epiblast of the pre-gastrula embryo and subsequently in the posterior half of the primitive streak (Liu *et al*., 1999, Fig. 2e). In *Hnf3ß, Lim1* and *Otx2* mutant embryos, *Wnt3* expression was restricted to the proximal region of the gastrula and showed very little distal extension on the posterior side of the embryo (Figs. 2 f-h).

Fgf8 is expressed first in the epiblast adjacent to the prospective site of the primitive streak in the pre-streak embryo. During gastrulation it continues to be expressed in the epiblast adjacent to and in the posterior 2/3 of the primitive streak (Crossley and Martin, 1995; Fig. 2i). In *Hnf3ß, Lim1* and Otx2 null mutant embryos, *Fgf8* expression was greatly expanded in the proximal and posterior regions that encompass more than half of the embryo (Fig. 2 j-l). However, there was no discrete localization of *Fgf8* activity to any primitive streak structure, although stronger expression was seen in the proximal epiblast.

The localization of primitive streak markers to the proximal region of the *Hnf3ß, Lim1* and *Otx2* mutant embryos reveals a previous unnoticed defect in the organization of the A-P body plan. Recent work has shown that the location at which a cell passes through the streak as well as the time of ingression correlates with the final destination of the cell within the A-P axis (Kinder *et al*., 1999). The incorrect positioning of the streak in the proximal region of the epiblast in all three mutant embryos may result in different positional cues being perceived by cells as they ingress through the streak compared with those in the normal primitive streak. This may lead to incorrect localization of the mesodermal and endodermal cells in the A-P axis, the lack of segmental pattern in the paraxial mesoderm of the *Hnf3*β null mutant and the loss of anterior axial mesendoderm in the *Lim1* and *Otx2* mutant embryos.

Primitive streak localization to the proximal region of the mutant gastrula raises the question whether the proper inductive interaction exists between the extraembryonic tissues and the epiblast. In the mouse, one member of the BMP family *Bmp4*, is expressed in the extraembryonic ectoderm on the boundary with the proximal

TABLE 2

EXPRESSION OF PRIMITIVE STREAK AND MESODERM MARKERS, *T, Wnt3* AND *Fgf8,* AND *Bmp4* IN 7.5-DAY *Hnf3ß-/-, Otx2-/-, Lim1-/-* AND *T/T* MUTANT EMBRYOS

Gene	Mutant	Expression pattern (N)
T	*Hnf3β-/-*	Proximal epiblast (3/7) Proximal epiblast and slight distal extension on the posterior side (1/7), *and Ang and Rossant, 1994* Posterior side of the embryo (3/7)
	Lim1-/-	Proximal epiblast (1/2) Posterior side of the embryo (1/2), *and Shawlot and Behringer, 1995*
	Otx2-/-	Proximal epiblast (2/3) Posterior side of the embryo (1/3), *and Ang et al., 1996* Posterior side, node and anterior midline (Acampora *et al*., 1995)
Wnt3	*Hnf3β-/-*	Proximal epiblast (3/5) Posterior side of the embryo (2/5)
	Lim1-/-	Proximal epiblast (3/3)
	Otx2-/-	Posterior side of the embryo (2/3) Proximal epiblast (2/3)
Fgf8	*Hnf3β-/-*	Expanded domain in the proximal region (3/3)
	Lim1-/-	Proximal region of the embryo (3/4) No expression (1/4)
	Otx2-/-	Proximal region of the embryo (5/5)
Bmp4	*Hnf3β-/-*	Distal extraembryonic tissues adjacent to the epiblast (4/4) *Extraembryonic mesoderm and amnion (Klingensmith et al., 1999)*
	Lim1-/-	Distal extraembryonic tissues adjacent to the epiblast and in the proximal germ layer tissues in the embryo (3/3)
	Otx2-/-	Distal extraembryonic tissues adjacent to the epiblast (3/3)
	T/T	Distal extraembryonic tissues adjacent to the epiblast (5/5)

(N), number of specimens showing the specific expression over total number examined. Results of other studies are in italics.

epiblast (Fig. 2m) and later in the extraembryonic mesoderm and the chorionic tissues of the gastrula embryo (Fig. 2n; Waldrip *et al*., 1998). *Bmp4* signalling activity has been shown to be essential for the formation of the mesoderm (Winnier *et al*., 1995; Mishina *et al*., 1995) and for lineage specification of proximal epiblast cells (Lawson *et al*., 1999). In the *Hnf3ß* and *Otx2* mutant gastrulae, *Bmp4* expression persisted in the extraembryonic tissue adjacent to the epiblast (Table 2; Figs 2 o,q), similar to that in the wild-type early gastrula (Fig. 2m). In more advanced *Hnf3ß* mutant embryos, *Bmp4* was expressed in the extraembryonic mesoderm and the chorionic ectoderm (data not shown; Klingensmith *et al*., 1999). In the early-streak *Lim1* mutant embryo, *Bmp4* was expressed in the extraembryonic ectoderm as in the wild-type embryo (data not shown). However, in gastrula-stage embryos, *Bmp4* expression was not restricted to the extraembryonic tissues but was expressed ectopically in the proximal embryonic germ layer tissues (Fig. 2p). In the *T/T* mutant embryo *Bmp4* expression was present in the extraembryonic tissues adjacent to the epiblast (Fig. 2r). These results show that in all four mutant embryos, BMP4 signalling that might mediate the inductive interaction between the epiblast and the extraembryonic tissues may have persisted for much longer than during normal development. Whether this may lead to abnormal regionalization or morphogenetic activity of the primitive streak is not known. Our preliminary results show that in the *Lim1* mutant embryo, the differentiation of tissue lineages that are specified during gastrulation such as the germ cells, allantoic mesoderm and lateral and intermediate mesoderm are affected (Tsang *et al.,* submitted). It is not known if the defective differentiation of these lineages may be associated with the ectopic *Bmp4* activity in the primitive streak.

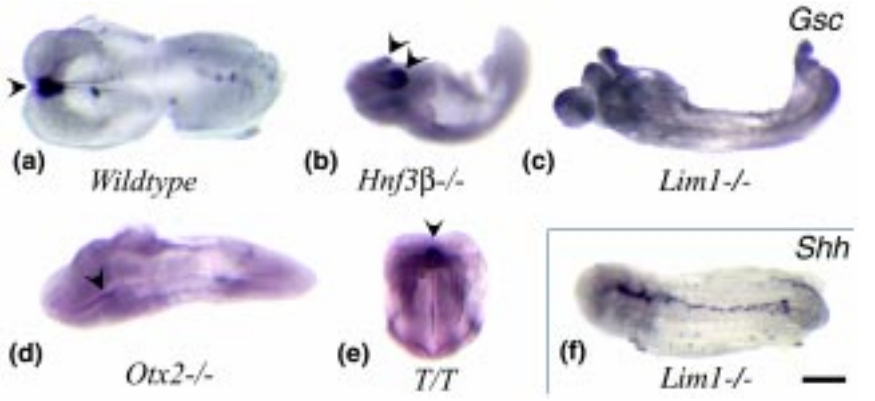

Fig. 4. Expression of *Gsc* and *Shh* in the axial mesendoderm. *Expression of Gsc (arrowheads) in the early-somite stage* **(a)** *Wild-type,* **(b)** *Hnf3β,* **(c)** *Lim1,* **(d)** *Otx2 and* **(e)** *T/T mutant embryos.* **(f)** *Expression of Shh in the midline of the Lim1 mutant embryo. Anterior is to the left (a-d, f) or up (e). Scale bar, 400 μm.*

The localization of the organizer and its derivatives

The expression of *Chrd* was examined to reveal the localization of the organizer in mutant embryos. In the wild type embryo, *Chrd* is first expressed in the anterior primitive streak of mid-streak gastrula (data not shown) and later in the node and the axial mesendoderm immediately anterior to the node (Fig. 3a). In the *Hnf3*β mutant, no *Chrd* expression was found in the 7.5-day gastrula (Fig. 3b) but weak and diffuse expression has been observed in the posterior epiblast in the early gastrula (Klingensmith *et al*., 1999). The lack of *Chrd* and also *Nog* expression is consistent with the absence of any morphologically distinct node in the *Hnf3ß* mutant embryo (Ang and Rossant, 1994; Dufort *et al*., 1998; Klingensmith *et al*., 1999). In the *Lim1* mutant embryo, *Chrd* expression was localized to the posterior epiblast but at variable sites (Table 3; Fig. 3c). Previous studies on *Otx2* mutant embryos have shown that organizer genes such as *Chrd* (Rhinn *et al.,* 1998) and *Hnf3*β (Ang *et al.,* 1996) are correctly localized in the distal region of the gastrula embryo. However, in one of the four mutant embryos studied in the present series, *Chrd* was localized to an expanded domain in the proximal region of the embryo (Table 3: Fig. 3 d,i). This demonstrates that in a minority of *Otx2* mutants the organizer may be ectopically localized. Embryos that displayed distal localization of *Chrd* activity also showed expression in the anterior axial tissues (Fig. 3d ii). In the *T/T* mutant *Chrd* was localised to the node region and anterior midline as in wild-type embryos (Fig. 3e).

In view of the variable regionalization of *Chrd* activity among the three types of mutant embryos, we next studied the differentiation of the axial mesendodermal derivatives in the late-streak and early-somite stage embryo. *T* expressing cells were absent from the anterior midline in *Hnf3ß, Lim1* and *Otx2* mutant embryos (Fig. 2 b-d; Table 2; Acampora *et al*., 1995; Ang and Rossant, 1994). Expression of *Lim1* in the anterior mesendoderm was also absent in the *Hnf3ß* mutant (Fig. 3g; Ang and Rossant, 1994; Table 3), but was present in *Otx2* mutant embryos (Ang *et al*., 1996; Fig. 3h). *T/T* mutants showed normal *Lim1* expression in the node and anterior midline (Fig. 3i).

The absence of *T*-expressing cells in the anterior midline of the *Hnf3ß, Lim1* and *Otx2* mutant embryos raises the question of whether the more rostral segment of the axial mesendoderm that does not express *T* activity may be present. Contained in this segment of axial tissue is the prechordal mesoderm that characteristically expresses *Gsc* activity (Fig. 4a arrowhead; Belo *et al*., 1998). Surgical ablation of the axial mesendoderm from the 7.5 dpc wild-type embryo produces a phenocopy of the truncated neural tube found in the *Otx2* and *Lim1* null mutant embryos

TABLE 3

EXPRESSION OF *Chrd* AND *Lim1* IN ORGANIZER-DERIVED TISSUE IN 7.5-DAY *Hnf3ß-/-, Otx2-/-, Lim1-/-* AND *T/T* MUTANT EMBRYOS

Gene	Mutant	Expression pattern (N)
Chrd	*Hnf3β-/-*	No expression (4/4), *and Klingensmith et al., 1999*
	Lim1-/-	Variable sites in the posterior epiblast (3/4), *and Shawlot et al., 1999*
	Otx2-/-	Distal and anterior germ layer tissues (2/4) *Distal only (Rhinn et al., 1998)* Posterior side of the embryo (1/4) Proximal germ layer tissue (1/4)
	T/T	Distal epiblast (2/4) Posterior side of the embryo (2/4, early gastrula)
Lim1	*Hnf3β-/-*	Posterior epiblast and endoderm (4/4), *and Ang and Rossant, 1994*
	Otx2-/-	Posterior epiblast and endoderm (3/3) *Node and anterior mesendoderm (Ang et al., 1996)*
	T/T	Node and anterior mesendoderm (2/2)

(N), number of specimens showing the specific expression over total number examined. Results of other studies are in Italics.

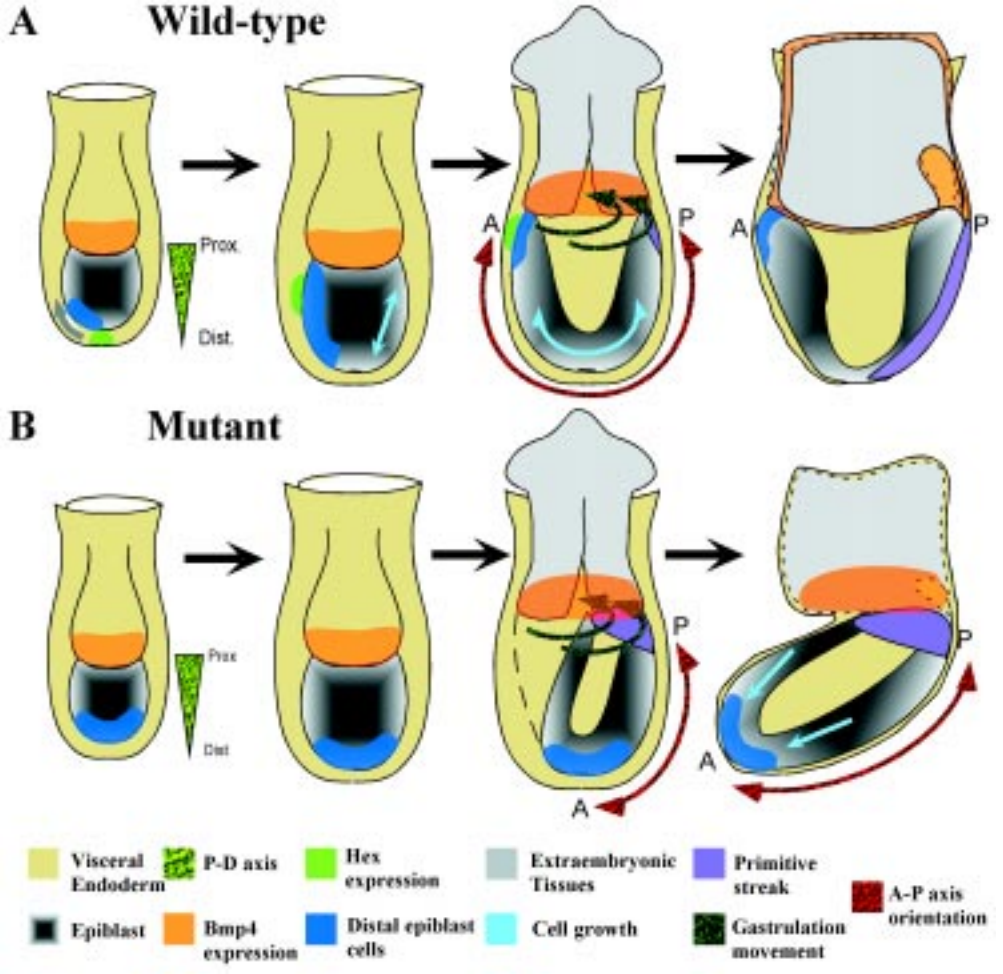

Fig. 5. Morphogenetic tissue movement and axis orientation in wild-type and mutant embryos. (A) *During pre-gastrulation development, endodermal cells in the distal region of the embryo are displaced towards the prospective anterior side of the embryo and contribute to the anterior visceral endoderm of the pre- and early-streak embryo. This results in the re-alignment of visceral endoderm from the distal (Dist.)-proximal (Prox.) axis to the prospective anterior-posterior (A-P) axis of the gastrula. A concerted displacement of the distal epiblast (the presumptive anterior neuroectoderm) in the same direction of the visceral endoderm may also take place, presumably due to difference in the growth of the epiblast between the anterior and posterior sides of the embryo. During gastrulation, cells are recruited first from the posterior region of the proximal epiblast to the primitive streak (for the formation of the extraembryonic mesoderm), followed later by those in the anterior region (for the formation of the amnion).* **(B)** *In the mutant embryo, the lack of anterior movement of the endoderm results in the retention of the presumptive anterior visceral endoderm in the distal region. At gastrulation, the primitive streak fails to extend along the posterior side of the embryo and is confined to proximal region of the epiblast next to the extraembryonic ectoderm (which continues to express Bmp4). The incorrectly positioned primitive streak may recruit cells from both the anterior and posterior regions of the proximal epiblast for germ layer formation. The inability to replenish the anterior-proximal epiblast cells that are lost precociously to abnormal gastrulation leads to a significant reduction in the tissue mass in the proximal region of the epiblast and might cause the formation of the constriction that is common to the three types of mutant embryos. Growth of the mutant embryo may be directed towards the distal-anterior pole and could lead to the increasing departure of the prospective anterior region of the embryo from the extraembryonic structures as the body axis elongates in the mutant embryo.*

(Camus *et al.*, 2000), thus pointing to a critical role of the mesendoderm in head development.

Gsc expression has been examined in the *Hnf3ß*, *Otx2,* and *Lim1* at early gastrulation. *Gsc*-expressing cells were found mainly in the proximal epiblast of the early-streak embryo (Table 4). We have examined *Gsc* expression in 8.5 dpc mutant embryos. *Gsc* was weakly expressed in only one of ten *Hnf3ß* mutant embryos studied, and was not localised to the midline (Table 4; Fig. 4b). The weak expression of *Gsc* in the *Hnf3*β mutant embryo suggests that some prechordal mesoderm might have been derived from the *Gsc*-expressing epiblast in the early gastrula. This contrasts with the absence of *Lim1, Shh* and *T*-expressing tissues (Ang and Rossant, 1994) that are normally found in the more caudal axial mesendoderm in the wild-type embryo. However, the expression of *Gsc* (Table 4) and *Lim1* activity (Fig. 3g, Table 3) in the *Hnf3ß* gastrula may be sufficient for the molecular patterning of the neural axis. No *Lim1* mutant embryos that formed a truncated head were found to express *Gsc* activity but *Shh* expressing cells were found in the midline tissues posterior to the level of axis truncation (Fig. 4 c,f). *Gsc*-expressing cells are found in the anterior axial tissues in some *Otx2* null mutant embryos but, like the *Hnf3ß* mutant embryo, the expression was weak (Fig. 4d). *T/T* embryos that form normal head structures displayed normal anterior *Gsc* expression (Fig. 4e).

Discussion

Abnormal gastrula morphology is associated with aberrations of primitive streak activity and morphogenetic tissue movement

Our *in situ* hybridization analysis of *T*, *Wnt3* and *Fgf8* activity has shown that these genes are expressed in the proximal region and not restricted to the posterior aspect of the mutant gastrula-stage embryo. The persistence of expression of these genes in the proximal germ layers is mirrored by that of *Bmp4* in the extraembryonic tissues juxtaposed to the epiblast. *Bmp4* is expressed in the distal part of the extraembryonic ectoderm of the pre-streak embryo, where *Sox2* and *Sox3* are also expressed, and as gastrulation proceeds it is expressed in the developing extraembryonic mesoderm and amnion (Lawson *et al.*, 1999). In wild type embryos, *T* and *Wnt3* are expressed early in the proximal region of the epiblast at the pre-streak stage, which is fated to form extraembryonic tissues (Lawson *et al.*, 1991). Later expression is

restricted to the posterior aspect of the embryo and finally to the primitive streak of the gastrula (Thomas *et al*., 1998; Liu *et al*., 1999). *Fgf8* expression is detected first in the prospective posterior region of the proximal epiblast and later found in the primitive streak and the nascent mesoderm (Crossley and Martin, 1995). The expression pattern of *T, Wnt3, Fgf8* and *Bmp4* in the mutant embryo is therefore reminiscent of the pre-gastrulation wild-type pattern. This might be accounted for by the delay in development of the mutant embryo such that the preceding expression pattern has persisted until a normal expression pattern can be subsequently established. However, this is unlikely because, although mutant embryos examined at 7.5 dpc may be variable in their developmental stages, they have already initiated gastrulation and normal morphology is never restored. This contrasts with the ability of wild-type embryos to regulate growth and morphogenesis in a compensatory response to tissue deficiency before the onset of gastrulation (Snow and Tam, 1979; Power and Tam, 1994). The heterotopic expression of these genes in the mutant embryo may instead be indicating the abnormal localization of the primitive streak. In the wild-type embryo, *Bmp4* activity in the extraembryonic ectoderm is required for the specification of cell fate in the proximal epiblast (Lawson *et al*., 1999), therefore it is possible that BMP4 signalling has an overriding influence in the specification of the mesoderm in the proximal epiblast. *Bmp4* null mutants lack primordial germ cells and mesoderm of the posterior body (Lawson *et al*., 1999) and in the absence of *Bmpr1* activity, the embryo fails to proceed with gastrulation (Mishina *et al*., 1995). The persistence of *Bmp4* expression in the extraembryonic tissues located closest to the epiblast may have confined the primitive streak to the proximal region of the mutant embryo. This ectopic localization of the primitive streak might have resulted in the precocious recruitment of the proximal epiblast to the germ layers. In normal embryos, cells are recruited first from the posterior-proximal epiblast to the extraembryonic mesoderm of the yolk sac, followed later by the more anterior-proximal population to the ectoderm of the amnion and the allantoic mesoderm (Lawson *et al*., 1991; Kinder *et al*., 1999). The precise fate of cells in the proximal epiblast of mutant embryos is not known, but the constriction of the embryo at the border of the epiblast and the extraembryonic tissue may be caused by the premature depletion of cells from the anterior region of the proximal epiblast to the extraembryonic mesoderm.

In some mutant embryos, the expression of *Cer1* or *Sox17* is localized predominantly in the distal region of the gastrula. Both are normally expressed in the AVE early, and later in the definitive endoderm of the prospective foregut. It is therefore not possible to distinguish, based on the distal localization of gene activity, whether this is due to the failure of the AVE precursors to relocate to the anterior region of the gastrula, or the abnormal congregation of the foregut endoderm in the distal region. The mouse *Cer1* gene has been shown to encode anti-BMP activity by animal cap assay (Biben *et al*., 1998). The localization of such activity to the AVE may be essential for the modulation of BMP activity emanating from the extraembryonic ectoderm, as well as in the positioning of the primitive streak and the organizer (Camus and Tam, 1999). The distal localization of *Cer1* and *Sox17*-expressing endodermal cells in mutant embryos may result in a lack of modulation of BMP signalling activity in the proximal region of the embryo. Consequently, the primitive streak and the organizer cannot be regionalised correctly in the mutant gastrula. The present findings of abnormal expression pattern of genes associated with the primitive streak and organizer are consistent with this hypothesis.

During normal development, the endoderm in the distal and posterior region of the pre-streak and early-streak embryo is displaced towards the anterior and proximal region by the definitive endoderm recruited from the primitive streak during gastrulation (Lawson *et al*., 1986; Lawson and Pedersen, 1987; Tam and Beddington, 1992). The morphogenetic movement of the definitive endoderm appears to be in concert with that of the mesoderm (Tam *et al*., 1997; reviewed by Tam and Behringer 1997). Analysis of the movement of clonal descendants of the epiblast reveals that some cells in the proximal epiblast are displaced towards the primitive streak (Lawson *et al*., 1991; Kinder *et al*., 1999) and their place is filled by cells that were previously localized in the anterior and distal region of the epiblast (Lawson *et al*., 1991; Quinlan *et al*., 1995). The persistent localization of *Cer1* and *Sox17*-expressing endoderm in the distal region of the mutant embryo suggests that there is a general lack of anterior-proximal displacement of the endoderm and the underlying germ layer tissues. This may be brought about by the lack of germ layer generation in the posterior region of the embryo as a result of the proximal localization of the primitive streak or by defects in the proliferation of epiblast cells. The failure to replenish the anterior-proximal epiblast population that is precociously recruited for gastrulation and the elevated apoptosis of the distal epiblast (as shown in the *Hnf3β* mutant embryo, Manova *et al.,* 1998) may have led to the formation of the constriction in the mutant gastrula (Fig. 5).

TABLE 4

EXPRESSION OF *Gsc* AND *Shh* IN THE AXIAL MESENDODERM IN 7.5- AND 8.5-DAY *Hnf3ß-/-, Otx2-/-, Lim1-/-* AND *T/T* MUTANT EMBRYOS

Gene	Mutant	Expression pattern (N)	
		Gastrula-stage	Early-somite-stage
Gsc	*Hnf3β-/-*	Proximal germ layer tissues (Ang and Rossant, 1994)	Anterior tissue clumps (1/10) No expression (9/10)
	Lim1-/-	*Proximal germ layer tissues (Shawlot and Behringer, 1995)*	No expression (10/10)
	Otx2-/-	*Proximal germ layer tissues (1/4) Ang et al., 1996* *No expression (3/4) Ang et al., 1996*	Anterior midline tissues (4/13) No expression (9/13)
	T/T	Not known	Anterior mesendoderm (2/2)
Shh	*Hnf3β-/-*	Not known	*Gut endoderm only (Ang and Rossant, 1994)*
	Lim1-/-	Not known	Axial mesendoderm and gut endoderm (2/3) Gut endoderm only (1/3)
	Otx2-/-	Not known	*Gut endoderm only (Acampora et al., 1995)*

(N), number of specimens showing the specific expression over total number examined. Results of other studies are in italics.

Defective organizer differentiation impacts on head development

The detection of *Cer1* and *Sox17*-expressing cells in mutant embryos suggests that the AVE population may be present in the distal region of the gastrula. In the *Cripto* mutant embryo, the distally localized AVE seems to be associated with the induction of neural gene activity in the distal ectoderm but the embryo never forms a proper neural axis (Ding *et al*., 1998). However, in the absence of the organizer or primitive streak derivatives, AVE

alone is not sufficient to induce anterior neural development in the *Cripto* and *Wnt3* null mutant embryos (Ding *et al.*, 1998; Liu *et al.*, 1999). No morphologically recognizable organizer such as the node or any localized expression of *Gsc* that may indicate the formation of the early gastrula organizer are found in the *Hnf3ß* or *Lim1* mutant embryos (this study, Shawlot and Behringer, 1995; Klingensmith *et al.*, 1999) although diffuse *Gsc* expression is seen in the *Hnf3ß* mutant epiblast at the early streak stage (Ang and Rossant., 1994). Cells that show *Lim1* activity are found in a diffuse domain in the epiblast of the *Hnf3ß* mutant embryo. The *Gsc*- or *Lim1*-expressing cells and their descendants might have provided the necessary organizing activity for the formation of the rudimentary neural axis in this mutant. *Chrd*-expressing cells are present but are not always properly regionalised in the *Otx2* and the *Lim1* mutant embryo. However, *Gsc*-expressing anterior mesendoderm that is associated with the rostral midline of the head fold and later with the prospective forebrain is absent in all three mutant embryo strains. Ablation of the *Gsc*-expressing rostral axial mesendoderm has been shown to result in the abnormal morphogenesis of the forebrain and midbrain (Camus *et al.*, 2000). The absence of a defined organizer and the loss of one of its derivatives, the anterior axial mesendoderm, may be the cause of the anterior truncation in the *Lim1* mutant embryo and the failure of head morphogenesis in the *Hnf3ß* mutant. Results of this study therefore highlight the critical requirement of the morphogenetic function as well as the proper regionalization of the activity of the gastrula organizer and the AVE for the formation of the AP axis.

Materials and Methods

Otx2 (Ang *et al.,* 1996), *Hnf3ß* (Ang and Rossant, 1994), *Lim1* (Shawlot and Behringer, 1995), and *T/T* (Chesley, 1935) mutant mice were used for this study. Embryos were collected at 7.5 and 8.5 dpc from heterozygous female mice of each strain that were crossed to heterozygous males of the respective mutant strain. *Otx2, Hnf3ß* and *Lim1* null mutant embryos were identified by their characteristic phenotype and in some cases confirmed by genotyping. For *T/T* mutant embryos, which do not display any abnormal phenotype at early stages, all embryos in the litter were processed for *in situ* hybridization using a mixed reagent that contained the riboprobe for *T* and the gene of interest. Embryos that showed no *T* hybridization signal were assumed of *T/T* genotype, when the majority of their littermates showed positive results in the same *in situ* hybridization experiment.

The protocol for *in situ* hybridization followed that of Wilkinson and Nieto (1993) with the following modifications. The Ampliscribe kit (Epicentre Technologies, Madison, Wisconsin, USA) was used in conjunction with Dig-11-UTP (Roche, Mannheim, Germany) to synthesize RNA probes, SDS was used instead of CHAPS in the hybridization solution and post-hybridization washes. Only 5x SSC was used in the hybridization solution, while post hybridization washes were at 70°C and excluded formamide. No RNA digestion was performed after hybridization. Following *in situ* hybridization embryos were photographed using a SPOT 2 digital camera and figures were prepared with Photoshop 5.5 and Illustrator 8.0.

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