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Interplay between FGF, *one-eyed pinhead,* and T-box transcription factors during zebrafish posterior development

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Abstract

The zebrafish T-box transcription factors *spadetail (spt)* and the *brachyury* ortholog *no tail (ntl)* are together essential for posterior mesoderm formation. In addition to being functionally redundant, *spt* and *ntl* also genetically interact with zygotic mutant alleles of *one-eyed pinhead (Zoep),* leading to synergistic mesodermal defects. Here we have used genetic and pharmacological assays to address the mechanism of these interactions. We show that *Zoep* and *ntl* are together required upstream of *spt* expression, accounting for the severity of the mesodermal defects in *Zoep;ntl* embryos. Since *Xenopus brachyury* is proposed to regulate *fgf* expression, and FGF signaling is required for *spt* expression, we analyzed the involvement of the FGF signaling pathway in these genetic interactions. Using a specific inhibitor of FGFR activity to indirectly assay the strength of FGF signaling in individual embryos, we found that *spt* and *ntl* mutant embryos were both hypersensitive to the FGFR inhibitor. This hypersensitivity is consistent with the possibility that Spt and Ntl function upstream of FGF signaling. Furthermore, we show that minor pharmacological or genetic perturbations in FGF signaling are sufficient to dramatically enhance the Zoep mutant phenotype, providing a plausible explanation for why *Zoep* genetically interacts with *spt* and *ntl*. Finally, we show that *Zoep* and *ace/fgf8* function are essential for the formation of all posterior tissues, including spinal cord. Taken together, our data provide strong in vivo support for the regulation of FGF signaling by T-box transcription factors, and the cooperative activity of Oep and FGF signaling during the formation of posterior structures.
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Keywords: Spadetail; Brachyury; No tail; EGF-CFC factors; Mesoderm formation

Introduction

The development of posterior structures (spinal cord, somites) in vertebrates involves the spatially and temporally controlled differentiation of small populations of multipotent progenitors, and is dependent upon FGF signaling. Inhibition of FGF signaling using a dominant negative FGF receptor (FGFR) prevents the formation of posterior mesoderm, and such embryos develop without posterior structures (Amaya et al., 1991; Griffin et al., 1995). Although these studies illustrate the requirement for FGF signaling in this process, it is not clear from them whether FGF signaling is required for the formation, maintenance, or behavior of posterior progenitors. A study of chick spinal cord development indicates that one important role of FGF signaling is to inhibit progenitor differentiation, thereby maintaining a population of stem cell-like cells (Mathis et al., 2001).

Genetic and molecular interventions have attempted to identify factors acting downstream of FGF in the mesodermal progenitor population, and have shown that at least one important role of FGF signaling is to regulate expression of certain T-box transcription factors (Amaya et al., 1993; Isaacs et al., 1994; Griffin et al., 1995, 1998; Schulte-Merker and Smith, 1995; Ruvinsky et al., 1998). In zebrafish, the T-box transcription factors *spadetail/tbx16 (spt)* and *no tail (ntl;* the ortholog of murine *brachyury)* are extensively coexpressed in mesodermal progenitors (Schulte-Merker et al., 1992; Griffin et al., 1998; Amacher et al., 2002), and their expression is lost when FGF signal-

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ing is blocked using the dominant negative FGFR (Griffin et al., 1995, 1998). *Spt* and *ntl* are together essential for the formation and/or maintenance of posterior mesodermal progenitors (Amacher et al., 2002). *Spt;ntl* double mutants do not form any posterior mesodermal derivatives, and closely resemble embryos in which FGFR signaling has been inhibited. In contrast, *spt* and *ntl* single mutant embryos do not show this severe phenotype, indicating that these factors are functionally redundant (Kimmel et al., 1989; Halpern et al., 1993). *Spt* and *ntl* mutants do, however, have more specific defects, affecting either trunk somitogenesis, or tail and notochord formation, respectively.

In addition to T-box transcription factors acting downstream of FGF signaling, evidence from *Xenopus* suggests that they may also act upstream of *fgf* ligand expression (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). It has been proposed that expression of *Xenopus brachyury (Xbra)* is regulated by an indirect autoregulatory loop involving FGF signaling. Simply, FGF signaling maintains *Xbra* expression and Xbra in turn activates *eFGF* expression. Consistent with this, a consensus T-box binding site is present in the *eFGF* promoter (Casey et al., 1998), and *eFGF* is coexpressed with *Xbra* (Isaacs et al., 1994). Despite the proposed tight linkage between T-box transcription factor function and the FGF pathway, this relationship has not yet been adequately tested using a genetic approach. Furthermore, there is genetic evidence that the situation may not be so straightforward. For example, the simplest interpretation of the "T-box → FGF → T-box" autoregulatory model predicts that *ntl/brachyury* expression should depend upon Ntl/Brachyury function. However, this is not the case either in zebrafish (Schulte-Merker et al., 1994a) or mouse (Schmidt et al., 1997), with the exception of expression in notochord progenitors. This indicates that either additional factors maintain FGF signaling in the absence of Ntl function, and/or that the regulation of *ntl* expression is more complex and involves additional signaling pathways.

In addition to the FGF pathway, the function of One-Eyed Pinhead (Oep) is also intricately associated with Spt and Ntl function. Oep, an extracellular EGF-CFC factor, is maternally as well as zygotically expressed. Due to the presence of maternal *oep,* zygotic *oep* mutant alleles *(Zoep)* merely attenuate Oep-dependent signaling and directly cause only endoderm and prechordal mesoderm defects. However, *Zoep* dramatically enhances the *spt* and *ntl* single mutant phenotypes (Schier et al, 1997; Griffin and Kimelman, 2002). Whereas *ntl* single mutants form trunk somites and blood, *Zoep;ntl* double mutant embryos fail to form blood, and somites are almost completely absent (Schier et al., 1997). Similarly, whereas *spt* single mutant embryos have reduced and disorganized trunk paraxial mesoderm, *Zoep;spt* double mutant embryos form no paraxial mesoderm whatsoever and have an unexpected midline progenitor defect (Griffin and Kimelman, 2002). Although the mechanisms of these genetic interactions are unclear, they nevertheless provide a glimpse into the genetic complexity of posterior mesoderm formation.

Here we have addressed the relationship between *oep,* FGF signaling, and the T-box transcription factors *spt* and *ntl*. We show that *Zoep* and *ntl* are together required to maintain expression of *spt,* and propose that the loss of Spt function is sufficient to account for the synergistic mesodermal deficiencies observed in *Zoep;ntl* embryos. Using a pharmacological inhibitor to manipulate FGFR signaling in different mutant backgrounds, we show that *spt* and *ntl* embryos have an apparent deficit in FGF signaling, consistent with these factors acting upstream of FGF ligand expression. In addition, reduction in FGFR signaling in *spt* and *ntl* may cause their genetic interactions with *Zoep,* since we show that *oep* and FGF signaling act synergistically in vivo, and are together required for the formation of posterior structures. These data provide insights into the genetic complexity of posterior mesoderm formation, and are suggestive of critical molecular interactions among tissues in the tail bud.

Methods

In situ hybridization and antibody staining

Whole mount in situ hybridization and antibody staining was performed as previously described (Griffin et al., 1998). Digoxygenin-labeled (Boehringer) RNA probes were prepared as previously described: *spt* (Griffin et al., 1998), *myoD* (Weinberg et al., 1996), *pax2.1* (Lun and Brand, 1998), and *flk-1* (Liao et al., 1997). MF20 antibody recognizes an epitope in myosin (Shimizu et al., 1985); MF20 supernatant was used at 1:50, and visualized with HRP-conjugated goat anti-mouse I (Sigma) secondary antibodies; HRP was visualized using DAB and standard reaction conditions (Westerfield, 1995).

Fish maintenance and mutant alleles

Wild-type fish were AB strain. All mutant strains were kept as heterozygous adults identified by random crossing, and were maintained by out-crossing to AB fish. Double-mutant lines were obtained by intercrossing heterozygous adults; doubly heterozygous F1 progeny were identified by random crosses. The following allele combinations were used: $Zoep^{in134}$ ntl^{b195}, $Zoep^{in134}$; ace^{ti282a}, $Zoep^{tz57}$; ace^{ti282a}. The *Zoep;ace* genotypes yielded similar phenotypes at 24 hpf; however, subsequent analysis was performed only using $Zoep^{m134}$; ace^{ti282a}.

SU5402 treatment

SU5402 (Mohammadi et al., 1997; Calbiochem) was dissolved in DMSO and stored at −80°C; prior to addition to embryos, stock SU5402 was diluted in embryo rearing

medium (1 × ocean salts). Embryos in their chorions were treated with SU5402 from the mid or late gastrulation stage and cultured overnight in the presence of the drug. Treatments were performed in 24-well plates, 20–25 embryos per well in 0.5 ml of medium. All experiments were performed at least twice with multiple concentrations of SU5402. Experiments with mutant embryos were performed as follows. Embryos were collected from heterozygous adults and divided into pools of 20–25. One pool was left untreated, to confirm the presence and proportion of homozygous mutant embryos. No effects were observed by exposure to DMSO vehicle alone. Treated embryos were collected at 24 h post fertilization, fixed in 4% paraformaldehyde in PBS, and processed for immunohistochemistry or in situ hybridization. Morphological criteria were used to determine the effects of SU5402 on homozygous *Zoep* and *spt* mutant embryos. In addition, SU5402-treated *ace* mutant embryos were also genotyped following photography. Individual embryos were digested with 0.5 mg/ml proteinase K in 0.1 M Tris, pH 8.0, 0.1% Triton X-100, and used as a template for PCR amplification using primers spanning the mutant base. The genotype was determined by direct sequencing of the PCR product.

Results

Zoep and ntl are together required to maintain expression of spt

Genetic analysis has shown that the zebrafish T-box transcription *no tail (ntl),* the ortholog of murine *brachyury,* is required for notochord and tail mesoderm formation, although it is expressed transiently by all mesodermal progenitors (Halpern et al., 1993; Schulte-Merker et al., 1994b). Zygotic *oep (Zoep)* is required for endoderm and prechordal mesoderm formation, but posterior mesoderm formation is remarkably normal (Schier et al., 1997; Gritsman et al., 2000). In contrast, embryos doubly mutant for *Zoep* and *ntl* have profound deficiencies in trunk paraxial mesoderm and blood, demonstrating that there is a combinatorial requirement for these factors in certain mesodermal tissues (Schier et al., 1997). We were interested in understanding why *Zoep* and *ntl* genetically interact. We first analyzed *Zoep;ntl* embryos for mesodermal derivatives that were not examined by Schier et al., (1997). We observed that myosin-expressing cardiac mesoderm was easily detected (Fig. 1E), as were vascular endothelial progenitors (Fig. 1D), which were extremely disorganized and may even be substantially increased in number. Thus only a specific subset of mesodermal tissues (blood, paraxial mesoderm) are defective in *Zoep;ntl* embryos.

Trunk paraxial mesoderm and blood, but not vascular endothelium, are both tissues that depend upon of the T-box transcription factor Spadetail (Spt; Kimmel et al., 1989; Thompson et al., 1998; Griffin and Kimelman, 2002). We therefore characterized *spt* expression in embryos derived from an intercross of *Zoep;ntl*/+ adults to ascertain if Spt was involved in the *Zoep;ntl* mesodermal defects (Fig. 2). Prior to the onset of gastrulation, *spt* expression appeared normal in all embryos from such a cross, indicating that the initiation of *spt* expression occurred normally in *Zoep;ntl* embryos (data not shown). However, after the onset of gastrulation (6.5 h, 60% epiboly), *Zoep, ntl,* and *Zoep;ntl* mutant embryos could be distinguished based on alterations in the expression of *spt*. In *ntl* single mutant embryos, *spt* expression was weak in cells adjacent to the notochord progenitors, as previously reported (Fig. 2B; Griffin et al., 1998), whereas in *Zoep* mutant embryos *spt* was undetectable in the migrating prechordal plate progenitors (Fig. 2C and E; confirmed using embryos derived from an *Zoep*/+ intercross, data not shown). *Zoep;ntl* mutant embryos were identifiable by additive changes in *spt* expression (Fig. 2D and F). Beginning at midgastrulation (8 h post fertilization, hpf), however, *spt* expression in *Zoep;ntl* mutant embryos began to decline (data not shown) and, by the end of gastrulation, was barely detectable (10 hpf; Fig. 2H). This indicates that *Zoep* and *ntl* are together required to maintain *spt* expression during the formation of trunk mesoderm. To determine if this was solely an effect of Nodal signaling we compared *spt* expression in *Zoep;ntl* embryos with *cyc;sqt* double mutant embryos (Fig. 2I). At 10 hpf (bud stage), *spt* continued to be expressed at high levels in the tail bud of *cyc;sqt* double mutant embryos. Similar results were obtained with *MZoep* embryos (not shown). This demonstrated that *spt* expression is not exclusively regulated by Nodal signaling but also depended upon Ntl function. Furthermore, it demonstrates that the loss of *spt* expression in *Zoep;ntl* embryos was not simply due to defective Nodal signaling. Similar results were obtained with *MZoep* embryos (not shown). Since the presence of Spt protein correlates well with the distribution of *spt* mRNA (Amacher et al., 2002), the decline in *spt* mRNA presumably represents a late-onset loss of Spt function. The early decline in *spt* expression in *Zoep;ntl* embryos is therefore sufficient to account for the deficits in paraxial mesoderm and blood.

Inhibition of FGFR signaling with SU5402 causes developmental defects

Xenopus T-box transcription factors are implicated in an autoregulatory loop via eFGF signaling (Isaacs et al., 1994; Schulte-Merker and Smith, 1995; Casey et al., 1998). If *spt* and *ntl* also autoregulate via FGF signaling, then *spt* or *ntl* mutant embryos might have decreased FGFR activity, which in turn may play an important role in the genetic interaction with *oep*. To test this, we needed a sensitive assay to compare the levels of FGFR signaling found in individual wild-type or mutant embryos. Preliminary experiments using whole mount antibody staining to detect phosphorylated MAP kinase (Shinya et al., 2001), or whole mount in situ hybridization to detect the FGF-regulated

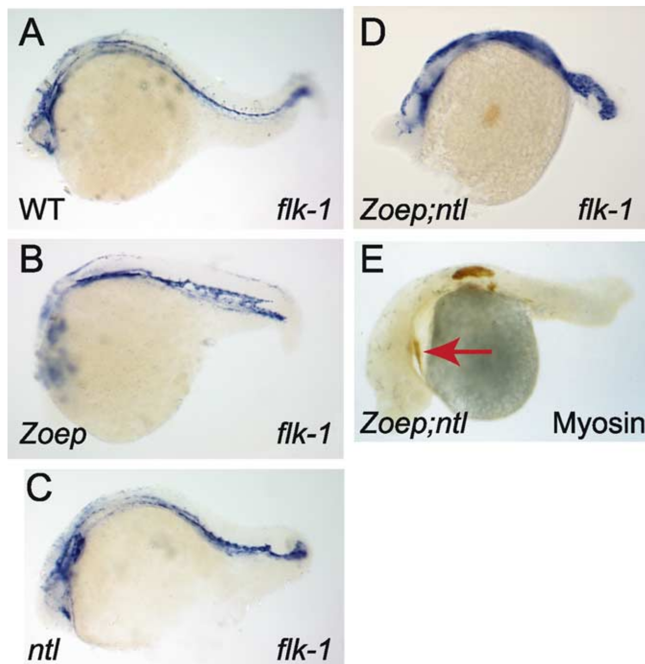

Fig. 1. *Zoep* and *ntl* are not required for the formation of cardiac mesoderm or vascular endothelium. Anterior, left; dorsal, uppermost; genotypes as indicated (bottom left) (A–D) *flk-1* expression. Note the presence of *flk-1* expressing cells in the *Zoep;ntl* embryo, which are disorganized and possibly more numerous than in wild-type or either single mutant embryos. (E) Myosin staining (brown) detects the cardiac primordium (arrow), as well as somitic tissue in the anterior trunk, as previously reported.

genes *pea3* and *erm* (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001), did not reveal any major changes between wild-type and embryos injected with an *ntl* morpholino (data not shown), but these techniques may not be sensitive enough to detect small changes. We therefore developed an alternate method based on a pharmacological challenge, which is more specific to FGFR signaling than phosphorylated MAP kinase staining, and more sensitive than in situ hybridization or antibody staining.

SU5402 is a specific, dose-dependent inhibitor of FGFR signaling in cell culture, but does not appreciably inhibit other tyrosine kinase receptors at doses of up to 100 μM (Mohammadi et al., 1997). We characterized the effects of increasing concentrations of SU5402 added to embryos at the mid or late gastrulation stage on the development of wild-type zebrafish embryos. SU5402 induced defects in cerebellum and posterior development, both of which are known to depend upon FGF signaling (Griffin et al., 1998; Reifers et al., 1998). An acerebellar phenotype was typically observed at 8 μM SU5402 (data not shown), whereas significant tail mesoderm defects began to be observed at 15 μM, and trunk mesoderm defects at higher doses still (Fig. 3A–D). Defects were also observed in neuroectodermal derivatives such as the retina at higher concentrations, but these were not specifically characterized (data not shown). Embryos treated with 30 μM SU5402 formed only anterior trunk paraxial mesoderm (Fig. 3D) and are similar to embryos expressing the dominant negative FGF receptor (Griffin et al., 1995). Some variability in the dose-response was observed using different strains (data not shown). Since at least some *myoD* and myosin-positive cells were detected in embryos treated with 30 μM SU5402, the posterior defects observed at this and lower concentrations of SU5402 are unlikely to be caused by inhibition of muscle terminal differentiation, but rather by inhibition of critical events earlier in the mesoderm formation pathway. These defects are consistent with SU5402 acting as a specific inhibitor of FGFR signaling in the zebrafish embryo. In addition, the dose-response relationship we observed is in accord with the dose-inhibition relationship defined in vitro, where 50% inhibition occurred at 15–20 μM (Mohammadi et al., 1997).

FGFR inhibition selectively enhances the ace/fgf8 mutant phenotype

We wished to use sensitivity to SU5402 treatment as an indirect assay of FGFR signaling activity in mutant embryos. To test the feasibility of this approach, we analyzed the effects of SU5402 treatment on embryos with a defined defect in FGF signaling. *Acerebellar (ace)* is a hypomorphic mutant allele of *fgf8* affecting the splicing of exons 2 and 3 (Reifers et al., 1998). *Ace* mutant embryos fail to develop the cerebellum but have relatively normal posterior development (Fig. 3E), either due to residual amounts of correctly spliced *fgf8* mRNA (Reifers et al., 1998; Draper et al., 2001), or functional redundancy with other FGF ligands (Draper et al., 2003). Whatever the basis, we reasoned that the reduction in FGF signaling in *ace* mutant embryos should make posterior development hypersensitive to SU5402 when compared with wild-type or heterozygous siblings. Fig. 3 shows representative embryos from an intercross of *ace*/+ adults treated with the 5 μM SU5402, a dose that only rarely causes acerebellar defects and never causes posterior defects in wild-type embryos. In this experiment, embryos with normal midhindbrain formation had relatively normal posterior development (Fig. 3F), whereas embryos that were acerebellar (Fig. 3G) also had severe posterior mesodermal defects that could be as severe as the defects in wild-type embryos treated with 20–30 μM SU5402 (Fig. 3C and D). Genotyping was performed on embryos from one such experiment ($n = 30$), demonstrating that inheritance of the *ace* mutant allele significantly increased the phenotypic severity due to minor FGFR inhibition ($P \leq 0.001$; Table 1). This demonstrated that minor inhibition of FGFR signaling could be used to induce pronounced patterning defects in embryos in which FGFR signaling was already compromised.

Spt and ntl mutants are hypersensitive to SU5402

The hypersensitivity of *ace* mutant embryos to SU5402 demonstrated that reductions in FGFR signaling could be phenotypically enhanced using this pharmacological ap-

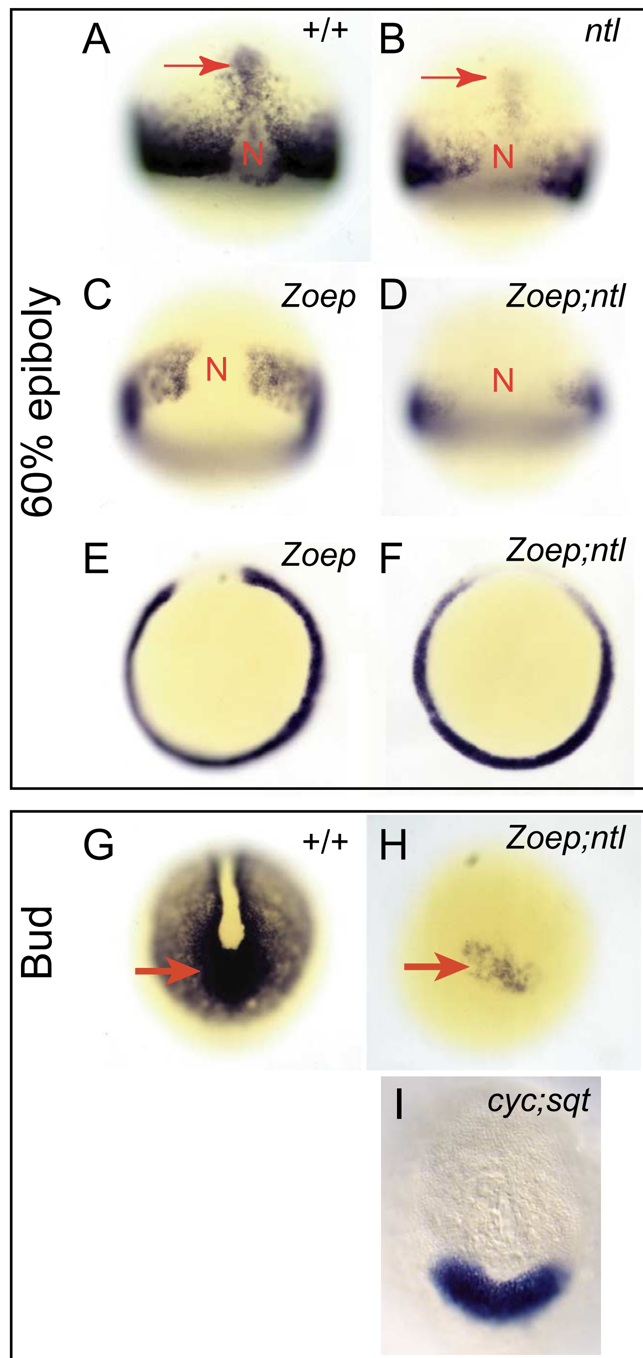

Fig. 2. *Zoep* and *ntl* are together required to maintain *spt* expression. (A–D) seven hours (60% epiboly), dorsal view, animal pole uppermost. (E and F) seven hours (60% epiboly), vegetal view. (G–I) Ten hours (bud stage), posterior view of the tail bud, dorsal is up. (A) Wild-type embryo. *Spt* is expressed in paraxial mesoderm progenitors involuting at the margin and the migrating prechordal mesoderm (arrow), but is excluded from the notochord progenitors (N). (B) *Ntl* mutant embryo; *spt* is expressed in paraxial and prechordal mesoderm (arrow), but is weaker in paraxial mesoderm adjacent to the notochord progenitors and the border of *spt* expression lacks the defined edge observed in wild-type embryos. (C) *Zoep* mutant embryo; *spt* expression is normal in cells at the margin, but is not observed in prechordal mesoderm progenitors. (D) *Zoep;ntl* double mutant embryo; *spt* is expressed at the margin but is weaker adjacent to the notochord and is not detected in prechordal mesoderm progenitors. (E and F) Vegetal view, dorsal side uppermost, of embryos in C and D, showing the distribution of *spt* expression in mesoderm at the margin. (G) Expression of *spt* in a wild-type embryo. Note the intense staining in the tail bud (arrow) and segmental plate cells either side of the nonexpressing notochord. (H) *Zoep;ntl* double mutant embryo; *spt* expression is only observed weakly in a small number of cells in the tail bud (arrow). (I) *cyc;sqt* double mutant embryo. Note the intense expression of *spt* in the tail bud.

Table 1

	Affected[a]	Unaffected
ace[b] +/+ & +/−	6	15
−/−	9	0
P^c	<0.001	
spt +/?	3	34
−/−	11	4
P^c	<0.0001	

[a] In the experiment using embryos derived from *ace*/+ adults, embryos were classified as "affected" if posterior development was similar to either the mild or severe syndromes characterized in experiments using wild-type fish (Fig. 3). *Spt* −/− embryos were classified as affected if the postanal tail was significantly diminished, and there was a significant reduction in myosin staining relative to untreated *spt* −/− embryos. In these experiments, untreated *spt* −/− embryos had substantial amounts of myosin staining.

[b] Determined by genotyping.

[c] Based on χ^2 test to determine deviation from expected if the mutant alleles did not influence sensitivity to SU5402.

proach. We therefore used SU5402 to determine if *ntl* or *spt* mutant embryos might be similarly sensitive to SU5402. Embryos from intercrosses of *spt*/+ or *ntl*/+ heterozygous adults were exposed to a range of low concentrations of SU5402, and assayed for the presence of paraxial mesoderm using myosin staining at 24 hpf (Fig. 4). Approximately 25% of embryos (10 of 41) from an intercross of *ntl*/+ adults treated with 4 μM SU5402 had severe defects in posterior development, and myosin was only detected in the anterior-most region of the trunk (Fig. 4B). The remaining 75% of the embryos were the same as wild-type embryos treated with this dose of SU5402 (Fig. 4C). Since embryos with the typical appearance of *ntl* single mutants (Fig. 4A) were not observed in the treated group, but were present in the untreated control sibling embryos, the severely affected embryos were likely to be *ntl* mutant embryos that had been affected by the FGFR inhibitor.

Similarly, the formation of paraxial mesoderm in *spt* mutant embryos treated with 7 μM SU5402 was dramatically decreased compared to untreated controls (Fig. 4D and E), whereas defects in paraxial mesoderm formation in wild-type or heterozygous sibling embryos were infrequently observed (Fig. 4F). (SU5402-treated-*spt* mutant embryos were identifiable at 24 hpf by the characteristic presence of mesenchymal cells at the tip of the tail.) *Spt* mutant embryos were significantly more likely than wild-type or heterozygous embryos to be affected by 7 μM SU5402 ($P \leq 0.0001$; Table 1). The hypersensitivity of the *spt* and *ntl* mutant embryos to the FGFR inhibitor suggested that re-

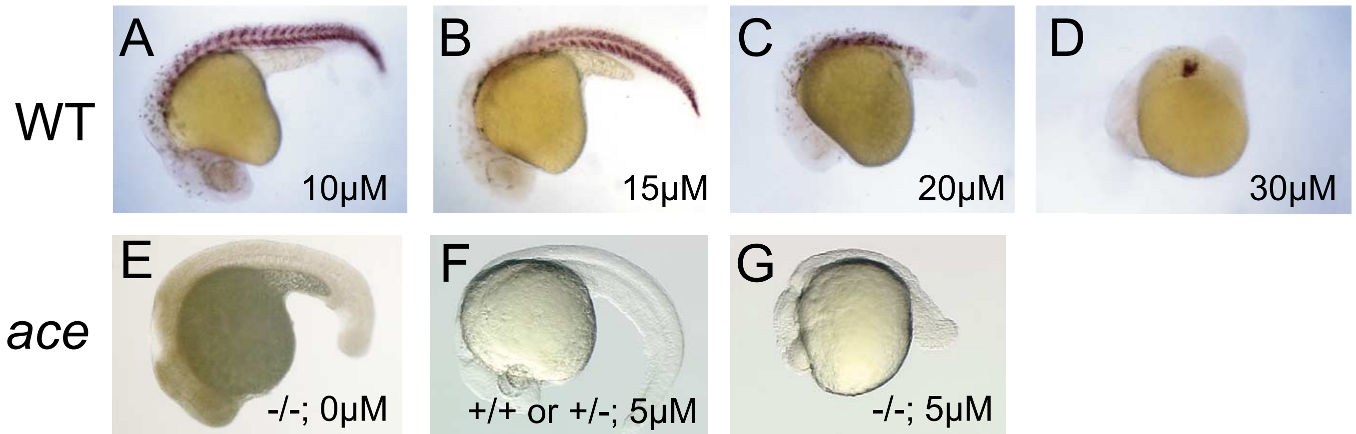

Fig. 3. Effect of the FGFR inhibitor SU5402 on posterior development in wild-type and *ace/fgf8* mutant embryos. (A–D and F) Wild-type and (E and G) *ace* mutant embryos. All embryos are 24–32 h, anterior to the left. Embryos are stained with an antibody to detect myosin, except E–G, which are unstained. (A–D) Increasing the concentration of SU5402 led to a dose-dependent loss in muscle staining, beginning with the tail. Only tail muscle defects were observed at 15 μM, whereas trunk and tail muscle defects were observed at 30 μM. (E–G) Embryos from obtained from *ace*/+ adults. (E) Untreated *ace* mutant embryo, showing typically good posterior development. (F) Wild-type embryo from *ace*/+ parents treated with 5 μM Su5402; (G) *ace* mutant embryo treated with 5 μM SU5402 showing severe posterior defects.

duced FGFR signaling was a common feature of the *spt* or *ntl* mutant phenotypes, consistent with the expression of these factors being regulated, at least in part, by indirect autoregulatory loops involving FGF signaling.

Zoep and FGF signaling interact synergistically in posterior development

If the reduction in FGFR signaling in *spt* and *ntl* embryos was important for the genetic interactions with *Zoep,* then mild perturbations in FGF signaling should be sufficient by themselves to cause synergistic posterior defects in *Zoep* mutant embryos. We tested this pharmacologically using the FGFR inhibitor SU5402, and genetically using the *ace/fgf8* mutant allele. Embryos obtained from *Zoep*/+ adults were treated with a variety of doses of SU5402 and the amount of paraxial mesoderm assayed at 24 hpf (Fig. 5). Since cyclopia was never induced by SU5402 at any dose tested (up to 50 μM, data not shown), cyclopia was used to identify *Zoep* homozygous mutant embryos. Paraxial mesoderm formation in *Zoep* mutant embryos was very sensitive to SU5402. At 10 μM SU5402, trunk and tail paraxial

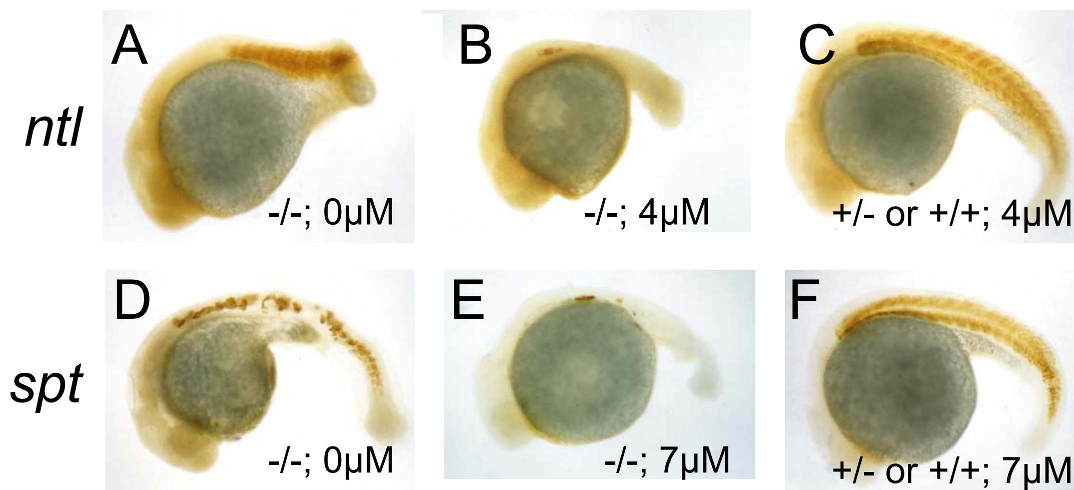

Fig. 4. *Spt* and *ntl* mutant embryos are hypersensitive to FGFR inhibition. (A–C) Embryos from *ntl*/+ adults. (A) Myosin staining in an untreated *ntl* mutant embryo. (B) Presumptive *ntl* mutant embryo treated with 4 μM SU5402; myosin staining is dramatically reduced relative to (A) and (C) *ntl* +/+ and +/− embryos treated with 4 μM SU5402. (D–F) Embryos obtained from *spt*/+ adults. (D) Untreated *spt* mutant embryos have patchy myosin staining in the trunk and segmented staining in the tail. (E) *spt* mutant embryos treated with 7 μM SU5402 are almost devoid of myosin staining, whereas (F) *spt* +/+ and +/− embryos treated with 7 μM SU5402 appear similar to untreated wild-type embryos.

Fig. 5. The *Zoep* mutant phenotype is enhanced by FGFR inhibition. Embryos at 24 h of development, anterior to left, hybridized to detect *myoD* expression (brown stain). (A–C) *Zoep* +/?; (D–F) *Zoep* −/−. Defects in wild-type tail somitic mesoderm are first observed at 15 μM (C), as described in Fig. 3. (D) Untreated *Zoep* mutant embryo. (E) *Zoep* mutant embryos treated with 10 μM show aberrations in the number of *myoD*-positive cells. Unlike the defects observed in wild-type embryos treated with 10 μM SU5402, loss of muscle did not occur strictly from posterior to anterior, as indicated by the gap in *myoD* expression in the posterior trunk. Muscle staining in the tail was continuous across the midline, indicating the absence of posterior notochord. (F) *Zoep* mutant embryos treated with 15 μM; *myoD* staining is only detected in the anterior trunk.

mesoderm was reduced (Fig. 5E), and at 15 μM SU5402 muscle was only detected in the anterior trunk (Fig. 5F). In contrast, wild-type and heterozygous sibling embryos had normal posterior development at 10 μM and only tail defects were observed at 15 μM SU5402 (Fig. 5B and C).

As an additional test of the relationship between *Zoep* and FGF signaling, and to specifically address the role of the Fgf8 ligand, we analyzed the phenotype of *Zoep;ace* double mutant embryos (Fig. 6). At 48 h, *ace* and *Zoep* single mutant embryos have only minor defects in trunk and tail mesoderm formation (Fig. 6B and C). *Zoep;ace* double mutant embryos were easily identified by the combination of cyclopia and abnormal midhindbrain morphology. In contrast to either single mutant, *Zoep;ace* embryos had extremely poor posterior development that appeared to affect mesodermal and ectodermal derivatives (Fig. 6D). At earlier stages, extensive cell death was apparent throughout the tail bud and posterior mesoderm (Fig. 6N). The *Zoep;ace* mutant phenotype was always much more severe than merely additive but some variability was observed, probably reflecting variability in the inheritance of maternal *oep,* and possibly also variability in the extent of processing of the *ace* transcript. Typically, *myoD*-expressing cells were only observed in the anterior trunk, and in severely affected embryos *myoD* expression was barely detectable (Fig. 6H). Notochord was present, but was only observed in the anterior trunk. Expression of *pax2.1* demonstrated that there were additive defects in eye and midhindbrain, but synergistic defects in otic vesicle, nephric mesoderm, and spinal cord, which was severely truncated posteriorly (Fig. 6L). Taken together, these data demonstrate that *Zoep* synergistically interacts with the FGF signaling pathway, and that *Zoep* and *fgf8* are together essential for the formation of tail bud-derived tissues (somites, notochord, and spinal cord).

Discussion

We are interested in understanding the molecular and genetic pathways underlying the formation of posterior mesoderm. Among the factors known to be required for posterior development in zebrafish are: FGF signaling (Griffin et al., 1995), Nodal signaling (Feldman et al., 1998), and Oep (Gritsman et al., 1999), as well as at least two members of the T-box transcription factor family, Spt and Ntl (Kimmel et al., 1989; Halpern et al., 1993; Schulte-Merker et al., 1994b; Griffin et al., 1998). Primarily, the roles of these factors have been established using either genetic analysis or misexpression and dominant negative studies analyzing the importance of single factors or pathways. However, in the normal course of development, cells are exposed to multiple signals simultaneously and coexpress multiple transcription factors influencing cell fate, each of which may obscure or alter the function of other factors with which they are expressed (Goering et al., 2003). Complex genetic networks are likely therefore to be the rule rather than the exception. Ultimately, we need to understand how combinations of factors interact in the formation of a particular tissue. Here we have addressed how mesodermally expressed T-box transcription factors genetically interact with Zoep, and how FGF signaling is involved.

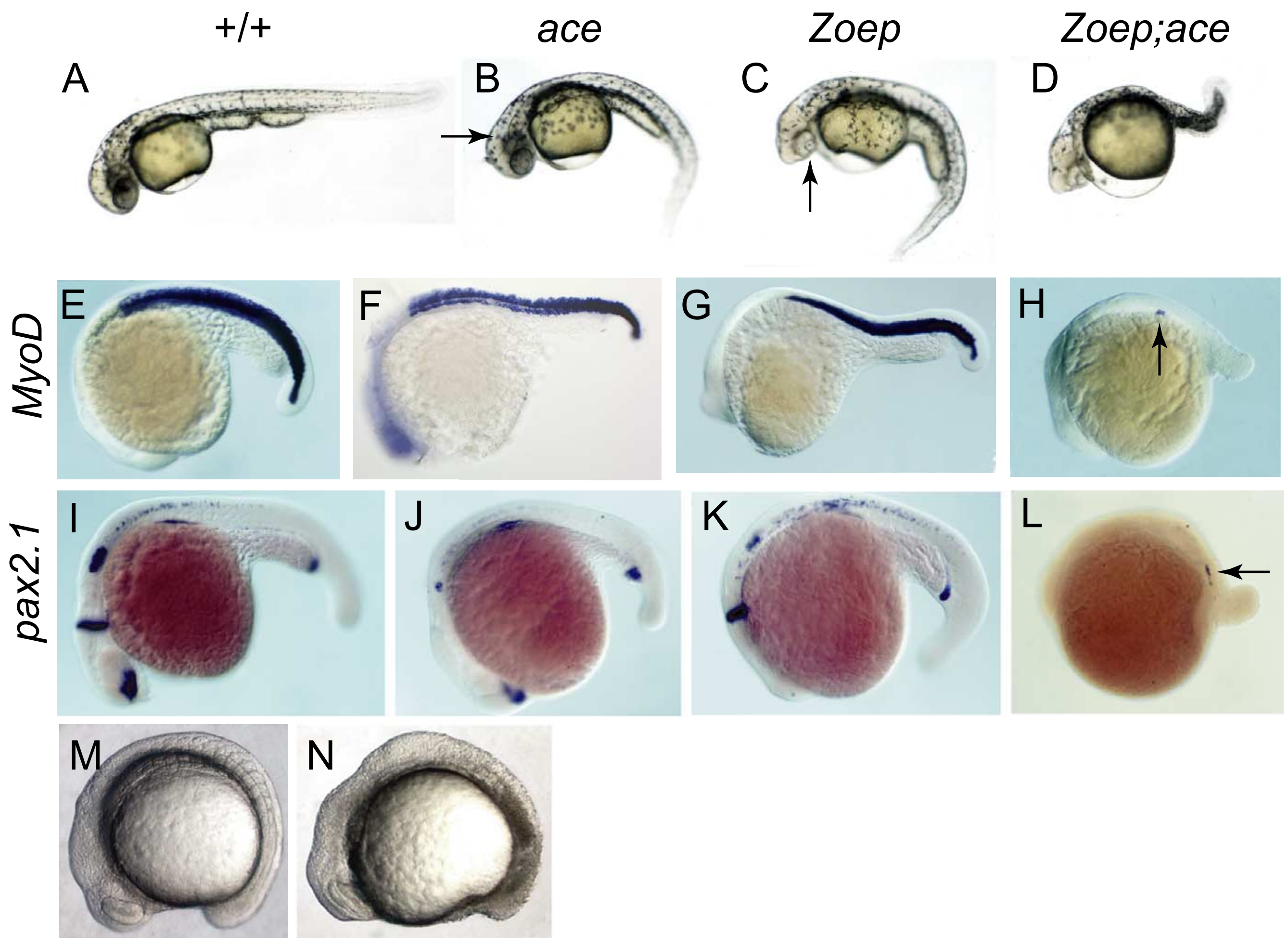

Fig. 6. *Zoep* genetically interacts with *ace/fgf8*. All embryos are shown in lateral view, anterior to the left. (A, E, I, and M) wild-type; (B, F, and J) *ace* mutants; (C, G, and K) *Zoep* mutants; (D, H, L, and N) *Zoep;ace* double mutants. (A–D) Live embryos at 48 h. (A) Wild-type embryo. (B) *ace* mutant embryos lack the cerebellum and have an enlarged tectum (arrow). (C) *Zoep* mutant embryos are obviously cyclopic (arrow). Both *ace* and *Zoep* single mutants have well developed posterior mesoderm and neuroectoderm. (D) *Zoep;ace* double mutant embryo; the phenotype was additive in anterior neuroectoderm. Posterior development in *Zoep;ace* embryos was extremely poor, and mesodermal derivatives (somites and notochord) were difficult to distinguish morphologically. (E–H) *MyoD* expression (24 h). (E) Wild type, (F) *ace,* and (G) *Zoep* mutant embryos show strong *myoD* expression throughout the somites. (H) Severely affected *Zoep;ace* embryo; *myoD* is expressed only in a few cells in the anterior trunk (arrow) and not more posterior to this. (I–L) *Pax2.1* expression (24 h). (I) In wild-type embryos, *pax2.1* is expressed in the retina, midhindbrain border, otic vesicle, dorsal spinal cord, and pronephric mesoderm. (J) In *ace* mutant embryos, *pax2.1* expression is absent from the midhindbrain border and is reduced in the retina and otic vesicle. (K) In *Zoep* mutant embryos with a strong phenotype, *pax2.1* expression is absent from the retina, and is reduced in the otic vesicle. (L) Severely affected *Zoep;ace* mutant embryo; *pax2.1*-expressing cells are only detected in the anterior spinal cord (arrow). (M and N) Midsomitogenesis live embryos, anterior to left. Note the large numbers of opaque dead or dying cells in posterior tissues of the *Zoep;ace* embryo (N), which are not apparent in the wild-type embryos (M).

Oep and Ntl are required to maintain spt expression

Schier et al. (1997) observed that *Zoep;ntl* double mutant embryos had mesodermal defects that were not observed in either single mutant, notably the near total absence of paraxial mesoderm and blood. The molecular basis for this genetic interaction was unknown, and was not clarified by the discovery that *oep* encodes an extracellular factor essential for Nodal signaling (Zhang et al., 1998; Gritsman et al., 1999). Here we have shown that *Zoep* and *ntl* are essential to maintain high levels of expression of the T-box transcription factor *spt*. The failure to maintain *spt* expression in the *Zoep;ntl* mutant background is sufficient to account for the synergistic mesodermal defects in somitic mesoderm and blood for the following reasons. Spt plays an important role in blood formation, is functionally redundant with *ntl* in the formation of posterior mesodermal progenitors, and in combination with *Zoep* is essential for the formation of somitic mesoderm. Furthermore, vascular endothelium was unaffected by the interaction between *Zoep* and *ntl,* and is not dependent upon Zoep and Spt function (Thompson et al., 1998; Griffin and Kimelman, 2002).

We have previously shown that *Zoep* and *spt* are together required for the formation of myocardial cells (Griffin and Kimelman, 2002). It is interesting therefore that myocardial cells are present in *Zoep;ntl* embryos at 24 hpf, despite the

fact that they lack *Zoep* and, indirectly, Spt function. Since *spt* expression is initially normal in *Zoep;ntl* embryos, *Zoep;ntl* embryos only have a late-onset defect in Spt function. Taking this into consideration, we suggest that Spt is likely to play an early role in cardiac mesoderm formation. In contrast, since Spt is also required for blood formation (Thompson et al., 1998) and this is defective in *Zoep;ntl* embryos, the role of Spt in blood development is likely to be significantly later, after gastrulation.

Spt and ntl embryos have enhanced sensitivity to reduced FGFR signaling

We have used hypersensitivity to SU5402, a specific inhibitor of FGFR activity (Mohammadi et al., 1997), to indirectly assay the overall strength of FGFR signaling in different mutant backgrounds. We found this to be an effective tool with which to uncover impairments in the FGF signaling pathway that alone do not yield a significant phenotype. In general, this approach could be used in many other contexts, using any of the increasingly large number of specific inhibitors of signal transduction pathways.

Using this approach, we have shown that paraxial mesoderm formation in *spt* and *ntl* mutant embryos is hypersensitive to FGFR inhibition, suggesting that reduced FGFR signaling is an important feature of these mutant phenotypes. A likely explanation for reduced FGFR signaling in *spt* and *ntl* mutants is that Spt and Ntl regulate expression of *fgf* ligands, as suggested for Brachyury in *Xenopus* early mesoderm (Isaacs et al., 1994; Schulte-Merker and Smith, 1995), and in the limb buds of mouse and chick embryos (Liu et al., 2003). In *Xenopus, Xbra* expression is maintained by direct regulation of *efgf* expression by Xbra, which in turn activates *Xbra* expression (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). Although our data are consistent with similar T-box/FGF-positive feedback loops involving *spt* and *ntl,* their regulation is likely to be much more complex and subtle than the simple model from *Xenopus* suggests. For example, with the exception of the notochord, *ntl/brachyury* expression does not depend upon Ntl/Brachyury function (Schulte-Merker et al., 1994a; Schmidt et al., 1997). While our work supports the existence of these FGF loops, they must involve multiple downstream factors regulating the feedback and are likely to involve multiple FGF ligands and/or interactions across tissue layers, such as occurs in the chick limb bud (Ohuchi et al., 1997; Xu et al., 1998).

Posterior mesoderm requires Oep and FGF signaling, acting synergistically

Using the FGFR inhibitor as well as a genetic approach, we have shown that Oep acts synergistically with FGF signaling, specifically Fgf8, in the formation of posterior tissues. *Zoep* and *ace/fgf8* were especially useful for this demonstration since both mutant alleles cause only hypomorphic reductions in activity through their respective signaling pathways, thereby sensitizing the embryo to reductions in factors acting in parallel or downstream. In *Zoep* mutant embryos, the presence of maternally inherited Oep permits sufficient Oep-dependent signaling to support posterior axial and paraxial development. Similarly, as described above, the *ace* mutant allele is hypomorphic, and in addition there is functional redundancy between Fgf8 and another mesodermally expressed FGF ligand (Draper et al., 2003). However, in *Zoep;ace* double mutant embryos, the combination of reduced Oep and hypomorphic Fgf8 signaling caused a synergistic posterior defect. This interaction between *Zoep* and the FGF pathway suggests an attractive explanation for the genetic interactions between *Zoep* and *ntl* or *spt* (Fig. 7). Since *spt* and *ntl* mutant embryos may have reduced FGFR activity, the alterations in the signaling environment in *Zoep;spt* and *Zoep;ntl* mutant embryos may resemble the signaling environment in *Zoep;ace* mutant embryos, and *Zoep* mutant embryos treated with the FGFR inhibitor SU5402.

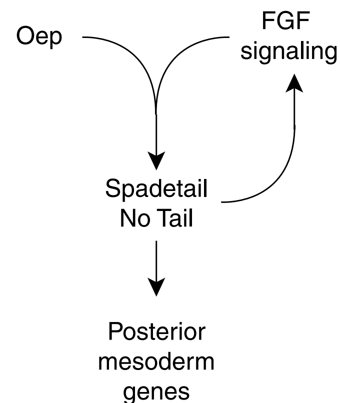

Fig. 7. Simplified scheme depicting the relationships between Oep, FGF signaling, and the T-box transcription factors Spadetail and No Tail during posterior development. Spadetail and No Tail are both upstream and downstream of FGF signaling, due to putative positive feedback loops. Oep and FGF signaling act cooperatively during posterior development, rendering *oep* mutant embryos sensitive to alterations in FGF signaling from any of the following causes: FGFR inhibition, hypomorphic Fgf8 function, or mutations in either *spadetail* or *no tail*.

Our data implicate Spt and Ntl in the regulation of FGF signaling (Fig. 7), but a major question is which signaling pathway Oep is involved with in these mutant scenarios? Although Oep is strongly implicated in signaling by certain TGFβs such as the Nodal ligands Cyclops and Squint (Gritsman et al., 1999) as well as Vg1 and GDF-1 (Cheng et al., 2003), there is also evidence from *Xenopus* that EGF-CFC proteins are directly involved with the FGF pathway. Recent work has implicated the *Xenopus* Oep-related protein FRL-1 with FGF signaling in two contexts—convergent extension, via the FGFR1 (Yokota et al., 2003), and in neural induction (Yabe et al., 2003), and was originally identified as an atypical FGFR ligand (Kinoshita et al., 1995). Consistent with the latter, neural induction by FRL-1

involves MAP kinase signaling and requires active FGFR signaling (Yabe et al., 2003). Although Oep is able to rescue the *Xenopus* FRL-1 depletion phenotype, there is strong circumstantial evidence that Oep mediates TGFβ signals during zebrafish posterior development. Interference with two transcriptional effectors of TGFβ signaling, *Bon* (Griffin and Kimelman, 2002) and *schmalspur* (Rojo et al., 2001), a mutant allele of *foxH1,* in combination with *spt* and/or *ntl* mutant alleles phenocopies the posterior mesodermal defects observed in *Zoep;ntl* and *Zoep;spt*. Furthermore, we were unable to detect any effect of Oep on FGFR activity in a *Xenopus* oocyte assay, with or without addition of eFGF ligand (unpublished observations). Thus, it is likely that the interactions between *Zoep* and *spt, ntl,* and the FGF pathway (Fig. 7) in zebrafish represent synergy between TGFβ and FGF signaling, consistent with a variety of in vitro models of mesoderm induction (Kimelman and Kirschner, 1987; Green et al., 1992; Kimelman et al., 1992). However, without more definitive proof, the role of Oep in these contexts remains controversial.

In comparison with many other aspects of early development our understanding of how the tail bud functions is extremely rudimentary, although some studies have begun to demonstrate its complexity and the important role of tissue interactions within this structure (Agathon et al., 2003). In particular, a study in the chick clearly demonstrated the special properties of a small group of cells located at the juxtaposition of axial and paraxial progenitor (the axial-paraxial hinge). Surgical removal of the axial-paraxial hinge secondarily caused loss of chordoneural hinge-derived tissues (floor-plate and notochord) and, subsequently, massive apoptosis throughout the spinal cord and somites (Charrier et al., 1999). The *Zoep;ace* phenotype is remarkably similar to this syndrome of defects. It will be very interesting to determine the specific roles of Oep and Fgf8 signaling pathways in tissue interactions in the zebrafish tail bud.

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