Development 125, 4495-4506 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV4076

The *Polycomb*-group gene *eed* is required for normal morphogenetic movements during gastrulation in the mouse embryo

Cynthia Faust[1], Kirstie A. Lawson[2], Nicholas J. Schork[3], Bonnie Thiel[3] and Terry Magnuson[1,*]

[1]Department of Genetics, Case Western Reserve University, Cleveland, OH 44106-4955 USA
[2]Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands
[3]Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, OH 44106-4955 USA
*Author for correspondence (e-mail: trm4@po.cwru.edu)

Accepted 2 September; published on WWW 20 October 1998

SUMMARY

We have characterized an induced mutation, called *embryonic ectoderm development* or *eed,* that disrupts A-P patterning of the mouse embryo during gastrulation. Positional cloning of this gene revealed it to be the highly conserved homologue of the *Drosophila* gene *extra sex combs,* which is required for maintenance of long-term transcriptional repression of homeotic gene expression. Mouse embryos homozygous for loss-of-function alleles of *eed* initiate gastrulation but display abnormal mesoderm production. Very little embryonic mesoderm is produced; in contrast, extraembryonic mesoderm is relatively abundant. These observations, along with mRNA in situ hybridization analyses, suggested a defect in the anterior primitive streak, from which much of the embryonic mesoderm of the wild-type embryo is derived. To analyse this defect, we initiated clonal analysis of the pre-streak epiblast in *eed* mutant embryos, using the lineage tracer horseradish peroxidase (HRP). The results of these studies indicate that epiblast cells ingress through the anterior streak, but the newly formed mesoderm does not migrate anteriorly and is mislocalized to the extraembryonic compartment. Abnormal localization of mesoderm to the extraembryonic region did not appear to be due to a restriction and alteration of distal epiblast cell fate, since the majority of clones produced from regions fated to ingress through the anterior streak were mixed, displaying descendants in both embryonic and extraembryonic derivatives. *eed* mutant embryos also fail to display proper epiblast expansion, particularly with respect to the A-P axis. Based on patterns of clonal spread and calculated clone doubling times for the epiblast, this does not appear to be due to decreased epiblast growth. Rather, epiblast, which is normally fated to make a substantial contribution to the axial midline, appears to make mesoderm preferentially. The data are discussed in terms of global morphogenetic movements in the mouse gastrula and a disruption of signalling activity in the anterior primitive streak.

Key words: Mammalian embryogenesis, Mouse, Mutants, Germ layer formation, Primitive streak, Cell fate, Cell lineage, Microinjection, HRP

INTRODUCTION

In the mouse, gastrulation begins approximately midway through embryonic day (E) 6 (for reviews, see Tam and Behringer, 1997; Tam et al., 1993). At this time, the embryo consists of a single layer of primitive ectoderm (epiblast) covered by a layer of primitive endoderm. On the posterior side of the embryo, a disruption in the epithelial basal lamina defines a transient structure called the primitive streak, through which epiblast cells ingress and differentiate into mesoderm and definitive endoderm. Mesodermal cells exiting the primitive streak migrate anteriorly around the surface of the epiblast, contributing to head and trunk mesoderm; mesoderm also moves proximally into the extraembryonic region, contributing to the formation of the amnion, chorion, yolk sac and allantois, tissues that are critical for the proper development of the maternal-fetal interface.

The rapidly growing number of mutations affecting various aspects of gastrulation is beginning to allow for elucidation of a molecular basis for this dynamic process (reviewed in Tam and Behringer, 1997). We have characterized an induced mutation, called *embryonic ectoderm development* or *eed*, that disrupts A-P patterning of the mouse embryo during gastrulation (Faust et al., 1995; Niswander et al., 1988). Positional cloning of this gene revealed it to be the highly conserved homologue of the *Drosophila* gene *extra sex combs* (*esc*), which is required for maintenance of long-term transcriptional repression of homeotic gene expression (Schumacher et al., 1996). Mouse embryos homozygous for loss-of-function alleles of *eed* (hereafter referred to as *eed* mutant embryos or *eed* mutants) initiate but fail to complete gastrulation and display abnormal mesoderm production (Faust et al., 1995; Niswander et al., 1988). Very little embryonic mesoderm is present, and it does not differentiate into the

normal axial and paraxial derivatives, i.e., the notochord and somites. In contrast, abundant extraembryonic mesoderm is produced, forming an allantois and the mesodermal lining of the amnion, chorion and yolk sac. Accompanying reduced embryonic mesoderm production is a complete lack of anterior development: embryos display an underdeveloped embryonic ectoderm with no morphological or molecular evidence of neural induction (Schumacher et al., 1996). Because *eed* mRNA is normally expressed in all tissues of the gastrulating embryo, it is unclear whether lack of anterior development in *eed* mutant embryos is directly related to or independent of abnormal mesoderm production.

Extensive fate mapping of the wild-type mouse gastrula and primitive streak (Lawson et al., 1991; Tam and Beddington, 1987) suggests that the gross deficiency of embryonic mesoderm and absence of axial and paraxial derivatives in *eed* mutant embryos reflect a defect in the anterior primitive streak. This hypothesis is supported by lack of formation of a morphologically defined node, which is the mouse gastrula organizer (Beddington, 1994). In addition, *Nodal* mRNA fails to be localized to the streak and there is a loss of expression of *Fgf4* and *Gsc* in the anterior streak (Faust et al., 1995). *Evx1*, which is normally expressed in the streak in a graded fashion with highest levels posteriorly (Dush and Martin, 1992), appears to be expressed at high levels throughout the *eed* mutant streak (Schumacher et al., 1996). Taken together, the data suggest a disturbance in the anterior primitive streak of *eed* mutant embryos. In contrast, the apparently unimpeded production of extraembryonic mesoderm and primordial germ cells (Faust et al., 1995) suggests that epiblast cells are able to traverse the posterior streak.

We wished to define further the anterior primitive streak defect in *eed* mutant embryos. The presence of sparse axial mesoderm (Faust et al., 1995) suggested that some initial ingression occurs, but might be subsequently blocked. Alternatively, continuous ingression of epiblast cells through the anterior streak might occur, but mesodermal cells leaving the streak move aberrantly into the extraembryonic region. In order to test these hypotheses, we initiated clonal analysis of the pre-streak epiblast in *eed* mutant embryos, using the lineage tracer horseradish peroxidase (HRP). Using this technique, the morphogenetic movements of *eed* epiblast cells through the streak and localization of their descendants in the embryo have been determined. Clonal analysis also enabled us to investigate if individual epiblast cell fate is altered, as well as to examine cell proliferation in the *eed* mutant embryos. The results of these studies indicate that there is no block in epiblast cell ingression through the anterior streak; instead, nascent mesoderm does not migrate anteriorly and is subsequently mislocalized to the extraembryonic region of *eed* mutant embryos.

MATERIALS AND METHODS

Embryos

The *eed* mutant mice used in this study (*l(7)Rn5*^{3354SB}) originated at the Oak Ridge National Laboratory (Rinchik and Carpenter, 1993) and were maintained as described (Faust et al., 1995). Heterozygous *Tyr*$^{c\text{-}ch}$, +/*Tyr*^{c}, *l(7)Rn5*^{3354SB} mice were crossed inter se to obtain embryos. Noon of the day of the appearance of a vaginal plug was designated E0.5. Embryos were dissected at approximately E6.7. At this time, embryos were classified as either pre-streak or early streak. Pre-streak embryos were either radially symmetrical or exhibited a thickened posterior epiblast, preceding the site of primitive streak formation. Early streak embryos displayed some mesoderm formation. Only pre-streak embryos were used for cell labelling.

Cell labelling

Embryos were labelled iontophoretically as described (Lawson et al., 1991), with minor modifications. The embryo injection assembly consisted of a single-chambered LabTek II slide (Nunc) in which the chamber had been removed. Embryos were injected in a large drop of Dulbecco's modified minimal essential medium (DMEM) containing 10% fetal calf serum and 20 mM Hepes. Labelling was performed on a Nikon Diaphot microscope fitted with differential interference contrast (DIC) optics and epifluorescence. The microscope was attached to an image intensifier (Dage/MTI), standard CCD camera and video monitor to allow visualization of the embryos. One epiblast cell per embryo was injected with a mixture of 8% HRP (Boehringer Mannheim Biochemicals) and 2% lysinated-tetramethyl rhodamine dextran (LRDX, M_r 10×10^3; Molecular Probes) in 0.05 M KCl, at 3 nA discontinuous current (0.5 second duty cycle) for 15 seconds. Occasionally two adjacent epiblast sister cells were labelled due to leakage via cytoplasmic bridges (14% and 16% of the time for mutant and wild-type embryos, respectively). These embryos were included in the analysis and did not significantly affect interpretation of the data. An additional injection was made in one extraembryonic visceral endoderm cell at 3 nA discontinuous current for 12 seconds to identify retrospectively the 'position of longitude' of the injected epiblast cell in the egg cylinder (Lawson et al., 1991). The positions of the injected, fluorescently labelled cells were recorded with a Sony black and white videographic printer attached to the video monitor.

Embryo culture

Embryos were cultured in DMEM containing 50% rat serum as described (Lawson et al., 1986). Embryos were cultured for either 1 day (range 15-31.5 hours, average time 23.6 hours) or 2 days (range 34.5-45.5 hours, average time 39.6 hours). The percentage normal development was 83% and 80% for wild-type and *eed* mutant embryos, respectively, after 1 day in culture. The percentage normal development after 2 days in culture was 75% and 65% for wild-type and *eed* mutant embryos, respectively.

Detection of HRP-labelled cells and scoring of embryos

Detection of HRP-labelled cells was performed as described (Lawson et al., 1991). After staining, embryos were fixed in 2.5% glutaraldehyde in phosphate-buffered saline, dehydrated, cleared in benzyl alcohol: benzyl benzoate (1:2) and photographed under DIC optics. After clearing, those embryos displaying an abnormal phenotype and/or resembling homozygous *eed* mutants were subjected to genotype analysis. A portion of the yolk sac was removed, lysed and PCR was performed using primers flanking the *l(7)Rn5*^{3354SB} point mutation (Schumacher et al., 1996). *eed* homozygous embryos were identified by an *Alu*I restriction polymorphism; the mutant allele disrupts this site. All embryos displaying normal development with respect to various morphological features (Table 1) were classified as 'wild type.'

To confirm the number of descendants of the labelled cells and precisely determine their location, embryos were embedded in glycolmethacrylate (JB4; Polysciences), serially sectioned and stained with 0.2% methylene blue. Only those embryos wherein all of the descendants were evenly stained were used to calculate clone doubling times for labelled cells, using a previously established formula (Lawson and Pedersen, 1987; Lawson et al., 1991). Furthermore, clone doubling times were calculated using only those embryos cultured for 1 day, due to the potential loss of descendants from dilution of HRP after 2 days in culture (Lawson et al., 1986).

Statistical analysis

The two-sample *t*-test (Zar, 1984, pp.126-128) was used to determine whether the differences for mean dimensions (Table 1) and clone doubling times between wild-type and mutant clones were statistically significant. To determine if the percentage distribution of descendants to different embryonic regions differed significantly between wild-type and mutant embryos (Tables 3, 4, 6), Chi-square and the Wilcoxon rank-sum tests were performed using PROC FREQUENCY and PROCNPAR1WAY software (SAS Institute, Inc., Cary, NC). For all calculations, the level of significance employed was ≤5%.

RESULTS

Embryo injection and development in vitro

For the analyses reported here, single epiblast cells of wild-type and *eed* mutant embryos from the same litters were injected with HRP at the pre-streak stage and cultured for either 1 or 2 days before staining the descendants. Wild-type and *eed* mutant embryos yielded epiblast clones at similar efficiencies (Table 1).

At the time of injection, wild-type and *eed* mutant embryos displayed similar embryonic dimensions (Table 1) but, with careful observation, *eed* mutant embryos could be identified. In general, the diameter of the *eed* mutant extraembryonic region was narrower than wild type and the ratio of embryonic height to extraembryonic height was often greater than the 1:1 ratio evident in wild-type embryos (compare Fig. 1A,B). After 1 day in culture, most wild-type embryos developed to the late streak stage and had formed an amnion, chorion and allantoic bud (Fig. 1C; Table 1). In contrast, *eed* mutant embryos were often smaller and, although the primitive streak was elongated, it never extended to the distal tip of the embryo (Fig. 1D). Varying degrees of mesoderm production were observed, but no amnion or chorion were formed (compare Fig. 1C,D).

After 2 days in culture, most wild-type embryos possessed 3-6 somites (Fig. 1E; Table 1). While *eed* mutant embryos continued to grow between 1 and 2 days in culture and demonstrated some degree of A-P axial extension (Table 1), they were significantly smaller than their wild-type littermates. Mutant embryos displayed a well-developed extraembryonic region, with an enlarged yolk sac and formation of an amnion and chorion (Fig. 1F). A large mass of extraembryonic mesoderm was present that extended from the posterior streak into the exocoelomic cavity in a proximoanterior direction, reminiscent of an enlarged allantoic bud. Indeed, this mesoderm showed signs of allantoic differentiation in its distal-most region and often displayed fusion with the chorion (Fig. 1F). Blood islands were occasionally present in the yolk sac. In summary, the development of *eed* mutant embryos in culture was identical to their development in utero; this was confirmed molecularly by in situ hybridization analysis of genes expressed in the primitive streak and its derivatives (Faust et al., 1995; C. F. and T. M., unpublished data).

Experimental strategy

Wild-type fate maps constructed by clonal analysis were based on the division of the epiblast into 11 zones (Lawson et al., 1991). Considerable overlap in cell fate among different zones was observed. For this reason, and to simplify the presentation of the results reported in this study, we have focused our efforts on selected zones and pooled the data obtained from different zones, where applicable, into five distinct regions along the anterior-posterior axis (Fig. 2). The proximal two-fifths of the epiblast, which includes regions A and B, primarily contributes to embryonic ectoderm, proximal embryonic mesoderm and extraembryonic mesoderm after 1 day in culture, and to surface and amnion ectoderm as well as extraembryonic mesoderm after 2 days in culture (Fig. 2; Lawson et al., 1991; Lawson and Pedersen, 1992b). The middle two-fifths of the epiblast, which includes regions C and D, primarily contributes to embryonic ectoderm and mesoderm after 1 day in culture, and neural ectoderm and/or mesodermal derivatives after 2 days in

Table 1. Development of embryos in vitro after intracellular injection of HRP

		0 hours[a]		1 day						
				Embryos with HRP-labeled cells (%)			Developmental stage[e]			
Embryo type	Total embryos[b]	Height[c] (μm)	Diameter[d] (μm)		Height (μm)	Diameter (μm)	MS	LS	NP	HF
wild type	100	174±22	147±18	35(35)	257±38	320±102	8	23	2	2
eed	77	186±40	134±26	32(42)	242±48	259±46*		NA		

			2 days					
			Embryos with HRP-labeled cells (%)			Developmental stage		
Embryo type	Total embryos			Height (μm)	Diameter (μm)	NP	HF	Som.
wild type	103		35(34)	323±86	825±130	1	9	25
eed	85		25(29)	261±108	359±103*		NA	

[a]Measurements taken at the time of embryo culture are combined for those embryos cultured 1 and 2 days.

[b]Total embryos are all injected embryos demonstrating normal growth in culture; see Materials and Methods for further details.

[c]The distance from the junction of the epiblast and extraembryonic ectoderm to the distal tip of the egg cylinder, or from the anterior junction of embryonic ectoderm and amnion to the distal tip of the embryo. Mean ± standard deviation.

[d]The greatest width of the embryonic part of the egg cylinder when the embryo is viewed laterally, reflecting the length of the A-P axis after 2 days in culture. Mean ± standard deviation.

[e]For embryos yielding HRP clones. Given as number of embryos per developmental stage. The wild-type developmental stages of mid-streak (MS), late-streak (LS), neural plate (NP), and headfold (HF) were defined as described (Lawson et al., 1987). Som, 3-6 somites present; NA, not applicable.

*Value is significantly different from wild type (see Materials and Methods).

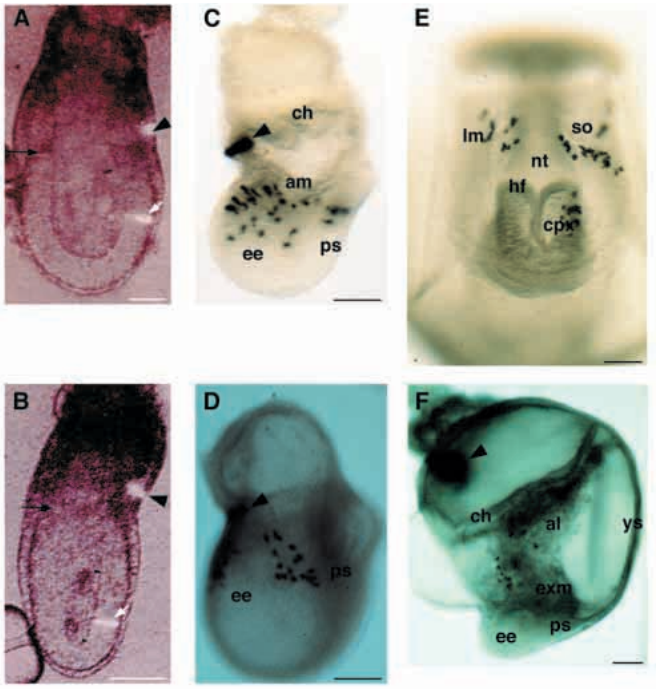

Fig. 1. Clonal analysis of wild-type and *eed* mutant epiblast. Wild-type (A,C,E) and *eed* mutant embryos (B,D,F) immediately following injection (A,B), 1 day after injection (C,D) and 2 days after injection (E,F). Black arrowheads indicate injected extraembryonic visceral endoderm cells in (A,B) and their descendant clones in (C,D,F). White arrows indicate injected epiblast cells and black arrows indicate the junction between embryonic and extraembryonic ectoderm in A,B. Note the narrower extraembryonic region and the increase in the ratio of embryonic to extraembryonic height in the *eed* mutant in B. (C,D) Lateral view of embryos injected in region A (see Fig. 2). Anterior is to the left, posterior to the right. The descendants (darkly stained cells) are found in the epiblast (ee) spanning back toward the primitive streak (ps). In the *eed* mutant embryo in D, three more descendants, out of focus, are present near the anterior midline. (E,F) Embryos injected in region C (see Fig. 2). (E) Ventral view (anterior at the bottom, posterior at the top) of a wild-type embryo reveals descendants in the lateral plate mesoderm (lm), somites (so) and cranial paraxial mesoderm (cpx). (F) Lateral view (posterior to the right) of an *eed* mutant reveals descendants in the extraembryonic mesoderm (exm) and allantois (al). Other abbreviations: ch, chorion; am, amnion; nt, neural tube; hf, headfold; ys, yolk sac. Scale bars: 50 μm in A,B; 100 μm in C-F.

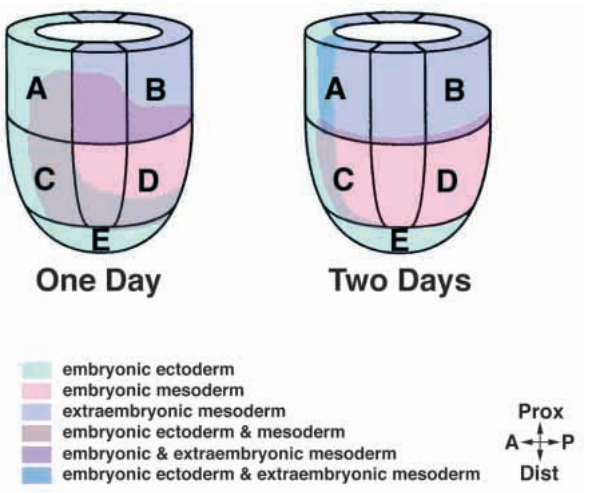

Fig. 2. Simplified fate maps of the pre-streak mouse epiblast, based on (Lawson et al., 1991; Lawson and Pedersen, 1992a,b) and this study. Separate fate maps are drawn to show derivatives 1 and 2 days after injection (corresponding to day 7.5 and day 8.5 of development). The original fate maps were constructed based on dividing the epiblast into 11 zones (Lawson et al., 1991): region A consists of zones I and II; region B, zones IV and V; region C, zones VI and VII; region D, zones IX and X; and region E, zone XI. Orientation is shown at the lower right of the figure. Abbreviations: Prox, proximal; Dist, distal, A, anterior, P, posterior.

culture (Fig. 2; Lawson et al., 1991; Lawson and Pedersen, 1992a,b). The distal-most portion of the epiblast, region E, contributes primarily to embryonic ectoderm after 1 day in culture and its derivative, neural ectoderm, after 2 days in culture (Fig. 2; Lawson et al., 1991; Lawson and Pedersen, 1992a).

Besides illuminating epiblast cell fate, clonal analysis yielded important information regarding the temporal and spatial incorporation of epiblast cells into the primitive streak, findings that are particularly relevant to this study (Lawson et al., 1991). At the time of primitive streak formation, the first cells to ingress lie at the posterior midline in regions B and D. As these cells leave the streak, they are replaced by progressively more lateral and anterior epiblast cells, including regions A and C. Thus, epiblast growth appears to be directed posteriorly towards the streak. Once epiblast cells reach the streak, they ingress, resulting in primitive streak extension and continuous mesoderm production. By the onset of neurulation at E7.5, the bulk of the epiblast initially located laterally and posteriorly (including regions B and D) will have been incorporated into mesoderm, while a substantial number of epiblast cells initially located more anteriorly (including regions A and C) will give rise to descendants that do not ingress through the streak. These cells will contribute to surface (regions A and C) and neural (region C) ectoderm.

Furthermore, clonal analysis revealed important information regarding growth of the epiblast and expansion of the A-P axis. In wild-type embryos, the A-P axis increases 3.5-fold in length during gastrulation, primarily due to elongation of the primitive streak and expansion of the area of the epiblast just anterior to the primitive streak (Lawson et al., 1991; Lawson and Pedersen, 1992a). As cells of regions A-D are progressively incorporated into the streak, the streak elongates. In addition, some descendants from region D remain in the ectoderm, inserting at the midline and contributing to the extension of the A-P axis (Lawson et al., 1991; Lawson and Pedersen, 1992a). As a result, descendants of the distal cap region E are propelled anteriorly, contributing to anterior expansion of the embryonic ectoderm.

The results obtained from clonal analysis of wild-type embryos enabled us to propose several hypotheses and make the following predictions regarding the fate and behavior of cells in these different regions of the epiblast of *eed* mutant embryos.

(1) The presence of extraembryonic mesoderm in *eed* mutant embryos suggests that proximal epiblast cells from regions A and B undergo posteriorly directed growth and ingress through

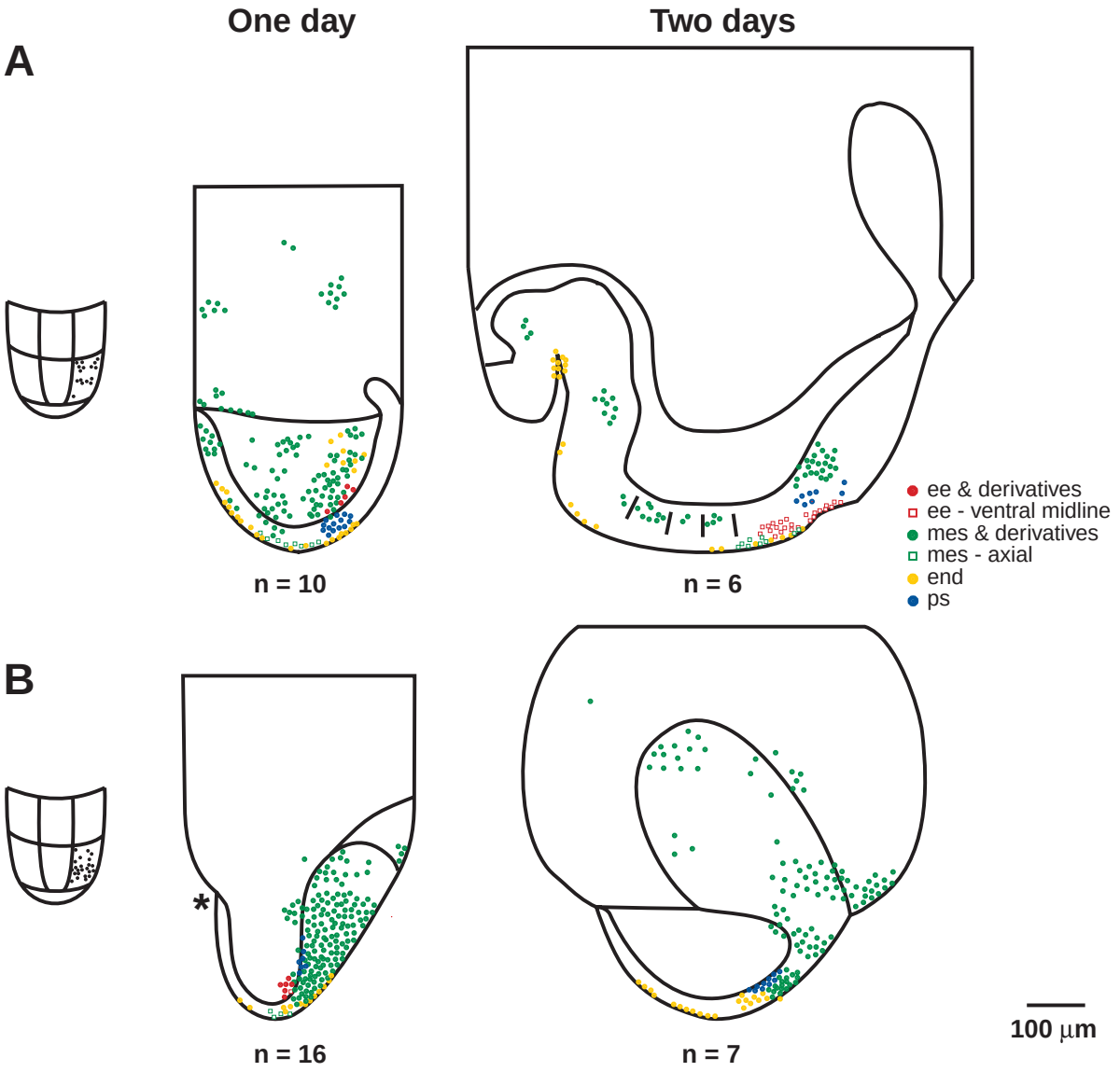

Fig. 3. Localization of descendants of region D in wild-type (A) and *eed* mutant (B) embryos. The diagrams at the left of each figure illustrate the location of the injections, with each dot representing an individual epiblast cell (and a different embryo). The middle diagrams represent embryos cultured 1 day (wild type, late-streak stage) and the diagrams on the right represent embryos cultured 2 days (wild type, ≥3 somites). Each colored dot in these embryos represents an individual descendant and all descendants obtained from multiple injections are shown. Dots are color coded to indicate presence in a particular germ layer. The asterisk in B indicates the boundary between the extraembryonic and embryonic regions in *eed* mutant embryos, which have not yet formed an amnion after 1 day in culture. The drawings depicting embryos after 1 and 2 days in culture are drawn to scale. Orientation is the same as for Fig. 2. *n*=number of embryos injected. Abbreviations: ee, embryonic ectoderm; mes, mesoderm; end, endoderm; ps, primitive streak.

the posterior primitive streak. Thus, cells of regions A and B would be expected to display similar fates in wild-type and *eed* mutant embryos.

(2) Sparse embryonic mesoderm production in *eed* mutant embryos is consistent with an abnormal anterior primitive streak. We propose two hypotheses to explain this defect in *eed* mutant embryos. One possibility is that epiblast cells ingress through the anterior streak, but as mesodermal cells exit, they move aberrantly into the extraembryonic region. If correct, clonal analysis of regions C and D in *eed* mutants would reveal a greater degree of contribution to extraembryonic mesoderm than observed in wild-type embryos. An alternative hypothesis is that ingression through the anterior primitive streak does not occur, or is greatly reduced. This was an attractive possibility because the small size of the epiblast of *eed* mutant embryos suggested a cell proliferation problem. If this were the case, we would predict seeing little posteriorly directed growth of the epiblast from anterior region C, and reduced or no contribution to mesoderm from this and posterior region D.

(3) The diminished epiblast expansion evident in *eed* mutant embryos might arise from either a general defect in epiblast proliferation, decreased primitive streak elongation, lack of incorporation of cells of region D into the midline ectoderm or a combination of these events. Clonal analysis was used to

Table 2. Incidence of embryos injected in regions A and B displaying labelled descendants in different embryonic and extraembryonic regions after culture

	Epiblast region							
	1 day				2 days			
	A		B		A		B	
	wild type	*eed*	wild type	*eed*	wild type	*eed*	wild type	*eed*
Total no. of embryos injected	5	3	5	3	4	5	7	2
Region								
Embryonic								
Ectoderm	3	3	0	1	2	3	0	0
Primitive streak	1	0	0	0	1	2	0	0
Mesoderm	2	0	3	3	2	3	1	0
Endoderm	0	0	0	0	0	0	1	0
Extraembryonic								
Amnion ectoderm	0	0	0	0	3	2	0	0
Extraembryonic mesoderm	0	0	5	1	2	2	7	2

investigate these possibilities. To characterize epiblast expansion in *eed* mutant embryos further, we also examined the fate of cells in region E.

Cells traversing the posterior streak display normal fates

As predicted from our morphological and molecular analyses of *eed* mutant embryos, *eed* mutant epiblast cells from regions A and B displayed similar fates to those of their wild-type counterparts, with some minor differences. As in wild-type embryos, after 1 day in culture descendants of region A were spread posteriorly in the epiblast towards the streak (Fig. 1C,D; Table 2). In addition, contribution to embryonic ectoderm and mesoderm, primitive streak, amnion ectoderm and extraembryonic mesoderm was observed after 2 days in culture (Table 2 and data not shown).

In wild-type embryos, region B, which contains cells traversing the streak very early in gastrulation, contributed to proximal embryonic (3/5 embryos injected), as well as extraembryonic mesoderm (5/5) after 1 day in culture (Table 2 and data not shown). In contrast, 3/3 *eed* mutant embryos injected in this region contributed to the proximal posterior mesoderm, but only 1/3 contributed to the extraembryonic mesoderm after 1 day in culture (Table 2). This difference may be due to a 6-12 hour delay in the onset of gastrulation observed in *eed* mutant embryos. Nevertheless, after 2 days in culture, both *eed* mutant and wild-type embryos contributed extensively to extraembryonic mesoderm (Table 2 and data not shown).

Cells traversing the anterior streak display abnormal fates

(i) Region D

Epiblast cells from region D traverse the primitive streak early in gastrulation and give rise to the bulk of the paraxial and axial mesoderm and definitive endoderm of the embryo; their descendants also make a significant contribution to ventral midline neurectoderm (Lawson et al., 1991; Lawson and Pedersen, 1992a,b; K. A. L., unpublished results). Rarely are descendants of these cells found in extraembryonic mesoderm. Analysis of region D in *eed* mutant embryos revealed marked differences in colonization by descendants (Fig. 3). For example, after 1 day in culture, the majority of descendants of wild-type region D were found in embryonic mesoderm, with fewer contributing to embryonic ectoderm, primitive streak, definitive endoderm and extraembryonic mesoderm (Fig. 3A). Descendants of *eed* mutant region D were also found in the embryonic mesoderm, streak, embryonic ectoderm and definitive endoderm. However, the embryonic mesoderm descendants lay posteriorly in the embryo (Fig. 3B). Transverse histological sections revealed that, while mesoderm cells readily exited the streak, they accumulated posterior to it

Table 3. Region D: percentage distribution of descendants and incidence of embryos contributing to different embryonic and extraembryonic regions after culture

	1 day		2 days	
	wild type	*eed*	wild type	*eed*
Total no. embryos injected	10	16	6	7
Total no. descendants in all regions	175	185	116	138
Region				
Embryonic ectoderm[c]	2.9[a] (3/10)[b]	3.8 (2/16)	20.7 (5/6)	0 (0/7)
Primitive streak	8 (3/10)	4.9 (5/16)	6.9 (4/6)	7.2 (2/7)
Embryonic (non-axial)[d] mesoderm	52.6 (6/10)	70.3 (14/16)	44 (4/6)	30.4 (5/7)
Axial mesoderm	4.6 (3/10)	2.2 (3/16)	7.8 (2/6)	0 (0/7)
Endoderm	16.6 (5/10)	7.0 (5/16)	20.7 (3/6)	13.0 (3/7)
Extraembryonic mesoderm[e]	15.4 (2/10)	11.9 (5/16)	0 (0/6)	49.3 (5/7)

[a]Percentage of descendants, calculated by dividing the number of descendants found in the region by the total number of descendants displayed in all regions.

[b]Incidence of embryos displaying descendants in the region.

[c]In wild-type embryos cultured for 2 days, includes ventral midline neurectoderm and node.

[d]In wild-type embryos cultured for 2 days, includes cranial, somitic, trunk paraxial and post-nodal (posterior to the node) mesoderm.

[e]For eed mutant embryos: after 1 day in culture, defined as that mesoderm lying proximal to the embryonic/extraembryonic ectoderm junction. After 2 days in culture, defined as allantoic bud-like mesoderm extending from the posterior streak into the exocoelom, differentiated allantois and mesoderm lining the yolk sac, amnion and chorion.

Table 4. Region C: percentage distribution of descendants and incidence of embryos contributing to different embryonic and extraembryonic regions after culture

	1 day		2 days	
	wild type	*eed*	wild type	*eed*
Total no. embryos injected	6	4	7	5
Total no. descendants in all regions	97	42	253	87
Region				
Embryonic ectoderm[c]	38.1[a] (4/6)[b]	71.4 (3/4)	36.4 (4/7)	26.4 (3/5)
Primitive streak	10.3 (2/6)	0 (0/4)	2.8 (1/7)	16.1 (3/5)
Embryonic (non-axial)[d] mesoderm	51.5 (3/6)	28.6 (1/4)	58.1 (4/7)	30.0 (3/5)
Extraembryonic mesoderm[e]	0 (0/6)	0 (0/4)	2.8 (2/7)	27.6 (3/5)

[a,b,d,e]See Table 3 legend.
[c]In wild-type embryos cultured for 2 days, includes neural, surface and postnodal ectoderm.

Table 6. Region E: percentage distribution of descendants and incidence of embryos contributing to different embryonic and extraembryonic regions after culture

	1 day		2 days	
	wild type	*eed*	wild type	*eed*
Total no. embryos injected	4	5	7	6
Total no. descendants in all regions	30	41	179	74
Region				
Embryonic ectoderm[c]	86.7[a] (4/4)[b]	65.8 (4/5)	97.2 (7/7)	58.1 (5/6)
Primitive streak	0 (0/4)	7.3 (1/5)	0 (0/7)	5.4 (2/6)
Embryonic (non-axial)[d] mesoderm	0 (0/4)	24.4 (2/5)	2.8 (1/7)	33.8 (4/6)
Endoderm	13.3 (2/4)	2.4 (1/5)	0 (0/7)	1.4 (1/6)
Extraembryonic mesoderm[e]	0 (0/4)	0 (0/5)	0 (0/7)	1.4 (1/6)

[a,b,e]See Table 3 legend.
[c]In wild-type embryos cultured for 2 days, includes neural and postnodal ectoderm.
[d]In wild-type embryos cultured for 2 days, includes postnodal mesoderm.

and demonstrated very little anterolateral migration away from it (data not shown). Cell mixing in *eed* mutant embryos was prevalent, in that descendants in the mesoderm were often dispersed widely among unstained cells. In some cases (2/16 embryos), clonal spread was extensive, spanning from the most distal embryonic mesoderm into the extraembryonic region. Other embryos displayed clones encompassing proximal embryonic and extraembryonic mesoderm (3/16 embryos), proximal embryonic ectoderm (2/16), distal embryonic mesoderm (6/16), or both (3/16).

The differences in contribution to mesoderm were amplified after 2 days in culture. In wild-type embryos, descendants from region D that after 1 day were found in the embryonic mesoderm, were now located in the cranial paraxial mesoderm and somites (Fig. 3A). Localization to the primitive streak, definitive endoderm, node and ventral midline neurectoderm was also observed. Descendants of *eed* mutant region D were still found in the embryonic mesoderm, primitive streak and definitive endoderm, but the bulk of the mesodermal descendants localized extraembryonically, in the allantoic bud-like mesoderm, as well as the differentiated allantois and yolk sac (Fig. 3B). 2/7 embryos contained clones wherein descendants were restricted to the extraembryonic region. In the remaining embryos examined, clones spanned either the proximal embryonic mesoderm and extraembryonic mesoderm (3/7) or extended from the distal into the proximal embryonic mesoderm (2/7). Again, transverse sections revealed very limited lateral migration away from the primitive streak (data not shown). Interestingly, once mesodermal cells reached the extraembryonic region, they were able to move more laterally and anteriorly, as descendants were found widely dispersed throughout the yolk sac and in the allantoic bud-like mesoderm and differentiated allantois (Fig. 3B).

Table 5. Incidence of embryos yielding clones encompassing embryonic and/or extraembryonic lineages after two days in culture

	wild type (%)	eed (%)
Total embryos injected[a]	13	12
Embryonic derivatives[b]	11 (84.6)	4 (33.3)
Extraembryonic derivatives[c]	0	3 (25)
Embryonic and extraembryonic derivatives	2 (15.4)	5 (41.7)

[a]Only embryos from regions C and D used for analysis.
[b]Embryos displaying descendants in embryonic derivatives of any of the three primary germ layers.
[c]Embryos displaying descendants in extraembryonic mesoderm (includes mesoderm lining the amnion, chorion and yolk sac, allantois or allantoic bud-like mesoderm and/or blood islands).

The percentage distribution of descendants and incidence of embryos contributing to different embryonic and extraembryonic derivatives of region D are described in Table 3. Chi-square analysis indicated that the overall distribution of descendants of region D to these different derivatives after culture was significantly different between wild-type and *eed* mutant embryos. After 1 day in culture, a small percentage of descendants localized to the embryonic ectoderm, primitive streak and axial mesoderm in both wild-type and *eed* mutant embryos; however, the incidence of mutant embryos displaying descendants in embryonic ectoderm and axial mesoderm was lower than wild type. Although the frequency of descendants contributing to embryonic mesoderm was similar for both wild-type and *eed* mutants, the incidence of *eed* mutant embryos contributing to embryonic mesoderm was much higher (14/16 compared to 6/10 embryos). The percentage distribution of descendants found in the endoderm was decreased in *eed* mutant embryos (7%, as compared to 17% for wild type). Both *eed* mutant and wild-type embryos displayed significant contribution to the extraembryonic mesoderm; however, although wild-type embryos exhibited a similar percentage of descendants in extraembryonic mesoderm, only 2/10 embryos contributed to this tissue, with one of these

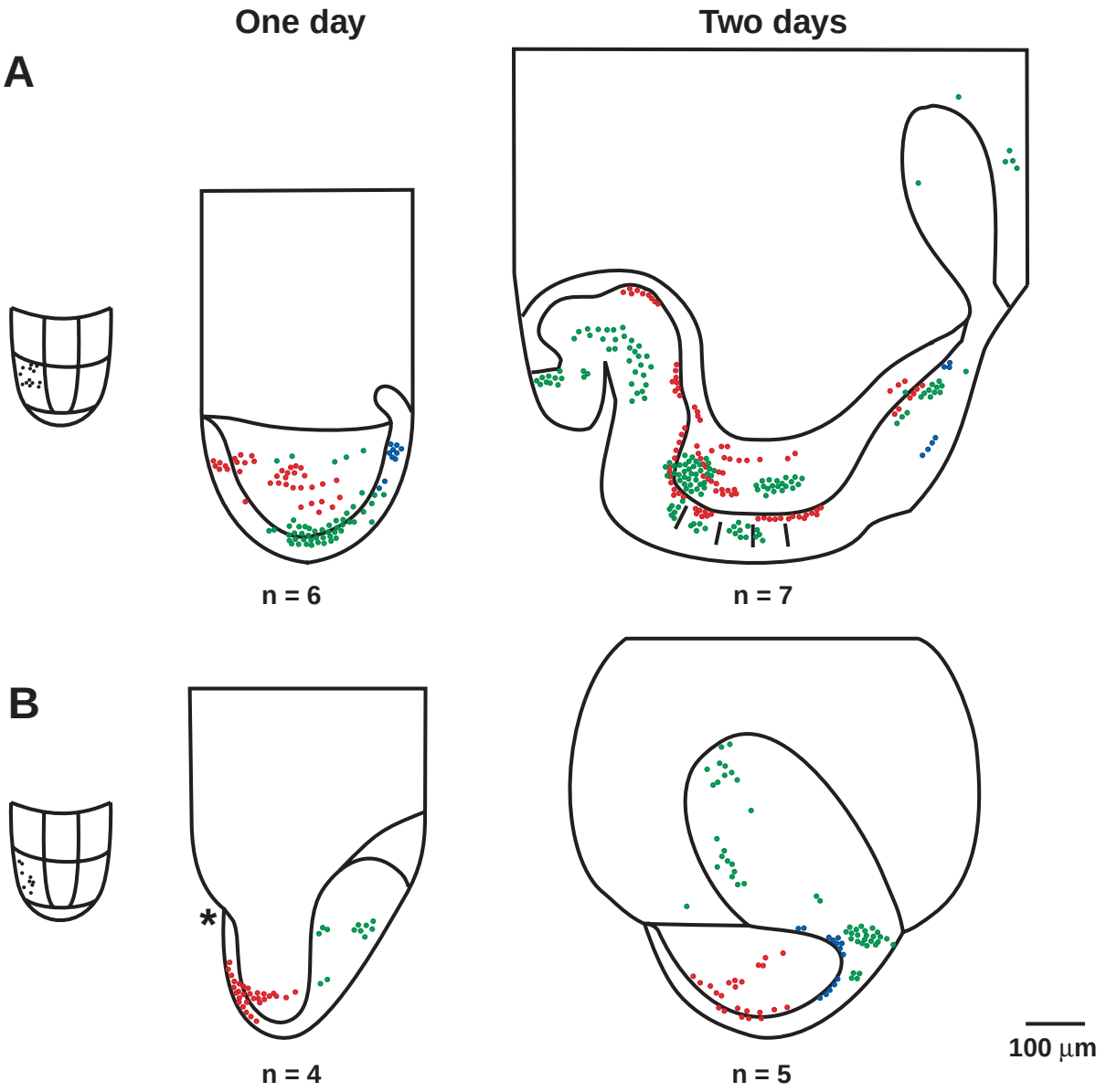

Fig. 4. Localization of descendants of epiblast region C in wild-type (A) and *eed* mutant (B) embryos. See Fig. 3 for details.

yielding a pure extraembryonic clone containing 24 descendants (accounting for 90% of the descendants in this region).

After 2 days in culture, 5/6 wild-type embryos contributed to embryonic ectoderm derivatives (ventral midline neurectoderm and/or the node; 21% of all descendants), while no contribution to embryonic ectoderm was seen in *eed* mutants (Table 3). The Wilcoxon rank-sum test (see Materials and Methods) indicated that this difference in contribution was highly significant. Both wild-type and *eed* mutant embryos continued to display a small contribution to the primitive streak. *eed* mutant embryos still contributed to embryonic mesoderm, although the percentage of descendants found here was decreased compared to both wild-type controls cultured 2 days and *eed* mutant embryos cultured for 1 day. Concomitantly, there was both an increase in the incidence of *eed* mutant embryos contributing to, as well as the percentage of descendants colonizing, the extraembryonic mesoderm in *eed* mutant embryos (5/7 versus 5/16 embryos; 49% of descendants versus 12% colonizing after 1 day in culture). In contrast, 0/6 wild-type embryos contributed to extraembryonic mesoderm after 2 days in culture; this difference in incidence was determined to be highly significant using the Wilcoxon rank-sum test. The percentage of descendants found in endoderm was still lower in *eed* mutant embryos and no contribution to axial mesoderm was seen. Whether the lack of *eed* mutant embryos displaying descendants in axial mesoderm after 2 days in culture is significant cannot be determined because of the low incidence of wild-type embryos contributing to this tissue (Table 3; K. A. L., unpublished results). Overall, the data indicate that epiblast cells from region D ingress through the anterior streak, but as their mesodermal descendants exit, they move extraembryonically.

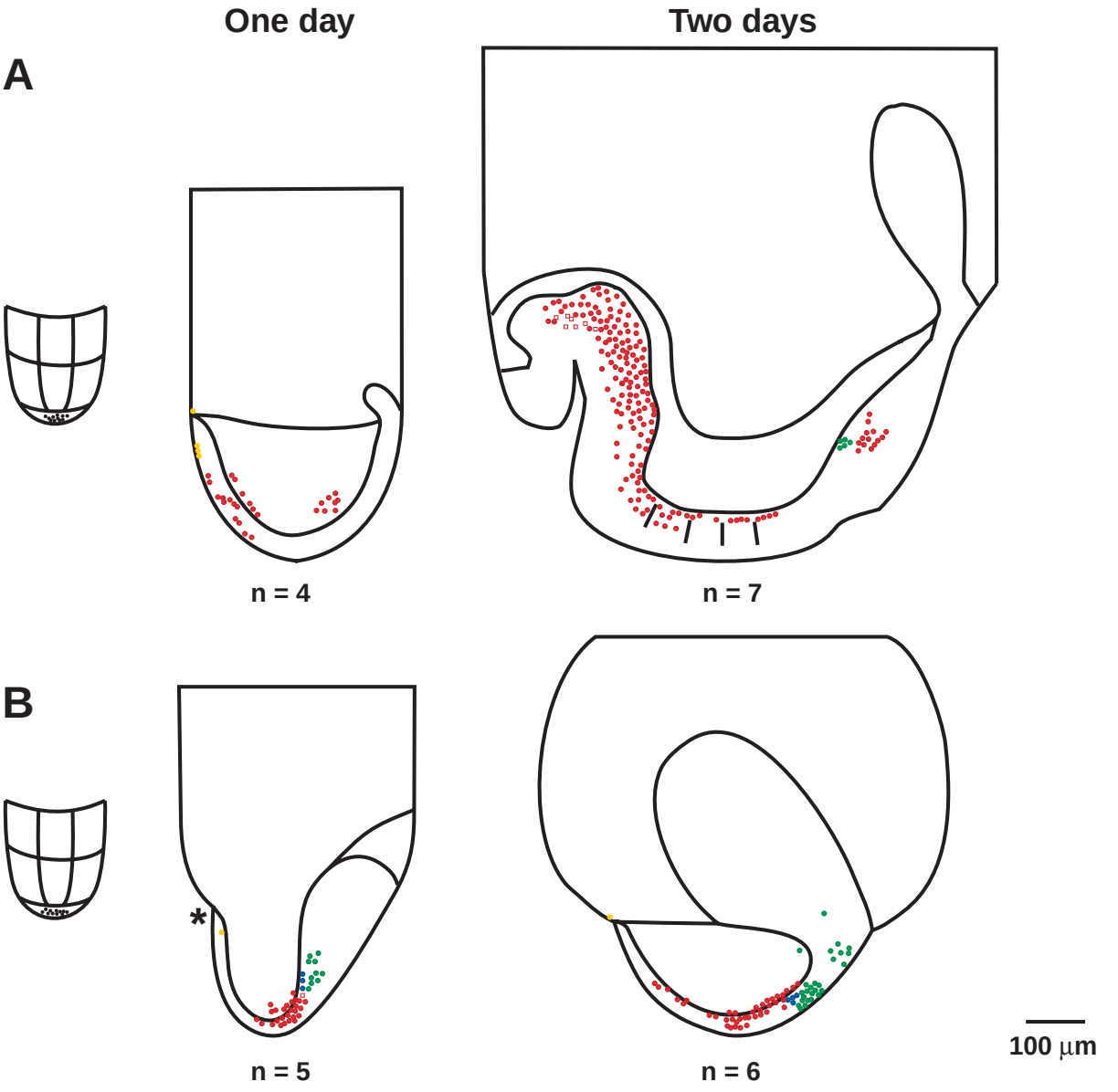

Fig. 5. Localization of descendants of epiblast region E in wild-type (A) and *eed* mutant (B) embryos. See Fig. 3 for details.

(ii) Region C

Having established that epiblast cells from region D ingress through the streak and contribute to mesoderm formation in *eed* mutant embryos, we next analysed the fate of region C. As stated earlier, it was unclear whether cells from this anterior region would undergo proper growth toward the streak and subsequent ingression and, as a result, contribute to the loss of embryonic mesoderm. The localization of descendants from region C is depicted in Fig. 4; the percentage distribution of descendants and incidence of embryos contributing to different embryonic and extraembryonic derivatives are described Table 4. Chi-square analysis indicated that the overall distribution of descendants of region C to these different derivatives after culture was significantly different between wild-type and *eed* mutant embryos. In wild-type embryos after 1 day in culture, descendants of region C were found in the epiblast spread posteriorly towards the streak; epiblast cells initially lying more posteriorly in region C also gave rise to descendants in the primitive streak and mesoderm (Fig. 4A; Table 4). Although a similar incidence of *eed* mutant embryos displayed descendants in the embryonic ectoderm, a much higher percentage of descendants was found in this region as compared to wild type (Table 4). In addition, the bulk of the descendants were localized more anteriorly in *eed* mutants (compare Fig. 4A,B). Furthermore, both the incidence of *eed* mutant embryos contributing, and the percentage of descendants found in the primitive streak and mesoderm, were lower than wild type. These results could reflect the above-mentioned delay in the onset of gastrulation evident in *eed* mutant embryos.

After 2 days in culture, a significant percentage of descendants of wild-type region C did not ingress through the primitive streak; instead they contributed to embryonic ectodermal derivatives, the neural and surface ectoderm (Fig.

4A; Table 4). In *eed* mutant embryos injected in region C and cultured for 2 days, similar contribution to the embryonic ectoderm was observed (Table 4), and, like wild type, descendants spanned posteriorly toward the streak (Fig. 4B).

Descendants of wild-type epiblast cells initially lying more posteriorly in region C contributed substantially to lateral plate mesoderm, as well as paraxial and posterior (postnodal) mesoderm after 2 days in culture (Figs 1E, 4A; Table 4). Epiblast cells lying posteriorly in *eed* mutant region C also gave rise to descendants in the embryonic mesoderm, albeit at a lower percentage (30%, as compared to 58% for wild type). In contrast to descendants of wild-type region C, all embryonic mesoderm descendants were found proximally, near the embryonic/extraembryonic junction and either posterior or just lateral to the primitive streak. Furthermore, a higher incidence of *eed* mutant embryos displayed contribution to the extraembryonic mesoderm (3/5 embryos; 28% of descendants, compared to 2/7 embryos, 3% of descendants for wild type) (Figs 1F, 4B; Table 4). Thus, as in wild-type embryos, descendants of *eed* mutant region C are able to reach the streak and produce mesoderm; however, as was seen with mutant region D, very little anterolateral migration out of the streak occurs and the mesoderm tends to be displaced extraembryonically.

Descendants of *eed* mutant regions C and D that localized to the extraembryonic region of *eed* mutant embryos were usually found in more than one extraembryonic derivative. Descendants were found in either allantoic bud-like mesoderm (6/10 embryos, 28% of extraembryonic descendants), the mesoderm lining the yolk sac (7/10 embryos, 54% of extraembryonic descendants) and/or allantoic mesenchyme (2/10 embryos, 18% of extraembryonic descendants).

Mislocalization of mesoderm extraembryonically is not due to an inherent restriction of distal epiblast cell fate

It has been previously established that the epiblast of the pre-streak mouse embryo displays no regional restriction in cell fate (Lawson et al., 1991). In *eed* mutant embryos, mislocalization of descendants to the extraembryonic mesoderm could reflect an alteration and premature restriction of epiblast cell fate, such that distal epiblast cells now become restricted to an extraembryonic, rather than embryonic, fate. If this occurred, we would only expect to see clones wherein all of the descendants were localized either to embryonic or extraembryonic derivatives. This was not the case (Table 5). Although 25% of *eed* mutant embryos injected in regions C and D displayed clones wherein all of the descendants were localized strictly to the extraembryonic region, 42% displayed mixed clones, wherein descendants were found in extraembryonic mesoderm, as well as embryonic mesoderm and/ or endoderm, in a single clone. In contrast, only 15% of wild-type embryos injected in regions C and D displayed mixed clones containing both embryonic and extraembryonic derivatives after 2 days in culture. Thus, the propensity to form extraembryonic mesoderm in *eed* mutant embryos appears to be due to a tendency for most cells leaving the streak to move extraembryonically and not due to an inherent restriction of distal epiblast cell fate.

Epiblast cells at the distal cap display abnormal morphogenetic movements

Compared to wild-type embryos, the epiblast of *eed* mutant embryos does not expand well during gastrulation, particularly with respect to the A-P axis (Table 1). Reduced axial extension was associated with a lack of incorporation of descendants of *eed* mutant region D into the ectoderm, particularly at the ventral midline (compare Fig. 3A,B, 2 days). In addition, although we could not detect a significant difference in the ability of descendants of *eed* mutant regions C and D to colonize the primitive streak (see Tables 3, 4), decreased primitive streak elongation was apparent in *eed* mutant embryos. Normally the streak constitutes about 30% of the total axial length at the early streak stage, and by the onset of neurulation it accounts for approximately 50% of the entire axis (Lawson et al., 1991). The elongation of the primitive streak in *eed* mutants varied, but never constituted more that 40% of the embryonic axis, even after 2 days in culture (data not shown).

To characterize the lack of epiblast expansion further, we compared the fate of mutant and wild-type region E. Descendants of these cells contribute to anterior expansion of the embryonic ectoderm, presumably as a result of axial extension from the insertion of descendants of region D at the distal midline (Lawson et al., 1991; Lawson and Pedersen, 1992a). In wild-type embryos after 1 day in culture, the majority of descendants contributed to the epiblast anterior to the node (Fig. 5A). After 2 days in culture descendants were found spread widely throughout the head and trunk neural ectoderm. Rarely did descendants contribute to ectoderm posterior to the node or to embryonic mesoderm (Fig. 5A; Table 6). As with regions C and D, chi-square analysis indicated that the overall distribution of descendants of *eed* mutant region E was significantly different from wild type. After 1 day in culture, most of the descendants were found in ectoderm posterior to the distal tip and 2/5 embryos displayed contribution to embryonic mesoderm (Fig. 5B; Table 6). The posterior localization and contribution to mesoderm were striking after 2 days in culture (Fig. 5B; Table 6). Thus, anterior expansion of the epiblast, normally illustrated by the anterior spread of region E descendants, is very limited in *eed* mutant embryos.

Embryo growth

In a previous clonal analysis of epiblast cell fate, it was determined that the wild-type epiblast demonstrated a homogeneous proliferation rate, dividing about every 7.5 hours (Lawson et al., 1991). Consistent with these results, the clone doubling time obtained for labelled epiblast cells of wild-type embryos in this study was 7.4±1.5 hours (n=17 embryos; see Materials and Methods for further details). Mutant epiblast cells demonstrated a clone doubling time of 7.5±1.1 hours (n=17 embryos), not a statistically significant different value from wild-type controls. Clone-doubling times from region D alone (n=5 wild-type and n=9 mutant embryos) were similar to the whole embryo values. Because of the paucity of embryos suitable for proliferation analysis from other regions of the epiblast (see Materials and Methods), we were unable to determine if there were statistically significant differences in proliferation between wild-type and *eed* mutant embryos within these other regions.

DISCUSSION

We have used clonal analysis to determine that loss of embryonic mesoderm in *eed* mutant embryos is not due to lack

of its formation. Rather, embryonic mesoderm is produced, but does not migrate anteriorly and accumulates in the extraembryonic compartment over time. Both the proximodistal clonal spread of descendants in mesoderm, as well as the gain of extraembryonic mesoderm at the expense of embryonic mesoderm between 1 and 2 days in culture support this notion.

Region D, in addition to the area of the epiblast just anterior to it and distal region B, gives rise to the bulk of the definitive endoderm in the gastrulating embryo (Lawson et al., 1991). In *eed* mutant embryos, contribution to this tissue was lower as compared to wild-type controls. Production of definitive endoderm ensues very early in gastrulation (Tam and Beddington, 1992), and it is possible that diminished contribution to this tissue in *eed* mutants simply reflects a delay in the onset of gastrulation. The fact that definitive endoderm is indeed produced in *eed* mutant embryos suggests that at least some of the appropriate conditions for the production of this tissue from the anterior primitive streak are present.

Abnormal localization of mesoderm to the extraembryonic region did not appear to be due to a restriction and alteration of distal epiblast cell fate, since the majority of clones produced from regions fated to ingress through the anterior streak displayed descendants in both embryonic and extraembryonic derivatives. Within these mixed clones, some descendants were able to adopt their normal fates (i.e., axial mesoderm and/or definitive endoderm) after having ingressed through the anterior streak. However, it is unknown if mesodermal cells moving into the extraembryonic region have truly adopted an extraembryonic mesodermal fate. If the mislocalized mesodermal cells exiting the anterior streak maintained their normal fates as they entered the extraembryonic region, we might expect to see misexpression of genes specific to embryonic mesoderm and anterior primitive streak derivatives. However, none of the mesoderm located in the extraembryonic region expresses *Lim1*, a marker of embryonic mesoderm (C. F. and T. M., unpublished data; Barnes et al., 1994; Shawlot and Behringer, 1995), or markers of the anterior streak and its derivatives, such as *Fgf4*, *Mox1*, *Hnf3β* or *Shh* (Faust et al., 1995; Schumacher et al., 1996, and references therein). Thus, while the gene expression data are suggestive of an alteration in fate as mesodermal cells move extraembryonically, they do not conclusively show the adoption of an extraembryonic cell fate.

eed mutant embryos fail to display proper epiblast expansion, particularly with respect to the A-P axis. Based on patterns of clonal spread from anterior regions A and C and calculated clone doubling times for the epiblast, this does not appear to be due to decreased epiblast growth. In wild-type embryos, extension of the A-P axis is primarily due to elongation of the primitive streak and expansion of the area of the epiblast just anterior to it, including region D (Lawson et al., 1991; Lawson and Pedersen, 1992a). A substantial portion of these cells remain in the epiblast, rather than ingress through the streak; moreover, they tend to colonize the ventral midline neurectoderm, resulting in axial extension (Lawson et al., 1991; Lawson and Pedersen, 1992a,b; K. A. L., unpublished results). Although after 1 day in culture a small number of descendants of *eed* mutant region D remained in the ectoderm just anterior to the streak, it is notable that by 2 days, no descendants were observed in embryonic ectoderm derivatives. These data indicate that posterior epiblast cells tend to ingress through the streak, rather than contribute to embryonic ectodermal derivatives, particularly ventral midline neurectoderm. Thus, preferential mesoderm production from region D, perhaps combined with decreased elongation of the streak, might reduce axial extension in *eed* mutant embryos. Consequently, descendants of region E localize posteriorly and many ingress through the streak, probably exacerbating the defect in epiblast expansion.

In summary, *eed* mutant embryos display complex defects in gastrulation. First, it appears that mutant epiblast cells preferentially make mesoderm rather than contribute to expansion of the epiblast. Consistent with this observation, studies with *eed* mutant ES cells have revealed a tendency to differentiate prematurely into mesoderm in embryoid body differentiation assays (C. F. and T. M., unpublished results). The second major defect observed in *eed* mutant embryos is the inability of mesoderm to migrate anteriorly and its subsequent mislocalization to the extraembryonic region. Preliminary analysis of chimeric embryos produced by injection of *eed* mutant ES cells into wild-type host embryos suggests that the defect in anterior migration could be intrinsic to the mesoderm (C. F. and T. M., unpublished results).

Expansion of mesoderm in the gastrulating mouse embryo appears to be due to a variety of forces. Besides propulsion away from the streak by the rapidly dividing and ingressing epiblast cells, short-term time-lapse micrography of gastrulating mouse embryos has revealed that individual mesodermal cells actively migrate away from the streak in an anterodistal direction (Nakatsuji et al., 1986). Furthermore, grafting experiments have indicated that all but the most distal-third of the mesoderm present at the mid-streak stage is eventually displaced extraembryonically and that this displacement is greatly diminished by the onset of neurulation (Parameswaran and Tam, 1995).

An important question that remains is how preferential movement of mesoderm to the extraembryonic region in *eed* mutant embryos is implemented. Does mesoderm actively migrate into the extraembryonic region or is it passively displaced extraembryonically as new mesoderm propels it out of the streak? While the experiments presented here do not directly assess the ability of mutant mesodermal cells to migrate, the extensive cell mixing and proximodistal clonal spread of descendants seen in many embryos suggests that mesodermal cells might be able to migrate into the extraembryonic region, even though they are inhibited in their anterior migration. Alternatively, it is possible that a decreased ability of mesoderm to migrate anteriorly, coupled with limited epiblast expansion, results in excessive displacement of mesoderm proximally in *eed* mutant embryos, leading to preferential production of extraembryonic mesoderm.

When grafted to ectopic sites, the distal posterior epiblast of the early-streak-stage embryo, as well as the mid-streak-stage node, exhibit organizer activity, such that they can recruit host tissues into a secondary neural axis (Beddington, 1994; Tam et al., 1997). These findings, coupled with mutational analysis and regionalized gene expression in the primitive streak, support a model wherein the anterior tip of the streak emits a combination of morphogenetic signals that play a role in determination of neurectodermal/mesodermal cell fates, as well as pattern mesodermal cells as they leave the streak

(Chapman and Papaioannou, 1998; Ciruna et al., 1997; Sasaki and Hogan, 1993; Yamaguchi et al., 1994; Yoshikawa et al., 1997). Based on their proximity to these signals, mesodermal cells leaving the streak are patterned accordingly: cells exiting the anterior streak differentiate into axial and paraxial derivatives, cells exiting the more medial regions of the streak differentiate into lateral and intermediate mesoderm, and cells exiting the posterior streak differentiate into extraembryonic mesoderm. If these proposed anterior streak-derived morphogenetic signals are disrupted, patterning of mesoderm leaving the streak, as well as proper determination of neurectodermal/mesodermal cell fate, might be disrupted.

Loss of the *eed* gene clearly affects transcriptional activity in the primitive streak (Faust et al., 1995; Schumacher et al., 1996). Furthermore, based on the function of its *Drosophila* homologue as a negative regulator of gene expression, it is possible that *eed* negatively regulates transcriptional activity in the posterior streak. Without *eed*, gene expression normally restricted to the posterior streak might be constitutively expressed throughout it; as a result, signalling in the anterior streak might be disrupted. Thus far, the strongest candidate for negative regulation by the *eed* gene is the homeobox-containing gene *Evx1* (Schumacher et al., 1996). Direct evidence that *Evx1* affects mesoderm patterning in the mouse awaits the production of conditional knockouts, since embryos homozygous for a null allele of *Evx1* die prior to implantation (Spyropoulos and Capecchi, 1994). However, several studies strongly suggest that other vertebrate homologues of *Evx1* are involved in posterior mesodermal patterning (Ahringer, 1996; Barro et al., 1995; Ruiz i Altaba et al., 1991; Ruiz i Altaba and Melton, 1989). Thus, it is possible that high levels of *Evx1* in the anterior streak of *eed* mutant embryos might disrupt the normal signalling that occurs here, the results of which may be two-fold: the preferential production of mesoderm at the expense of ventral midline neurectoderm and/or the inability of cells leaving the anterior streak to respond to the appropriate morphogenetic signals required for the patterning of embryonic mesoderm.

We thank Dr Alfred Malouf for invaluable technical advice, members of our laboratory for many helpful discussions, and Drs Patrick Tam and Jaime Rivera-Perez for critical reading of the manuscript. This work was supported by a grant from the NIH (#HD24462) to T. M.

REFERENCES

Ahringer, J. (1996). Posterior patterning by the *Caenorhabditis elegans even-skipped* homolog *vab-7*. *Genes Dev.* **10**, 1120-1130.

Barnes, J. D., Crosby, J. L., Jones, C. M., Wright, C. V. E. and Hogan, B. L. M. (1994). Embryonic expression of *Lim-1*, the mouse homolog of *Xenopus XLim-1*, suggests a role in lateral mesoderm differentiation and neurogenesis. *Dev. Biol.* **161**, 168-178.

Barro, O., Vriz, S., Joly, J.-S., Joly, C., Condamine, H. and Boulekbache, H. (1995). Widespread expression of the *eve1* gene in zebrafish embryos affects the anterior-posterior axis pattern. *Dev. Genet.* **17**, 117-128.

Beddington, R. S. P. (1994). Induction of a second neural axis by the mouse node. *Development* **120**, 613-620.

Chapman, D. L. and Papaioannou, V. E. (1998). Three neural tubes in mouse embryos with mutations in the T-box gene *Tbx6*. *Nature* **391**, 695-697.

Ciruna, B. G., Schwartz, L., Harpal, K., Yamaguchi, T. P. and Rossant, J. (1997). Chimeric analysis of *fibroblast growth factor receptor-1* (*Fgfr1*) function: a role for FGFR1 in morphogenetic movement through the primitive streak. *Development* **124**, 2829-2841.

Dush, M. K. and Martin, G. R. (1992). Analysis of mouse *Evx* genes: *Evx-1* displays graded expression in the primitive streak. *Dev. Biol.* **151**, 273-287.

Faust, C., Schumacher, A., Holdener, B. and Magnuson, T. (1995). The *eed* mutation disrupts anterior mesoderm production in mice. *Development* **121**, 273-285.

Lawson, K. A., Meneses, J. J. and Pedersen, R. A. (1986). Cell fate and cell lineage in the endoderm of the presomite mouse embryo, studied with an intracellular tracer. *Dev. Biol.* **115**, 325-339.

Lawson, K. A., Meneses, J. J. and Pedersen, R. A. (1991). Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development* **113**, 891-911.

Lawson, K. A. and Pedersen, R. A. (1987). Cell fate, morphogenetic movement and population kinetics of embryonic endoderm at the time of germ layer formation in the mouse. *Development* **101**, 627-652.

Lawson, K. A. and Pedersen, R. A. (1992a). Clonal analysis of cell fate during gastrulation and early neurulation in the mouse. *Ciba Found. Symp.* **165**, 3-26.

Lawson, K. A. and Pedersen, R. A. (1992b). Early mesoderm formation in the mouse embryo. In *Formation and Differentiation of Early Embryonic Mesoderm* (ed. R. Bellairs et al.), pp. 33-46. New York: Plenum Press.

Nakatsuji, N., Snow, M. H. L. and Wylie, C. C. (1986). Cinemicrographic study of the cell movement in the primitive-streak-stage mouse embryo. *J. Embryol. Exp. Morph.* **96**, 99-109.

Niswander, L., Yee, D., Rinchik, E. M., Russell, L. B. and Magnuson, T. (1988). The albino deletion complex and early postimplantation survival in the mouse. *Development* **102**, 45-53.

Parameswaran, M. and Tam, P. P. L. (1995). Regionalisation of cell fate and morphogenetic movement of the mesoderm during mouse gastrulation. *Dev. Genet.* **17**, 16-28.

Rinchik, E. M. and Carpenter, D. A. (1993). N-ethyl-N-nitrosourea-induced prenatally lethal mutations define at least two complementation groups within the embryonic ectoderm development (*eed*) locus in mouse Chromosome 7. *Mamm. Genome* **4**, 349-353.

Ruiz i Altaba, A., Choi, T. and Melton, D. A. (1991). Expression of the Xhox3 homeobox protein in *Xenopus* embryos: blocking its early function suggests the requirement of Xhox3 for normal posterior development. *Dev. Growth Diff. 3***3**, 651-669.

Ruiz i Altaba, A. and Melton, D. A. (1989). Involvement of the Xenopus homeobox gene Xhox3 in pattern formation along the anterior-posterior axis. *Cell* **57**, 317-326.

Sasaki, H. and Hogan, B. L. M. (1993). Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. *Development* **118**, 47-59.

Schumacher, A., Faust, C. and Magnuson, T. (1996). Positional cloning of a global regulator of anterior-posterior patterning in mice. *Nature* **383**, 250-253.

Shawlot, W. and Behringer, R. R. (1995). Requirement for *Lim1* in head-organizer function. *Nature* **374**, 425-430.

Spyropoulos, D. and Capecchi, M. R. (1994). Targeted disruption of the *even-skipped* gene, *evx1*, causes early postimplantation lethality of the mouse conceptus. *Genes Dev.* **8**, 1949-1961.

Tam, P. P. L. and Beddington, R. S. P. (1987). The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis. *Development* **99**, 109-126.

Tam, P. P. L. and Beddington, R. S. P. (1992). Establishment and organization of germ layers in the gastrulating mouse embryo. *Ciba Found. Symp.* **165**, 27-49.

Tam, P. P. L. and Behringer, R. R. (1997). Mouse gastrulation: the formation of a mammalian body plan. *Mech. Dev.* **68**, 3-25.

Tam, P. P. L., Steiner, K. A., Zhou, S. X. and Quinlan, G. A. (1997). Lineage and functional analyses of the mouse organizer. *Cold Spring Harbor Symp. Quant. Biol.* **62**, 135-144.

Tam, P. P. L., Williams, E. A. and Chan, W. Y. (1993). Gastrulation in the mouse embryo: ultrastructural and molecular aspects of germ layer morphogenesis. *Microsc. Res. Tech.* **26**, 301-328.

Yamaguchi, T., Harpal, K., Henkemeyer, M. and Rossant, J. (1994). *Fgfr-1* is required for embryonic growth and mesodermal patterning during mouse gastrulation. *Genes Dev.* **8**, 3032-3044.

Yoshikawa, Y., Fujimori, T., McMahon, A. P. and Takada, S. (1997). Evidence that absence of *Wnt-3a* signaling promotes neuralization instead of paraxial mesoderm development in the mouse. *Dev. Biol.* **183**, 234-242.

Zar, J. (1984). *Biostatistical Analysis.* Englewood Cliffs, NJ: Prentice-Hall.