

# Signals from the Yolk Cell Induce Mesoderm, Neuroectoderm, the Trunk Organizer, and the Notochord in Zebrafish

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We have analyzed the role of the zebrafish yolk cell in the processes of mesoderm induction and establishment of the organizer. By recombining blastomere-free yolk cells and animal cap tissue we have shown that the yolk cell itself can induce mesoderm in neighboring blastomeres. We further demonstrate the competence of all blastomeres to form mesoderm, suggesting the endogenous mesoderm inducing signal to be locally restricted. Ablation of the vegetal third of the yolk cell during the first 20 min of development does not interfere with mesoderm formation in general, but results in completely ventralized embryos. These embryos lack the notochord, neuroectoderm, and the anterior-most 14–15 somites, demonstrating that the ablation affects the formation of the trunk-, but not the tail region of the embryo. This suggests the presence of a trunk organizer in fish. The dorsalized mutant *swirl* (*zbmp-2b*) shows expanded dorsal structures and missing ventral structures. In contrast to the phenotypes obtained upon the ablation treatment in wild-type embryos, removal of the vegetal-most yolk in *swirl* mutants results in embryos which do form neuroectoderm and anterior trunk somites. However, both wild-type and *swirl* mutants lack a notochord upon vegetal yolk removal. These ablation experiments in wild-type and *swirl* mutant embryos demonstrate that in zebrafish dorsal determining factors originate from the vegetal part of the yolk cell. These factors set up two independent activities: one induces the notochord and the other is involved in the formation of the neuroectoderm and the trunk region by counteracting the function of *swirl*. In addition, these experiments show that the establishment of the anteroposterior axis is independent of the dorsoventral axis. © 1999 Academic Press

**Key Words:** zebrafish; mesoderm induction; dorsal determinant; dorsoventral polarity; neuroectoderm; trunk organizer; organizer; maternal; anteroposterior axis; yolk cell.

## INTRODUCTION

A central question in developmental biology is how the basic body plan of vertebrates is generated. As in invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans*, where maternally driven processes lay down the information determining the embryonic axes already in the oocyte (Bowerman, 1998; Ray and Schüpbach, 1996), the same seems to be the case in vertebrates. Here, the equivalent processes are understood best in *Xenopus laevis* (reviewed in Heasman, 1997; Slack, 1994), where it has been shown that dorsal specification is maternally controlled (Wylie *et al.*, 1996).

Very little is known about these events in other verte-

brate systems. We have chosen the zebrafish to investigate the nature of these early processes in teleosts. Zebrafish, as with most teleosts, produce large, yolky eggs where cleavage takes place in a blastodisc on top of the yolk. Initially yolk and cytoplasm are intermixed; soon after fertilization the cytoplasm separates and streams to the animal pole forming the blastodisc. During early cleavage and blastula stages the cytoplasm becomes cellular and forms the blastoderm which eventually gives rise to the embryo. The yolk cell is anuclear until the most marginal blastomeres collapse into the yolk cell and form the yolk syncytial layer (YSL; Kimmel and Law, 1985). This happens roughly around midblastula transition (MBT; Kane and Kimmel, 1993). The animal-vegetal axis defines the only visible polarity of the early embryo, and neither dorsoventral nor anteroposterior polarity can be correlated with the first cleavage plane (Abdelilah *et al.*, 1994). The developing zebrafish embryo is

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morphologically radially symmetric until the beginning of gastrulation, when the deep layer cells start to involute at the dorsal side of the embryo (Schmitz and Campos-Ortega, 1994). This is the site where soon afterward the embryonic shield, the homologue of the amphibian organizer, forms (Ho, 1992; Oppenheimer, 1936a,b; Shih and Fraser, 1996). According to the fate map of the blastula stage embryo, the mesodermal as well as the endodermal precursors are located equatorially, next to the yolk cell, whereas the ectodermal precursors are located in the animal pole region of the embryo (Kimmel *et al.*, 1990; Warga and Nüsslein-Volhard, 1998). Recombination experiments, placing a largely blastomere-free yolk cell onto the animal pole of another embryo, suggest that mesoderm is induced in the marginal zone of the late zebrafish blastula by the yolk cell (Mizuno *et al.*, 1996). Apart from a possible role in mesoderm induction, the yolk cell is believed to be involved in the establishment of the dorsoventral axis, as suggested by experiments performed in trout (Long, 1983). In these experiments blastoderms from younger embryos were transplanted onto gastrula-stage yolk cells and dorsal structures formed on the dorsal side of the yolk cell. Furthermore, microsurgical analysis of teleost eggs has shown that if the vegetal-most part of the yolk cell is removed during the first cell cycle, the embryos are strongly ventralized (Koshida *et al.*, 1998; Mizuno *et al.*, 1997; Tung *et al.*, 1945), suggesting that dorsal determinants are located in the vegetal half of the yolk cell just after fertilization and then transported to the future dorsal side of the embryo. The asymmetric translocation of the determinant to the overlying blastomeres appears to be dependent on an array of parallel microtubules at the vegetal pole (Jesuthasan and Strähle, 1996). This was shown by using reduction of temperature or nocodazole as a microtubule-depolymerizing agent, resulting in ventralized embryos (Jesuthasan and Strähle, 1996). In teleosts, a cortical rotation, similar to that in *X. laevis*, initially aligning the microtubules, has not been reported.

In this paper, we have analyzed the role of the yolk cell in the establishment of the basic body plan in the zebrafish embryo. Recombinates between blastomere-free yolk cells and animal cap tissue, representing presumptive ectoderm, show that the yolk cell, and not marginal blastomeres, is the source of the mesoderm-inducing signal. Blastoderm cultures revealed that the mesoderm-inducing signal comes from a ring-like source, probably the external YSL.

Removing the vegetal-most part of the yolk cell immediately after fertilization served as an assay for characterizing the developmental relevance of a localized determinant from this part of the zebrafish embryo. The resulting embryos are completely ventralized. We demonstrate that this vegetally located determinant establishes the organizer, the notochord, the nonaxial trunk mesoderm, and the neuroectoderm.

We have extended these experiments to *swirl* (*swr*) mutant embryos, which are deficient in the zebrafish homologue of the murine BMP-2 gene *zbmp-2b* (Kishimoto *et al.*,

1997). We demonstrate an interaction between the vegetally located determinant and *zbmp-2b*, as well as revealing a trunk organizer in zebrafish.

## MATERIAL AND METHODS

### Fish Embryos

Zebrafish (*Danio rerio*) were kept as previously described (Mullins *et al.*, 1994). Embryos were obtained through natural matings or, for production of ventralized embryos, by *in vitro* fertilization (Pelegrini and Schulte-Merker, 1998). Unless otherwise noted, embryos were kept in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, 0.33 mM MgSO<sub>4</sub>) containing gentamycin (20 mg/liter, GIBCO). Fish strains used were Tübingen wild-type, *golden<sup>bl</sup>* and *swr<sup>ta72</sup>*.

### Mesoderm Induction Assays

Blastomere-free yolk cells were obtained by manually removing all blastomeres from 1000-cell to high-stage embryos in Ca<sup>2+</sup>-free Ringer's (116 mM NaCl, 2.9 mM KCl, 5 mM Hepes, pH 7.2; Westerfield, 1993). Yolk cells were then transferred into Ringer's (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl<sub>2</sub>, 5 mM Hepes, pH 7.2; Westerfield, 1993). An animal cap from a sibling embryo was then attached to the animal pole of the yolk cell and fixed there with the help of a metal stalk. The recombinates were fixed and stained after 2–3 h of incubation. The blastomere and YSL nuclei were visualized by incubating the fish in DAPI (4,6-diamidino-2-phenylindole; 5 mg/liter) for 2 h.

Marginal zone–animal cap conjugates were dissected and cultured in Ringer's, fixed after 3 h of incubation, and stained for Ntl protein. The donor embryos of both tissue types were 4–4.5 h old at the time of dissection. Donor embryos for marginal zone tissue were dye-labeled by injection of lysine fixable fluorescein dextran [2% (w/v) in 0.2 M KCl; Molecular Probes] at the one-cell stage. Donor embryos for animal cap tissue were not labeled. Conjugates were examined by using a digital camera (Hamamatsu) and MetaMorph software.

### Tissue Cultures

Dechorionated embryos were dissected in Ringer's with an eyelash knife according to the experimental scheme (Fig. 2A) or as mentioned in the text. All dissections and culturing experiments were performed on agarose-coated dishes.

### Generation of Ventralized Embryos

Females were squeezed as described (Pelegrini and Schulte-Merker, 1998). Embryos were dechorionated manually with watchmaker forceps 5 min after fertilization, and, using a fine hair-loop, the most vegetal part of the yolk was removed. This procedure allows removal of a small amount of yolk of discrete size, without any yolk oozing out. The operation was always finished within 20 min postfertilization (mpf). Sibling embryos were cultured in the same dish and served as a staging reference. Experimental embryos developed slightly slower than controls until tailbud stages and normal afterward as judged either by the onset of expression of marker genes or, after 15 hpf, by observing somite development.

Removal of the lateral yolk following the above-described regime was performed as a control.

### Whole-Mount *In Situ* Hybridization and Immunohistochemistry

*In situ* hybridizations were carried out as previously described (Kishimoto *et al.*, 1997). Double stainings were carried out by detecting the digoxigenin-labeled probe first, using BM-purple (Boehringer-Mannheim) as a substrate. The reaction was stopped by washing the specimen in PBST (PBS/0.1% Tween 20) several times, followed by one wash with 100 mM glycine (pH 2.2) for 10 min. Embryos were washed again in PBST and then blocked for at least 1 h in 5% blocking reagent (Boehringer-Mannheim) in MABT buffer (150 mM NaCl, 100 mM maleic acid, 0.1% Tween 20, pH 7.5). Incubation with anti-fluorescein antibody (Boehringer-Mannheim, 1:500 in MABT) was carried out for a minimum of 3 h at room temperature, followed by four washes with MABT. Fast red (Boehringer-Mannheim) was used as the substrate for detecting the fluorescein-labeled probe.

No tail (Ntl) and Engrailed (Eng;  $\alpha$ -4D9 antibody from Developmental Studies Hybridoma Bank recognizes all three zebrafish engrailed proteins; Ekker *et al.*, 1992) protein was detected as described previously (Schulte-Merker *et al.*, 1992), with the following modifications: the blocking reagent (Boehringer-Mannheim) was used at 5% in MABT buffer for blocking, MABT buffer was used for all washes, and a peroxidase-coupled secondary antibody (1:2000, Dianova) in MABT was used for detecting the primary antibody. In cases where detection of Ntl was combined with *in situ* hybridizations, specimens were processed for *in situ* hybridizations and simultaneously incubated with both anti-Ntl/anti-4D9 and anti-digoxigenin antibodies. Ntl or Eng detection was always carried out first in those cases.

Probes used were *zbmp-4* (Nikaido *et al.*, 1997), *din* (Schulte-Merker *et al.*, 1997), *nwk* (Koos and Ho, 1998), *anf* (Kazanskaya *et al.*, 1997), *otx-2* (Li *et al.*, 1994), *gsc* (Schulte-Merker *et al.*, 1994), *krox-20* (Oxtoby and Jowett, 1993), *myoD* (Weinberg *et al.*, 1996), *sna-1* (Hammerschmidt and Nüsslein-Volhard, 1993), and *gata-1* (Detrich *et al.*, 1995). Embryos were photographed either in 70% glycerol or in benzylalcohol:benzylbenzoate (2:1).

## RESULTS

### The Yolk Cell Induces Mesoderm in the Zebrafish Embryo

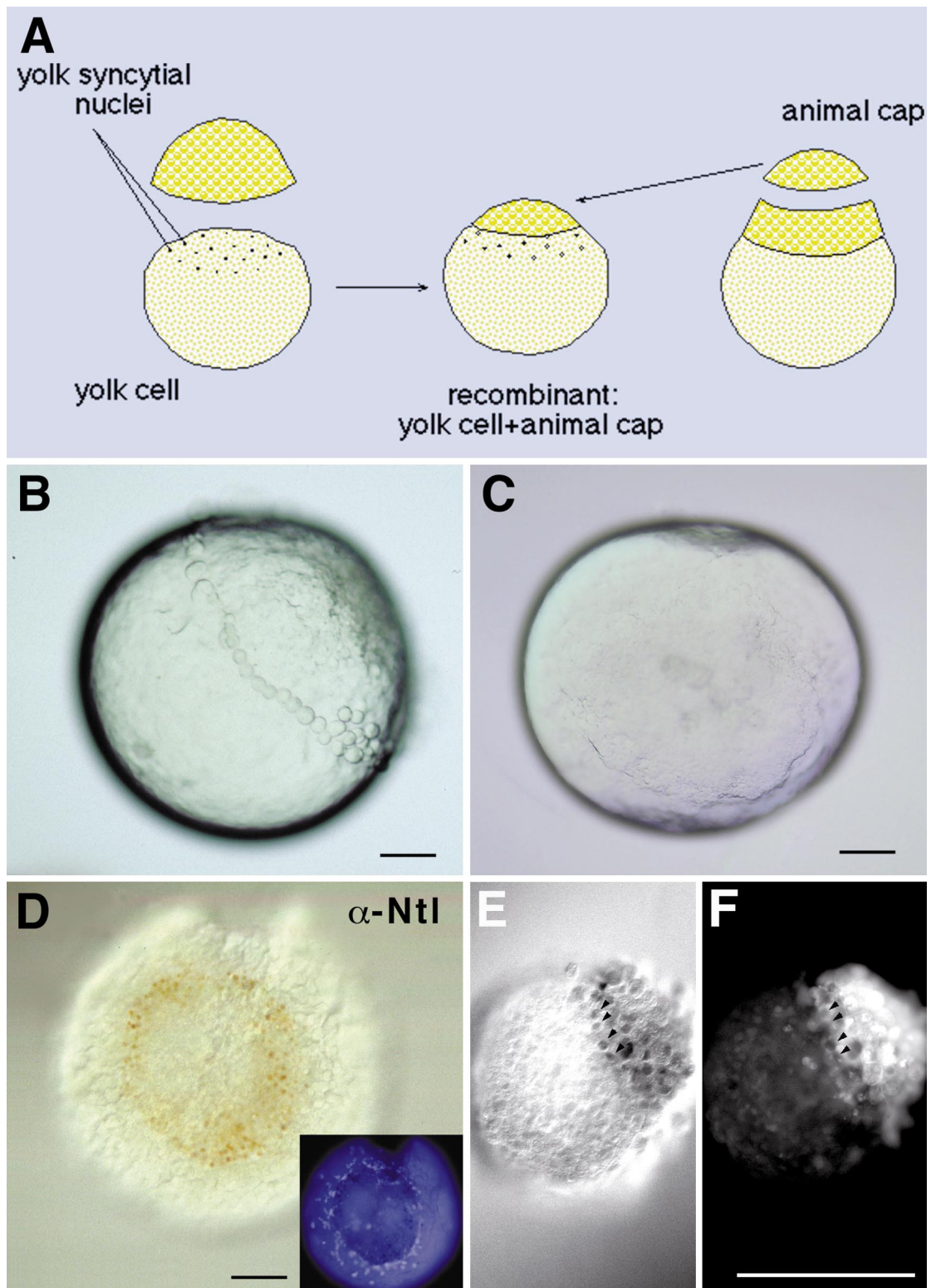
The source of mesoderm-inducing signals in *Xenopus* has been elegantly demonstrated by Nieuwkoop in his conju-

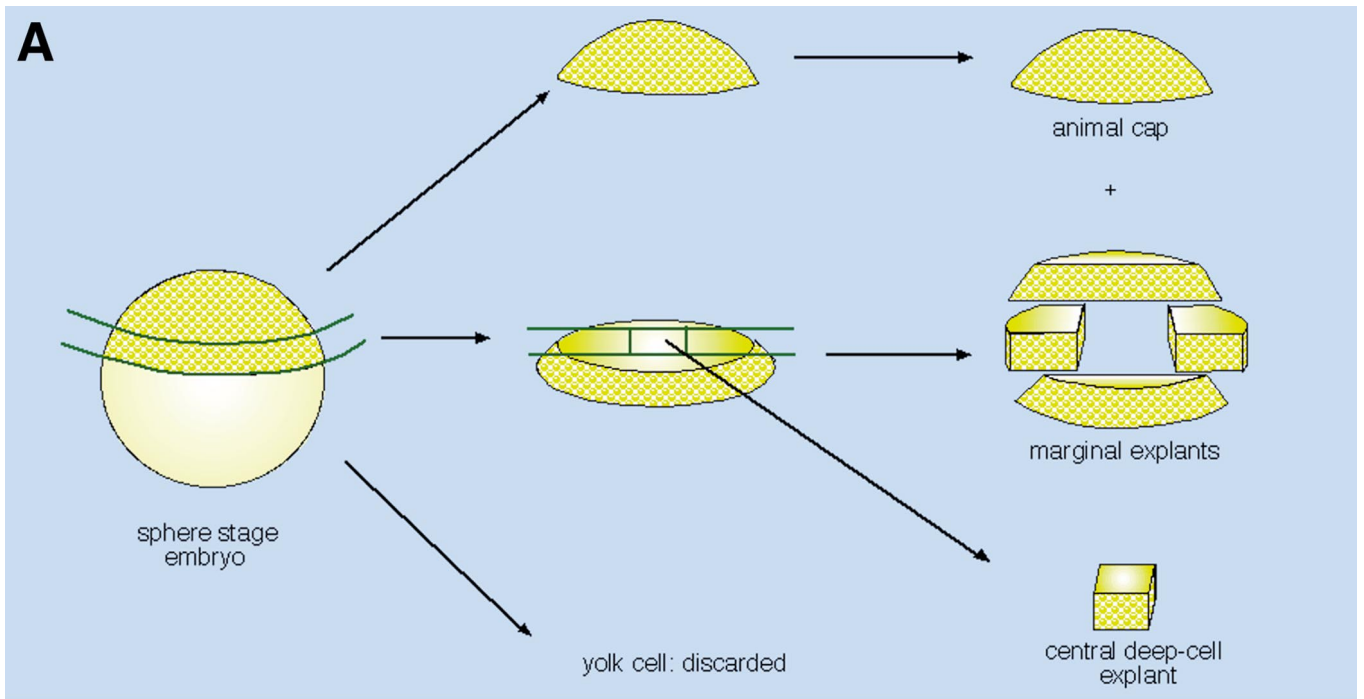
gate experiments (Nieuwkoop, 1969). If vegetal cells and animal cap cells are removed from the embryo and cultured in isolation, they will differentiate into yolky endoderm and ciliated epidermis, respectively. If both tissues are brought into contact, however, then the animal cap cells are induced by the cells of the vegetal pole to differentiate into mesoderm.

Mesoderm in zebrafish forms, as in *Xenopus*, in the equatorial region of the embryo. Therefore, we have asked whether the vegetal part of the embryo, the huge uncleaved yolk cell, also has inductive capacity. This was suggested by Mizuno *et al.* (1996), who placed a largely blastomere-free yolk cell onto the animal pole of a second embryo and observed mesoderm induction. However, as the yolk cells they used in their experiments were not completely devoid of blastomeres, one cannot exclude that the marginal blastomeres which are still attached to the yolk cell have mesoderm-inducing activity. To circumvent this problem, we created the equivalent of a Nieuwkoop conjugate by placing animal cap tissue on a completely blastomere-free yolk cell (Fig. 1A).

Our assay contains two important differences to the procedure by Mizuno *et al.* (1996): First, we have juxtaposed isolated animal cap tissue to the yolk cell, thereby creating a true Nieuwkoop conjugate (Fig. 1A). In contrast to the experiments by Mizuno *et al.* (1996), in which the animal cap tissue is still in contact with the rest of the embryo as well as an additional yolk cell, the animal cap tissue in our assay receives signaling only from a single yolk cell. Second, we have succeeded in removing all blastomeres from the yolk cell to demonstrate beyond doubt that it is the yolk cell, and not adhering blastoderm, which is the source of mesoderm-inducing signals. This is technically difficult, as the most vegetal and peripheral cells of the sphere-stage embryo adhere to the yolk cell membrane via tight junctions (Betchaku and Trinkaus, 1978). All other blastomeres can be easily removed from the yolk cell by incubating the embryo in  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -free medium, but a significant number of cells are resistant to this treatment (Fig. 1B). Only physical removal using watchmaker forceps allows the generation of yolk cells without any adhering blastomeres (Fig. 1C). The removal of the blastoderm causes a slight contraction of the animal part of the yolk cell (data not shown). The yolk cells were obtained from 1000-cell to high-stage embryos and were capable of inducing mesoderm

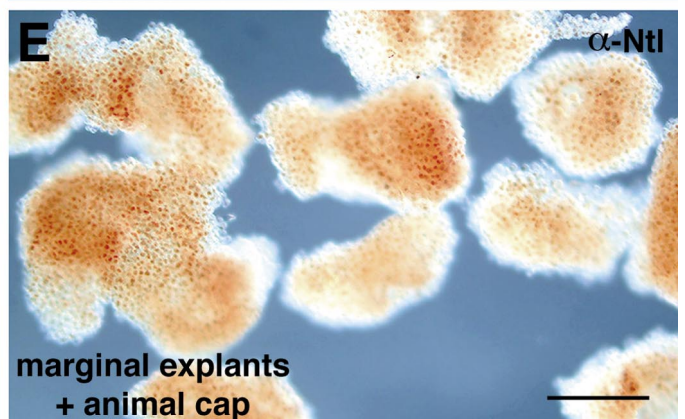
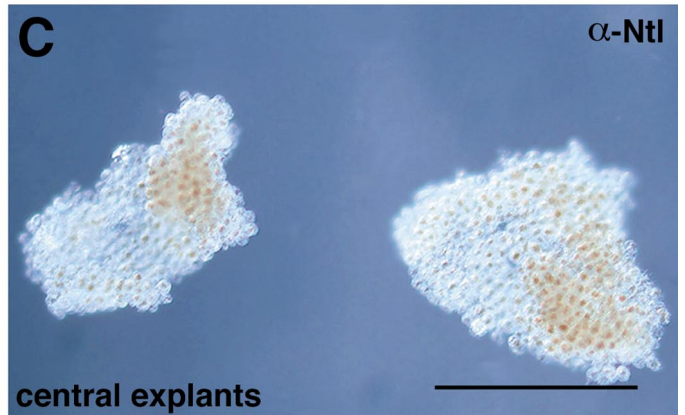
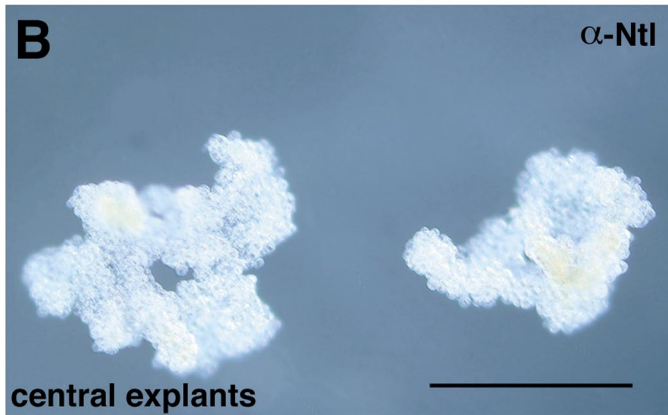
**FIG. 1.** The yolk cell, but not marginal zone tissue, can induce mesoderm in animal cap tissue. (A) Scheme of the experimental procedure. All blastomeres were removed from a high-stage embryo. This yolk cell was then recombined with an animal cap removed from a sibling embryo between high- and sphere-stage. (B) Lateral view of a high-stage embryo, which was incubated in  $\text{Ca}^{2+}$ -free Ringer's; animal pole is up. Only the marginal-most blastomeres remain attached to the yolk cell, via tight junctions. (C) Animal view of a completely blastomere-free yolk cell from a high-stage embryo. (D) Animal view of one conjugate. Blastomeres at the periphery of the cap express the pan-mesodermal marker Ntl. (Inset) Animal view of the same conjugate incubated with DAPI showing the external YSL nuclei arranged in a ring-like fashion around the animal cap. The nuclei of the YSL are larger than those of the animal cap blastomeres. (E, F) An animal cap-marginal zone conjugate stained for Ntl protein. The endogenous Ntl expression in the lineage-labeled marginal zone tissue (F) is detected in brown (E), but Ntl expression is not induced in the animal cap explant. Arrowheads point out corresponding nuclei. Scale bars: 100  $\mu\text{m}$ .





without activin

with activin



as assayed by expression of the pan-mesodermal marker Ntl (Fig. 1D). Ntl expression is restricted to the peripheral blastomeres of the animal cap in a ring-like fashion ( $n = 23$ ; 12 independent experiments). Other mesodermal markers such as *gooseoid*, expressed by the dorsal mesoderm (Schulte-Merker *et al.*, 1994), and *fkf-2*, expressed in the marginal zone and the YSL (Odenthal and Nüsslein-Volhard, 1998), were also induced ( $n = 4$ ; 3 independent experiments; data not shown). These findings clearly demonstrate that a blastomere-free yolk cell can induce mesoderm of at least two different dorsoventral identities.

Counterstaining of the recombinates with the DNA stain DAPI reveals that mesoderm induction always occurs precisely in those cells of the animal cap that are in closest proximity to the external YSL (Fig. 1D, inset). In addition, DAPI staining shows that blastoderm removal has caused contraction not only of the surface of the yolk cell but also of the external YSL neighboring the peripheral animal cap blastomeres. Furthermore, in the few cases where no Ntl staining was observed in the recombinates, subsequent DAPI staining demonstrated that the nuclei of the YSL had disintegrated, presumably due to unfavorable culturing conditions, suggesting that mesoderm induction occurs only in the presence of an intact YSL. These observations have prompted us to ask whether the source of the mesoderm-inducing signal, at this stage of development, is restricted to a ring-like region of the yolk cell neighboring the marginal-most blastomeres.

Mesoderm-inducing competence of marginal blastomeres was examined by placing dye-labeled marginal tissue next to unlabeled animal cap tissue and staining for Ntl expression ( $n = 13$ ; three independent experiments). We never observed any Ntl staining in unlabeled animal cap tissue, which was in contact with Ntl expressing marginal zone tissue (Figs. 1E and 1F). These results suggest that marginal zone blastomeres derived from a sphere-stage embryo are not competent to induce Ntl expression in neighboring blastomeres in this experimental assay.

Recently it has been shown that injury or surgical manipulation of tissues causes a transient (<60 min) activation of ERK/MAPK (Christen and Slack, 1999), as well as being the consequence of TGF $\beta$  or FGF signaling. We can exclude that the observed marginal expression of Ntl represents a healing artifact, since we always culture our recombinates at least 2 h and we never observe ectopic Ntl expression in the periphery of explants in Figs. 2B and 2D.

### All Deep Cells Are Competent to Form Mesoderm

As the yolk cell is competent to induce mesoderm, why is mesoderm derived only from hypoblast cells at the margin and not from all deep cells that are in contact with the yolk cell? There are two possibilities: either only the blastomeres at the margin are competent to respond to mesoderm-inducing signals or all blastomeres are competent, but the endogenous signal is locally restricted. To distinguish between these two options, we have asked whether central deep cells have the ability to form mesoderm. As shown in Fig. 2A, after removal of the animal cap marginal explants and central explants were prepared from the three or four cell layers that sit right on top of the yolk cell. These were cultured either in a simple salt medium or in a medium containing activin, a TGF $\beta$  family member which has been shown to be a strong mesoderm inducer *in vitro* in both *Xenopus* (Green *et al.*, 1992) and zebrafish (Schulte-Merker *et al.*, 1992). All explants were fixed after 3 h of culture and expression of Ntl protein was analyzed ( $n = 27$ ; four independent experiments). Central explants express Ntl only after incubation with activin, but not in medium alone (Figs. 2B and 2C), demonstrating that central cells are indeed capable of forming mesoderm. Marginal explants express Ntl in some cells (those which had already been induced to form mesoderm) if cultured without activin and in all cells if incubated with activin (Figs. 2D and 2E). Animal caps which were cocultured with the marginal explants expressed Ntl ubiquitously, if cultured in the presence of activin, but did not express Ntl if cultured without activin. These explants served as controls for appropriate culturing conditions. These results clearly show that all cells of the sphere-stage embryo can respond to mesoderm-inducing signals. It is therefore very likely that the endogenous signal is spatially restricted in a ring-like fashion, and more central cells do not form mesoderm because they are not exposed to the signal.

### Removal of the Vegetal Pole Immediately after Egg Deposition Leads to Completely Ventralized Embryos

To further localize the source of mesoderm-inducing signals, we have removed about 75% of the vegetal yolk mass at various stages of early development, from the one-cell to the eight-cell stage. The blastoderm, with remnants of the yolk attached, was then cultured and assayed for mesoderm formation at 6 hpf. Regardless of how early we carried out this procedure, we never found experimental

**FIG. 2.** All deep cells are competent to respond to a mesoderm-inducing signal such as activin. (A) Scheme of the experimental procedure. (B) Explants of the central deep cells dissected at sphere stage and cultured until siblings had reached shield stage show no endogenous expression of Ntl. (C) Explants of the central deep cells cultured in activin (8 U/ml) show Ntl expression in all cells. (D) Explants of the marginal blastomeres and animal caps show endogenous Ntl expression only in the marginal explants. (E) Explants of marginal blastomeres and animal caps cultured in activin (8 U/ml) show Ntl expression in all cells. Scale bars: 100  $\mu$ m.

**TABLE 1**  
Effect of Removing the Vegetal Pole in Both Wild-Type and *swr* Mutant Embryos on Different Tissues and Structures

Genotypes	+/+		<i>swr</i> -/-	
	+/+	+/+ vegetal pole removed	<i>swr</i> -/-	<i>swr</i> -/- vegetal pole removed
Dorsal mesoderm (notochord)	+	-	+	-
Ventral mesoderm (blood precursors)	+	+	-	-
Trunk somites	+	-	+	+
Tail somites	+	+	+	+
Neuroectoderm	+	-	+	+

embryos without Ntl expression in the marginal zone ( $n = 47$ ; seven independent experiments; data not shown), indicating that mesoderm-inducing signals are, already at the one-cell stage, located very close to the forming blastoderm at the animal pole.

While induction of Ntl expression was undisturbed, we have found that removal of the vegetal-most third of the yolk during the first 20 min postfertilization leads to a complete ventralization of the embryo (Fig. 3; Table 1), confirming recent findings of Koshida *et al.* (1998). In total we removed the vegetal yolk cell of 121 embryos in 31 independent experiments resulting in 86% of the cases in embryos that are completely ventralized ( $n = 104$ ). The other 14% showed gastrulation defects and lysed. Removing approximately one-fifth of the yolk cell from the *lateral* side of the newly fertilized egg ( $n = 8$ ; 3 independent experiments) results in embryos without any morphological defect.

At 6 hpf, due to involution and dorsal convergence, the embryonic shield forms at the dorsal side of control embryos (Fig. 3B). In experimental embryos with the vegetal pole removed an embryonic shield never forms (Fig. 3C). At tailbud stages, the majority of all blastomeres has migrated to the dorsal side of the embryo (Fig. 3D) in control sibling embryos, with the future anterior neuroectoderm populating the former animal pole of the embryo. In experimental cases, the embryo is radially symmetrical, and there are no signs of dorsal convergence movements (Fig. 3E). Involution

appears to be normal, as judged by the appearance of a germ ring (Fig. 3C) as well as by the occurrence of a hypoblast (Fig. 3E). Strikingly, there is an apparent lack of cells at the animal pole of the embryo suggesting the absence of neuroectoderm (Fig. 3E). At later stages of development there are cells which populate the anterior-most region of the embryo, but we have never observed any differentiated neural structures such as eyes or the midbrain-hindbrain boundary (Figs. 3F-3I).

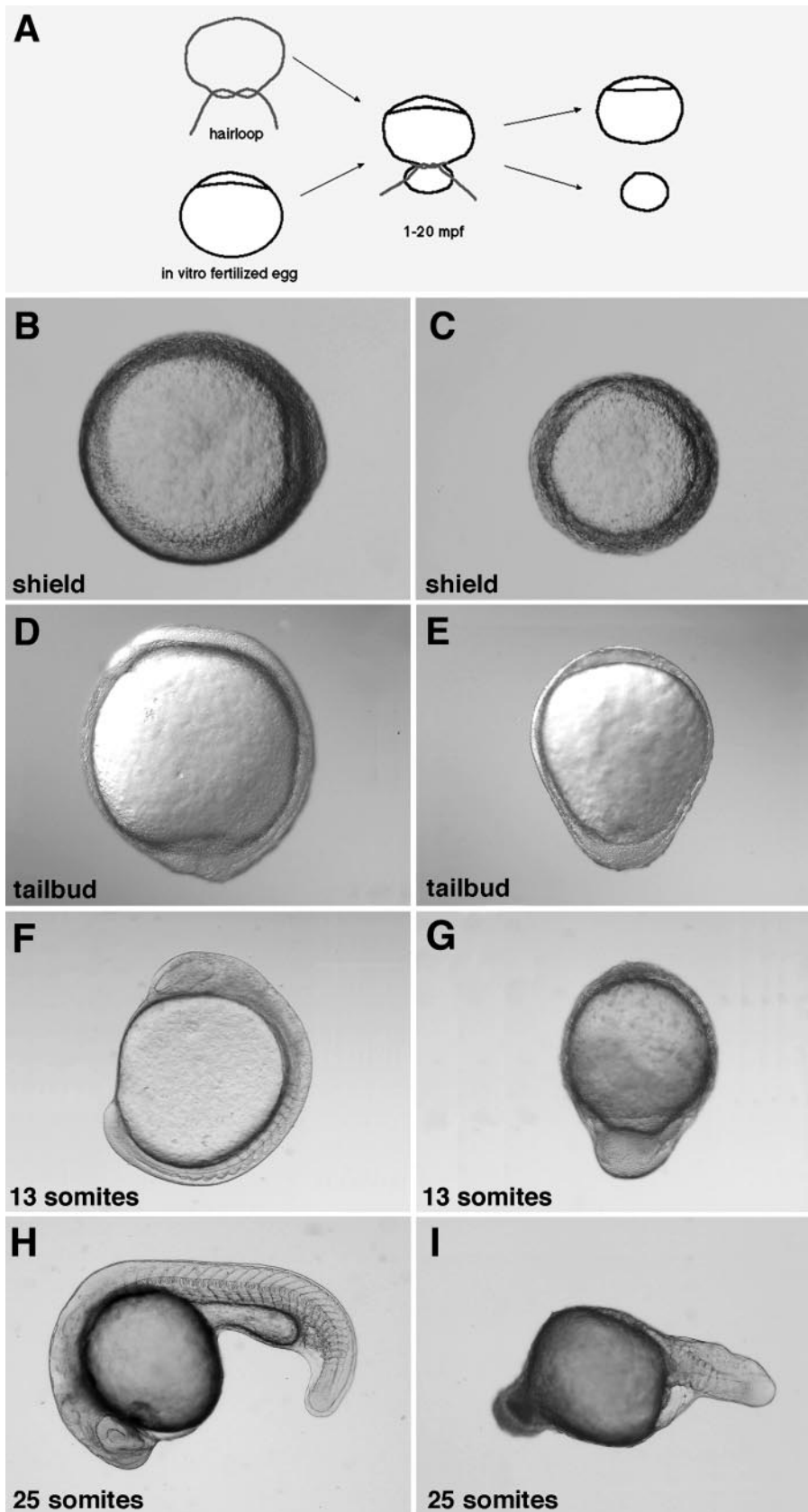
### **Evidence for a Trunk Organizer: Anterior, but Not Posterior Somites Are Absent in Ventralized Embryos**

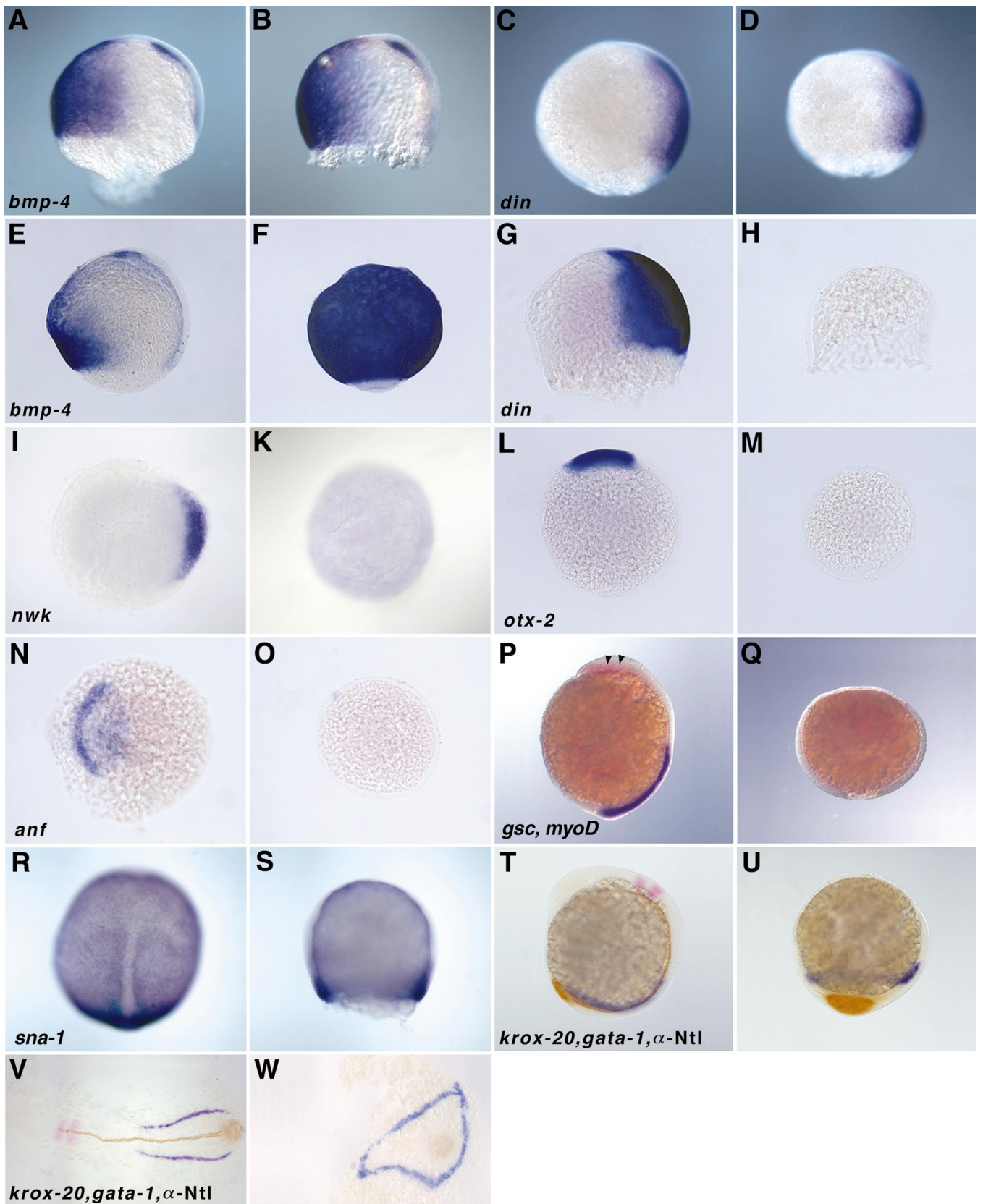
While the early effects of removing the vegetal-most part of the yolk cell have been described previously, we have asked how removal of the vegetal-most part of the yolk cell affects later events in zebrafish development, an issue which has not been addressed previously (Koshida *et al.*, 1998; Mizuno *et al.*, 1997). In experimental cases, head structures are missing, but an enlarged tailbud forms (Figs. 3F and 3G) which is even more pronounced than the tailbud observed in the strongly ventralized embryos deficient in *chordino* function (Hammerschmidt *et al.*, 1996; Schulte-Merker *et al.*, 1997). Another striking difference is the lack of somite formation in the experimental cases during the time when in sibling controls the first 14-15 anterior somites formed. After this stage of development, somitogenesis commences and proceeds with the normal rate of one pair of somites forming every 30 min. These data strongly suggest the existence of a separately controlled trunk organizer (anterior trunk region) versus tail organizer (posterior trunk region) in fish, as suggested for other vertebrates (Spemann, 1931).

### **Molecular Characterization of Ventralized Embryos Reveals a Complete Lack of Neural Tissue and Anterior Trunk Somites**

To further understand the defects caused by removal of the vegetal-most yolk cell, we analyzed the expression of numerous genes. We examined the degree of ventralization by using *zbmp-4* (Nikaido *et al.*, 1997) and *gata-1* (Detrich *et al.*, 1995) as ventral markers; *chordino* (*din*; Schulte-Merker *et al.*, 1997), *nieuwkoid* (*nwk*; Koos and Ho, 1998), *gooseoid* (*gsc*; Schulte-Merker *et al.*, 1994), and  $\alpha$ -Ntl

**FIG. 3.** The vegetal part of the yolk cell contains all information necessary to establish dorsoventral polarity and the anterior region of the embryo. (A) The most vegetal part of the yolk cell can be ligated off within the first 20 mpf using a hairloop. (B, C) After 6 h of development morphogenetic movements form a shield at the future dorsal side of an untreated embryo (B), whereas in a ventralized embryo only involution occurs, resulting in a completely radialized embryo. (D, E) Convergence and extension movements lead to the formation of an axis at the dorsal side of the embryo (D), whereas experimental embryos (E) are completely radialized. (F, G) Thirteen somites have formed after 15 h of development in untreated sibling embryos (F), whereas no somites have developed in embryos lacking the vegetal-most part of the yolk cell (G). (H, I) At a stage when the untreated embryo has formed 25 somites, only 11 somites are developed in the experimental case. (B, C) Animal view, dorsal to the right; (D-G) lateral view, dorsal to the right; (H, I) lateral view, dorsal up.





(Schulte-Merker *et al.*, 1992) as dorsal markers; and *snail-1* (*sna-1*; Hammerschmidt and Nüsslein-Volhard, 1993) and *myoD* (Weinberg *et al.*, 1996) as markers for anterior paraxial mesoderm and muscle.

In control embryos where the lateral and not the vegetal yolk was removed, expression of the dorsal gene *din* and the ventral gene *zbmp-4* was not affected (Figs. 4A–4D).

In wild-type embryos, *zbmp-4* is expressed in the ventral hemisphere at 70% epiboly (Fig. 4E), as well as in a few cells of the prechordal plate. Its antagonist *din* is expressed in a complementary fashion in both the dorsal mesoderm and the presumptive neuroectoderm (Fig. 4G; see also Schulte-Merker *et al.*, 1997). In experimental cases, we found *zbmp-4* expression to be grossly expanded to all regions of the embryo (Fig. 4F), while *din* expression was completely abolished (Fig. 4H). Expression of *nwk*, a gene expressed in the blastomeres and the YSL at the dorsal side (Fig. 4I), was absent in ventralized embryos (Fig. 4K). We have also never detected, at gastrula and early somitogenesis stages, expression of *gsc* or *myoD* in those embryos where the vegetal pole had been removed early (Fig. 4Q). *Sna-1* expression in the paraxial mesoderm is absent in ventralized embryos at 90% epiboly, but still present in the involuting hypoblast (Fig. 4S). Similarly, the presumptive notochord as depicted by expression of the nuclear antigen Ntl was never present in experimental embryos (Figs. 4N and 4O, and 4P and 4Q), while *gata-1*, expressed in presumptive blood cells in a bilateral fashion, was radialized in experimental cases (Figs. 4N and 4O, and 4P and 4Q). These data clearly indicate a complete ventralization of embryos after early removal of the vegetal-most yolk.

As both *din* and *gsc* are expressed not only in the mesoderm, but also in the presumptive neuroectoderm, we have employed other markers to test for the presence of neuroectoderm. Both *otx-2* and *anf* are expressed in the anterior-most neuroectoderm in wild-type embryos (Figs. 4L and 4N), but were never expressed in experimental embryos (Figs. 4M and 4O). *Krox-20*, a marker for rhom-

bomeres 3 and 5 (Figs. 4T and 4V), was also never detected in experimental embryos (Figs. 4U and 4W). In summary, removal of the vegetal yolk results in a complete lack of neuroectoderm. In this respect, as well as in the degree of ventralization, these embryos are much more severely affected than embryos mutant in *din* (Hammerschmidt *et al.*, 1996).

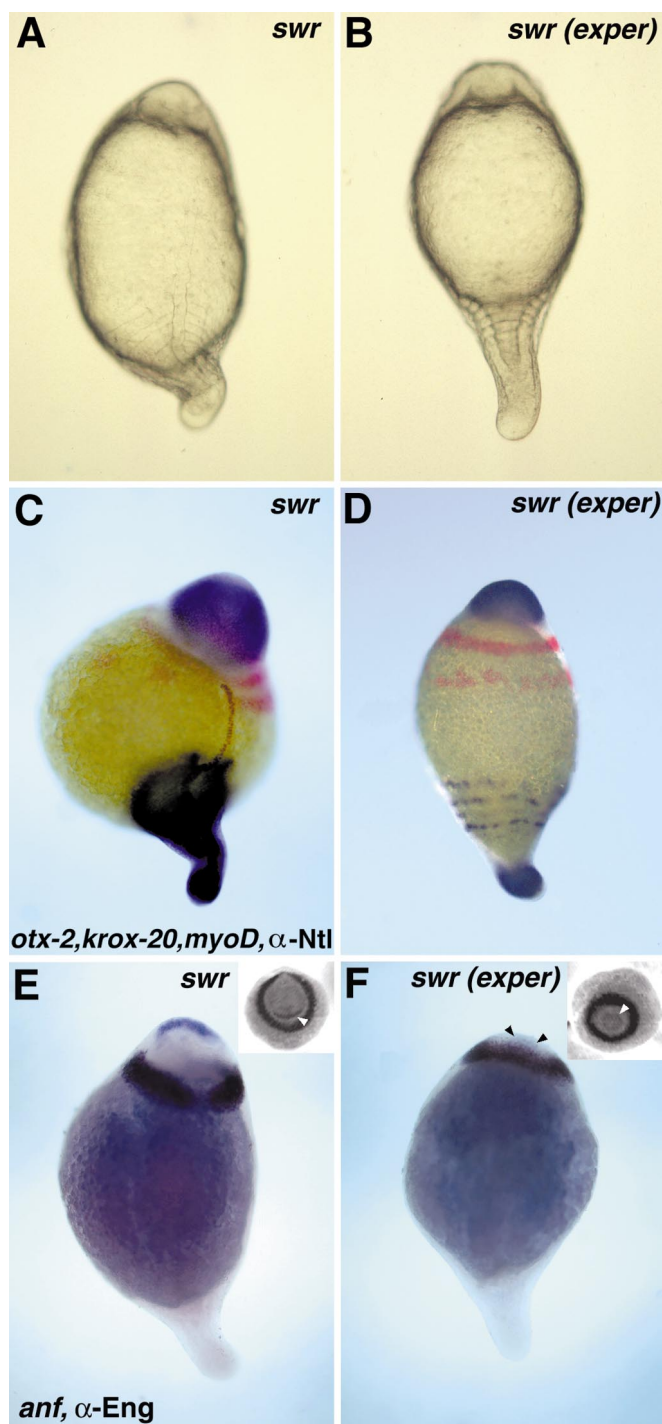
### Removal of the Vegetal Cytoplasm from *swr* Mutant Embryos

We have analyzed the effect of vegetal yolk removal in embryos mutant for *swr* in order to understand whether establishing the early, maternally governed dorsoventral polarity is dependent on *swr* function. *Swr* is the zebrafish homologue of BMP-2 (Kishimoto *et al.*, 1997), and embryos mutant for *swr* are severely dorsalized (Mullins *et al.*, 1996), lacking ventral structures such as *gata-1* expressing presumptive blood cells. Injection of *swr* mRNA is able to rescue the mutant phenotype, resulting in viable homozygous mutant adults (Kishimoto *et al.*, 1997). These homozygotes, when mated, produce in turn exclusively mutant progeny, which were used for these experiments.

Removal of the vegetal yolk in *swr* mutants leads to a significant rescue of the ventralized phenotype compared to wild-type embryos after the same treatment ( $n = 20$ ; six independent experiments). *Swr* mutants, in which the vegetal yolk had been removed, developed somites as assayed by live observation (Figs. 5A and 5B; Table 1) and *myoD* expression. Somite formation commenced at the same time as in sibling controls and in the radial manner typical for *swr* mutant embryos. However, a presumptive notochord was never observed (Fig. 5B). This was confirmed by examining Ntl expression which was only present in the tailbud, but missing in the presumptive notochord cells (Fig. 5D).

Remarkably, neural tissue was present in experimental *swr* mutants, in contrast to experimental wild-type embryos. *Otx-2*-positive cells as well as *krox-20*-expressing cells were observed in all cases in a manner very similar to

**FIG. 4.** Gene expression in embryos with lateral (A–D) or vegetal (E–W) yolk removed detected by *in situ* hybridization and immunohistochemistry. In embryos with removed lateral yolk the expression pattern of *zbmp-4* (B) and *din* (D) is not altered compared to the siblings (A, C). (E, F) *zbmp-4* is at 70% epiboly expressed in the ventral epi- and hypoblast and dorsal prechordal plate mesoderm (E), whereas it is ubiquitously expressed in the experimental embryo (F). (G, H) *din* is expressed at 70% epiboly in the axial mesoderm and dorsal ectoderm (G) and absent in embryos which lack the vegetal pole (H). (I, K) At 30% epiboly *nwk* is expressed in the blastomeres and the YSL of the future dorsal side (I), but is absent in ventralized embryos (K). (L, M) At 90% epiboly *otx-2* is expressed in the anterior-most neuroectoderm (L), but is absent in ventralized embryos (M). (N, O) *anf* is expressed at 90% epiboly in the anterior-most neuroectoderm (N) and is absent in the experimental cases (O). (P, Q) At tailbud stage, *gsc* (red, arrowheads) is expressed in the anterior prechordal plate and *myoD* (blue) in the paraxial mesoderm (P). Transcripts of both genes are abolished in embryos lacking the vegetal pole (Q). At the end of gastrulation *sna-1* expression is found in the paraxial mesoderm and the marginal zone (R). In a ventralized embryo paraxial mesoderm expression is absent (S). (T–W) *krox-20* (red) is expressed in rhombomeres 3 and 5, *gata-1* (blue) marks the blood precursor cells, and Ntl (brown) stains the nuclei of the notochord and the tailbud at the five somite stage in untreated embryos (T, V). In experimental cases (U, W) *krox-20* and axial Ntl expression are missing, whereas one can still find Ntl expression in the tailbud. *gata-1* is expressed in a radial fashion. (A–H, L, M, and P–U) Lateral view, dorsal to the right (only in R and S is dorsal to the front); (I–K, N, O, V, and W) animal view, anterior to the left.



**FIG. 5.** Performing ventralization experiments in *swirl* embryos rescues neural fates and restores formation of the trunk region. (A) Untreated *swr* embryo around 13 h of development: the notochord and eight ventrally expanded somites have formed. (B) In an experimental *swr* embryo a notochord fails to form and the somites are completely radialized. (C) Untreated *swr* embryo expresses *otx-2* (anterior-most staining) most anteriorly, followed by radialized *krox-20* (red) in rhombomeres 3 and 5. *MyoD* is expressed by the radialized somites and brown marks the nuclear localization of

untreated *swr* mutant embryos (Figs. 5C and 5D). *Anf* and *Eng* are expressed in the anterior-most neuroectoderm and in the domain of the midbrain–hindbrain boundary, respectively (Fig. 5E). Both are expressed around the whole circumference of the embryo with slightly lower levels at the dorsal side. In contrast, in experimental *swr* embryos expression of both genes is completely radialized (Fig. 5F). Although the anteroposterior order of gene expression is correct in experimental *swr* embryos, it seems as if there is less tissue anterior to the midbrain–hindbrain boundary compared to untreated *swr* mutants (Figs. 5C–5F). These findings show that the balance between the signal from the vegetal pole and *swr* determines the formation of nonneural versus neural ectoderm. The induction of the most dorsal mesoderm, the presumptive notochord, depends only on the determinant localized at the vegetal part of the yolk cell shortly after fertilization and is independent of *swr*. The data further demonstrate the existence of a trunk organizer established by the determinant from the vegetal yolk cell and a tail organizer independent of this factor. Strikingly, these experiments suggest that the anteroposterior axis is set up independently of the dorsoventral axis.

## DISCUSSION

In this paper we have shown the zebrafish yolk cell to be an important source of inductive signals for mesoderm formation and dorsoventral as well as neural patterning. We have demonstrated that signals from the external YSL can induce mesoderm at late blastula stages. We have also shown that a signal localized at the vegetal pole of the yolk cell is essential for organizer induction, neuroectoderm formation, and formation of the trunk region of the embryo: these processes do not occur if the vegetal-most part of the yolk cell is removed from the embryo immediately after fertilization. Performing the same experiment in a *swr/zbmp-2b* mutant background reveals that these processes are counteracted by *swr* signaling in the zebrafish embryo. Notochord formation, however, depends on the determinant localized at the vegetal pole and is independent of *swr*.

Ntl. (D) In experimental *swr* embryos expression of *otx-2*, *krox-20*, and *myoD* is not changed compared to the untreated *swr* embryo, whereas axial Ntl expression is absent and only the nuclei of the tailbud still express Ntl. In untreated *swr* embryos expression of *anf* (blue) in the anterior-most neuroectoderm and *Eng* (brown) in the midbrain–hindbrain boundary is radialized, showing a slight reduction dorsally (E). In experimental *swr* embryos expression of both genes is completely radialized (F); arrowheads mark *anf* expression. Insets show anterior views of the same embryos; the *anf* expression domain is pointed out by arrowheads. (A, B) Dorsal view of living embryos, anterior up; (C–F) dorsal slightly to the right, anterior up.

### **The Yolk Cell Is the Source of a General Mesoderm-Inducing Signal**

We have designed an experimental system that allowed us to place zebrafish animal caps onto yolk cells completely stripped of blastomeres, thereby mimicking the experimental setup of Nieuwkoop. While the outcome of our experiments confirm the data of Mizuno *et al.* (1996), we present unambiguous evidence that the yolk cell, and not any adhering blastomeres, provides mesoderm-inducing signals. Mesoderm induction was always correlated to an intact yolk syncytial layer, suggesting strongly that, after MBT, the nuclei of the YSL are responsible for maintaining the mesoderm-inducing capacity of the yolk cell. Further evidence for this notion stems from the finding that all blastomeres of the embryo are competent to respond to activin at blastula stages (this study), a potent mesoderm inducer in both frogs and zebrafish (Green *et al.*, 1992; Schulte-Merker *et al.*, 1992). Assuming that activin mimics the *in vivo* inducer, this suggests that the mesoderm-inducing signal is restricted to the margin of the embryo, coinciding with the position of the external YSL. We have demonstrated that marginal blastomeres are not competent to induce Ntl expression when juxtaposed to animal cap tissue. Therefore, we exclude the possibility that either a left-behind blastomere or a signal released by marginal blastomeres and deposited onto the yolk cell induces mesoderm in the overlying animal cap.

Two *nodal*-related genes (*znr*) with mesoderm-inducing capacity have been identified in zebrafish: *znr1* and *znr2* (Erter *et al.*, 1998). Candidate gene approaches have revealed the mutant *cyclops* (*cyc*) corresponding to the *znr1* locus (Rebagliati *et al.*, 1998; Sampath *et al.*, 1998) and the mutant *squint* (*sqt*) corresponding to the *znr2* locus (Feldman *et al.*, 1998). Both genes are expressed during early gastrulation stages in the entire marginal zone of the embryo. In addition, *znr2* is expressed at low levels maternally and in the YSL (Erter *et al.*, 1998; Feldman *et al.*, 1998). Therefore, *znr2/sqt* is a good candidate to signal from the yolk cell to the overlying blastomeres to induce mesoderm. However, overexpression studies and mutant analysis suggest that *znr2/sqt* is not sufficient to induce mesoderm of all dorsoventral identities (Erter *et al.*, 1998) and must act in concert with other factors, e.g., *znr1/cyc* (Feldman *et al.*, 1998). Future studies will show if *znr2* is indeed the mesoderm-inducing signal emanating from the yolk cell.

In *Xenopus*, mesoderm-inducing signals are secreted by vegetal pole cells (reviewed in Heasman, 1997; Slack, 1994), even though it is impossible to draw the line between signaling cells and responding cells. Although early amphibian and teleost embryos are quite different in structure, mesoderm induction in both phyla seems to be controlled by factors residing in the most vegetal part of the respective embryos. There might be another similarity, namely that both the yolk in zebrafish and the vegetal cells in *Xenopus* can be considered extraembryonic, as both do not contrib-

ute to the embryo, but rather end up in the gut. It will be interesting to find molecular markers specific to the YSL and to examine the distribution of their homologues in frog embryos and other vertebrates.

Finally, some teleosts such as sturgeons (Bolker, 1993) are very similar to amphibian embryos in that they are not telolecithal and that they form a gray crescent. Mesoderm induction in teleosts and amphibia is likely to have a common evolutionary origin and to represent variations of one scheme.

### **Notochord Induction and Dorsal Specification Are Independently Governed by a Vegetally Localized Component**

In the course of our studies we have found that removal of the vegetal part of the yolk cell within the first 20 min after fertilization leads to completely ventralized embryos in the zebrafish. This confirms findings in goldfish and zebrafish (Koshida *et al.*, 1998; Mizuno *et al.*, 1996), where a similar treatment abolishes *gsc* staining. Control embryos in which the lateral and not the vegetal yolk was removed showed no developmental defects. These findings show that the ventralization of the experimental embryos is caused by removing the dorsal determinant located at the vegetal pole and not as a consequence of the embryos having too little cytoplasm. Our observations confirm and significantly extend these earlier studies: First, we have used a wide variety of markers to analyze this phenotype in greater detail. Second, we show not only that D-V polarity is disturbed, but also that neural tissue is completely missing in these embryos. Third, we have analyzed the ventralized phenotype at later stages and demonstrate the existence of a trunk organizer which depends on this vegetally localized signal.

Removal of the vegetal yolk cell leads to slightly smaller embryos, which develop normally during cleavage and blastula stages. They undergo involution and form mesoderm, but seem to lack dorsal convergence movements. An embryonic shield consequently never forms (Fig. 3C). Analysis with molecular markers such as *nwk* (Koos and Ho, 1998), *din*, and *gsc*, all of which are expressed before the onset of dorsal convergence, shows that the absence of dorsal convergence movements is likely due to a failure in determining the dorsal side.

Upon fertilization a rearrangement of microtubules at the vegetal pole of the zebrafish zygote results in the ordered arrangement of a parallel array of microtubules (Jesuthasan and Strähle, 1996). Fluorescent beads which are injected at the vegetal pole immediately after fertilization are transported rapidly to the marginal region of the zygote, presumably along these microtubules (Jesuthasan and Strähle, 1996). Taken together with data suggesting that in medaka there might be transport of vesicles to the dorsal side of the zygote (Trimble and Fluck, 1995), it is tempting to speculate that in teleosts dorsal specification occurs through transport of a determinant that is located at the vegetal pole at fertilization to the dorsal margin of the zygote. Removing

the vegetal yolk by experimental manipulation also removes this determinant, leading to complete ventralization of the embryo.

Both *myoD* staining and morphological examination show that the anterior somites are missing in experimental embryos, a finding that has not been reported previously. Somitogenesis commences only at a point in time when sibling controls have reached the 15-somite stage. At this time, somitogenesis in experimental cases proceeds with the same speed as in controls, indicating that the lack of anterior somites is not due to a slowing down in somitogenesis, but rather to a complete absence of anterior somite specification in ventralized embryos. This finding is supported by the absence of *sna-1* expression in the paraxial mesoderm at the end of gastrulation in ventralized embryos. These results show that the formation of the anterior 14 to 15 somites, the trunk somites, and the posterior tail somites are regulated differently. The anterior trunk formation is dependent on the determinant from the vegetal pole, whereas the posterior tail region is not. While our observations provide the first embryological evidence for a trunk organizer in teleosts, there exists experimental evidence for our hypothesis: Injection of dominant-negative FGF-receptor mRNA results in embryos lacking trunk and tail structures (Griffin *et al.*, 1995). As the mutant phenotype of the FGF-regulated gene *no tail* (*ntl*) lacks the tail and notochord but has a normal trunk, the authors suggest trunk development as being dependent on an unidentified, FGF-regulated gene, which they have putatively named "no trunk" (Griffin *et al.*, 1995). The phenotype of the *spadetail* (*spt*) mutant is consistent with *spt* encoding "no trunk" (Griffin *et al.*, 1998; Kimmel *et al.*, 1989), and it will be interesting to see whether *spt* is a downstream target of the vegetally localized determinant which we have identified as the trunk organizer.

Ventralized embryos are also completely devoid of neural tissue. We have employed a variety of markers, ranging from anterior markers such as *otx-2* and *anf* to posterior markers such as *krox-20*. This finding is consistent with the observation that all cells of these embryos seem to express *zbmp-4*, which is known to be a strong repressor of neural tissue (Wilson and Hemmati-Brivanlou, 1995).

The effect of removing the vegetal yolk results in completely ventralized embryos with a much more severe phenotype than that observed in embryos mutant for *chordino*. In mutant *chordino* embryos, neural tissue is somewhat reduced and anterior somites are smaller, but both tissues are clearly present (Hammerschmidt *et al.*, 1996). This strongly suggests that *chordino* is not the only dorsal determinant in the zebrafish embryo and that other gene products such as Noggin (Smith and Harland, 1992) and Follistatin (Hemmati-Brivanlou *et al.*, 1994) can partially substitute for *chordino* function.

### **Lack of *swr* Can Substitute for the Trunk Organizer and Neuroectoderm Formation**

In ventralized embryos, dorsal structures such as notochord and anterior muscle are missing (Figs. 3G, 4Q, and 4U), while *gata-1*, a marker for blood precursors, is radialized (Figs. 4U and 4W). In embryos mutant for *swr*, the opposite situation can be found: dorsal structures are expanded, while *gata-1*-positive tissue is absent (Mullins *et al.*, 1996). We have asked about the epistatic relationship of the dorsal determinant and *swr* signaling and found that both neural tissue and trunk structures are restored in *swr* mutant embryos in which the vegetal yolk had been removed (Fig. 5), whereas the dorsal-most mesoderm, the notochord, is not restored in these embryos. This finding has four important implications.

First, the determinant which is located at the vegetal pole of the yolk cell and which is essential for notochord induction in wild-type embryos is still required in the absence of *swr*, meaning that it is acting independently of *swr* function in notochord induction.

Second, the vegetally localized determinant which is essential for neuroectoderm formation in wild-type embryos is not required in the absence of *swr*. This means that in fish, as has been suggested for *Xenopus* (Wilson and Hemmati-Brivanlou, 1995), neural is likely to be the default state of animal pole tissue: in the absence of *swr*, neural inducers such as that emanating from the vegetal pole neutralize the epiblast, while in the absence of the neuralizing activity from the vegetal pole *swr* signaling is sufficient to counteract any remaining neuralizing activity in the embryo, and neural tissue is not specified at all.

Third, our experiments suggest that the default state of the mesoderm is somitic and that the trunk region is under the influence of two activities, namely *swr* and the determinant from the vegetal pole. We have identified *swr* as an essential repressor of trunk somite formation. In wild-type embryos with the vegetal yolk removed, *swr* activity is not counteracted by the activity of the determinant from the vegetal pole and consequently is able to suppress the formation of trunk somites completely. In *swr* mutant embryos with the vegetal yolk removed, the absence of both *swr* and of the determinant from the vegetal pole leads to the formation of trunk somites, according to the default state of these cells.

Fourth, we could show that the establishment of the anteroposterior axis in zebrafish is independent of the induction of the dorsoventral axis. This is most clearly shown in ventralized *swr* embryos which only exhibit lateral identity concerning the dorsoventral axis, but they establish an anteroposterior polarity, although the anterior-most neuroectoderm is slightly reduced in size. These results are supported by transplantation studies showing that the anteroposterior value of induced neural tissue is dependent on its animal-vegetal position rather than on the organizer (Koshida *et al.*, 1998; Woo and Fraser, 1997).

## CONCLUSIONS

We have demonstrated that the zebrafish yolk cell is the source of mesoderm-inducing signals at sphere stage. Furthermore, mesoderm always forms in a ring-like fashion even though all blastomeres are competent to respond to mesoderm induction. This strongly suggests the external YSL to be the localized source of these inductive signals in the embryo.

By removing the vegetal-most part of the yolk cell, we can show that there is a localized signal which is essential for organizer induction, neuroectoderm formation, and formation of the anterior somites of the embryo. Embryos lacking this signal are completely ventralized. Performing this experiment in a *swr* mutant background rescues the formation of neuroectoderm, as well as of the head and trunk region, demonstrating that *swr* counteracts the dorsal determinant in a wild-type embryo. The formation of neuroectoderm and anterior somitic mesoderm in embryos lacking the dorsal-most and ventral-most information suggests neuroectoderm and lateral mesoderm to resemble their default state. The notochord, however, which is induced by the determinant from the vegetal pole, forms independently of *swr* signaling. This suggests two signaling cascades induced by this signal, one dependent on *swr* and one independent of *swr*.

With these experiments we further demonstrate that the anteroposterior axis is established independently of the dorsoventral axis in the zebrafish embryo.

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