Development 121, 273-285 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

The *eed* mutation disrupts anterior mesoderm production in mice

Cynthia Faust, Armin Schumacher, Bernadette Holdener and Terry Magnuson*

Department of Genetics, Case Western Reserve University, Cleveland, Ohio 44106-4955, USA

*Author for correspondence (Internet trm4@pop.cwru.edu)

SUMMARY

Mouse embryos homozygous for the mutation embryonic ectoderm development (*eed*) exhibit a growth defect and fail to gastrulate normally. While extraembryonic mesoderm is produced extensively, very little embryonic mesoderm is detected in *eed* mutant embryos, and there is no subsequent organization of mesoderm into node, notochord, or somites. The phenotype is consistent with a defect in the distal primitive streak. Here we report additional phenotypic analyses that include mRNA in situ hybridization of genes whose expression reflects the function of different regions of the primitive streak and their derivatives. These studies have confirmed that mesoderm derived from the proximal primitive streak is specified appropriately. Despite the absence of a morphologically distinct node, sparse axial mesoderm cells in *eed* mutant embryos are specified, as reflected by expression of *Brachyury* (*T*), *Sonic hedgehog*, and *Tcf3b*/*HNF-3*β, and definitive endoderm is produced. Specification of these cell types is also independent of correct expression of *nodal*, *Fgf4*, and *gsc*. Finally, *T* and *Evx1* display ectopic expression in cells not normally fated to ingress through the primitive streak. The data presented are discussed in terms of mechanisms for establishment of the *eed* phenotype, and are consistent with the *eed* gene product playing an early role in primitive streak formation and/or organization.

Key words: mammalian embryogenesis, mouse mutants, gastrulation, mesoderm induction, primitive streak, germ cell lineage, node, axial mesoderm, *eed*, *Brachyury (T)*, *Evx1*

INTRODUCTION

Gastrulation is a process through which the definitive embryonic germ layers – the ectoderm, mesoderm, and endoderm – are formed, resulting in establishment of the basic body plan. In mouse, gastrulation occurs from approximately embryonic day (E) 6.5-7.5, as cells of the primitive ectoderm (epiblast) delaminate on the posterior side of the embryo and ingress through the primitive streak. The fate of epiblast cells of pre- and early-streak mouse embryos has been determined via clonal analysis of epiblast cells in situ (Lawson et al., 1991). This and previous work (Snow, 1977) demonstrate that increased epiblast cell proliferation likely directs growth towards the primitive streak, as well as epiblast cell ingression. As the primitive streak extends to the distal tip of the embryo, mesodermal cells migrate anteriorly around the surface of the epiblast, contributing to head and trunk mesoderm; mesoderm migrating into the extraembryonic region of the embryo drives the formation of the amnion, chorion, and allantois.

While mouse gastrulation is morphologically well defined, its molecular control is poorly understood. Numerous homologues of genes implicated in *Xenopus laevis* mesoderm induction, organization, or specification demonstrate specific expression patterns in the mouse gastrula (reviewed by Beddington and Smith, 1993; Faust and Magnuson, 1993). These include, for example, members of the fibroblast growth factor (*Fgf*) gene family and their receptors (Hébert et al., 1991; Orr-Urtreger et al., 1991; Niswander and Martin, 1992; Yamaguchi et al., 1992); *nodal*, a member of the transforming growth factor (TGF)-β superfamily (Zhou et al., 1993; Conlon et al., 1994); and putative transcription factors, including *Brachyury* (*T*) (Wilkinson et al., 1990), *even-skipped homeobox gene-1* (*Evx1*) (Bastian and Gruss, 1990; Dush and Martin, 1992), *goosecoid* (*Gsc*) (Blum et al., 1992), and *transcription factor 3*β (*Tcf3b*), also called *hepatocyte nuclear factor-3*β (*Hnf3b*)[1] (Ang et al., 1993; Monaghan et al., 1993; Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993).

Mutational analysis is critical for establishing essential roles for these genes. For example, targeted gene disruption via homologous recombination in embryonic stem cells has verified the importance of *Tcf3b* during gastrulation and node formation (Ang and Rossant, 1994; Weinstein et al., 1994). However, analysis of gene function by this approach can be complicated if the gene has an additional, essential role earlier in development, as has been shown for *Evx1* (Spyropoulos and Capecchi, 1994). A strategy that has successfully yielded the identification of novel genes involved in gastrulation has been analysis of spontaneous or induced mutations producing dramatic phenotypes during gastrulation and early organogenesis. This approach has lead to the characterization of *nodal* and *T*, which are required for normal primitive streak formation and function (Iannaccone et al., 1992; Conlon et al., 1994; Herrmann, 1994).

[1]In keeping with the nomenclature established by the Committee on Standardized Genetic Nomenclature for Mice [(1993) Mouse Genome, vol. 91, pp. 149-150], *Hnf3b* will herein be referred to as *Tcf3b*.

We have taken the latter approach and characterized a deletion mutant, *eed* (*e*mbryonic *e*ctoderm *d*evelopment), which exhibits a dramatic phenotype during gastrulation (Niswander et al., 1988). Embryos homozygous for deletions removing *eed* are able to initiate mesoderm production, but the primitive streak appears abnormal.

The *eed* phenotype is most obvious at E8.5 (see Fig. 1): although wild-type embryos at this stage have undergone extensive neural and axial development (Fig. 1A), *eed* mutants possess a smaller, presumably undifferentiated embryonic ectoderm, and exhibit sparse embryonic mesoderm (Fig. 1B). Furthermore, there is no organization of mesoderm into somites, node, or notochord, and there is no morphological evidence of neural induction. In contrast, production of mesoderm in the proximal part of the embryo is undiminished, with extensive formation of the extraembryonic structures – amnion, chorion, and allantois. By E9.5 no mutant embryos are observed in litters derived from *eed*/+ inter se matings. Fate mapping studies of the wild-type mouse gastrula suggest that the deficiency of embryonic mesoderm and absence of axial structures (node, notochord, somites) reflect a defect in the distal primitive streak (Lawson et al., 1991). Consistent with this hypothesis is the unimpeded production of extraembryonic mesoderm, which is derived from epiblast cells traversing the proximal primitive streak (Lawson et al., 1991).

The *eed* mutation maps to the albino deletion complex on mouse chromosome 7. Because of the complexity of the phenotype and large size of the *eed* region (≤170 kilobases; B. Holdener and T. Magnuson, unpublished data), the possibility exists that more than one deleted gene is responsible for the *eed* phenotype. Indeed, two loci (*l(7)5Rn* and *l(7)6Rn*) mapping to the *eed* interval have been identified via an N-ethyl-N-nitrosourea (ENU) saturation mutagenesis screen (Rinchik, 1991; Rinchik and Carpenter, 1993). Three *l(7)5Rn* alleles have been isolated, and embryos homozygous for one of these alleles ($l(7)5Rn^{3354SB}$) demonstrate a similar phenotype as *eed* deletion homozygotes (B. Holdener and T. Magnuson, unpublished data). Since ENU-induced mutations are generally intragenic (Popp et al., 1983; Russell and Rinchik, 1987; Peters et al., 1990; Russell et al., 1990; Provost and Short, 1994), these data suggest that the *eed* phenotype probably results from a single-gene defect.

To explore the nature of the gastrulation defect in *eed* mutants, we have undertaken additional phenotypic analyses that include mRNA *in situ* hybridization of gene expression relevant to this process. These studies have confirmed that tissues derived from the proximal part of the primitive streak are correctly specified. Moreover, sparse mesodermal cells derived from the more distal regions of the primitive streak also appear to be appropriately specified in *eed* mutant embryos. Head process (notochord precursor) mesoderm, as well as definitive endoderm, are present, and their production is independent of correct expression of *nodal*, *Fgf4*, and *Gsc*. Furthermore, the primitive streak markers *T* and *Evx1* have expanded their domains of expression in *eed* mutant embryos to include cells not normally fated to traverse the primitive streak. The findings presented here are consistent with the *eed* gene product acting early in gastrulation to regulate genes required for proper primitive streak formation and/or organization.

MATERIALS AND METHODS

Mouse strains and embryos

The c^{AL} albino-deletion and $l(7)5Rn^{3354SB}$ mice used in this study originated at the Oak Ridge National Labs (Russell et al., 1982; Rinchik and Carpenter, 1993) and were obtained from Drs L. B. Russell and E. M. Rinchik. These mice have been maintained as closed colony, heterozygous stocks with chinchilla (c^{ch}). c^{AL}/c^{ch} and $c\ */\ c^{ch}$ + (where * = 3354SB mutation) stocks are a dilute chinchilla coat color as compared to a full chinchilla color evident in c^{ch}/c^{ch} mice. For experimental purposes, the stocks were expanded by crossing heterozygous mutant stocks with chinchilla mice to produce c^{AL}/c^{ch} or $c\ */\ c^{ch}$ + (dilute chinchilla) and c^{ch}/c^{ch} (chinchilla) offspring. Dilute chinchilla offspring were crossed inter se to produce experimental embryos. No significant differences in gene expression were detected between embryos homozygous for the ENU-induced allele and deletion homozygotes.

Noon of the day of the appearance of the vaginal plug was designated E0.5.

Histology and in situ hybridization to sectioned embryos

Pregnant females were sacrificed at 6.5-9.0 days of gestation and embryos left intact in their decidua for processing. For in situ hybridization, embryos were fixed in 4% paraformaldehyde overnight, dehydrated, embedded in Paraplast (Oxford), and sectioned according to standard procedures (Zeller, 1989). An entire litter was embedded in a single block, from which 6 μm sections were cut. At this point E8.5 and some E7.5 *eed* mutant embryos were identified by their unique morphology, as described in the Introduction and Results. To identify E6.5-7.5 *eed* mutants not displaying an obvious phenotype, hybridization was performed using a cDNA clone (ϕmid2) which maps to the region removed by the c^{AL} deletion (A. Schumacher and T. Magnuson, unpublished data). In situ analysis revealed that this cDNA (ϕmid2) was ubiquitously expressed in wild-type embryos at E6.5-8.5 but no transcripts were detected in E8.5 *eed* mutant embryos (C. Faust, A. Schumacher, and T. Magnuson, unpublished data). To genotype E6.5-7.5 *eed* mutants, embryos were sectioned either sagittally or transversely and probed for ϕmid2 expression and the gene of interest on adjacent 6 μm sections.

For identification of primordial germ cells, embryos were fixed in 90% ethanol overnight and subsequently processed as described above. Embryo sections were stained for non-specific alkaline phosphatase activity using the protocol of McGadey (McGadey, 1970).

mRNA in situ hybridization to sectioned embryos

Probe preparation, in situ hybridization and autoradiography were performed as per Sassoon and Rosenthal (Sassoon and Rosenthal, 1993). Antisense strand probes were prepared via in vitro transcription using T3, T7, or Sp6 polymerase and α-^{35}S-UTP (>1000 Ci/mmol, NEN). To ensure that hybridization was specific as per the washing conditions employed, sense strand probes were also prepared and hybridized to adjacent sections in the initial stages of this study.

Except for ϕmid2, the probes used in this study have been described previously. Probes for the following genes were obtained, and we gratefully acknowledge those investigators who gave them: *Flk1*, from J. Rossant (Yamaguchi et al., 1993); *Igf2*, from A. Efstratiadis (De Chiara et al., 1991); *Evx1*, from P. Gruss (Bastian and Gruss, 1990); *Fgf4*, from G. Martin (Niswander and Martin, 1992); *T*, from B. Herrmann (Herrmann et al., 1990); *Tcf3b* and *-3a*, from B. Hogan (Sasaki and Hogan, 1993); *Shh*, from A. McMahon (Echelard et al., 1993); *nodal*, from M. Kuehn (Zhou et al., 1993), and *Gsc*, from E. De Robertis (Blum et al., 1992). Probes were exposed for 1-2 weeks (*Flk1*, *T*, *Igf2*, *Gsc*, and *Shh*), or 3-4 weeks (*nodal*, *Evx1*, *Tcf3b* and *-3a*, and *Fgf4*).

Sectioned embryos were scored for expression using a Nikon Optiphot microscope equipped with dark-field optics. For publication, sections were imaged on a Biorad MRC-600 confocal microscope

using the transmitted light mode, with the reflection block in PMT1. Bright-field and dark-field images were scanned in the PMT2 channel, and merged in Adobe Photoshop to obtain the final image.

Whole-mount in situ hybridization and immunohistochemistry

Preparation of digoxigenin probes and whole-mount in situ hybridization were performed essentially as per Conlon and Herrmann (Conlon and Herrmann, 1993), with the following exceptions. The peroxide, glycine and borohydride steps were eliminated, and embryos were treated with 10 μg/ml proteinase K for 10 minutes (E7.5 wild type/E8.5 mutants) or 15 minutes (E8.5 wild type).

A polyclonal rabbit T antiserum, kindly provided by B. Herrmann, was used for whole-mount immunohistochemistry as per Kispert and Herrmann (Kispert and Herrmann, 1994). Whole-mount embryos were photographed on a layer of 1% agarose, using a Nikon SMZ-2T microscope and Kodak Ektachrome 160T film.

RESULTS

Previous work had indicated that a mutant class of *eed* homozygotes could not be identified until E8.5, when wild-type littermates were initiating organogenesis (Niswander et al., 1988; Fig. 1A, B); however, some mutant embryos exhibit a striking phenotype at E7.5 (compare Fig. 1C and D). These mutants appear lopsided, with large amounts of mesoderm located proximally in the embryo and very little residing distally. The mesoderm layer of the proximal primitive streak is abnormally thickened, and the streak also appears kinked. Analysis of multiple E7.5 litters demonstrated variation in the extent of development of the *eed* mutant phenotype. While some E7.5 embryos have a large excess of proximal mesoderm, the majority of mutants generally resemble an E6.0-6.5 egg cylinder-stage embryo. Embryos exhibit a well defined embryonic ectoderm, and a small amount of mesoderm is present proximally on one side of the embryo. In some embryos there appears to be a delay in the transition of cuboidal to squamous visceral endoderm. The size of the mutant embryos varies, from about one-fourth to two-thirds the size of their wild-type littermates.

At E8.5 *eed* homozygotes resemble E7.5 embryos with respect to overall size, the apparent lack of differentiation of embryonic ectoderm and mesoderm, and the extent of development of the extraembryonic structures (Fig. 1B,C). Like E7.5, there is some variation among mutant embryos with respect to size and the amount of embryonic mesoderm produced. Embryos often appear skewed, wherein the proximodistal axis is visibly longer anteriorly. While extraembryonic development is quite extensive, it is not completely normal – the allantois is exaggerated in size and often not well defined, and the amnion is abnormal, often contiguous with the extraembryonic mesoderm; nevertheless, epiblast cells traversing the proximal part of the primitive streak seem to be able to adopt their normal fates. To investigate this at a molecular level, we adopted two approaches: staining for the

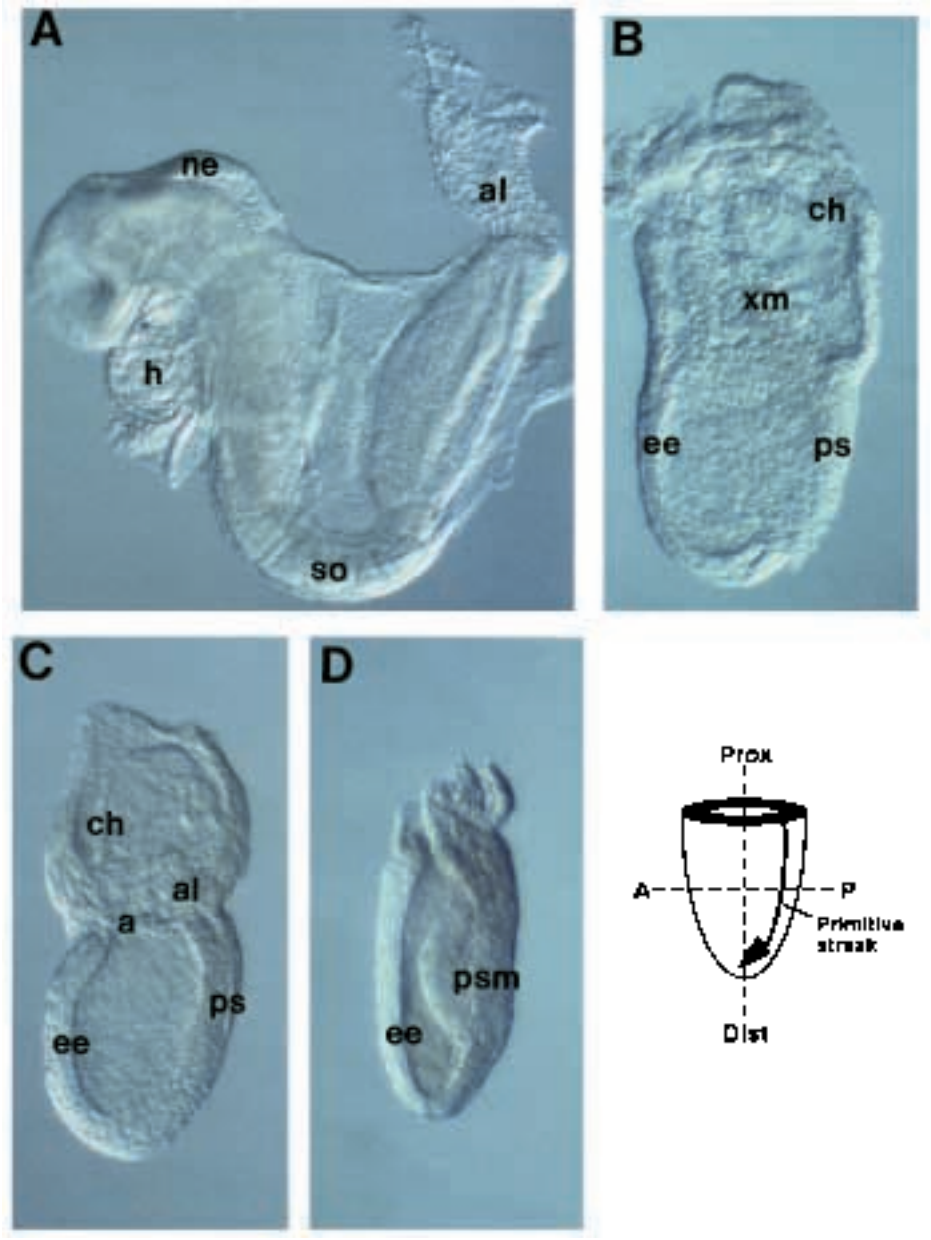
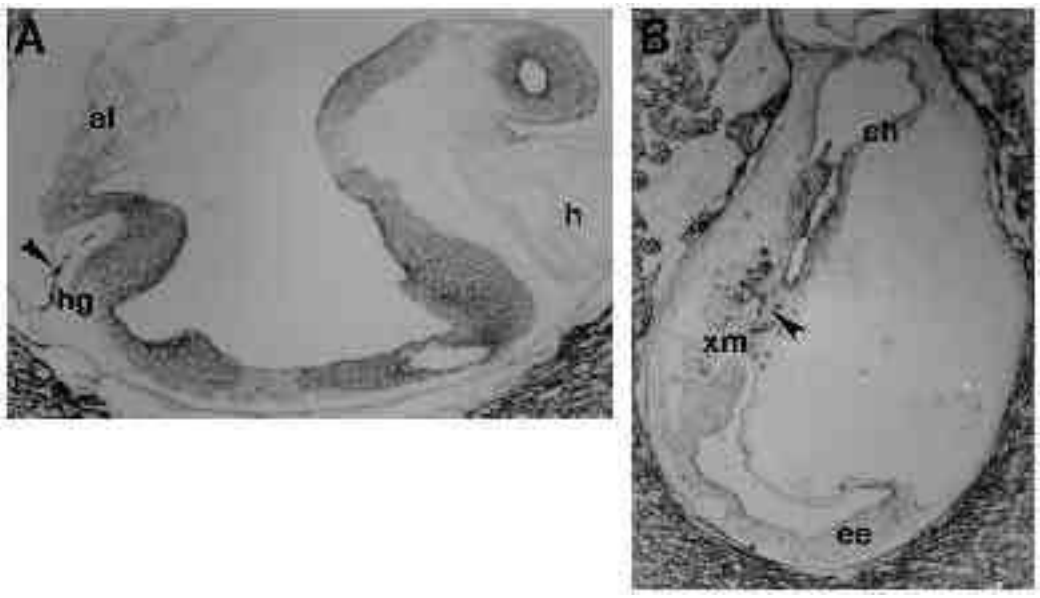


Fig. 1. The *eed* mutant phenotype. Whole-mount E8.5 wild-type (A) and *eed* mutant littermate (B); E7.5 wild-type (C) and mutant littermate (D). Magnification (A) 55×, (B-D) 70×. Abbreviations used in this and subsequent figures: A, anterior; P, posterior; Prox, proximal; Dist, distal; a, amnion; al, allantois; ch, chorion; ee, embryonic ectoderm (epiblast); h, heart; ne, neural ectoderm; ps, primitive streak; psm, primitive streak mesoderm; so, somite; xm, extraembryonic mesoderm.



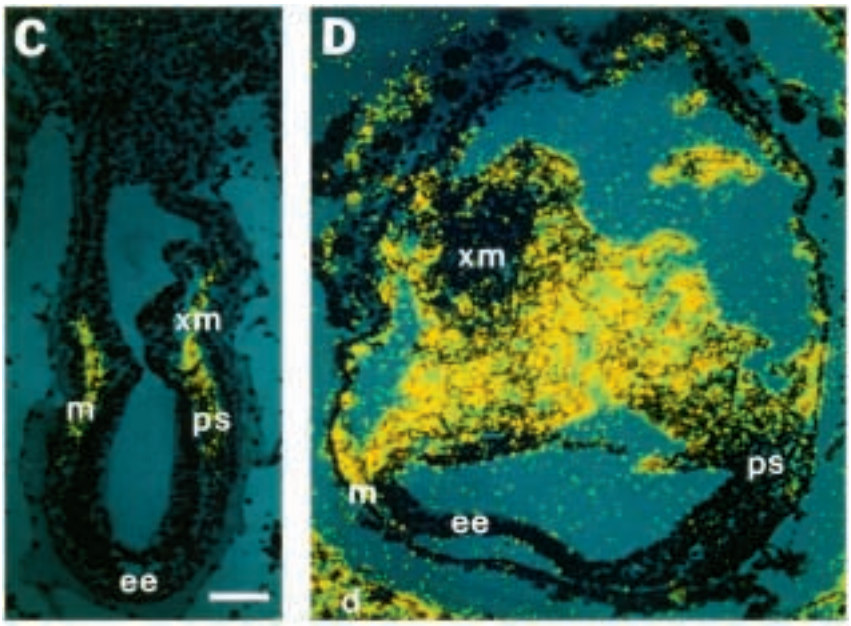


Fig. 2. Analysis of proximal primitive streak function. (A,B) Alkaline phosphatase staining of primordial germ cells. Arrowheads indicate regions of densely stained PGCs. (A) Near sagittal section of E8.5 wild-type embryo showing PGCs migrating into the hindgut (hg). (B) Frontal section of *eed* mutant littermate. PGCs are present in mass of extraembryonic mesoderm (xm) concentrated on one lateral side of the embryo. (C,D) *Flk1* expression. mRNA in situ hybridization was performed on E7.5-8.5 mouse embryo sections using an RNA probe corresponding to *Flk1* (Materials and Methods). Sagittal sections of E7.5 wild-type (C) and E8.5 *eed* (D) embryos. Posterior is to the right, proximal at top. Transcripts are detected in maternal deciduum (d), the nascent mesoderm of the primitive streak (ps), extraembryonic mesoderm (xm), and proximoanterior embryonic mesoderm (m). Bar, 100 μm.

presence of primordial germ cells, precursors of the germ cell lineage; and mRNA in situ analysis of genes whose expression reflects the function of the proximal primitive streak.

Primordial germ cells are specified in *eed* mutant embryos

Clonal analysis has indicated that primordial germ cells (PGCs), like extraembryonic mesoderm, descend from epiblast precursors that traverse the proximal primitive streak (Lawson and Hage, 1994). Thus, we were interested in determining if this cell type was specified in *eed* mutant embryos. In E7.0-7.5 wild-type embryos, PGCs, identified by their alkaline phosphatase (ALP) activity (Chiquoine, 1954; Ginsburg et al., 1990), are clustered at the base of the allantois; by E8.5, PGCs have migrated from the allantois into the embryonic hindgut (Ginsburg et al., 1990, and references therein; Fig. 2A). ALP-positive cells were identified in the extraembryonic mesoderm of E8.5 *eed* mutant embryos (Fig. 2B). The presence of these cells in *eed* mutant embryos indicates that epiblast cells traversing the proximal primitive streak are able to adopt a primordial germ cell fate.

Flk1 expression indicates that mesoderm derived from the proximal primitive streak is appropriately specified in *eed* mutant embryos

Flk1, an early marker of endothelial cell precursors (Yamaguchi et al., 1993), is initially expressed at E7.5 in mesoderm derived from cells traversing the most proximal part of the primitive streak (Lawson et al., 1991). These include the nascent mesoderm of the proximal primitive streak, extraembryonic yolk sac mesoderm, allantois, and proximoanterior embryonic mesoderm (see Fig. 2C). As development proceeds, *Flk1* expression is seen additionally in a variety of embryonic tissues undergoing vasculogenesis (Yamaguchi et al., 1993).

Flk1 expression in E8.5 *eed* mutants was similar to E7.5 wild-type, in that transcripts were detected in the proximal primitive streak, extraembryonic mesoderm, and proximoanterior embryonic mesoderm (Fig. 2D). These data indicated that specification of mesoderm derived from the proximal primitive streak is occurring normally. This conclusion was supported by expression analysis of *insulin-like growth factor II* (*Igf2*), which demonstrates a similar expression pattern to *Flk1* in wild-type embryos (Lee et al., 1990; data not shown).

The distal primitive streak markers *goosecoid* and *Fgf4* are not expressed in the primitive streak of *eed* mutant embryos

Having confirmed our previous morphological observations, i. e., that the proximal primitive streak appears to function normally, we turned to the analysis of markers of the distal primitive streak and its derivatives. The first marker examined was *goosecoid* (*Gsc*), which is expressed in a very defined window during gastrulation (Blum et al., 1992). Prior to any obvious delamination (E6.25-6.4), *Gsc* transcripts are localized to squamous visceral endoderm and the proximoposterior epiblast where the primitive streak will form (Blum et al., 1992; Conlon et al., 1994; Fig. 3A). As the streak forms and extends to the distal tip of the embryo (E6.5-7.5), *Gsc* transcripts are found in the distal streak, the node (lying at the distal-most tip of the streak), and axial

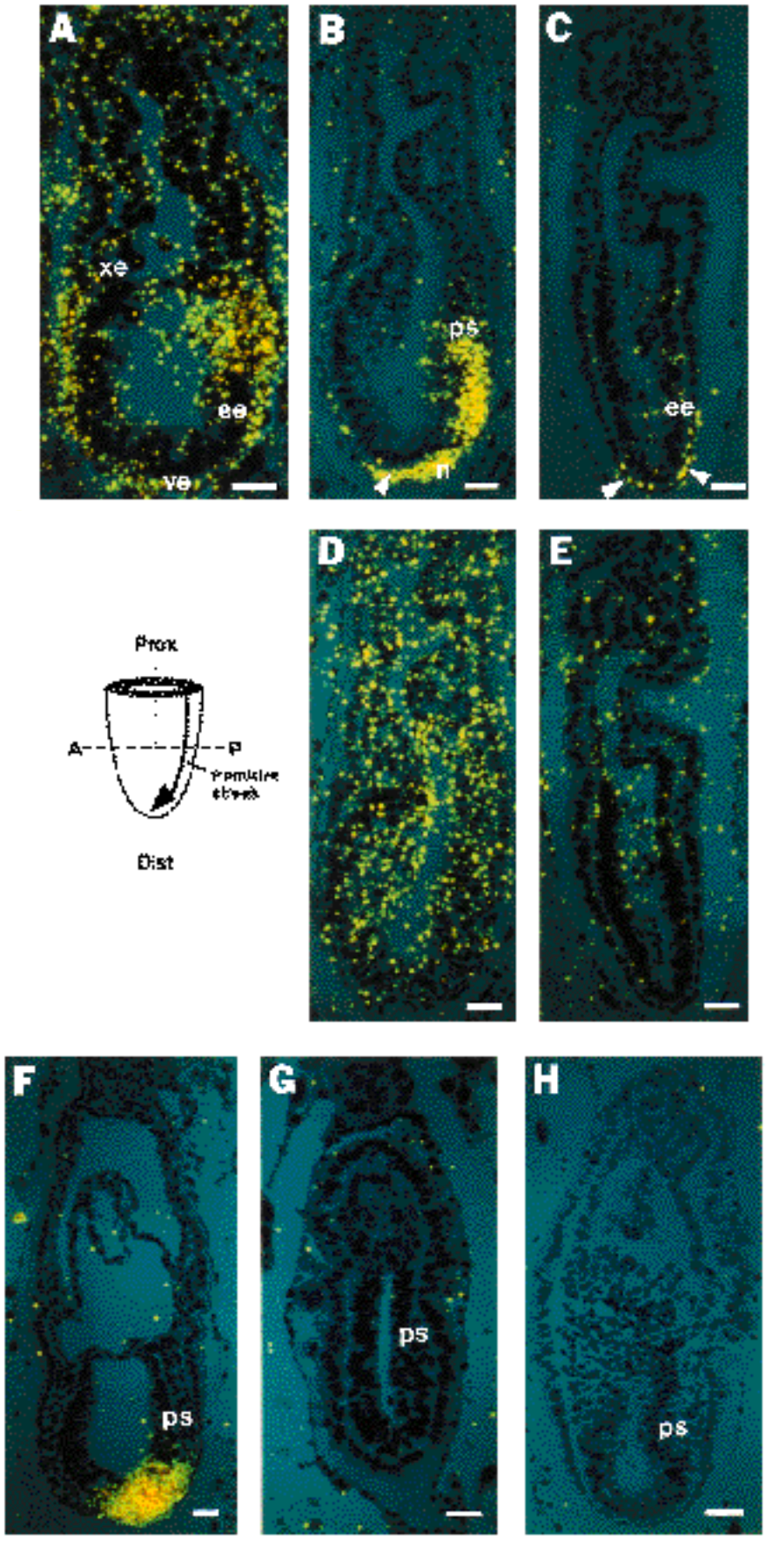


Fig. 3. Analysis of distal primitive streak function. mRNA in situ hybridization of *Gsc* (A-C); ϕmid2 (D,E); and *Fgf4* (F-H). (A-C) *Gsc* expression in sagittal sections of E6.4 (A) and E7.0 (B) wild-type, and E7.5 *eed* (C) embryos. (A) In E6.4 embryos, *Gsc* transcripts are detected in the posterior epiblast (ee) and squamous visceral endoderm (ve). xe, extraembryonic ectoderm. (B) At E7.0 *Gsc* expression in visceral endoderm continues, and expression is also seen in the distal primitive streak, node (n), and axial mesoderm (arrowhead). (C) In E7.5 *eed* embryos, only expression in the squamous visceral endoderm can be detected (arrowheads). (D-E) ϕmid2 expression in E7.0 wild-type (D) and E7.5 *eed* (E) embryos. (D) In E7.0 wild-type embryos ϕmid2 is ubiquitously expressed, but transcripts are absent from E7.5 *eed* embryos (E), permitting genotyping. The sections shown in D and E are 6 μm adjacent to B and C, respectively. (F-H) *Fgf4* expression in E7.5 wild-type (F), E7.5 *eed* (G), and E8.5 *eed* (H) embryos. (F) Oblique sagittal section demonstrating *Fgf4* expression in the distal primitive streak. (G, H) Midline sagittal sections of *eed* mutant embryos reveal no *Fgf4* transcripts in the primitive streak. Bar, 50 μm.

mesoderm extending anteriorly from the node (Fig. 3B). *Gsc* transcription ceases abruptly by approximately E7.75 (head fold stage), only to resume much later in development (Gaunt et al., 1993).

As expected, no *Gsc* transcripts were detected in E8.5 *eed* mutant embryos (data not shown). Interestingly, E6.5-7.5 mutant embryos (n = 7) demonstrated very little, if any, *Gsc* expression above background levels in the posterior epiblast, as determined by both sagittal (Fig. 3C) and transverse sections through the egg cylinder. Expression in squamous visceral endoderm, however, was maintained. To distinguish mutant embryos at this early stage, embryos were genotyped using a cDNA (ϕmid2) mapping to the *eed* region (A. Schumacher and T. Magnuson, unpublished data; see Materials and Methods). ϕmid2 transcripts were ubiquitously expressed in wild-type embryos (Fig. 3D), but were absent in mutant littermates (Fig. 3E).

In wild-type mouse embryos, *Fgf4* expression is initiated quite early, beginning in the late blastocyst stage (Niswander and Martin, 1992). After implantation *Fgf4* transcripts are detected throughout the epiblast, but at the onset of primitive streak formation, expression becomes restricted to the distal two-thirds of the streak (see Fig. 3F); expression continues in the streak at E8.5 (Niswander and Martin, 1992). E6.5-7.5 *eed* mutant embryos (n = 7), identified by a lack of ϕmid2 expression, exhibited no *Fgf4* gene expression (Fig. 3G). In addition, no *Fgf4* transcripts could be detected in E8.5 *eed* mutants (n =10; Fig. 3H). Serial 6 μm sections were taken to confirm that expression was not missed. In pre-streak mutant embryos (E6.0-6.25), *Fgf4* expression was detected throughout the epiblast, similar to wild-type littermates (data not shown). Thus, *Fgf4* expression prior to gastrulation appears normal in *eed* mutant embryos, but is not subsequently initiated in the primitive streak.

Sparse axial mesoderm cells are correctly specified in *eed* mutant embryos

While distal primitive streak gene expression is disrupted in *eed* mutant embryos, it is not completely ablated. This was indicated via whole-mount immunohistochemical analysis of Brachyury (T) protein. In E7.5 wild-type embryos, T protein is expressed throughout the primitive streak, node and notochord precursor cells (the headprocess/notochord precursor, herein referred to as the notochord precursor), which extends anteriorly from the node at the distal midline (Kispert and Herrmann, 1994; Fig. 4A, right). In E8.5 *eed* mutant embryos similar expression was detected in the primitive streak and sparse mesoderm cells extending anteriorly from the streak (Fig. 4A, left). These data suggest that a notochord precursor is formed in *eed* mutant embryos, despite the absence of a morphologically distinct node, the major source of these cell types (Selleck and Stern, 1991; Beddington, 1994).

Finding that T was expressed in axial mesoderm cells of *eed* mutant embryos was intriguing, and we were interested in learning to what extent this mesoderm is specified. Thus, two additional markers with specific expression in these tissues, *Tcf3b* and *Shh*, were examined. mRNA in situ hybridization using both whole mount and sectioned embryos was performed on E8.5 mutant and E7.5 wild-type embryos (Fig. 4B-D,F). At E7.5 *Tcf3b* is expressed in the node, notochord precursor, and prechordal mesoderm, which lies rostral to the notochord (Fig. 4B, left) (Ang et al., 1993; Monaghan et al., 1993; Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993). In mutant embryos *Tcf3b* transcripts were detected in the primitive streak and midline mesoderm extending anteriorly (Fig. 4B, right and 4F). Expression appeared to be in a band 3-5 cells wide at the midline.

In wild-type embryos *Shh* transcripts are first detected at E7.5 in the notochord precursor (Fig. 4C, right), and thus are present in a subset of the *Tcf3b* expressing domain. Later, during somite formation, *Shh* expression spreads caudally into the node, overlapping with *Tcf3b* (Echelard et al., 1993). In some *eed* mutant embryos, *Shh* transcripts were detected in a subset of the *Tcf3b*-expressing domain lying more anteriorly in the embryo, similar to wild-type expression at E7.5 (Fig. 4C, left; Echelard et al., 1993). In others, *Shh* expression overlapped more completely with *Tcf3b*, extending farther posteriorly. Variation in *Shh* expression likely reflects differences in the extent of development among E8.5 *eed* mutant embryos with respect to wild-type. Comparison of *Shh*, *T*, and *Tcf3b* gene expression in frontal sections of E8.5 *eed* embryos revealed that while all three genes are expressed in distal midline mesoderm (Fig. 4D-F), *Shh* and *Tcf3b* expression extends more anteriorly than *T* (data not shown). Such expression could reflect the presence of prechordal mesoderm, but this cannot be confirmed until a good marker restricted to this cell population is available.

eed mutant embryos are able to initiate production of definitive endoderm

Tcf3b and a related molecule, *Tcf3a* (also called *HNF-3α*; see Footnote in Introduction) are also markers of the definitive endoderm lineage (Ang et al., 1993; Monaghan et al., 1993; Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993). Definitive endoderm is derived in part from cells traversing the most distal region of the primitive streak (Lawson et al., 1991). A significant amount of definitive endoderm also arises from the node (Selleck and Stern, 1991; Beddington, 1994), where it emerges concomitantly with the notochord precursor, displacing embryonic visceral endoderm proximoanteriorly (Poelmann, 1981b; Lawson and Pedersen, 1987; Tam and Beddington, 1992). Analysis of sectioned mutant embryos revealed abundant expression of both *Tcf3b* (Fig. 4F) and *Tcf3a* (not shown) in the endoderm layer extending anteriorly from the distal tip of the embryo. Both *Tcf3b and Tcf3a* are expressed at low levels in primitive visceral endoderm (Ang et al., 1993), but expression is upregulated in definitive endoderm. Therefore, it is likely that strong expression of these genes indicates the presence of definitive endoderm in *eed* mutant embryos.

nodal is expressed throughout the epiblast of *eed* mutant embryos

While *eed* mutant embryos lack a morphologically distinct node, they are still able to produce and specify some axial mesoderm, suggesting that some node activity might be present. One gene that has been suggested to be important for the establishment and/or maintenance of this structure is *nodal*, a member of the TGFβ superfamily (Zhou et al., 1993). In pre- to early-streak wild-type embryos, prior to node formation, *nodal* is expressed throughout the proximal epiblast and visceral endoderm (Conlon et al., 1994; Fig. 5A).

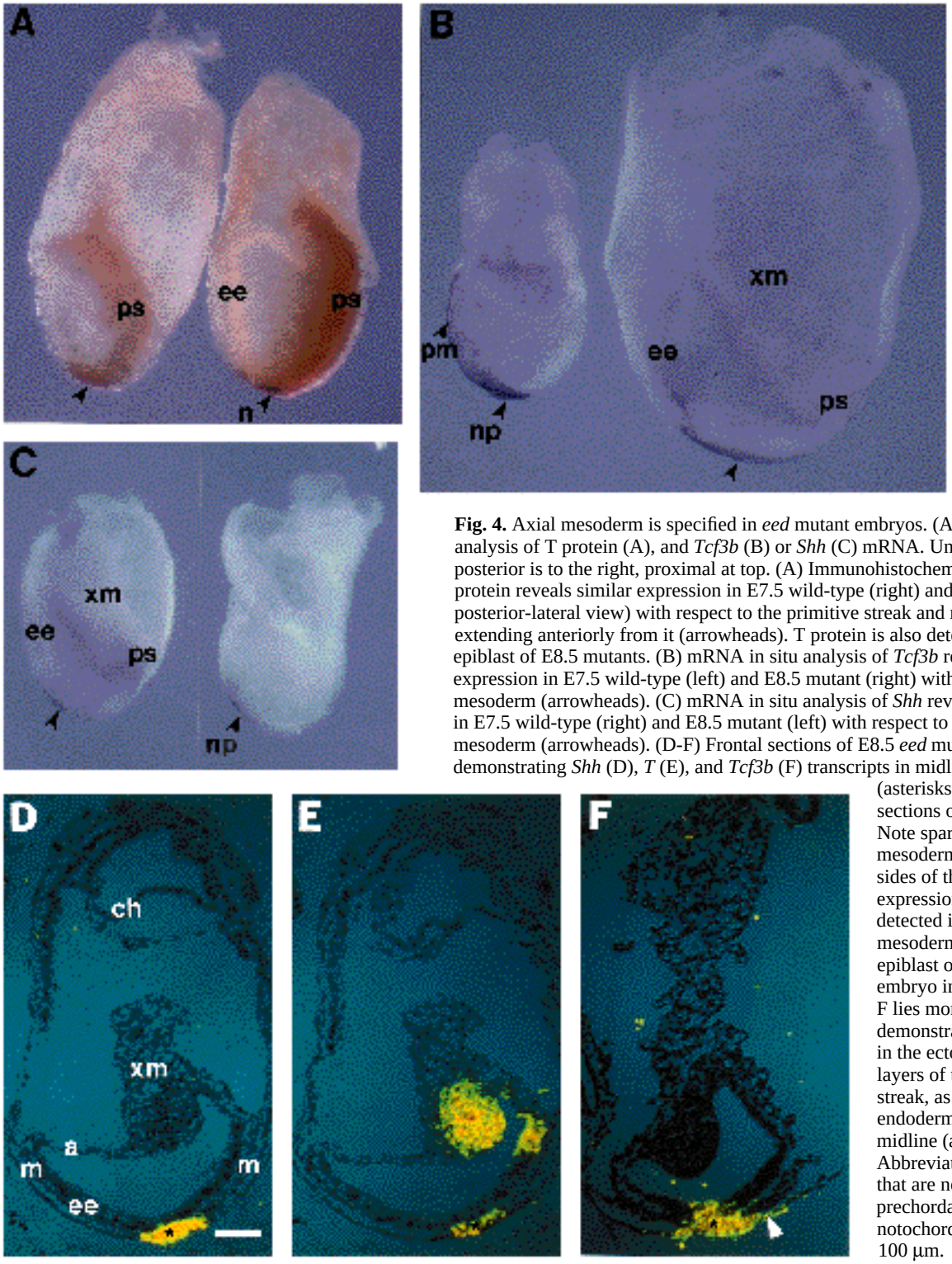


Fig. 4. Axial mesoderm is specified in *eed* mutant embryos. (A-C) Whole-mount analysis of T protein (A), and *Tcf3b* (B) or *Shh* (C) mRNA. Unless otherwise stated, posterior is to the right, proximal at top. (A) Immunohistochemical analysis of T protein reveals similar expression in E7.5 wild-type (right) and E8.5 mutant (left; posterior-lateral view) with respect to the primitive streak and midline mesoderm extending anteriorly from it (arrowheads). T protein is also detected in the proximal epiblast of E8.5 mutants. (B) mRNA in situ analysis of *Tcf3b* reveals similar expression in E7.5 wild-type (left) and E8.5 mutant (right) with respect to midline mesoderm (arrowheads). (C) mRNA in situ analysis of *Shh* reveals similar expression in E7.5 wild-type (right) and E8.5 mutant (left) with respect to anterior midline mesoderm (arrowheads). (D-F) Frontal sections of E8.5 *eed* mutant embryos demonstrating *Shh* (D), *T* (E), and *Tcf3b* (F) transcripts in midline mesoderm (asterisks). D and E are adjacent sections of the same embryo. Note sparse embryonic mesoderm present on the lateral sides of the embryos. *T* expression is additionally detected in extraembryonic mesoderm and the proximal epiblast on one lateral side of the embryo in E. Embryo section in F lies more posteriorly, demonstrating *Tcf3b* expression in the ectoderm and mesoderm layers of the distal primitive streak, as well as in definitive endoderm fanning out from the midline (arrowhead). Abbreviations used in this figure that are not listed elsewhere: pm, prechordal mesoderm; np, notochord precursor. Bar, 100 μm.

As gastrulation proceeds, *nodal* expression becomes limited to epiblast cells lying more posteriorly, so that by mid- to late-streak expression is detected only on the posterior side of the epiblast, adjacent to primitive streak mesoderm. Subsequently, epiblast expression is downregulated and *nodal* transcripts become restricted to cells surrounding the node (Fig. 5B). Examination of E7.5 *eed* mutants revealed that *nodal* was expressed throughout the proximal epiblast and visceral endoderm (data not shown). In E8.5 *eed* mutant embryos, *nodal* transcripts were still detected throughout the proximal part of the epiblast (Fig. 5C). Thus, *nodal* expression appears to be correctly initiated in *eed* mutant embryos, but subsequently expression is not properly downregulated in the epiblast.

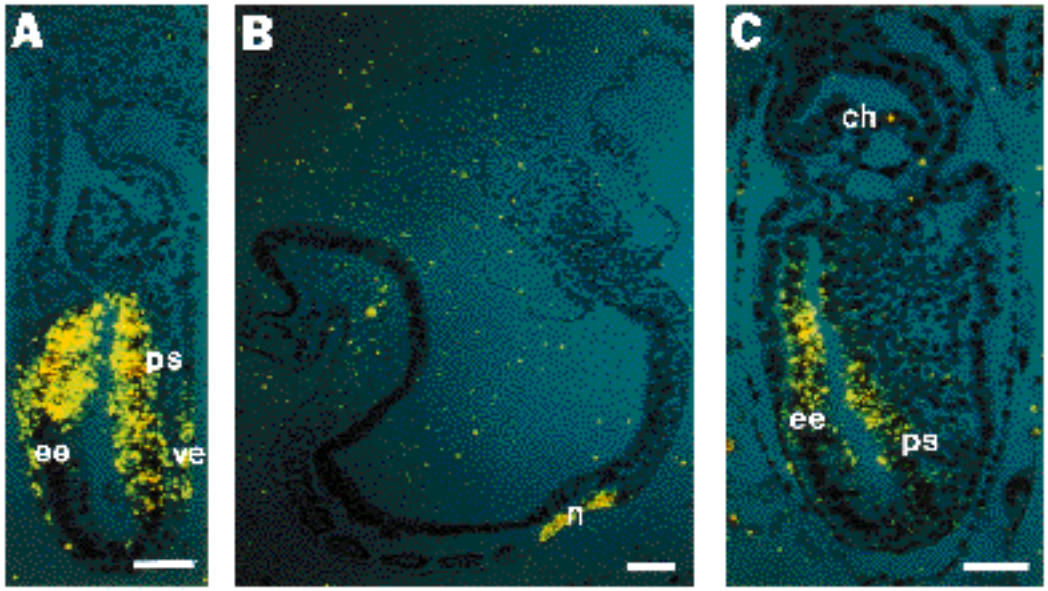


Fig. 5. *nodal* is inappropriately expressed in *eed* mutant embryos. mRNA in situ hybridization of *nodal* was performed on sagittal sections of E7.0 (A) and E8.5 (B) wild-type, and E8.5 *eed* (C) embryos. Posterior is to the right, proximal at top. (A) Section of an approximately E7.0 wild-type embryo demonstrating *nodal* expression in proximal epiblast and visceral endoderm. (B) In E8.5 embryos *nodal* is expressed specifically in cells surrounding the node. (C) E8.5 *eed* embryo in which transcripts are detected throughout the proximal epiblast; visceral endoderm-specific transcripts are not readily apparent in this particular section, but are present in more parasagittal sections of the same embryo. Bar, 100 μm.

Brachyury and *Evx1* are inappropriately expressed in cells normally fated to become surface ectoderm

While appropriate expression of *T* mRNA and protein was observed in the primitive streak and axial mesoderm, our analyses indicated an additional domain of expression in E8.5 *eed* mutant embryos (see Fig. 4A,E); expression was detected in the proximal epiblast on one side of the embryo, and appeared to extend anteriorly. Further analysis of E8.5 *eed* mutant embryo sections demonstrated *T* mRNA expression in the most proximoanterior epiblast (compare Fig. 6A and B), cells that do not normally ingress through the primitive streak, but are fated to give rise to surface ectoderm (Lawson et al., 1991; Lawson and Pedersen, 1992). Serial transverse sections through mutant embryos revealed *T* expression encompassing a 180° arc of epiblast cells extending from the primitive streak around one side of the epiblast to the anterior. The expression domain was approximately 5-8 cells wide along the proximodistal axis. E7.5 mutant embryos exhibited a similar expression pattern that was slightly expanded along the proximodistal axis (Fig. 6C).

Like *T*, *Evx1* transcripts are detected in the nascent mesoderm of the primitive streak; however, *Evx1* is expressed in a gradient, with the most abundant transcripts located more proximally (Dush and Martin, 1992; Fig. 6D). In E8.5 *eed* mutants, *Evx1* transcripts were detected in the proximal primitive streak and proximal mesoderm (Fig. 6E). In addition, *Evx1* exhibited ectopic expression in the proximoanterior epiblast, reminiscent of that observed for *T*. Similar expression was observed in E7.5 mutant embryos (Fig. 6F). Hybridization of *T* and *Evx1* probes to adjacent 6 μm sections of mutant embryos indicated that the ectopic expression patterns of these genes overlap. These data are intriguing, since misexpression of the *T* homologue *Xbra* in *Xenopus* embryos leads to the production of ectopic mesoderm expressing *Xhox3*, the *Xenopus* homologue of *Evx1* (Cunliffe and Smith, 1992). In E6.5 *eed* mutants, no *T* or *Evx1* transcripts could be detected (data not shown). This is likely to reflect a developmental delay as compared to wild-type littermates, which normally express these genes at E6.5.

DISCUSSION

Our earlier morphological work described the *eed* phenotype as a retardation of embryonic ectoderm growth and absence of axial structures (Niswander et al., 1988). Since the deficient tissues are normally derived from epiblast cells fated to traverse the distal primitive streak, we hypothesized that the defect might lie in the failure of their progenitors to ingress through the streak. The results of the mRNA in situ hybridization analyses presented here are consistent with a defect in distal primitive streak function, but they also suggest alternative mechanisms for the establishment of the *eed* phenotype. A comparison of gene expression between *eed* and wild-type embryos is summarized in Fig. 7.

Two lines of evidence strongly suggest that epiblast cells traversing the proximal primitive streak of *eed* mutant embryos are able to adopt their normal fates. First, *Flk1* and *Igf2*, two genes expressed in derivatives of the proximal primitive streak, are expressed normally with respect to the extraembryonic mesoderm cell types present in *eed* mutant embryos (see Fig. 7A). Second, PGCs, descendants of the presumptive extraembryonic mesoderm cells of the epiblast, are also present. Although PGCs are clearly specified, differences with wild-type embryos were observed: PGCs of *eed* mutant embryos were not always present in a cluster, and were often distributed more anteriorly. Such differences could reflect the overabundance and disorganization of extraembryonic mesoderm in *eed* mutant embryos as compared to wild-type.

Absence of detectable *Gsc* and *Fgf4* mRNA in the primitive streak of *eed* mutant embryos (see Fig. 7A,B) likely represents a defect in distal primitive streak function. Our analyses, however, do not distinguish whether lack of expression of these genes directly affects the establishment of the phenotype

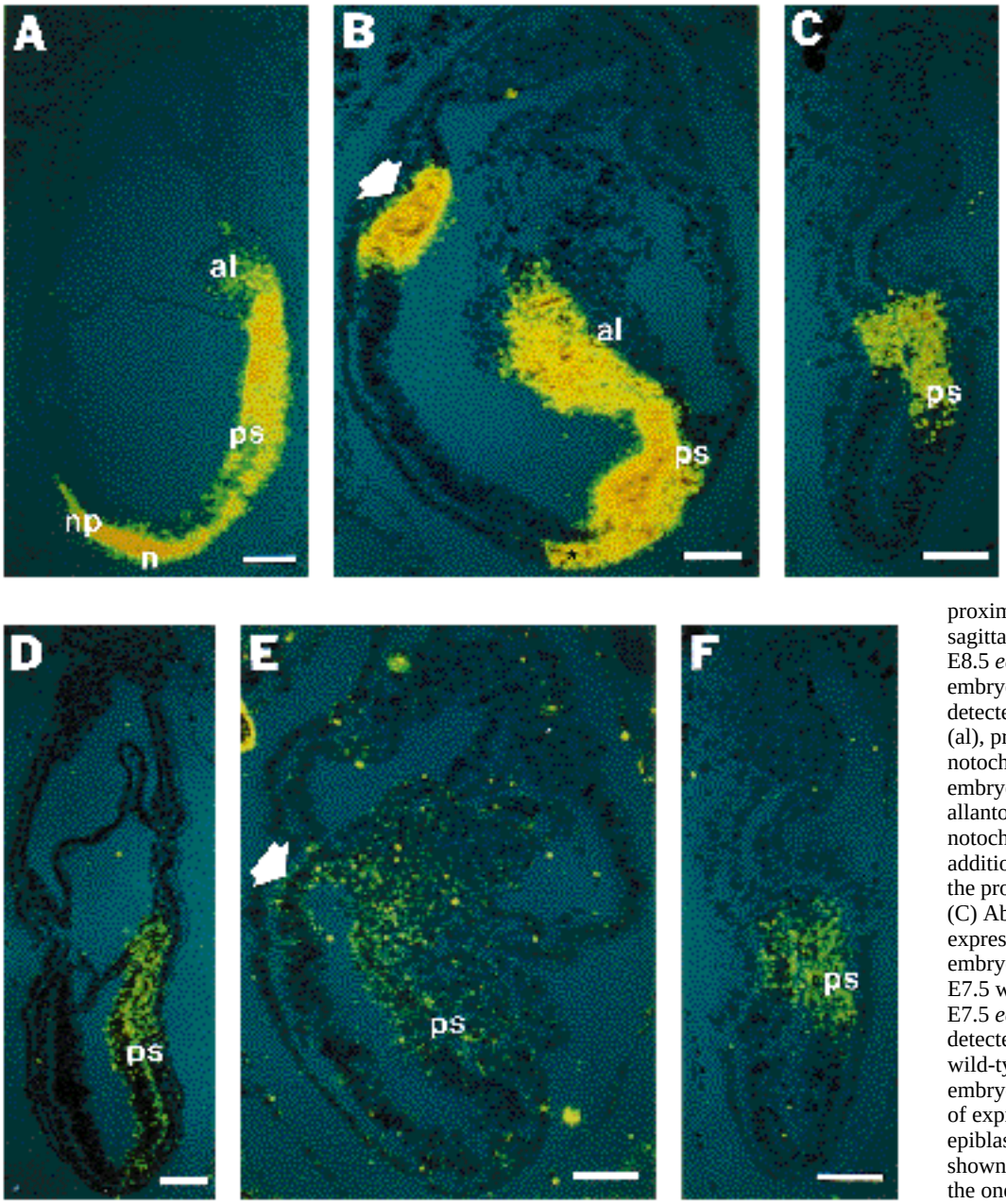


Fig. 6. *T* and *Evx1* are ectopically expressed in *eed* mutant embryos. mRNA in situ hybridization of *T* (A-C) and *Evx1* (D-F). Posterior is to the right, proximal at top. (A-C) *T* expression in sagittal sections of E7.5 wild-type (A), E8.5 *eed* (B) and E7.5 *eed* (C) embryos. (A) At E7.5 *T* transcripts are detected at the base of the allantois (al), primitive streak, node and notochord precursor. (B) In E8.5 *eed* embryos, expression is observed in the allantois, primitive streak and putative notochord precursor (asterisk). In addition, *T* transcripts are present in the proximoanterior epiblast (arrow). (C) Aberrant proximoanterior epiblast expression is also detected in E7.5 *eed* embryos. (D-F) *Evx1* expression in E7.5 wild-type (D), E8.5 *eed* (E), and E7.5 *eed* (F) embryos. Transcripts are detected in the primitive streak of wild-type and *eed* embryos. Mutant embryos exhibit an additional domain of expression in the proximoanterior epiblast (arrow in E). The section shown in F is 6 μm and is adjacent to the one in C. Bar, 100 μm.

or merely reflects an absence of a population(s) of distal primitive streak cells. In wild-type embryos both *Fgf4* and *Gsc* are expressed in the primitive streak very early in gastrulation (Blum et al., 1992; Niswander and Martin, 1992), but their potential functions in this process are unclear. The spatial and temporal expression of *Gsc* during gastrulation is highly conserved among vertebrates (Cho et al., 1991; Izpisua-Belmonte et al., 1993; Stachel et al., 1993; Schulte-Merker et al., 1994); nevertheless, *Gsc* expression does not appear to be required for mouse gastrulation, since embryos harboring a null mutation for *Gsc* survive well beyond this point without any apparent defects (R. Behringer, personal communication).

Anterior mesoderm production, while inhibited in *eed* mutants, is not completely abolished, perhaps due to the continued activity of other molecules, like *Tcf3b*, in specifying anterior fates early in gastrulation. Sparse midline mesoderm cells lying anterior to the primitive streak of E8.5 *eed* mutant embryos appear to be specified as notochord precursor, as exemplified by expression of *Tcf3b*, *T*, and *Shh* (see Fig. 7A). Furthermore, *Tcf3b and Tcf3a* expression in endoderm extending anteriorly from the streak suggest that mutant embryos are able to initiate definitive endoderm production.

The node represents a major source of notochord and definitive endoderm in the mouse embryo (Beddington, 1994). Furthermore, the node demonstrates organizing activity, such that when grafted to an ectopic site, it induces a secondary neural axis containing notochord, definitive endoderm, and somites (Beddington, 1994). Thus, the ability to form a notochord precursor and definitive endoderm in *eed* mutants indicates that some node activity is present, even though a morphologically distinct node structure is not apparent. This activity could be attributed to the appropriate expression of *Tcf3b*, which in

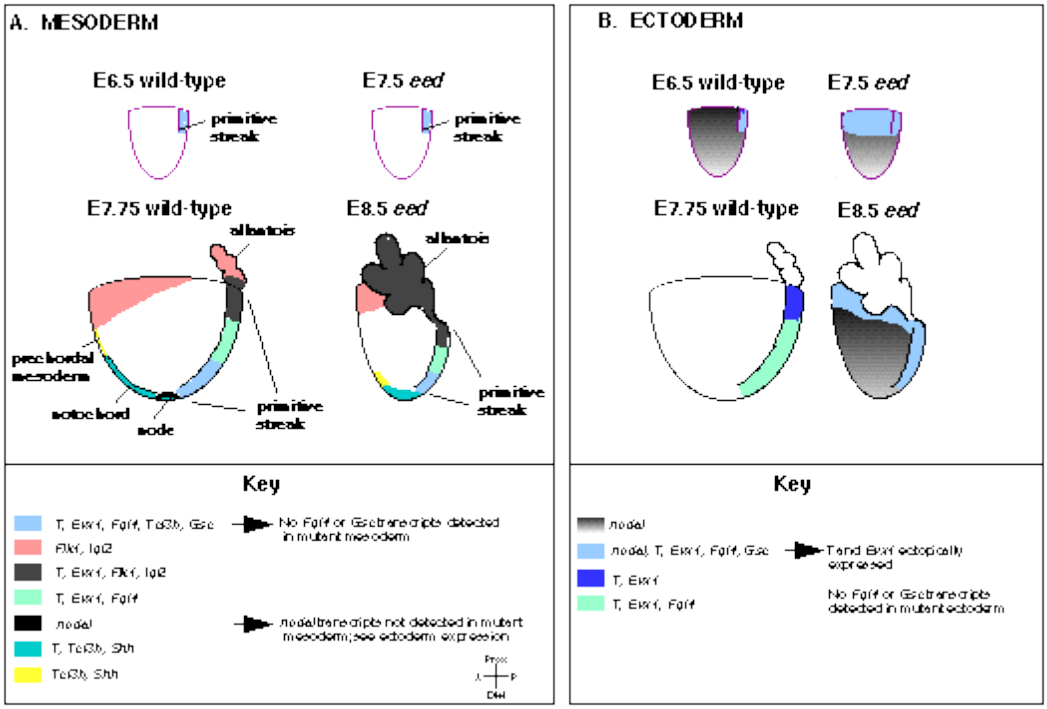


Fig. 7. Summary of gene expression in *eed* mutant embryos. Expression patterns of genes examined are compared between E6.5 (early-streak) wild-type and E7.5 mutant, and E7.75 (headfold stage) wild-type and E8.5 *eed* mutant. The germ layers have been separated for clarity, and only mesoderm and ectoderm gene expression are illustrated. Except for the allantois, the extraembryonic portion of the embryos is not shown. Different colored regions represent domains of gene expression as indicated in the key. *Tcf3b* was not analyzed in E6.5 wild-type/E7.5 mutants, and is not included in the ectoderm gene expression illustration, even though it is expressed in both wild-type and mutant ectoderm of the primitive streak at E7.75/E8.5. No expression gradients are implied, except for ectoderm-specific expression of *nodal*. Figure adapted from Sasaki and Hogan (1993).

wild-type embryos is required for node function. Targeted inactivation of *Tcf3b* results in both loss of morphological structure and functional activity of the node; *Tcf3b*$^{-/-}$ mutants do not initiate the formation of a notochord precursor (Ang and Rossant, 1994; Weinstein et al., 1994).

The paucity of axial mesoderm and definitive endoderm, as well as lack of further organization of these tissues in *eed* mutant are consistent with diminished node function. Because of its restricted expression pattern and putative nature as a signaling molecule, *nodal* might be important for the establishment and/or maintenance of a structurally definitive node (Zhou et al., 1993; see Sasaki and Hogan, 1993). Thus, it is tempting to speculate that inappropriate expression of *nodal* in E8.5 *eed* mutant embryos (Fig. 7A,B) affects node function. Consistent with its essential role early in gastrulation (Iannaccone et al., 1992; Conlon et al., 1994), initial expression of *nodal* in the *eed* mutant epiblast appears normal (Fig. 7B); however, at later stages it fails to be down-regulated and localized around the node. Further analysis is required to determine if *eed* is directly involved in the regulation of *nodal* in the epiblast, and whether or not absence of localized *nodal* expression results in failure to form a node.

The gene expression data presented thus far are consistent with a model in which the *eed* gene product plays a direct role in the production of mesoderm from the distal primitive streak. Perhaps without the *eed* gene product to induce complete anterior mesoderm formation, the embryo can still initiate gastrulation, but produces mesoderm of mostly posterior, or caudal, character. Support for this model comes from studies of *Xenopus laevis* embryos, where absence of early signals specifying dorsal fates leads to inhibition of proper organizer function during gastrulation, resulting in the default production of ventral/posterior mesoderm (see Gerhart et al., 1989; Kimelman et al., 1992; Slack and Tannahill, 1992). The problem with this model, however, is that it does not readily explain the morphologically apparent overproduction of extraembryonic mesoderm, as well as ectopic expression of *T* and *Evx1* observed in the proximal epiblast of *eed* mutant embryos (Fig. 7B). In fact, data regarding the functions of *T* and *Evx1* in both mouse and *Xenopus* embryos are consistent with a model in which their misexpression plays a dominant role in the establishment of the *eed* phenotype.

Besides being required cell autonomously for the differentiation and maintenance of notochord (Rashbass et al., 1991), a number of studies suggest that the *T* gene is required for proper epiblast cell ingression and migration of mesoderm cells from the proximal primitive streak (Herrmann and Kispert, 1994, and references therein). Additional support for the role of *T* in posterior mesoderm formation is provided by studies of the *Xenopus T* homologue *Xbra*. Injection of *Xbra* mRNA into the animal pole of one-cell stage embryos results in misexpression of *Xbra* throughout the embryo and a disruption of gastrulation; although blastopore formation occurs, no involution of mesoderm at the marginal zone is detected, and a layer of ectopic mesoderm contiguous with the marginal zone is formed over the animal hemisphere (Cunliffe and Smith, 1992). Older embryos often lack heads and develop an additional tail. The ectopic mesoderm is of posterior character, exhibiting small amounts of muscle and expressing *Xhox3*, the *Xenopus* homologue of *Evx1*. These data are consistent with a model in which *Xbra*/*T* is sufficient to act as a genetic switch from an ectodermal cell fate to a posterior/ventral mesodermal cell fate (Herrmann and Kispert, 1994).

The phenotype observed in *Xenopus* embryos misexpress-

ing *Xbra* is not unlike that of mouse *eed* mutants. Gastrulation is disrupted in both embryos, and posterior mesoderm appears to be produced at the expense of anterior. At the onset of gastrulation in *eed* mutant embryos, *T* is ectopically expressed in the proximoanterior epiblast, a region sharing a similar fate with animal hemisphere cells in *Xenopus* – that of surface ectoderm (Dale and Slack, 1987; Tam, 1989; Lawson et al., 1991). These cells do not normally ingress through the primitive streak, and one possibility is that in *eed* mutants ectopic *T* expression in these cells initiates a cellular program that influences them to ingress. Because of their proximal location in the mutant embryo, they would likely give rise to extraembryonic mesoderm after traversing the streak (Lawson et al., 1991), resulting in the large deposition of mesoderm observed proximally. Alternatively, they might undergo direct delamination to form mesoderm, bypassing the primitive streak. One way to determine how proximal epiblast cells behave in *eed* mutant embryos would be to perform lineage analysis like that used to construct a fate map of the pregastrula mouse embryo (Lawson et al., 1991). Furthermore, creation of $T^{-/-}$, *eed* double homozygotes will be helpful in elucidating the effect of ectopic *T* expression on the establishment of the *eed* phenotype.

Ectopic *Evx1* expression might also play a role in establishing the *eed* phenotype. Like the mouse, *Xenopus Xhox3* is expressed in a graded fashion along the anteroposterior axis (Ruiz i Altaba and Melton, 1989a). Inhibition of *Xhox3* function prevents the normal development of posterior structures (Ruiz i Altaba et al., 1991), while abolishing its normal expression gradient by ectopic expression anteriorly results in embryos in which anterior mesoderm patterning is inhibited (Ruiz i Altaba and Melton, 1989b). Inhibition of anterior development is similar to that seen upon misexpression of *Xbra*; however, ectopic mesoderm is not induced, and embryos undergo normal gastrulation movements, producing an apparently normal amount of embryonic mesoderm (Ruiz i Altaba and Melton, 1989b). Thus, evidence from *Xenopus* supports a role for *Xhox3* in specifying cell fates along the anteroposterior axis, rather than potentiating cell movement during gastrulation. If such a role is conserved in mouse, ectopic expression of *Evx1* in *eed* mutant embryos might reflect specification of a wider range of epiblast cells to a posterior fate. Assuming that a gradient of *Evx1* expression is normally involved in specifying positional values of nascent mesoderm, it is possible that disrupting this gradient by an increase in *Evx1* expression early in streak formation could lead to the inhibition of gene expression required for the specification of anterior mesoderm. In this manner the distal primitive streak defect hypothesized in *eed* mutant embryos might be a downstream effect of the dominant misexpression of *T* and *Evx1*. Furthermore, if ectopic expression of *T* and *Evx1* does indeed reflect a dominant posteriorization of the embryo, the normal role of the *eed* gene product during gastrulation would likely be in the negative regulation of one or both of these genes.

Finally, it is possible that, in addition to its effects on primitive streak function, the *eed* gene product has an independent role in controlling epiblast growth. Alternatively, growth inhibition could directly influence primitive streak function in *eed* mutants, since in the wild-type embryo epiblast cell ingression is thought to be driven by growth of the epiblast toward the primitive streak (Lawson et al., 1991). *eed* might directly regulate cell growth or affect expression of other gene(s) that direct epiblast cell growth and ingression during gastrulation. One gene whose expression is affected in *eed* mutants and might be involved in cell growth is *Fgf4*. Although the function of *Fgf4* in gastrulation is unknown, targeted disruption of the gene encoding the FGF type 1 receptor, which can bind the FGF4 protein (Mansukhani et al., 1990), has revealed that FGF signaling is required for proper embryonic growth, mesoderm production, and patterning during gastrulation (T. Yamaguchi and J. Rossant, personal communication). To implicate a growth defect as primary for the establishment of the phenotype, assays will be conducted to examine cell growth and proliferation in the *eed* mutant embryo (see Snow, 1977; Poelmann, 1981a,b; Lawson et al., 1991; Mac Auley et al., 1993).

The authors would like to thank the reviewers for helpful comments; Kirstie Lawson for helpful discussions, and Ron Conlon and members of our laboratory for critical reading of the manuscript. We also thank Terry Yamaguchi, Janet Rossant, and Richard Behringer for sharing data prior to publication; Karen Gustashaw for help with confocal microscopy, and Tracy Mourton for help with embryo sectioning. C. F. is a recipient of a post-doctoral fellowship from the American Cancer Society (no. PF-4091). A. S. is supported by a Feodor Lynen Fellowship from the Alexander von Humboldt Foundation. This work was supported by grants from the National Institutes of Health (no. HD-2446) and March of Dimes (no. F494-0874) to T. M.

REFERENCES

Ang, S.-L. and Rossant, J. (1994). *HNF-3*β is essential for node and notochord formation in mouse development. *Cell* **78**, 561-574.

Ang, S.-L., Wierda, A., Wong, D., Stevens, K. A., Cascio, S., Rossant, J. and Zaret, K. S. (1993). The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/*forkhead* proteins. *Development* **119**, 1301-1315.

Bastian, H. and Gruss, P. (1990). A murine *even-skipped* homologue, *Evx 1,* is expressed during early embryogenesis and neurogenesis in a biphasic manner. *EMBO J.* **9**, 1839-1852.

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

Beddington, R. S. P. and Smith, J. C. (1993). Control of vertebrate gastrulation: inducing signals and responding genes. *Curr. Opin. Genet. Dev.* **3**, 655-661.

Blum, M., Gaunt, S. J., Cho, K. W. Y., Steinbeisser, H., Blumberg, B., Bittner, D. and De Robertis, E. M. (1992). Gastrulation in the mouse: the role of the homeobox gene *goosecoid*. *Cell* **69**, 1097-1106.

Chiquoine, A. D. (1954). The identification, origin, and migration of the primordial germ cells of the mouse embryo. *Anat. Rec.* **118**, 135-146.

Cho, K. W. Y., Blumberg, B., Steinbeisseer, H. and De Robertis, E. M. (1991). Molecular nature of Spemann's Organizer: the role of the Xenopus homeobox gene *goosecoid*. *Cell* **67**, 1111-1120.

Conlon, F. L., Lyons, K. M., Takaesu, N., Barth, K. S., Kispert, A., Herrmann, B. and Robertson, E. J. (1994). A primary requirement for *nodal* in the formation and maintenance of the primitive streak in the mouse. *Development* **120**, 1919-1928.

Conlon, R. A. and Herrmann, B. G. (1993). Detection of messenger RNA by in situ hybridization to postimplantation embryo whole mounts. In *Guide to Techniques in Mouse Development* (*Methods in Enzymology*) vol. 225 (eds. P. M. Wassarman and M. L. DePamphilis), pp. 373-383. San Diego: Academic Press.

Cunliffe, V. and Smith, J. C. (1992). Ectopic mesoderm formation in *Xenopus* embryos caused by widespread expression of a *Brachyury* homologue. *Nature* **358**, 427-430.

Dale, L. and Slack, J. M. W. (1987). Fate map for the 32-cell stage of *Xenopus laevis*. *Development* **99**, 527-551.

De Chiara, T. M., Robertson, E. J. and Efstratiadis, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* **64**, 849-859.

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.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Faust, C. and Magnuson, T. (1993). Genetic control of gastrulation in the mouse. *Curr. Opin. Genet. Dev.* **3**, 491-498.

Gaunt, S. J., Blum, M. and De Robertis, E. M. (1993). Expression of the mouse *goosecoid* gene during mid-embryogenesis may mark mesenchymal cell lineages in the developing head, limbs, and body wall. *Development* **117**, 769-778.

Gerhart, J., Danilchik, M., Doniach, T., Roberts, S., Rowning, B. and Sterwart, R. (1989). Cortical rotation of the Xenopus egg: consequences for the anteroposterior pattern of embryonic dorsal development. *Development* **Supplement**, 37-51.

Ginsburg, M., Snow, M. H. L. and McLaren, A. (1990). Pimordial germ cells in the mouse embryo during gastrulation. *Development* **110**, 521-528.

Hébert, J. M., Boyle, M. and Martin, G. R. (1991). mRNA localization studies suggest that murine FGF-5 plays a role in gastrulation. *Development* **112**, 407-415.

Herrmann, B. G. and Kispert, A. (1994). The *T* genes in embryogenesis. *Trends Genet.* **8**, 281-286.

Herrmann, B. G., Labeit, S., Poustka, A., King, T. R. and Lehrach, H. (1990). Cloning of the *T* gene required in mesoderm formation in the mouse. *Nature* **343**, 617-622.

Iannaccone, P. M., Zhou, X., Khokha, M., Boucher, D. and Kuehn, M. R. (1992). Insertional mutation of a gene involved in growth regulation of the early mouse embryo. *Dev. Dynamics* **194**, 198-208.

Izpisua-Belmonte, J. C., De Robertis, E. M., Storey, K. G. and Stern, C. D. (1993). The homeobox gene *goosecoid* and the origin of organizer cells in the early chick blastoderm. *Cell* **74**, 645-659.

Kimelman, D., Christian, J. L. and Moon, R. T. (1992). Synergistic principles of development: overlapping patterning systems in *Xenopus* mesoderm induction. *Development* **116**, 1-9.

Kispert, A. and Herrmann, B. G. (1994). Immunohistochemical analysis of the *Brachyury* protein in wild-type and mutant mouse embryos. *Dev. Biol.* **161**, 179-193.

Lawson, K. A. and Hage, W. J. (1994). Clonal analysis and the origin of primordial germ cells in the mouse. In *Ciba Foundation Symposium: Germline Development* vol. 182 (eds. J. Marsh and J. Goode), pp. 68-91. Chichester: Wiley.

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. (1992). Clonal analysis of cell fate during gastrulation and early neurulation in the mouse. In *Ciba Foundation Symposium: Postimplantation Development in the Mouse*. vol. 165 pp. 3-26. Wiley.

Lee, J. E., Pintar, J. and Efstratiadis, A. (1990). Pattern of insulin-like growth factor II gene expression during early mouse embryogenesis. *Development* **110**, 151-159.

Mac Auley, A., Werb, Z. and Mirkes, P. E. (1993). Characterization of the unusually rapid cell cycles during rat gastrulation. *Development* **117**, 873-883.

McGadey, J. (1970). A tetrazolium method for non-specific alkaline phosphatase. *Histochemie* **23**, 180-184.

Mansukhani, A., Moscatelli, D., Talarico, D., Levytska, V. and Basilico, C. (1990). A murine fibroblast growth factor (FGF) receptor expressed in CHO cells is activated by basic FGF and Kaposi FGF. *Proc. Natl. Acad. Sci. USA* **87**, 4378-4382.

Monaghan, A. P., Kaestner, K. H., Grau, E. and Schutz, G. (1993). Postimplantation expression patterns indicate a role for the mouse forkhead/HNF3α, β, and γ genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. *Development* **119**, 567-578.

Niswander, L. and Martin, G. R. (1992). *Fgf-4* expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* **114**, 755-768.

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.

Orr-Urtreger, A., Givol, D., Yayon, A., Yarden, Y. and Lonai, P. (1991). Developmental expression of two murine fibroblast growth factor receptors, *flg* and *bek*. *Development* **113**, 1419-1434.

Peters, J., Jones, J., Ball, S. T. and Clegg, J. B. (1990). Analysis of electrophoretically detected mutations induced in mouse germ cells by ethylnitrosourea. In *Banbury Report* vol. 34 (eds. J. W. Allen, B. A. Bridges, M. F. Lyon, M. J. Moses and L. B. Russell), pp. 247-257. Plainview, NY: Cold Spring Harbor Laboratory Press.

Poelmann, R. E. (1981a). The formation of the embryonic mesoderm in the early post-implantation mouse embryo. *Anat. Embryol.* **162**, 29-40.

Poelmann, R. E. (1981b). The head-process and the formation of the definitive endoderm in the mouse embryo. *Anat. Embryol.* **162**, 41-49.

Popp, R. A., Bailiff, E. G., Skow, L. C., Johnson, F. M. and Lewis, S. E. (1983). Analysis of a mouse α-globin gene mutation induced by ethylnitrosourea. *Genetics* **105**, 157-167.

Provost, G. S. and Short, J. M. (1994). Characterization of mutations induced by ethylnitrosourea in seminiferous tubule germ cells of transgenic B6C3F_1 mice. *Proc. Natl. Acad. Sci. USA* **91**, 6564-6568.

Rashbass, P., Cooke, L. A., Herrmann, B. G. and Beddington, R. S. P. (1991). A cell autonomous function of *Brachyury* in *T*/*T* embryonic stem cell chimeras. *Nature* **353**, 348-351.

Rinchik, E. M. (1991). Chemical mutagenesis and fine-structure functional analysis of the mouse genome. *Trends Genet.* **7**, 15-21.

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 on mouse Chromosome 7. *Mammalian 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 Dif.* **33**, 651-669.

Ruiz i Altaba, A. and Melton, D. A. (1989a). Bimodal and graded expression of the *Xenopus* homeobox gene *Xhox3* during embryonic development. *Development* **106**, 173-183.

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

Ruiz i Altaba, A., Prezioso, V. R., Darnell, J. E. and Jessell, T. M. (1993). Sequential expression of *HNF-3*β and *HNF-3*α by embryonic organizing centres: the doral lip/node, notochord and floor plate. *Mech. Dev.* **44**, 91-108.

Russell, L. B., Montgomery, C. S. and Raymer, G. D. (1982). Analysis of the albino-locus region of the mouse. IV. Characterization of 34 deficiencies. *Genetics* **100**, 427-453.

Russell, L. B. and Rinchik, E. M. (1987). Genetic and molecular characterization of genomic regions surrounding specific loci of the mouse. In *Banbury Report* vol. 28 (eds. M. M. Moore, D. M. Demarini, F. J. de Serres and K. R. Tindall), pp. 109-121. Plainview, NY: Cold Spring Harbor Laboratory Press.

Russell, L. B., Russell, W. L., Rinchik, E. M. and Hunsicker, P. R. (1990). Factors affecting the nature of induced mutations. In *Banbury Report* vol. 34 (eds. J. W. Allen, B. A. Bridges, M. F. Lyon, M. J. Moses and L. B. Russell), pp. 271-285. Plainview, NY: Cold Spring Harbor Laboratory Press.

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.

Sassoon, D. and Rosenthal, N. (1993). Detection of messenger RNA by in situ hybridization. In *Guide to Techniques in Mouse Development* (*Methods in Enzymology*) vol. 225 (eds. P. M. Wassarman and M. L. DePamphilis), pp. 384-404. San Diego: Academic Press.

Schulte-Merker, S., Hammerschmidt, M., Beuchle, D., Cho, K. W., De Robertis, E. M. and Nusslein-Volhard, C. (1994). Expression of zebrafish *goosecoid* and *notail* gene products in wild-type and mutant *no tail* embryos. *Development* **120**, 843-852.

Selleck, M. A. J. and Stern, C. D. (1991). Fate mapping and cell lineage analysis of Hensen's node in the chick embryo. *Development* **112**, 615-626.

Slack, J. M. W. and Tannahill, D. (1992). Mechanism of anteroposterior axis specification in vertebrates: lessons from the amphibians. *Development* **114**, 285-302.

Snow, M. H. L. (1977). Gastrulation in the mouse: Growth and regionalization of the epiblast. *J. Embryol. exp. Morph.* **42**, 293-303.

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.

Stachel, S. E., Grunwald, D. J. and Meyers, P. Z. (1993). Lithium

perturbation and *goosecoid* expression identify a dorsal specification pathway in the pregastrula zebrafish. *Development* **117**, 1261-1274.

Tam, P. P. L. (1989). Regionalisation of the mouse embryonic ectoderm: allocation of prospective ectodermal tissues during gastrulation. *Development* **107**, 55-67.

Tam, P. P. L. and Beddington, R. S. P. (1992). Establishment and organization of germ layers in the gastrulating mouse embryo. In *Ciba Foundation Symposium: Postimplantation Development in the Mouse* vol. 165 pp. 27-49. Chichester: Wiley.

Weinstein, D. C., Ruiz i Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M. and Darnell Jr., J. E. (1994). The winged-helix transcription factor *HNF-3*β is required for notochord development in the mouse embryo. *Cell* **78**, 575-588.

Wilkinson, D. G., Bhatt, S. and Herrmann, B. G. (1990). Expression pattern of the mouse *T* gene and its role in mesoderm formation. *Nature* **343**, 657-659.

Yamaguchi, T., Dumont, D. J., Conlon, R. A., Breitman, M. L. and Rossant, J. (1993). *flk-1*, an *flt*-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development* **118**, 489-498.

Yamaguchi, T. P., Conlon, R. A. and Rossant, J. (1992). Expression of the fibroblast growth factor FGFR-1/*flg* during gastrulation and segmentation in the mouse. *Dev. Biol.* **152**, 75-88.

Zeller, R. (1989). Fixation, embedding, and sectioning of tissues, embryos, and single cells. In *Current Protocols in Molecular Biology* vol. 2 (eds. F. M. Ausubel), pp. 14.1.1-14.1.8. Boston: Wiley.

Zhou, X., Sasaki, H., Lowe, L., Hogan, B. L. M. and Kuehn, M. R. (1993). *Nodal* is a novel TGF-β-like gene expressed in the mouse node during gastrulation. *Nature* **361**, 543-546.

(Accepted 28 October 1994)