Nodal and Fgf pathways interact through a positive regulatory loop and synergize to maintain mesodermal cell populations

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Summary

Interactions between Nodal/Activin and Fibroblast growth factor (Fgf) signalling pathways have long been thought to play an important role in mesoderm formation. However, the molecular and cellular processes underlying these interactions have remained elusive. Here, we address the epistatic relationships between Nodal and Fgf pathways during early embryogenesis in zebrafish. First, we find that Fgf signalling is required downstream of Nodal signals for inducing the Nodal co-factor One-eyed-pinhead (Oep). Thus, Fgf is likely to be involved in the amplification and propagation of Nodal signalling during early embryonic stages. This could account for the previously described ability of Fgf to render cells competent to respond to Nodal/Activin signals. In addition, overexpression data shows that Fgf8 and Fgf3 can take part in this process. Second, combining zygotic mutations in *ace/fgf8* and *oep* disrupts mesoderm formation, a phenotype that is not produced by either mutation alone and is consistent with our model of an interdependence of Fgf8 and Nodal pathways through the genetic regulation of the Nodal co-factor Oep and the cell propagation of Nodal signalling. Moreover, mesodermal cell populations are affected differentially by double loss-of-function of Z*oep;ace*. Most of the dorsal mesoderm undergoes massive cell death by the end of gastrulation, in contrast to either single-mutant phenotype. However, some mesoderm cells are still able to undergo myogenic differentiation in the anterior trunk of Z*oep;ace* embryos, revealing a morphological transition at the level of somites 6-8. Further decreasing Oep levels by removing maternal *oep* products aggravates the mesodermal defects in double mutants by disrupting the fate of the entire mesoderm. Together, these results demonstrate synergy between *oep* and *fgf8* that operates with regional differences and is involved in the induction, maintenance, movement and survival of mesodermal cell populations.

Supplemental data available online

Key words: Nodal, Fgf, Zebrafish, Gastrulation, Mesoderm

Introduction

The concept of germ layers emerged from classical studies in experimental embryology. Ectoderm, mesoderm and endoderm layers are induced before they are shaped through gastrulation movements to give rise subsequently to specific differentiated cell types. A model for the formation of mesoderm was mainly derived from studies in amphibian embryos where mesoderm induction in the marginal zone relies on secreted molecules emanating from the vegetal pole (Nieuwkoop, 1973). This model can be readily applied to the zebrafish embryo where the extra-embryonic yolk syncytial layer and the blastoderm margin have been suggested to be the source of inducing signals (Mizuno et al., 1996; Rodaway et al., 1999; Chen and Kimelman, 2000).

At the molecular level, secreted molecules of the transforming growth factor β (TGFβ) family have been implicated in the formation of mesoderm in both organisms (Harland and Gerhart, 1997). In the *Xenopus* embryo, TGFβ family members such as Activin and Vg1 were initially thought to act as maternal factors. However, characterization of the maternal transcription factor VegT, localized at the vegetal pole, led to a model in which the TGFβ family members Vg1-related Derriere and Nodal-type Xnr1, Xnr2 and Xnr4 are required zygotically downstream of VegT for the formation of the entire mesoderm (Sun et al., 1999; Clements et al., 1999; Kofron et al., 1999; Yasuo and Lemaire, 1999; Agius et al., 2000; Hyde and Old, 2000). Although VegT does not seem to be involved in the early steps of mesoderm formation in mouse and zebrafish, the study of mutants affected in the Nodal pathway indicates a conserved role for Nodals in vertebrate mesoderm development.

In zebrafish, genetic analysis of the Nodal-related genes *squint* (*sqt*), *cyclops* (*cyc*) and of *oep*, which encodes a Nodal co-factor, reveals that Nodal signalling is implicated in both mesoderm and endoderm formation (Feldman et al., 1998; Gritsman et al., 1999). Indeed, embryos lacking maternal and zygotic Oep function (MZ*oep*), and *sqt;cyc* double mutants are devoid of endoderm and mesoderm in the head and trunk. However, the tail mesoderm is still induced and maintained in these mutants, which indicates that pathways other than the Oep-dependent Nodal pathway are implicated in formation of ventral mesoderm.

Fgf was identified in *Xenopus* as a mesoderm inducer (Kimelman and Kirschner, 1987; Slack et al., 1987) and overexpression of a dominant-negative form of a Fgf receptor impairs mesoderm induction in *Xenopus* and zebrafish gastrulae (Amaya et al., 1991; Griffin et al., 1995), which indicates that Fgf activity could be involved in mesoderm induction in vertebrate embryos. However, Fgf signalling is not sufficient for mesoderm induction in *Xenopus*, because embryos expressing a dominant-negative form of the activin receptor lack mesoderm but have an intact Fgf signalling pathway (Hemmati-Brivanlou and Melton, 1992). The current model, based largely on experiments in *Xenopus*, proposes that Fgf is required either in parallel to or downstream of Nodal/Activin signals to induce and maintain mesodermal fates (Cornell and Kimelman, 1994; Labonne and Whitman, 1994; Schulte-Merker et al., 1994; Labonne et al., 1995). However, the molecular basis of the interaction between the two pathways is largely unknown. One possibility is that Fgf acts downstream of Nodal signalling as a relay mechanism required for mesoderm induction (Rodaway et al., 1999).

In addition to their role in cell-fate induction, Nodal and Fgf signalling have been implicated in cell movements, but their possible cooperation in this matter has not been studied. In zebrafish, MZ*oep* cells do not ingress at the margin of the embryo (Carmany-Rampey and Schier, 2001), however they are able to involute in a wild-type environment (Aoki et al., 2002a). In addition, activation of Nodal signalling promotes cell-autonomous movements (David and Rosa, 2001). Analysis of *fgf8* and *fgfR1*-mutant mice revealed that Fgf signalling is involved in gastrulation movements (Ciruna and Rossant, 2001). In *Xenopus*, inhibiting Fgf signalling by overexpressing either a dominant-negative receptor or a MAP kinase phosphatase severely affects gastrulation (Isaacs et al., 1994; Labonne et al., 1995; Labonne and Whitman, 1997). The regulation of gastrulation movements through Fgf signalling is probably a common trait of amphibians and teleosts, because overexpression of either Fgf or a dominant-negative FgfR dramatically affects gastrulation in zebrafish (Griffin et al., 1995; Rodaway et al., 1999).

The analysis of a putative interaction between Nodal and Fgf signalling pathways is complicated by the fact that both are regulated through positive and negative feedback loops. It was shown in *Xenopus*, zebrafish and mouse, that Nodal promotes its own expression as well as the expression of its antagonist Lefty/Antivin (Meno et al., 1999; Thisse and Thisse, 1999; Hyde and Old, 2000). Similarly, Fgf promotes the expression of its antagonists *sprouty* and *sef* (Furthauer et al., 2001; Furthauer et al., 2002; Tsang et al., 2002), and eFgf positively regulates its own expression through the T-box transcription factor Xbra (Isaacs et al., 1994; Labonne et al., 1995; Schulte-Merker and Smith, 1995; Umbhauer et al., 1995; Casey et al., 1998).

Together, these data indicate the existence of a gene network involving Fgf and Nodal signalling components, the connectivity and function of which remain to be understood. In the present study, we show that intact Fgf signalling is required for the cell-autonomous and cell-nonautonomous induction of *oep* downstream of the Nodal pathway, and that intact Oep signalling is required for the cell-nonautonomous induction of the Activin/Nodal type I receptor Taram-A (Renucci et al., 1996). This regulation circuit provides a model for the involvement of the Fgf pathway in the cellular response to Nodal signals, and for the maintenance, amplification and cell-to-cell propagation of Nodal activity after the activation of the zygotic genome. Such a model predicts that lowering Fgf and Nodal levels will have cooperative, deleterious effects on the cell-nonautonomous induction of genes downstream of the Tar*/Nodal pathway. Indeed, this is what we observe for the regulation of the mesoderm marker *no tail* (*ntl*) expression in chimeric embryos. In addition, overexpression data and analysis of the *oep;ace* double-mutant phenotype show that Fgf8 takes part in this process. The genetic interaction between *oep* and *ace* differentially affects cell movements and survival of distinct mesodermal cell populations and is consistent with a synergistic activity of Nodal and Fgf8 in mesoderm cells through the genetic regulation of the Nodal co-factor Oep.

Materials and methods

Fish strains

Embryos were obtained from natural spawning of wild-type (TL) or mutant fish lines. The following mutant alleles were used: oep^{tz57} (Hammerschmidt et al., 1996), ace^{ti282a} (Brand et al., 1996). Embryos lacking both maternal and zygotic *oep* function were obtained by crossing $oep^{-/-}$ adults that had been rescued to viability by injection of *oep* RNA at the one-cell stage.

Microinjection and transplantation

Synthetic mRNAs were transcribed in vitro using the SP6 mMessage mMachine™ transcription kit (Ambion). Embryos were injected at the 1-4-cell stage with *tar** (2 pg), *sqt* (5 pg), *cyc* (5 pg), *fgf8* (40 pg) and *fgf3* (40 pg) synthetic mRNAs. *fgf3* (CATTGTGGCATGG-CGGGATGTCGGC 0.2 mM) and *fgf8* (GAGTCTCATGTTT-ATAGCCTCAGTA 0.5 mM) morpholino modified antisense oligonucleotides (GeneTools) were prepared and injected as described (Furthauer et al., 2001). For transplantation experiments, donor embryos were injected at the 1-4-cell stage with *LacZ* RNA (50 pg) and/or *gfp* RNA (50 pg) as lineage tracers in combination with *tar** (2 pg).

Labelling hypoblast cells

A solution of 10-kD DMNB-caged fluorescein (5 mg ml^{-1}) (Molecular Probes) was injected at the one-cell stage. When embryos reached the shield stage, the dye was activated in a few blastomeres by a microlaser beam, as described (Serbedzija et al., 1998). At the tail-bud stage, the location of the labelled cells was assessed by immunocytochemistry against fluorescein following in situ hybridization.

SU5402 treatment

Embryos were treated in the dark with either 10 μM or 30 μM SU5402 (Calbiochem) from the two-cell stage until they were fixed or rinsed with embryo medium to allow development to proceed.

Table 1. An intact Fgf pathway is required for the induction of *oep* downstream of Tar*/Nodal

Injected RNA	Probe	Wild type	Wild type + SU5402	MZ*oep*	MZ*oep* + SU5402	Wild type *fgf8* MO	MZ*oep fgf 8*MO
*tar**	*cyc*	55% (*n*=65) (auto)	41% (*n*=46) (auto)				
*tar**	*sqt*	52% (*n*=38) (auto)	75% (*n*=24) (auto)				
*tar**	*tar*	68% (*n*=38)	86% (*n*=45)	100% (*n*=17) (auto)	78% (*n*=18) (auto)	89% (*n*=19)	87% (*n*=38) (auto)
*tar**	*oep*	75% (*n*=87)	13% (trace) (*n*=62) (auto)	93% (*n*=42)	20% (trace) (*n*=35) (auto)	100% (weak) (*n*=18)	90% (weak) (*n*=44) (auto)
*tar**	*fgf3*	39% (*n*=31)	37% (*n*=17)				
*tar**	*fgf8*	71% (*n*=45)	68% (*n*=19)	35% (weak) (*n*=20) (auto)	33% (weak) (*n*=18) (auto)		
*tar**	*ntl*	73% (*n*=41)	0% (*n*=14)	54% (*n*=15) (auto)	0% (*n*=12)		
sqt	*tar*	90% (*n*=11)	100% (*n*=8)	0% (*n*=8)	0% (*n*=6)		
sqt	*oep*	93% (*n*=15)	44% (trace) (*n*=9) (auto)	0% (*n*=8)	0% (*n*=8)		
sqt	*ntl*	92% (*n*=14)	72% (trace) (*n*=11) (auto)	0% (*n*=6)	0% (*n*=7)		
cyc	*tar*	100% (*n*=17)	100% (*n*=11) (auto)				
cyc	*oep*	100% (*n*=23)	60% (trace) (*n*=17)				
cyc	*ntl*	95% (*n*=23)	70% (trace) (*n*=11) (auto)				

A few cells taken from donor embryos injected at the one-cell stage with *tar**, *cyc or sqt* synthetic mRNA were transplanted to the animal pole at the sphere stage into a host with the same genetic background. In some cases, a *fgf8* MO was injected into the recipient embryo and in other cases the embryos were treated with SU5402 (+ SU5402) immediately after transplantation. The qualitative effect of the SU5402 treatment or of the genetic background compared with wild-type embryos is indicated (weak or trace staining). In some cases, the staining with the probe was only cell-autonomous to the transplanted cells (auto).

Acridine Orange treatment

Live embryos were treated with 5 μg ml^{-1}Acridine Orange (Sigma) in embryo medium for 30 minutes, then washed with embryo medium and observed in epifluorescence.

Annexin V injection

Annexin-V-Alexa 488 (Nexins Research B.V., 2-5 nl, injected pure) was injected directly into the posterior hypoblast of embryos at the tail-bud stage with an eppendorf transjector (5246) and a pulled glass needle. 15 minutes after the injection, embryos were mounted in methylcellulose 3% in embryo medium and observed with a confocal microscope (Leica TCS SP2) with a 40× objective (Leica 40×/0.80 W).

In situ hybridisation and immunocytochemistry

In situ hybridisation was carried out as described (Hauptmann and Gerster, 1994) using the following mRNA: *myoD* (Weinberg et al., 1996); *ntl* (Schulte-Merker et al., 1992); *tbx6* and *fgf3* (Hug et al., 1997); *fgf8* (Furthauer et al., 1997); *tar* (Renucci et al., 1996); *oep* (Zhang et al., 1998); *cyc* (Rebagliati et al., 1998b); *sqt* (Feldman et al., 1998; Rebagliati et al., 1998a); *her5* (Müller et al., 1996); *spt* (Griffin et al., 1998); *hgg1* (Thisse et al., 1994); *sprouty4* (Furthauer et al., 2001); and *myhz1* (Xu et al., 2000). β-galactosidase was revealed with a rabbit polyclonal antibody (Cappel) at 1:1000 dilution and diaminobenzidine staining. Green fluorescent protein was revealed with a rabbit polyclonal antibody (Molecular Probes) at 1:1000 dilution and anti-rabbit CY3 (Jackson Immuno Research) at 1:500.

Genotyping

Embryos were genotyped after in situ hybridization using a PCR-based method. Single embryos were boiled in 10 μl lysis buffer (Tris-HCl 10 mM pH 7.3, KCl 50 mM, $MgCl_2$ 1.5 mM, Tween-20 3%, NP40 3%). They were then digested for 4 hours at 56°C with 1 mg ml^{-1} proteinase K. The latter was inactivated by boiling for 5 minutes. PCR reaction was performed using 2.5 μl of the embryo extract in a 25 μl reaction volume. The oep^{tz57} allele was identified using the primers tzup, 5′-AGATGGAGATGTTCTAATGGTGTTTTTGGG-3′, and tzdown, 5′-TGACAAATAATCACAGCAAACATCAAGAAC-3′. The PCR product was digested with MaeIII, which cuts the mutant allele only. The ace^{ti282a} allele was identified using the primers EcoRVup, 5′-CTTCGGATTTCACATATTTATGCCCGTATGTATG-CATATC-3′, and EcoRVdown, 5′-CAGTTTTAGTAAGTCAC-AAAAGTGATGACTTTTTCAGATA-3′. The PCR product was digested with Eco RV, which cuts the mutant allele only.

Results

The Fgf signalling pathway is a relay in the induction of *oep* by the Tar*/Nodal pathway

It was shown that the Nodal pathway might be regulated through positive and negative feedback (Meno et al., 1999; Thisse and Thisse, 1999; Hyde and Old, 2000; Dickmeis et al., 2001). To further elucidate the genetic interactions and cellular mechanisms involved, we examined the cell-autonomous and cell-nonautonomous induction of Nodal pathway components in cells with constitutively active intracellular Activin/Nodal signalling. This was achieved by transplanting wild-type cells expressing Tar*, a constitutively active form of the Activin/Nodal type I receptor Taram-A (Renucci et al., 1996), after the injection of *tar** RNA at the one-cell stage into the animal pole of wild-type embryos at the sphere stage. Assessed by in situ hybridization in the resulting chimeric embryos at the shield stage, Tar* activates cell-autonomously the expression of the genes encoding the Nodal ligands *sqt* and *cyc*, and activates both cell-autonomously and cell-nonautonomously the expression of *tar* and *oep* (Fig. 1A-D, Table 1). The type I receptor Tar was previously described as a potential receptor for the Nodal ligands Sqt and Cyc (Aoki et al., 2002b). In accord with this, we found that wild-type cells that express the Nodal ligands Sqt or Cyc have the same properties as Tar* expressing cells, activating the expression of *tar* and *oep* both cell-autonomously and cell-nonautonomously (Fig. S1A,B,G,H at http://dev.biologists.org/supplemental and Table 1). These observations indicate that the Tar*/Nodal signalling pathway can be amplified through a positive-feedback loop and that there is a relay mechanism for the cell-

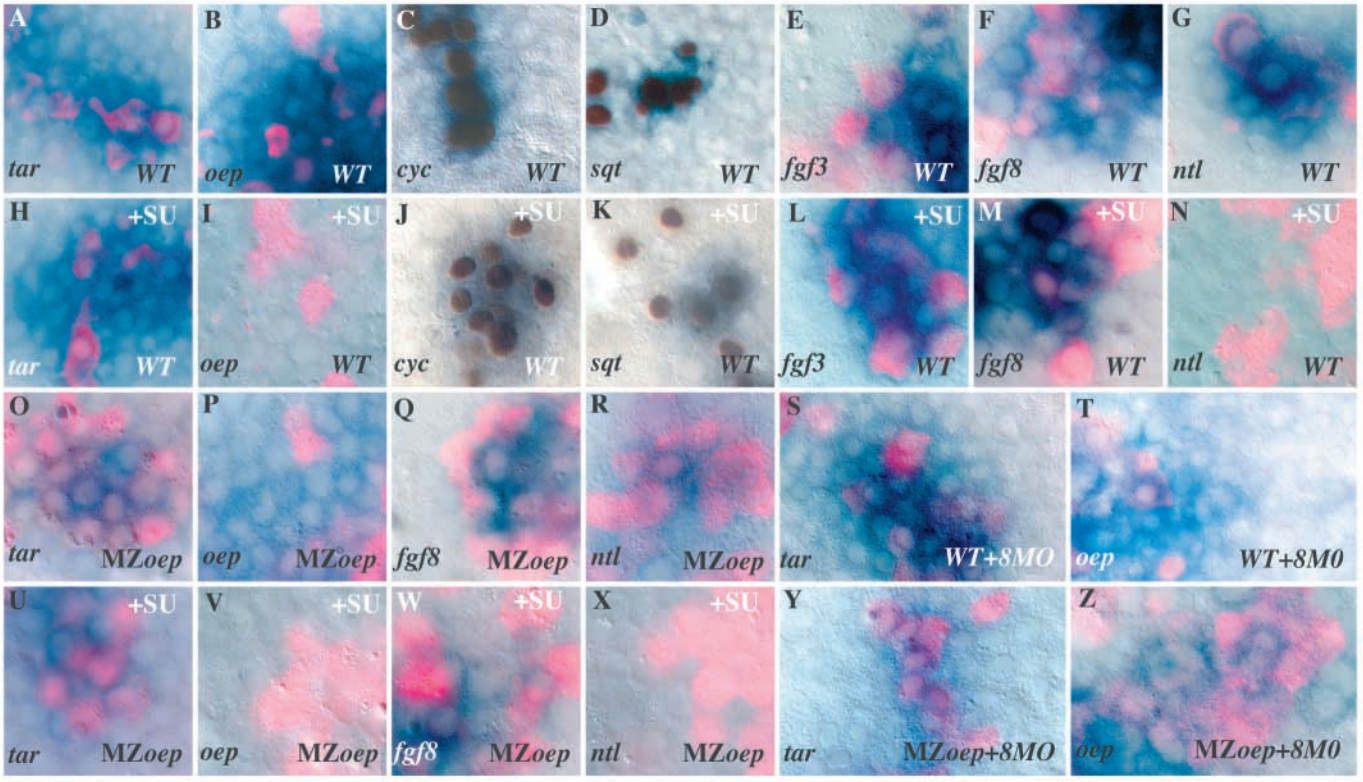

Fig. 1. Fgf signalling pathway serves as a relay for the cell-nonautonomous induction of *oep* by the Tar*/Nodal pathway. (A-Z) Flat mounts of chimeric embryos at the shield stage showing the expression of *tar*, *oep*, *cyc*, *sqt*, *ntl*, *fgf3* and *fgf8* in blue (the probe is indicated in the bottom left of each panel). The progeny of transplanted cells expressing Tar* is revealed by immunocytochemistry (red fluorescence staining the green fluorescent protein, except in C,D,J,K where brown staining reveals nls-β galactosidase). The genotype of the recipient embryo is shown in the bottom right of each panel. +SU, embryos treated with 10 μM SU5402 after transplantation. In some experiments, the recipient embryo was injected with a morpholino oligonucleotide directed against *fgf8* (8MO). All views are of the animal pole. For all panels except C,D,J,K, a Normarski image and an epifluorescence image were overlayed.

nonautonomous induction of *oep* and *tar* that is downstream of the Tar*/Nodal signalling pathway.

The secreted components of the Nodal pathway (Sqt and Cyc), which are themselves induced by Tar*, could act as a relay for the cell-nonautonomous induction of *oep* and *tar*. To investigate this, we assessed the induction of both genes in the MZ*oep*-mutant background. That these cells are unable to transduce Nodal signals (Gritsman et al., 1999) was confirmed by the inability of Sqt-expressing cells to induce the expression of *tar*, *oep* and the T-box gene *ntl* in the MZ*oep* mutant background (Fig. S1M-O at http://dev.biologists.org/supplemental and Table 1). Although we observed cell-autonomous induction of *tar* by Tar* in the mutant background, its cell-nonautonomous induction was abolished (Fig. 1O). This result indicates that Oep-dependent signalling is involved in the cell-nonautonomous induction of *tar*. By contrast, as in wild-type chimeras, we observed cell-autonomous and cell-nonautonomous induction of *oep* by Tar* in the MZ*oep*-mutant background (Fig. 1P, Table 1). This result indicates that a relay signal other than Nodal is involved in the cell-nonautonomous induction of *oep* by Tar*.

Previously, Fgf signalling was proposed to act as a relay signal from the Nodal-expressing mesendoderm into the mesoderm proper (Rodaway et al., 1999). Consistent with such a model, Tar* expressing cells, which have mesendodermal characteristics (Mathieu et al., 2002), could induce both cell-autonomous and nonautonomous expression of the mesoderm marker *ntl* in chimeric, wild-type embryos (Fig. 1G and Table 1). Cells that express Cyc or Sqt are also very potent inducers of *ntl* in wild-type chimeras (Fig. S1C,I at http://dev.biologists.org/supplemental). Therefore, we investigated whether Fgf signalling was involved in the response to Tar* and Nodals, using SU5402, a pharmacological inhibitor of FgfR activity (Mohammadi et al., 1997). The cell-nonautonomous induction of the mesoderm marker *ntl* by Tar*, Cyc or Sqt expressing wild-type cells was abolished by SU5402 treatment, and the cell-autonomous induction of *ntl* in the same chimeric embryos was strongly diminished (Fig. 1G,N, Fig. S1C,F,I,L at http://dev.biologists.org/supplemental and Table 1). In addition, the induction of *ntl* by Tar* expressing cells in MZ*oep* embryos, was abolished by SU5402 treatment (Fig. 1R,X, Table 1). These observations are in agreement with previous studies in *Xenopus* demonstrating that TGFβ-dependent induction of mesodermal genes such as *ntl* depends on FGF signalling (Cornell and Kimelman, 1994; Labonne and Whitman, 1994). In addition, they indicate that Fgf signalling might act as a relay signal from the Nodal-expressing cells into the neighbouring cells, and that Fgf signalling cooperates with Oep-dependent signalling to induce downstream genes.

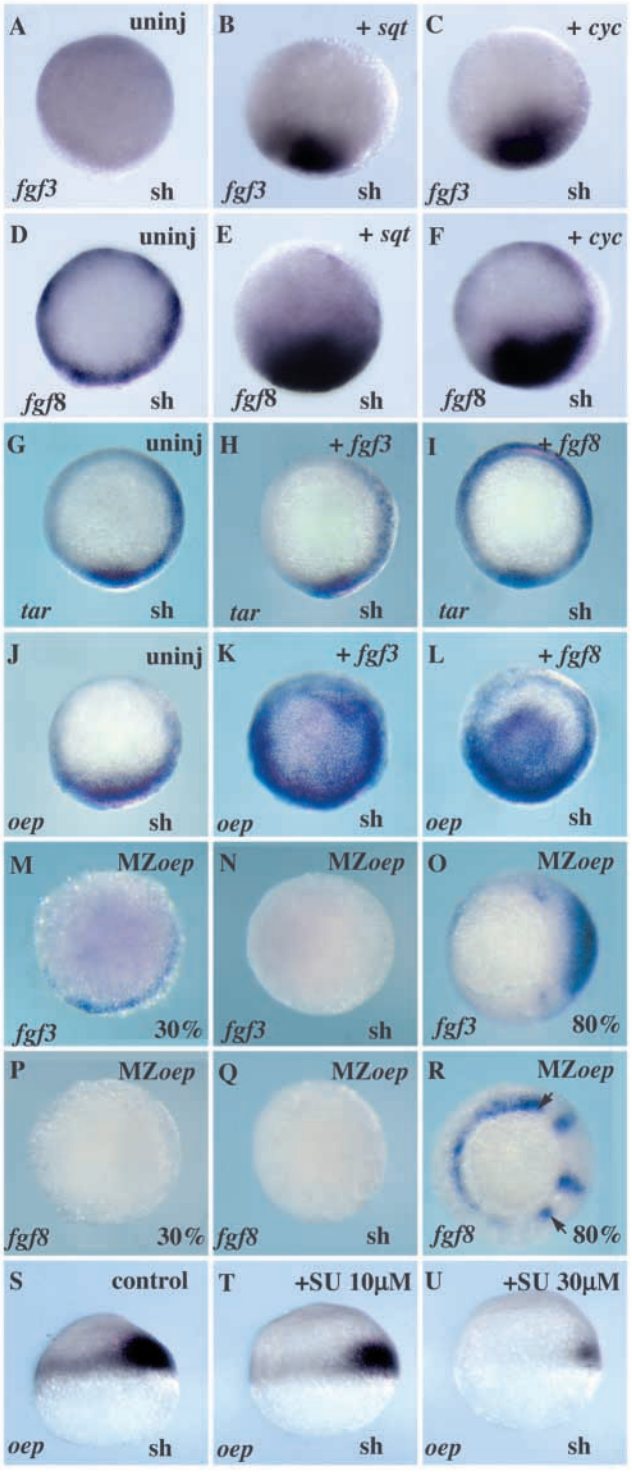

Fig. 2. Fgf3 and Fgf8 might account for the involvement of Fgf signalling pathway in the Nodal positive-feedback loop. (A-U) Whole mount embryos showing expression of *fgf3*, *fgf8*, *oep* and *tar* in blue (the probe is indicated in the bottom left and the stage at the bottom right of each panel: sh, shield stage: 80%, 80% epiboly). (A-L) Views from the animal pole, dorsal to the bottom of embryos uninjected (uninj) or injected with *sqt*, *cyc*, *fgf3* and *fgf8* synthetic mRNA (indicated top right). (M-R) Expression pattern of *fgf3* (M-O) and *fgf8* (P-R) throughout epiboly in the MZ*oep* background. Arrows in R point to the dorsal limit of the marginal staining. (S-U) Lateral views, dorsal to the right, of untreated (control) embryos or embryos treated with SU5402 (top right). 10 µM SU5402 (T) phenocopies the *ace* mutant phenotype and 30 µM SU5402 (U) abolishes the expression of *oep* at the blastoderm margin. Note that *oep* is still expressed on the dorsal side, consistent with the involvement of other pathways.

The activation of *oep* and *tar* expression by Tar*/Nodal differs in their requirements for Oep and FGF. The cell-nonautonomous induction of *oep* by Tar*, Sqt and Cyc in wild-type cells was abolished by the SU5402 treatment and there remained only traces of *oep* cell-autonomous expression in the chimeric embryos (Fig. 1I, Fig. S1E,K at http://dev.biologists.org/supplemental and Table 1). Similarly, cell-nonautonomous induction of *oep* by Tar* expressing cells was abolished in the presence of SU5402 in MZ*oep* background and there only remained traces of cell-autonomous induction (Fig. 1V). In contrast, the induction of *tar* by Tar* was not affected by the SU5402 treatment in either background (Fig. 1H,U, Table 1) and the induction of *cyc* and *sqt* by Tar* in the wild-type background, was not affected by the SU5402 treatment either (Fig. 1J,K, Table 1). In summary, these observations indicate that at least two relays act in the positive-feedback loop of the Nodal signalling pathway: Nodal, which is needed for non-autonomous *tar* expression; and Fgf, which is needed for autonomous and nonautonomous *oep* expression.

Fgf3 and Fgf8 are candidates for involvement in the Nodal positive-feedback loop

Because our data indicated that Fgf signalling is involved in a relay mechanism downstream of Nodal, we predicted that the expression of specific Fgf ligands should be regulated by Nodal, and that these ligands should be involved in the regulation of endogenous *oep*. Both *fgf3* and *fgf8* are expressed at early developmental stages in marginal blastomeres and could play such a role. To investigate this possibility, we tested whether *fgf3* and *fgf8* could be induced by overexpression of RNA encoding Nodal ligands Sqt and Cyc. Sqt and Cyc overexpression was sufficient to induce the expression of both *fgf3* and *fgf8* (Fig. 2A-F). In chimeric embryos, Tar*-expressing cells also induced *fgf3* and *fgf8* cell-autonomously and cell-nonautonomously (Fig. 1E,F, Table1). Treatment with SU5402 had little effect on the expression of these *fgf* genes by Tar*-expressing cells (Fig. 1L,M, Table 1). However, *fgf8* expression was induced in a higher percentage of embryos that expressed Tar* than *fgf3* (twice as much), which makes Fgf8 a better candidate to act downstream of Tar*. These observations indicate that activation of the Nodal pathway is sufficient to induce expression of Fgf ligands.

In addition, we found that although the cell-autonomous induction of *fgf8* by Tar*-expressing cells persisted, even on SU5402 treatment (Fig. 1M), *fgf8* cell-nonautonomous induction was abolished in the MZ*oep* background (Fig. 1Q). Thus, Nodal signalling is necessary for the nonautonomous induction of *fgf8* by Tar* in chimeric embryos. In addition, Nodal signalling is necessary for the early expression of *fgf3* and *fgf8* in uninjected embryos, particularly at the shield stage (Fig. 2N,Q). However, by mid-gastrulation, expression of *fgf8* in the posterior mesoderm partially recovers in MZ*oep* mutants (Fig. 2R). These observations indicate that activation of the

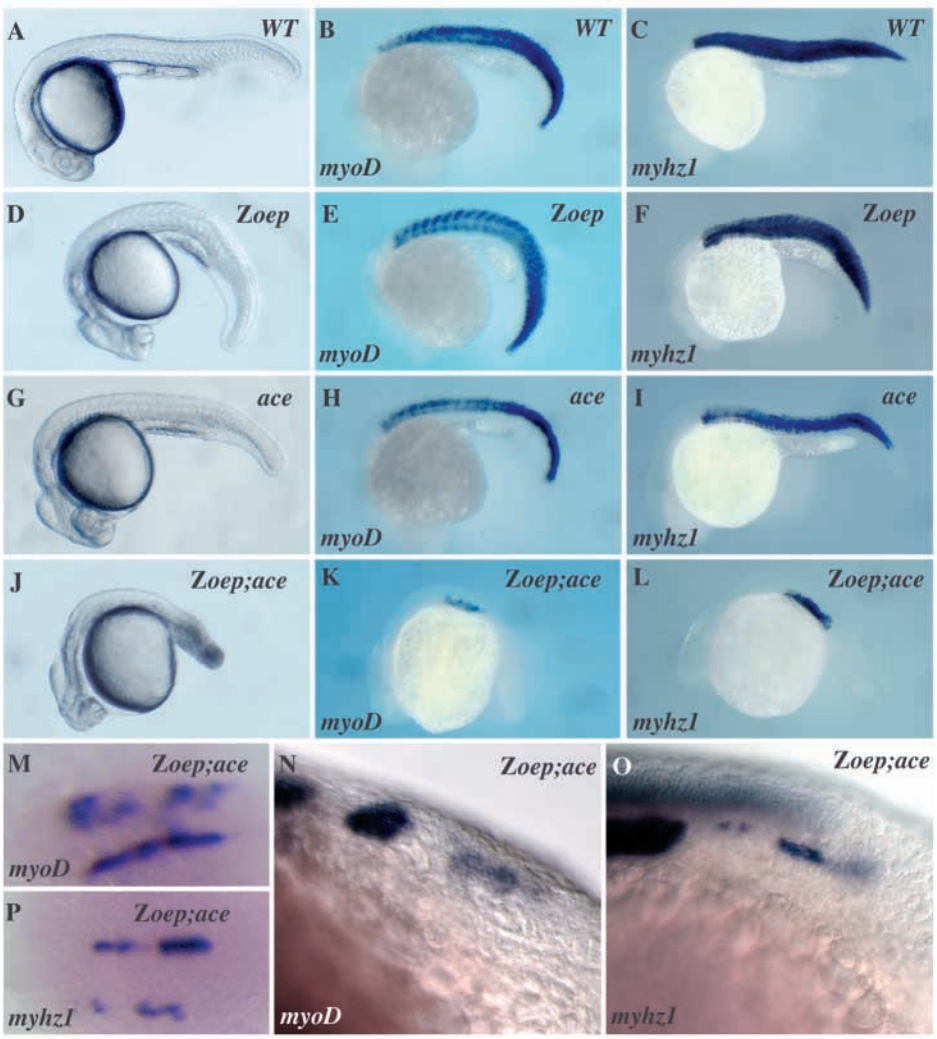

Fig. 3. *oep* and *fgf8* interact in vivo in the formation of mesoderm. (A,D,G,J) Lateral views of live embryos at 30 hours of development, taken from the progeny of Z*oep;ace* double-heterozygous parents. The genotype, which is inferred from the phenotype and statistical analysis, is indicated at the top right. (B-C,E-F,H-I,K-L) Lateral views of fixed embryos at 30 hours of development taken from the progeny of Z*oep;ace* double-heterozygous parents. The genotype, inferred from the phenotype and statistical analysis, is indicated top right. *myoD* or myosin heavy-chain (*myhz1*) staining in blue (the probe is indicated bottom left). (M,P) Dorsal view of embryos in K,L, respectively, note the bilateral staining. (N,O) Higher magnification of the embryos in K,L, respectively.

Nodal pathway is necessary and sufficient for the early expression of the Fgf ligands in the presumptive mesoderm, whereas the later expression of Fgf3 and Fgf8 in the neuroectoderm and Fgf8 in the posterior mesoderm depends on Nodal-independent factors.

We next asked whether Fgf signalling was involved in the regulation of endogenous *oep*. Again consistent with the hypothesis that Fgf3 and/or Fgf8 may act downstream of the Tar*/Nodal pathway to induce the cell-nonautonomous expression of *oep*, we observed that *fgf3* and *fgf8* overexpression induced the expression of *oep* but not *tar* (Fig. 2G-L) *sqt* and *cyc* (data not shown). In addition, impairment of Fgf signalling selectively affected the expression of *oep* but not *tar* as observed in embryos treated from the one-cell stage with 30 μM SU5402 (Fig. 2S-U and data not shown). However, impairment of both *fgf3* and *fgf8* using morpholino modified antisense oligonucleotides (MOs) reduced but did not abolish *oep* expression at early gastrula stages (Fig. S2 at http://dev.biologists.org/supplemental) indicating that other Fgfs might contribute to the regulation of *oep*. To further investigate the contribution of Fgf8 to the induction of *oep* downstream of Tar*/Nodal, we performed transplantation experiments in the context of impaired Fgf8 function by injection of a *fgf8* MO at the one-cell stage into wild-type or MZ*oep* donor and host embryos (Fig. 1S,T,Y,Z, Table 1). Induction of *Tar* was not affected in *fgf8* MO-injected embryos, and although *oep* expression was much weaker than in uninjected embryos, it was not lost. Thus, *fgf8* MO injection does not completely mimic the effects of SU5402 treatment.

Together, these results show that Fgf signalling is necessary and sufficient to trigger the induction of *oep* downstream of the Nodal pathway, and indicate that Fgf3 and Fgf8 are likely to be involved in such a relay mechanism, although other Fgfs may take part in this process.

oep and *fgf8* interact genetically in vivo in the formation of mesoderm

The previous experiments indicated the presence of a previously unsuspected regulatory interaction between the Oep/Nodal and Fgf pathways and indicated the involvement of Fgf8 in this process. To further address the interaction in vivo between Nodal and Fgf8, we examined the phenotype of Z*oep;ace* double mutants. The presence of maternal Oep protein enables some Nodal signalling to occur in embryos lacking Z*oep* function. Similarly, *ace*^{ti282}*a* is a mutant allele of *fgf8*, but the presence of correctly spliced full-length *fgf8* message renders *ace*^{ti282}-mutant embryos hypomorphic in Fgf8 signalling (Reifers et al., 1998). Because both these mutations reduce but do not eliminate the corresponding activities, they can be used as sensitised mutant backgrounds to uncover processes in which both pathways may be involved.

By day one of development, the double mutants were identified easily by the additive combination of brain defects, including the absence of hypothalamus, cerebellum and midbrain-hindbrain boundary. They also exhibited a profoundly altered morphology of mesoderm derivatives in the trunk and tail, contrasting with the phenotype of Z*oep* and *ace* single mutants (Fig. 3D,G,J). The posterior trunk and tail tissue of the

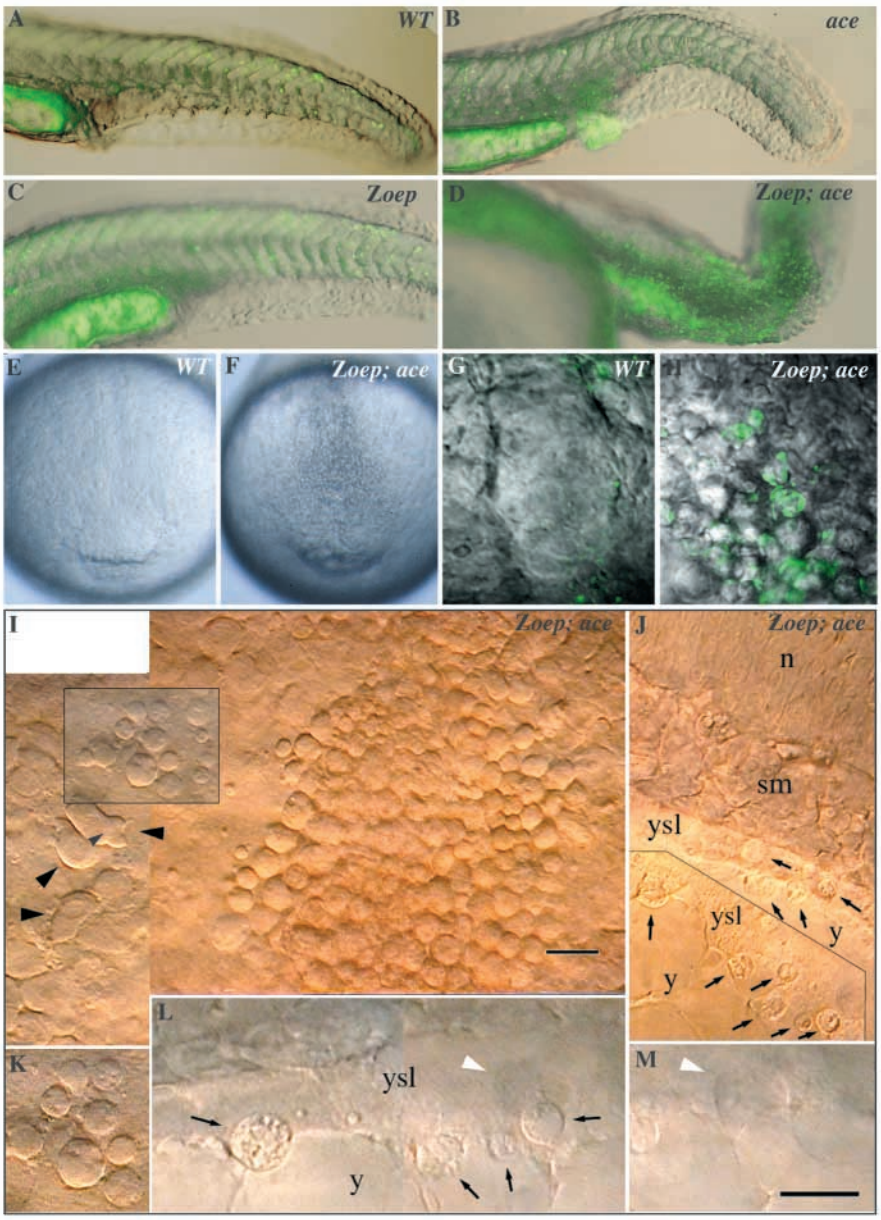

Fig. 4. The dorsal mesoderm of Z*oep;ace* embryos undergoes massive cell death at the end of gastrulation. (A-D) Lateral views (tail region) of live embryos at 30 hours of development stained with acridine orange. Dead cells stain green. The genotype, which is inferred from the phenotype and statistical analysis is indicated at the top right. (E,F) Views from the dorsoposterior region of live embryos at the shield stage (the inferred genotype is indicated top right). (G,H) Confocal sections of live embryos injected at the tail-bud stage with Annexin V Alexa 488 15 minutes before inspection (the inferred genotype is indicated top right). (I-M) Live Z*oep/ace* embryos inspected with Nomarski optics. (I) Focus on the dorsal (axial) and dorso-lateral hypoblast at tailbud stage, presented as three overlapping panels because of the curvature of the underlying yolk ball. Axially, notochord does not form; instead there is a large mass of dying cells that have morphology typical of apoptotic cells. Paraxially (left), 100-160 μm away from the axis, dead cell bodies of various sizes are also found (boxed region; enlarged in K) next to live cells of typical migrating (converging) non-axial hypoblast cell morphology (black arrowheads). (J) 4-somite stage, parasagittal view (caudal to the lower right) through the neuroectoderm, somitic mesoderm, yolk syncytial layer and yolk core. The inset shows the yolk syncytial layer and yolk portion appearing at a shallower focus. Both focal planes reveal numerous cell corpses (arrows) in the yolk syncytial layer, some of which abut and penetrate the underlying yolk core. (K-L) Higher-magnification views of cell corpses. (K) Dead cell bodies in the paraxial/lateral hypoblast at tailbud stage, from panel I inset. (L) Cell corpses (arrows) at 4-somite stage inside the yolk syncytial layer, one of them (left) indenting the underlying yolk mass. A yolk syncytial layer nucleus (white arrowhead), seen here slightly out of focus behind a dead cell body, is in focus in panel M. Abbreviations: n, neuroectoderm; sm, somitic mesoderm; y, yolk core; ysl, yolk syncytial layer. Scale bar: 20 μm.

double-mutants appeared necrotic and was heavily stained by acridine orange (Fig. 4A-D). However, remnants of notochord and somites could be detected in the anterior trunk. Furthermore, we found by in situ hybridization that a variable number of trunk cells in most double-mutant embryos expressed *myoD* (Fig. 3K,M,N) and *myhz1* myosin heavy chain RNA (Fig. 3L,O,P), indicating that the differentiation of some muscle fibres occurred in a region extending at most until somite 8.

These observations show that mesodermal cells are affected by the Z*oep;ace* loss-of-function and that some do not survive until 24 hours of development in the double-mutant background, indicating a synergistic activity of *oep* and *ace* in mesoderm formation.

The dorsal mesoderm of Z*oep;ace* embryos undergoes massive cell death at the end of gastrulation

We further investigated the timing of the mesodermal cell death observed in Z*oep;ace* mutants by inspecting live embryos during gastrulation by Nomarski video microscopy. We observed that dorsal hypoblastic cells underwent a dramatic morphological change by the end of gastrulation, losing their transparency and displaying the typical morphology of apoptotic cells (Fig. 4E-J). These cells were stained neither by acridine orange nor by TUNEL, which only marked small vesicles dispersed in the overlying ectoderm (data not shown), but they were stained by Annexin V Alexa injected directly into the extracellular space (Fig. 4G,H). The latter observation indicated that these hypoblastic cells were undergoing an apoptotic process (van den Eijnde et al., 1997). More laterally, cells with a typical hypoblastic morphology were found 100 μm from the bulk of dying cells (Fig. 4I). As development proceeded, somite condensation and notochord formation occurred in the anterior trunk and the mass of dead cells was pushed caudally (Fig. 4J and data not shown). Concomitantly, numerous cell corpses were

incorporated into the yolk cell, as indicated by Nomarski images (Fig. 4J,L).

We conclude from these observations that the mesoderm is subdivided into populations that differ in their requirement for cooperation between the Oep and Fgf8 pathways. Most of the dorsal mesoderm undergoes massive cell death by the end of gastrulation in Z*oep;ace* mutants and this aspect of the double-mutant phenotype is fully penetrant. More lateral mesoderm cells, although somewhat scattered on the yolk syncytium, displayed a normal morphology by the end of gastrulation (Fig. 4I).

Cooperation between *oep* and *ace* is required before the onset of gastrulation to maintain axial mesoderm fate

As described above, the dorsal mesoderm underwent massive cell death by the end of gastrulation in Z*oep;ace* mutants. However, some cells were able to contribute to anterior notochord and somites and we hypothesized that they expressed mesoderm markers. To investigate this, we analyzed the expression of the axial and adaxial mesoderm markers *ntl* and *myoD*, respectively, at the end of gastrulation (Fig. 5A,B). In the double mutant, *myoD* was undetectable by this stage (Fig. 5B). Staining of *ntl* was detected at the blastoderm margin and was very limited and scattered in the embryonic axis (Fig. 5B). In addition, we found that the anterior limit of *ntl* staining along the rostro-caudal axis did not reflect the hypoblast involution that occurred in the double mutants. The hypoblast involution was assessed in wild-type and Z*oep;ace* embryos by labelling cells in the embryonic shield. This was achieved by locally uncaging caged-fluorescein injected at the one-cell stage in the progeny of double-heterozygous fish. By the end of gastrulation, treated embryos were stained with *ntl* and uncaged fluorescein was revealed by immunocytochemistry (Fig. 5C,D). From the observation of the cell tracer, we concluded that the rostro-caudal extension of the axial hypoblast in the double-mutants (Fig. 5D) was significantly less than in wild-type (Fig. 5C) and single mutants (data not shown). However, the defects in involution do not readily explain the loss of *ntl* and *myoD* expression in the hypoblast of Z*oep;ace* mutants. Rather, we hypothesize that Oep and Fgf8 cooperate in maintaining dorsal mesodermal markers.

We further investigated the timing of a putative cooperation between Oep and Fgf to maintain *ntl* expression earlier during gastrulation by treating the progeny of Z*oep* heterozygous fish with SU5402 and by varying time windows of exposure to the drug. Our experiments revealed a crucial period between sphere stage and 50% epiboly (onset of gastrulation) during which intact Fgf signalling is required for Z*oep* embryos to retain a normal *ntl*-expression domain, whereas wild-type embryos are essentially unaffected by this specific treatment (Fig. 5E-H).

The phenotype of Z*oep;ace* mutants reveals a cooperation between Oep and Fgf8 pathways differentially affecting the morphogenesis of various mesodermal territories. In particular, we conclude that the axial hypoblast requires cooperation between Oep and Fgf8 before the onset of gastrulation to maintain the expression of the notochord marker *ntl* and insure the survival of dorsal mesodermal cells.

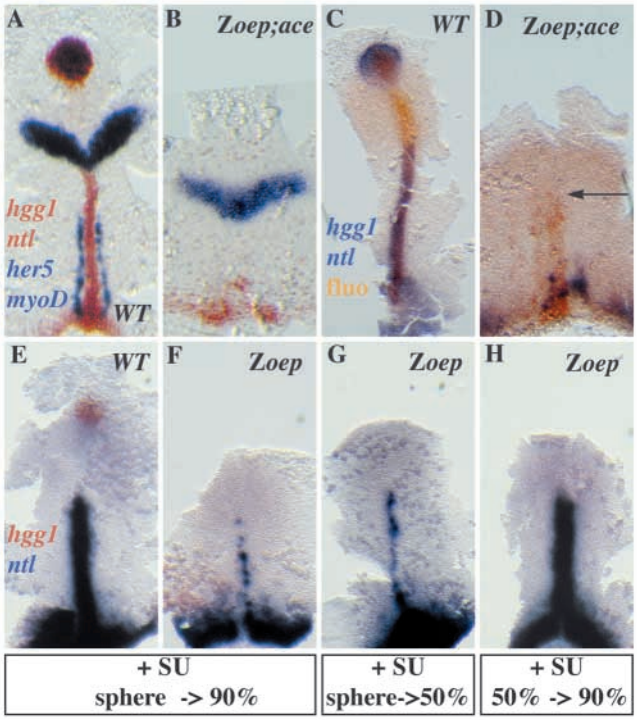

Fig. 5. Cooperation between *oep* and *fgf* is required before the onset of gastrulation for the maintenance of dorsal mesoderm. (A-D) Flat mounts of embryos taken from the progeny of Z*oep;ace* double-heterozygous parents (the genotype, which is inferred from the phenotype and statistical analysis, is indicated top right). (A-B) Embryos at 90% epiboly, *ntl* staining occurs at the notochord and presumptive mesoderm at the margin of the blastoderm and *hgg1* staining at the presumptive hatching gland (red), *myoD* staining labels adaxial cells, and *her5* staining the presumptive midbrain-hindbrain boundary (blue). (C-D) Embryos injected with caged dextran-fluorescein at the one-cell stage, laser irradiated at the shield stage at the level of the embryonic shield, fixed and stained at the end of gastrulation for *ntl* and *hgg1* (blue) and fluorescein (brown). Arrow points to the anterior limit of migration of the labelled cells in D. (E-H) Flat mounts of embryos taken from the progeny of *oep* heterozygous parents (the genotype, inferred from the phenotype and statistical analysis, is indicated top right), treated with 10 μM SU5402 for various periods of time (as shown), fixed at 90% epiboly and stained for *ntl* (blue) and *hgg1* (red). Wild-type embryos appear to be unaffected by SU5402 treatment.

Cooperation between *oep* and *fgf8* acts differentially on the induction of mesoderm markers but is mainly required for the maintenance of mesodermal fates

The above results did not address whether Oep and Fgf8 act cooperatively in the initial phase of mesoderm induction. To investigate this, we analyzed the expression of the T-box mesoderm markers *ntl*, *tbx6* and spadetail (*spt)* before the onset of gastrulation in Z*oep;ace* mutants (Fig. 6). No difference was seen between wild-type and single mutants, but the latter differed slightly from Z*oep;ace* double mutants in the levels of expression of *ntl* at 30% epiboly (Fig. 6A,G) and of *tbx6* at 30% epiboly and shield stage (Fig. 6C,I,F,L). By contrast, the expression of *spt* was not obviously affected at either stage (Fig. 6D,E).

Correlating with their overall normal morphology (Fig. 4I

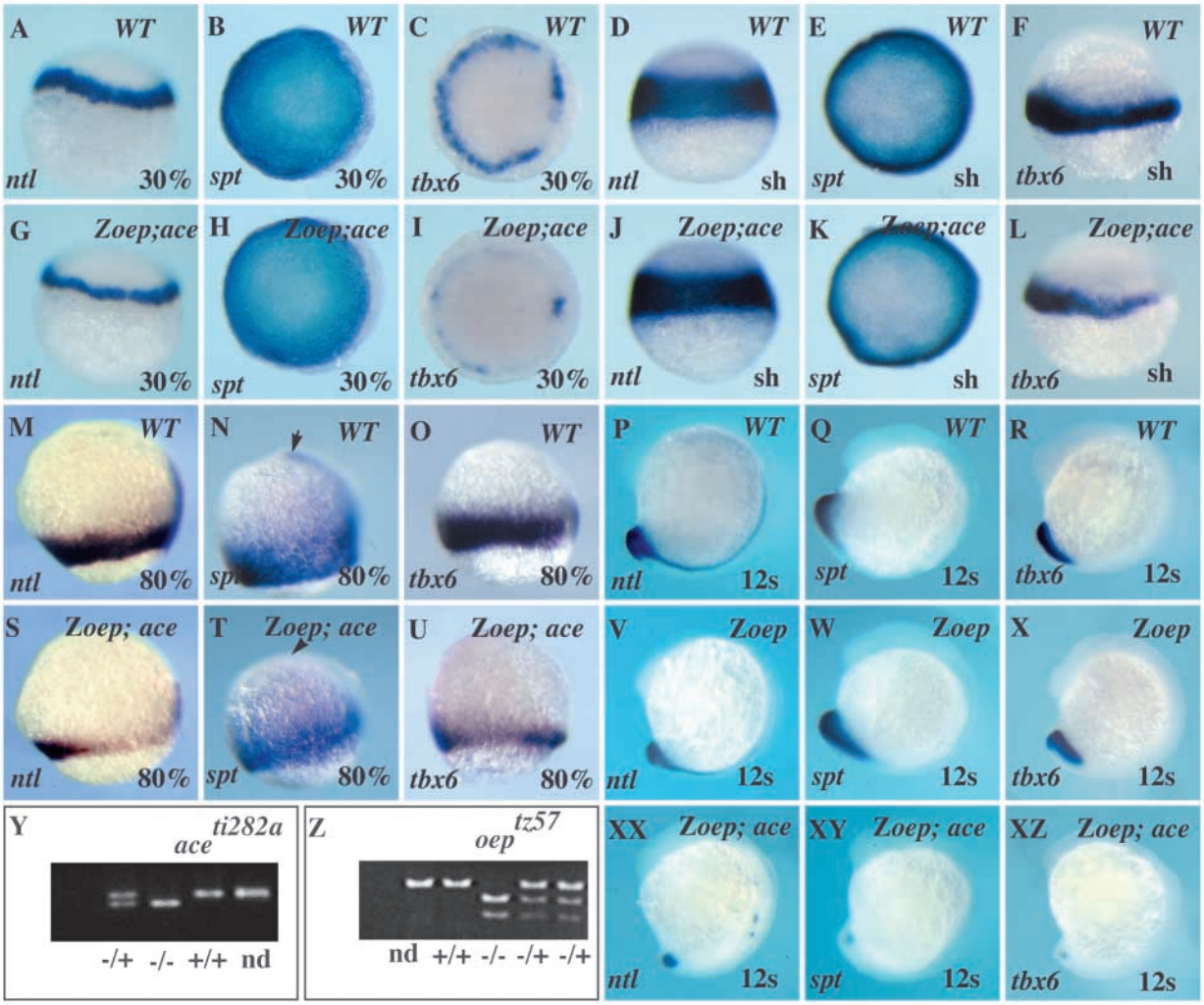

Fig. 6. T-box genes expression is down regulated in Z*oep;ace* mutants. (A-X,XX-XZ) All mount, in situ hybridization of embryos were taken from the progeny of double-heterozygous parents. The genotype (indicated top right) is inferred from either the phenotype (P-R,V-X,XX-XZ) or PCR-based genotyping. The embryonic stage is shown at bottom right (30%, 30% epiboly; sh, shield; 80%, 80% epiboly; 12s, 12 somites) and the probe (*ntl*, *tbx6* and *spt* in blue) is indicated at bottom left. (A,G) Lateral views. (D,F,J,L,M-X,XX-XZ) Lateral views, dorsal to the right. (B,C,E,H,I,K) Animal pole views. (N,T) Arrowhead points to the prechordal plate, which is stained in N and lost in T. Note that the double-mutant phenotype is otherwise similar to wild-type. (Y-Z) PCR-based genotyping of embryos after in situ hybridization. Digested and nondigested (nd) PCR products were run on a 1.5% agarose gel.

and data not shown), lateral mesoderm cells expressed the T-box markers *ntl*, *tbx6* and *spt* (Kimelman and Griffin, 2000) during gastrulation in Z*oep;ace* mutants. However, their expression domains were markedly reduced in the double mutants at 80% epiboly compared to wild-type or single mutant embryos (Fig. 6M-O,S-U). At the 12-somite stage, very faint expression of the three markers was retained in the protruding tail bud of the double mutants (Fig. 6V-X,XX-XZ).

These observations indicate that *oep* and *fgf8* act synergistically on the maintenance of all the mesoderm markers tested. Their combined action on induction of T-box genes is rather subtle but suggests a differential regulation with no effect on *spt* expression, a delay in the amplification of *ntl* and a downregulation of *tbx6* that can be detected at all stages.

However, the induction of mesoderm still occurs in the absence of the zygotic contribution of *oep* and *fgf8*. We hypothesized that Oep maternal contribution rescued earlier aspects of the formation of mesoderm. To assess this, we investigated the phenotype of MZ*oep;ace* embryos. By 30 hours of development they were devoid of somites in the tail (Fig. 7B) unlike MZ*oep* mutants (Fig. 7A), confirming that Oep and Fgf8 also cooperate in the maintenance of the presumptive tail mesoderm and consistent with our observation that *fgf8* is expressed in the posterior mesoderm during gastrulation (Fig. 2R).

Regarding early steps of mesoderm formation, *ntl* expression was diminished by 30% epiboly in MZ*oep;ace* embryos compared to MZ*oep* siblings (Fig. 7C,H). Although the difference was stronger than that observed at the same stage comparing Z*oep;ace* embryos and their wild-type siblings (Fig. 6), the enhanced effect of MZ*oep;ace* on *ntl* was not detected by the shield stage (Fig. 7D,I). Expression of *tbx6* was highly variable in MZ*oep* mutants throughout epiboly

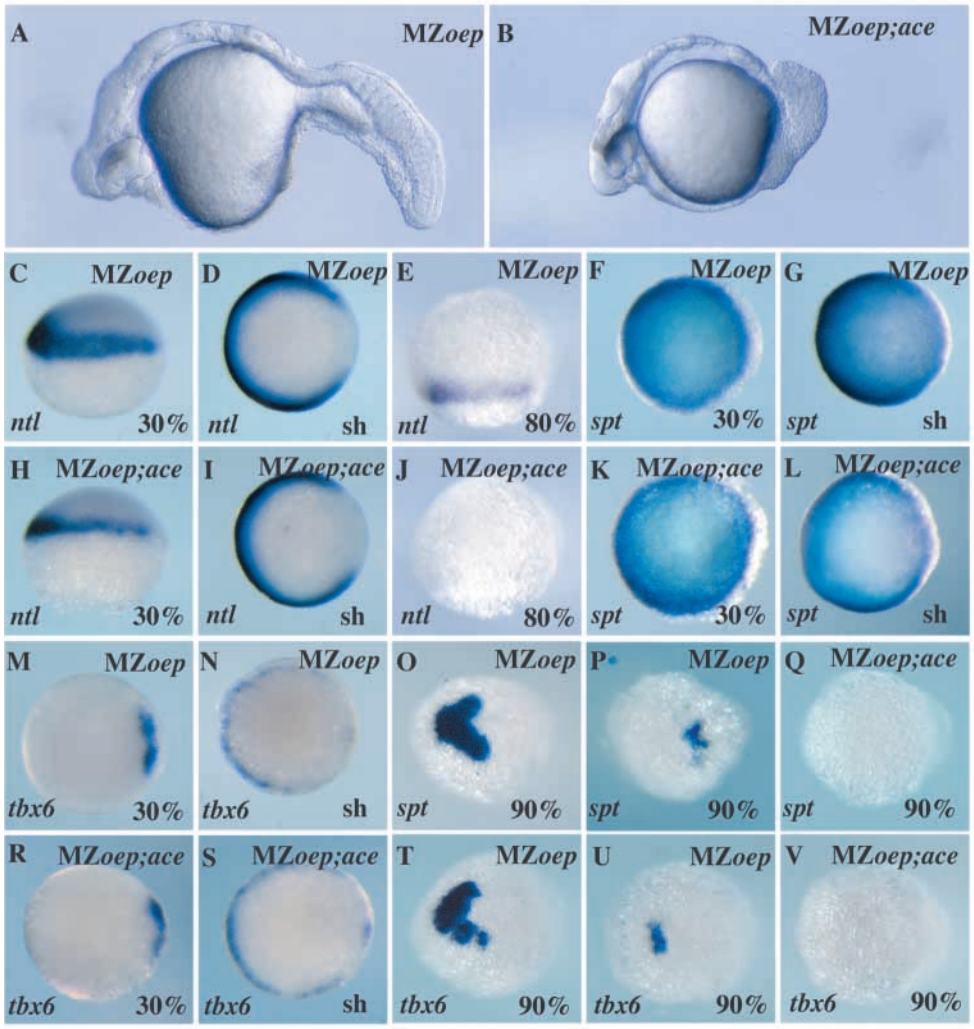

Fig. 7. Posterior mesoderm is still induced but not maintained in MZ*oep;ace* mutants. (A-B) Live embryos at 30 hours of development taken from the progeny of *oep*$^{-/-}$;*ace*$^{-/+}$ parents (genotype top right). (C-V) Whole mount in situ hybridization of embryos taken from the progeny of *oep*$^{-/-}$;*ace*$^{-/+}$ parents and *oep*$^{-/-}$ parents (genotype top right). MZ*oep;ace* embryos were genotyped for the *ace* allele. We systematically compared the phenotypes of the progeny of *oep*$^{-/-}$;*ace*$^{-/+}$ parents with those of the progeny of *oep*$^{-/-}$ parents. The stage is shown bottom right and the probe is indicated bottom left. Dorsal is to the right. Lateral views of embryos (C,E,H,J). Animal-pole views (D,F,G,I,K-N,R,S). Vegetal-pole views (O-Q,T-V).

(Fig. 7M,N and data not shown), and removing zygotic *ace* activity did not obviously affect this aspect of the mesodermal phenotype at earlier stages (Fig. 7R,S and data not shown). The strong, ubiquitous expression of *spt* prior gastrulation did not allow us to distinguish differences between MZ*oep* mutants and MZ*oep;ace* siblings (Fig. 7F,K). By the onset of gastrulation, we found that *spt* was expressed at slightly lower levels in MZ*oep;ace* mutants compared to MZ*oep* embryos (Fig. 7G,L). In summary, analysis of the expression of T-box genes in MZ*oep;ace* mutants at the end of gastrulation reinforced our conclusion that cooperation between *oep* and *fgf8* acts mainly to maintain mesodermal fates rather than initiate mesoderm induction. Indeed, expression of *ntl*, *spt* and *tbx6* was abolished by 90% epiboly in MZ*oep;ace* mutants (Fig. 7J,Q,V).

Discussion

Several transduction pathways operate during early embryogenesis to generate cell diversity and coordinate cell behaviours. In particular, Activin/Nodal and Fgf are involved in the formation of mesoderm. It has been proposed that Fgf signalling renders cells competent to respond to Activin (Cornell and Kimelman, 1994; Labonne and Whitman 1994), but the underlying mechanisms have remained elusive. Here we propose a mechanism accounting for this aspect of the interaction between Fgf and Nodal pathways. In our model, Fgf serves as a relay downstream of Nodal, which regulates the expression of *oep*. Such a process is involved in our transplantation experiments and is also likely to act in normal embryos. This would mean that Fgf signalling is required for the maintenance, amplification and cell-to-cell propagation of the Nodal pathway as part of its positive-regulatory loop. In addition, a parallel can be made between the mesendoderm-mesoderm interaction and chimeric embryos where *tar**-expressing cells act on their neighbours. Indeed, cells that express tar* are fated to mesendoderm (Peyrieras et al., 1998; Mathieu et al., 2002) and are likely to mimic some of its properties (Rodaway and Patient, 2001). This is illustrated by the requirement for Fgf and Nodal signalling as relays for the cell-nonautonomous induction by Tar*-expressing cells of *oep* and *tar* respectively that parallels the simultaneous requirement for Fgf and Nodal signalling in the mesoderm proper. Such a model is consistent with the phenotypic defects observed in *oep* and *ace* single and double mutants, which show that Nodal and Fgf8 act synergistically in mesoderm formation. We further demonstrate that mesodermal cell populations differ in their requirement for the cooperation between *oep* and *fgf8*, indicating regional differences in the dynamics of the Nodal network.

Fgf is required for the amplification and propagation of Nodal signalling through a positive regulatory loop

Our overexpression data indicates a model in which Fgf

signalling is required both cell-autonomously and cell-nonautonomously for the activation of *oep* expression downstream of Tar*/Nodal. In this model, the mechanisms and dynamics of the positive regulation loop of the Nodal pathway depend on the role of Oep. Analysis of zebrafish and mouse mutants indicates that this EGF-CFC co-factor is essential for Nodal function during early embryogenesis (Whitman, 2001). However, it has been suggested recently that Nodal signalling might function in the absence of EGF-CFC co-factors (Reissmann et al., 2001). Such a hypothesis is not supported by our transplantation experiments. Indeed, cells that expressed *sqt* had no detectable inducing activity in the MZ*oep* background. In any case, the cell-nonautonomous induction of both *tar* and *oep* by Tar*-expressing cells indicates that Nodal signalling might propagate to the neighbouring cells via the secreted ligand Sqt because the latter can act 10 cells away from its source (Chen and Schier, 2001). However, Tar*-expressing cells induce the expression of *cyc* and *sqt* cell-autonomously only, indicating that the activity of Nodal signalling in neighbouring cells is modulated, and that the propagation of Nodal signalling is restricted. This could result from the negative regulation of the pathway. Indeed, the Nodal antagonist Antivin is induced downstream of Tar*/Nodal (Meno et al., 1999; Thisse and Thisse, 1999; Hyde and Old, 2000) and has been shown to limit the range of action of Sqt (Meno et al., 2001).

We assessed the potential role of Fgf signalling downstream of Nodal by using SU5402, inhibitor of the Fgf pathway (Mohammadi et al., 1997). Any Fgf molecules present between the sphere stage and 50% epiboly at the margin of the blastoderm in the mesodermal precursors could, potentially, account for the drug effects. Although we have reasons to implicate Fgf8, the high doses of SU5402 required to inhibit *oep* expression at the onset of gastrulation indicate contributions by other Fgfs. We explored the possibility of a combined role of FGF3 and FGF8 by using specific morpholinos (Maroon et al., 2002) and concluded that Fgf3 and Fgf8 account for at least part of the Fgf activity in early steps of mesoderm formation, but that other, as yet unidentified, Fgfs might be involved.

Oep/Nodal and Fgf8 signalling pathways act synergistically and their cooperation differentially affects the expression of mesoderm-marker genes

We discussed above a role for Fgf downstream of Nodal and suggested that *tar*, *fgf* and *oep* are connected directly through a cascade of gene activation. Several mechanisms operating at the genetic and epigenetic level might account for a strict requirement of *fgf8* downstream of, or in parallel to *oep*. By contrast, analysis of the zebrafish *oep* and *ace* single and double-mutant phenotypes demonstrates that Oep and Fgf8 cooperate in the formation of mesoderm. This means that both pathways also act in parallel, but in a redundant way rather than with a strict requirement for each other. We suggest that both types of interaction between *oep* and *fgf8* coexist within the *nodal/fgf* molecular network. Cooperation between Nodal and Fgf pathways might be achieved at the genetic level, through the interaction of their downstream components with the regulatory sequences of common target genes. This might happen for the regulation of the T-box genes. This hypothesis fits with our expression data in the Z*oep* and *ace* single and double-mutant backgrounds.

Analysis of single and double mutants of *oep* and *ace* indicates that several aspects of mesoderm formation depend on the Nodal/Fgf8 cooperation. However, the latter is not crucial for induction of mesoderm and initiation of the expression of the T-box genes *ntl*, *spt* and *tbx6*, although it does modulate their level of expression, probably through an amplification step. However, the Nodal/Fgf8 interaction is essential for the maintenance of mesodermal cell populations. This is shown by the loss of T-box gene expression during somitogenesis in Z*oep;ace* mutants, and even earlier in MZ*oep;ace* mutants. The timing of *oep* and *fgf8* interaction, determined from our SU5402 incubation experiments, reveals an early requirement, which correlates with the later maintenance of *ntl* expression in the embryonic axis. It fits with the detection of *fgf8* RNA at the margin of the blastoderm before gastrulation. Later, *fgf8* staining is excluded from the embryonic axis during gastrulation and retained in the presomitic mesoderm. At these stages, the Oep-Fgf8 cooperation might occur at the blastoderm margin where both are expressed, and act on the maintenance of nonaxial mesoderm.

Z*oep;ace* and *oep;ntl* double mutants have a dramatic deficit in the formation of mesoderm (Schier et al., 1997). The similarities between the phenotypes of the two mutants indicate that *ntl* has a major role downstream of *fgf8* and probably also parallel to it through a positive regulatory loop, as shown for eFgf and Xbra (Cornell and Kimelman, 1994; Labonne and Whitman, 1994; Schulte-Merker et al., 1994; Labonne et al., 1995). This also fits with the hypothesis that the T-box genes *ntl*, *spt* and *tbx6* are interconnected, either as common targets of *oep* and *fgf8*, or because they interact at the genetic level to regulate each others expression (Kimelman and Griffin, 2000).

Expression of *myoD* reveals other aspects of the cooperation between Oep and Fgf8

As in *ntl* mutants, the early phase of *myoD* expression in the adaxial cells (Weinberg et al., 1996), which extends from midgastrula to prior to somite formation, is abolished on combined zygotic reduction of function of *oep* and *ace*. However, *myoD* expression occurs at later stages in Z*oep;ace* mutants. Notably, by one day of development the *myoD*-expression domain reveals a transition in the trunk mesoderm at the 6–8-somite level. This rostro-caudal transition is distinct from the trunk-tail transition at the level of somite 18, which has been discussed previously (Kimelman and Griffin, 2000), and is reminiscent of that described in mouse embryos (Soriano, 1997). Recently, it has been suggested (Holley et al., 2002) that in both mouse and zebrafish mutants affected in the formation of somites show an anterior to posterior polarity in phenotype, with anterior regions less severely affected than posterior regions. This rostro-caudal transition may reflect profound morphogenetic differences between the anterior and posterior somites. The first six somites are known to form more rapidly and display a more synchronous morphogenesis than the posterior somites and it has been suggested that their formation might be independent of oscillating gene expression (Holley et al., 2002). Here, we suggest that this rostro-caudal transition also affects the axial mesoderm because the notochord does not form at more posterior rostro-caudal levels in Z*oep;ace* mutant embryos.

Differential requirement for the synergy between *ace* and *oep* defines distinct mesodermal territories

Our data point to regional differences in the behaviour of mesodermal cells that correlate with their requirement for the cooperation between *oep* and *ace*. The fate of lateral mesoderm is maintained until mid-somitogenesis in the absence of *oep* and *fgf8* zygotic components, but it is disrupted during gastrulation when maternal *oep* function is also removed.

Except for a few cells that either form notochord or undergo somite condensation and myogenic differentiation, cells of the dorsal mesoderm require cooperation between zygotic *oep* and *fgf8* before the onset of gastrulation in order to survive. Indeed, we observed that in a domain that probably encompasses the notochord and paraxial mesoderm territories, cells undergo a dramatic morphological change at the time of epiboly completion. Our Annexin V binding data indicates that these hypoblastic cells might undergo an apoptotic process with the exposure of phosphatidyl-serine on the outer lipid leaflet of the cell membrane. The link between this cell behaviour and disruption of the Oep and Fgf8 pathways remains to be elucidated. Subsequent morphogenesis in the Z*oep;ace* mutants is profoundly affected by the presence of this mass of dying cells, even though the yolk cell can engulf numerous cell corpses.

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