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The role of the yolk syncytial layer in germ layer patterning in zebrafish

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SUMMARY

Formation of the three germ layers requires a series of inductive events during early embryogenesis. Studies in zebrafish indicate that the source of these inductive signals may be the extra-embryonic yolk syncytial layer (YSL). The characterization of genes encoding the nodal-related factor, Squint, and homeodomain protein, Bozozok, both of which are expressed in the YSL, suggested that the YSL has a role in mesendoderm induction. However, these genes, and a second *nodal*-related factor, *cyclops*, are also expressed in the overlying marginal blastomeres, raising the possibility that the marginal blastomeres can induce mesendodermal genes independently of the YSL.

We have developed a novel technique to study signaling from the YSL in which we specifically eliminate RNAs in the YSL, thus addressing the in vivo requirement of RNA-derived signals from this region in mesendoderm induction. We show that injection of RNase into the yolk cell after the 1K cell stage (3 hours) effectively eliminates YSL transcripts without affecting ubiquitously expressed genes in the blastoderm. We also present data that indicate the stability of existing proteins in the YSL is unaffected by RNase injection. Using this technique, we show that RNA in the YSL is required for the formation of ventrolateral mesendoderm and induction of the *nodal*-related genes in the ventrolateral marginal blastomeres, revealing the presence of an unidentified inducing signal released from the YSL. We also demonstrate that the dorsal mesoderm can be induced independently of signals from the YSL and present evidence that this is due to the stabilization of β-catenin in the dorsal marginal blastomeres. Our results demonstrate that germ layer formation and patterning in zebrafish uses a combination of YSL-dependent and -independent inductive events.

Key words: Zebrafish, Squint, Yolk syncytial layer, Bozozok, Germ Layer

INTRODUCTION

Induction and patterning of the three germ layers are two of the earliest and most crucial events of embryogenesis. Studies in *Xenopus* have demonstrated that a vegetally localized maternal T-box transcription factor, VegT, is required for the transcription of inducing factors that position the endoderm at the bottom of the embryo, and the mesoderm in the overlying cells (reviewed in Kimelman and Griffin, 1998). In zebrafish, the mechanism of mesendoderm induction is less clear. Transplantation experiments demonstrate that the extra-embryonic yolk cell, and an associated syncytial layer of cytoplasm and nuclei known as the yolk syncytial layer (YSL), is capable of ectopically inducing mesoderm and endoderm when transplanted to the animal pole of a host embryo (Mizuno, 1996; Ober and Schulte-Merker, 1999; Rodaway et al., 1999). This suggests that the mesendoderm is induced by YSL-derived signals.

Although it is clear that the YSL is sufficient to induce mesoderm and endoderm, whether it is required to do so was not known. Consistent with the ability of the YSL to induce dorsal mesoderm, the nodal-related factor Squint (Sqt; Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998) and the homeodomain protein Bozozok (Boz; Fekany et al., 1999; Koos and Ho, 1998, 1999; Yamanaka et al., 1998), both of which have been shown to be necessary and sufficient for dorsal mesoderm induction, are expressed in the dorsal YSL (Erter et al., 1998; Fekany et al., 1999; Feldman et al., 1998; Koos and Ho, 1998; 1999; Rebagliati et al., 1998; Yamanaka et al., 1998). However, both of these genes are also expressed in the marginal blastomeres (Erter et al., 1998; Feldman et al., 1998; Koos and Ho, 1998; Rebagliati et al., 1998), suggesting that these blastomeres contain sufficient information to induce dorsal mesoderm without a contribution from the YSL. Endoderm formation also requires Sqt function, as shown by the double mutant of *sqt* and another nodal-related factor, *cyclops* (*cyc*; Gritsman et al., 2000; Rebagliati et al., 1998; Rodaway et al., 1999). *cyc* is expressed only in the marginal blastomeres (Gritsman et al., 2000; Rebagliati et al., 1998), again suggesting that the marginal blastomeres contain the necessary factors to induce endoderm, and may not require additional signals from the YSL. *sqt* and *boz* mutants do not address whether or not the expression of each is required in the YSL for the induction of dorsal mesoderm or endoderm, since these mutants eliminate the function of each gene in both the YSL and marginal blastomeres. In order to address whether or

not the expression of *sqt* and *boz* in the YSL is required for dorsal mesoderm and endoderm formation, it is necessary to specifically eliminate the expression of these genes in the YSL.

In order to determine whether or not the YSL is required for establishing the mesendoderm, we chose to eliminate all RNA transcripts from the YSL by injecting RNase into the yolk cell at the time that the YSL forms. Formation of the YSL at the 1K cell stage (3 hours) marks the time at which gap junctions between the yolk cell and the overlying marginal blastomeres are closed, resulting in the inability of large proteins to freely pass between the extra-embryonic and embryonic compartments (Kimmel and Law, 1985). Therefore, injection of RNase was expected to target transcripts in the YSL specifically, thus addressing whether or not the expression of *sqt* and *boz* in this region is required for dorsal mesoderm and endoderm formation. As no factors have yet been identified that are required for ventrolateral mesoderm induction, injection of RNase into the yolk cell should also indicate whether transcripts in the YSL are required for all mesoderm formation.

We show that injection of RNase specifically eliminated RNA in the YSL within 20 minutes, and we present evidence that protein stability is not affected. We demonstrate that the YSL is required for ventrolateral mesendoderm induction, and that RNA in the YSL is required between the 1K cell stage (3 hours) and sphere stage (4 hours) for ventrolateral *ntl* expression. We also show that the expression of *sqt* and *cyc* in the ventrolateral blastomeres depends on the YSL. In contrast, we find that dorsal mesendoderm induction occurs independently of the YSL via stabilization of β-catenin in the dorsal blastomeres. These results demonstrate a required role for the YSL in establishing the germ layers, and indicate that an unidentified signal in the YSL is needed to induce ventrolateral mesendoderm.

MATERIALS AND METHODS

Embryos

Adult AB strain zebrafish and embryos were raised at 28.5°C as described (Westerfield, 1989). Embryonic stages were determined by observation (Westerfield, 1989). Embryos were dechorionated in 2 mg/ml Pronase (Roche Molecular Biochemicals) prior to injection (Westerfield, 1989).

Dye, RNase and GFP injections

All injections were made into the yolk cell of dechorionated 1K cell stage (3 hours) embryos, unless otherwise indicated, using a Picospritzer II (Parker Hannifin Corporation). Approximately 1 nl was injected per embryo.

Lysine fixable biotinylated dextran or rhodamine-labeled biotinylated dextran (10kMW, Molecular Probes) was dissolved in water to a 20 mg/ml stock solution, and injected at 4 mg/ml, with or without RNase. Dextrans were visualized using biotin-avidin peroxidase staining (Vector Laboratories). RNase, DNase free (0.5 mg/ml, Roche Molecular Biochemicals) was diluted 1/50 in water, for a final concentration of 10 μg/ml and injected with 4 mg/ml dextran. Recombinant green fluorescent protein (GFP; Roche Molecular Biochemicals) was diluted to 0.5 mg/ml in water, and injected into embryos with or without RNase and dextran.

Inactivation of RNase with DEPC

A 25 μl volume of 0.2 mg/ml RNase, with or without 280 μM diethyl pyrocarbonate (DEPC; Sigma) was incubated in closed eppendorf tubes for approximately 20 hours in a 37°C air incubator. The tubes were then transferred to a 37°C heat block and incubated for 10 minutes with the caps open to allow volatile DEPC to escape. The overnight incubated RNase, with or without DEPC, was diluted 1:2 in water with dextran such that the final concentration of RNase and dextran was 10 μg/ml and 4 mg/ml. This mixture was then injected into embryos. For in vitro analysis of RNase activity, the overnight incubated RNase with or without DEPC was diluted 1:2 in water, and 6 μl of this was added to 1 μg of capped RNA (synthesized using the Ambion Message Machine System) and incubated at room temperature for 1 hour.

In situ hybridization

Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense RNA and visualized using anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Roche Molecular Biochemicals) as previously described (Griffin et al., 1995; Melby et al., 1997). Double staining was carried out by detecting the flourescein-labeled probe first, using Fast Red (Sigma) as a substrate. The reaction was stopped by washing the embryo in PBST (PBS/0.1% Tween) several times, followed by two washes in 100 mM glycine, pH 2.5, for 10 minutes each. Embryos were rinsed several times in PBST, and blocked for at least 1 h in 2% goat serum and 2 mg/ml BSA in PBST. The second probe was visualized using anti-digoxigenin Fab fragments.

In situ probes used: *boz* (Koos and Ho, 1998; Yamanaka et al., 1998), *ntl* (Schulte-Merker et al., 1992), *gsc* (Stachel et al., 1993), *gta5* (Rodaway et al., 1999), *sqt* (Erter et al., 1998; Rebagliati et al., 1998) and *cyc* (Rebagliati et al., 1998; Sampath et al., 1998). Embryos were photographed in either Permount (Fisher) or 70% glycerol.

Dorsalization with LiCl

Embryos were treated in their chorions with 0.3M LiCl for 9 minutes at the 64-cell stage (2 hours) (Stachel et al., 1993). The embryos were then Pronase treated, washed and incubated at 28.5°C.

RESULTS

To address the requirement of the YSL for mesendoderm induction, we wanted to specifically affect the ability of the YSL to send signals to the blastoderm. Studies in *Xenopus* indicate that the mesoderm-inducing signals are zygotically expressed (reviewed in Kimelman and Griffin, 1998). As the YSL forms at the 1K cell stage (3 hours), soon after zygotic transcription is initiated at the midblastula transition (MBT; Kane and Kimmel, 1993), injection of RNase into the yolk cell at the 1K cell stage (3 hours) should eliminate all zygotic transcripts before they are translated. Proteins can not diffuse into the blastomeres after the YSL forms (Kimmel and Law, 1985) therefore RNase injected as a protein should result in the rapid degradation of RNAs specifically within the YSL. We chose to inject DNase-free RNase (Roche Molecular Biochemicals), which contains a mixture of different RNases, although we have also individually injected RNase A and RNase T1 and obtained similar results (data not shown). We found that the results obtained by injecting this RNase were specific to the activity of the RNases (see below) and determined that it is necessary to inject a carrier along with the RNase to obtain consistant results. As it was useful to detect the location of the injected material, the carriers we used were either rhodamine-labeled biotinylated dextran, or unlabeled biotinylated dextran. By injecting the RNase into the yolk cell,

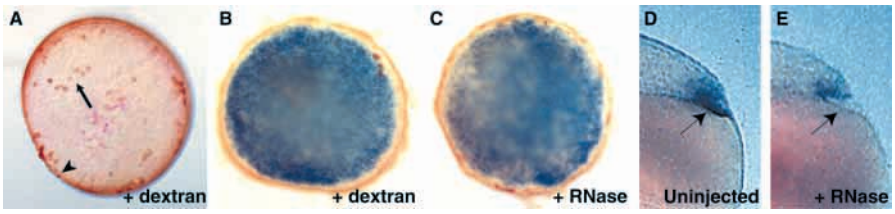

Fig. 1. Injected RNase is limited to the YSL. (A) 30% epiboly (4.7 hours) embryo injected into the yolk cell at the 1K cell stage (3 hours) with biotinylated dextran. Labeling is restricted to the YSL (arrowhead, in brown) except for some marginal cells and their descendants (arrow). (B,C) *spt* expression in sphere stage (4 hours) embryos injected with (B) dextran or (C) dextran and RNase. No change in *spt* expression is apparent in the RNase injected embryo compared to the dextran-injected embryo. (D) Uninjected embryo, fixed 10 minutes after siblings were injected with RNase at oblong stage (3.7 hours), expresses *boz* in both the YSL and dorsal marginal blastomeres. (E) Embryo fixed 10 minutes after injection with RNase expresses *boz* only in the dorsal marginal blastomeres. Arrows in D and E point to the YSL. Embryos in panels A-C are animal pole views, D and E are side views, dorsal is to the right.

we could specifically target RNAs in the YSL and ask whether or not the YSL is required for mesendoderm induction.

Injected RNase is limited to the YSL

Until the YSL is completely formed at the 1K cell stage (3 hours), marginal blastomeres retain cytoplasmic bridges with the yolk cell (Kimmel and Law, 1985). We wanted to ensure that material injected into the YSL at this stage would not be inherited by the marginal blastomeres, which are also a source of inducing signals, such as *sqt* and *cyc* (Erter et al., 1998; Feldman et al., 1998; Gritsman et al., 2000; Rebagliati et al., 1998). To determine the extent to which an injection of RNase into the yolk cell at the 1K cell stage (3 hours) would populate marginal blastomeres, we injected a similarly sized biotinylated dextran molecule, which was visualized by biotin-avidin peroxidase staining. We observed that the injected dextran was mostly limited to the YSL, although a few marginal cells and their descendents were labeled by 30% epiboly (4.7 hours; Fig. 1A). Since the labeled blastomeres represented a very small percentage of the marginal blastomeres, we concluded that even if the RNase were to enter a few marginal blastomeres, there were a large number of marginal cells remaining that could induce mesendodermal genes, if they had the capacity to do so.

Although the dextran injections revealed that injected RNase was likely to be restricted to the YSL, it was also important to establish that the expression of RNAs in the blastoderm that are not dependent on signals from the YSL are unaffected by the injection. As *spadetail* (*spt*; Griffin et al., 1998) is ubiquitously expressed at sphere stage (4 hours), we used it as a marker for YSL-independent gene expression. The expression of *spt* was indistinguishable in embryos that had RNase injected into their yolk cells when compared with embryos injected only with dextran (Fig. 1B,C). As a second control, we examined the expression of *boz* (Koos and Ho, 1998; Yamanaka et al., 1998) in embryos injected with RNase at oblong stage (3.7 hours), when *boz* is expressed in both the YSL and dorsal blastomeres. *boz* is normally expressed only in the dorsal blastomeres at the 1K cell stage (3 hours), then expands to include both the dorsal blastomeres and YSL until sphere stage (4 hours), after which it is restricted to the dorsal YSL (Koos and Ho, 1998; Yamanaka et al., 1998). Whereas *boz* is observed in both the YSL and blastomeres of uninjected embryos (Fig. 1D), RNase injection into the YSL abolished the expression of *boz* in the YSL without affecting its expression in the dorsal blastomeres (Fig. 1E). These results, in addition to those examining *spt* expression, demonstrated that we could selectively eliminate RNAs from the YSL without degrading transcripts in the blastomeres.

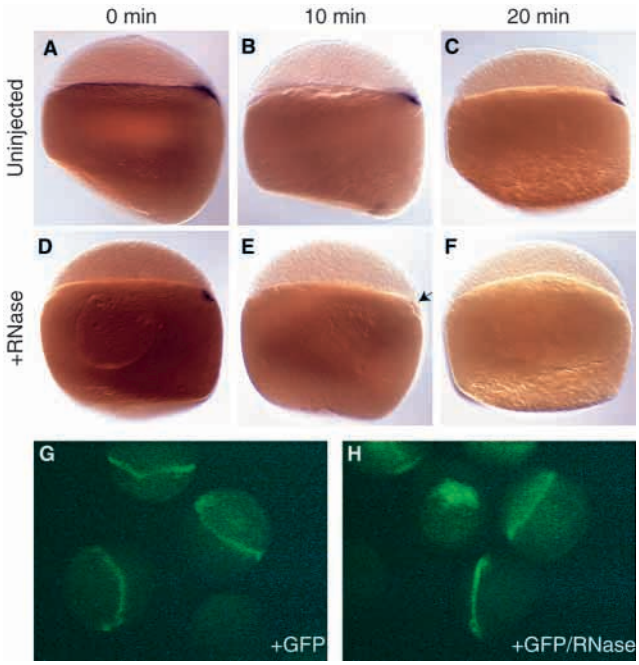

Fig. 2. RNase quickly and specifically eliminates RNA in the YSL. (A-C) *boz* expression in uninjected embryos fixed 0 min (A), 10 min (B) and 20 min (C) after siblings were injected with RNase into the yolk cell at at sphere stage (4 hours). (D-F) *boz* expression in embryos fixed 0 min (D), 10 min (E) and 20 min (F) after injection with RNase. Expression of *boz* is greatly reduced 10 min after injection (E; arrow) and completely eliminated 20 min after injection (F). Embryos in panels A-F are side views with dorsal to the right, when distinguishable. (G) Embryos injected with GFP protein. (H) Embryos injected with GFP and RNase. Injection of RNase did not change the level of GFP fluorescence compared with embryos injected with GFP alone

Effects of RNase injection on epiboly

Previous studies have suggested a role for the YSL in driving epibolic movements (Solnica-Krezel and Driever, 1994; Strahle and Jesuthasan, 1993). As was observed with the microtubule inhibitor nocodazole, RNase injections in the YSL resulted in slow epibolic movements and the blastoderm lifting off the yolk cell (data not shown). These results suggested that one or more transcripts in the YSL are necessary for the

microtubule-driven process of epiboly. In order to ensure that the effects on epiboly did not affect our analysis of mesendoderm induction, the studies on mesendodermal markers described below were conducted prior to or at 50% epiboly (5.3 hours), before severe morphogenetic effects of RNase injections were observed.

RNase rapidly and specifically eliminates RNAs in the YSL

To establish that RNase injection eliminates YSL RNAs and to determine how quickly this process occurs, we injected RNase and examined the expression of *boz* at a time when it is restricted to the YSL. Injections were made into the yolk cell of sphere stage (4 hours) embryos and then the embryos were fixed varying times after injection. We found that *boz* expression was significantly reduced within 10 minutes of injection, and completely eliminated 20 minutes after injection (Fig. 2A-F). This demonstrated that RNAs in the YSL are degraded within 20 minutes of RNase injection.

To examine whether or not RNase injections might have a general effect on protein stability, we injected recombinant GFP protein with or without RNase into the yolk, and examined the embryos from oblong stage (3.7 hours) to 30% epiboly (4.7 hours) for the presence of green fluorescent protein (GFP) by fluorescence microscopy. There was no apparent change in the level of GFP fluorescence over this time period in control injected embryos, and there was no difference in GFP intensity between embryos that were injected with or without RNase (Fig. 2G,H). Since we could not detect injections of five times less GFP, it seemed likely that there was not a significant amount of degradation occurring in the embryos. These results suggest that existing proteins in the YSL are not affected by the injection of RNase, and that any effects of RNase injection on mesoderm induction are due to eliminating existing transcripts in the YSL and/or preventing new transcription.

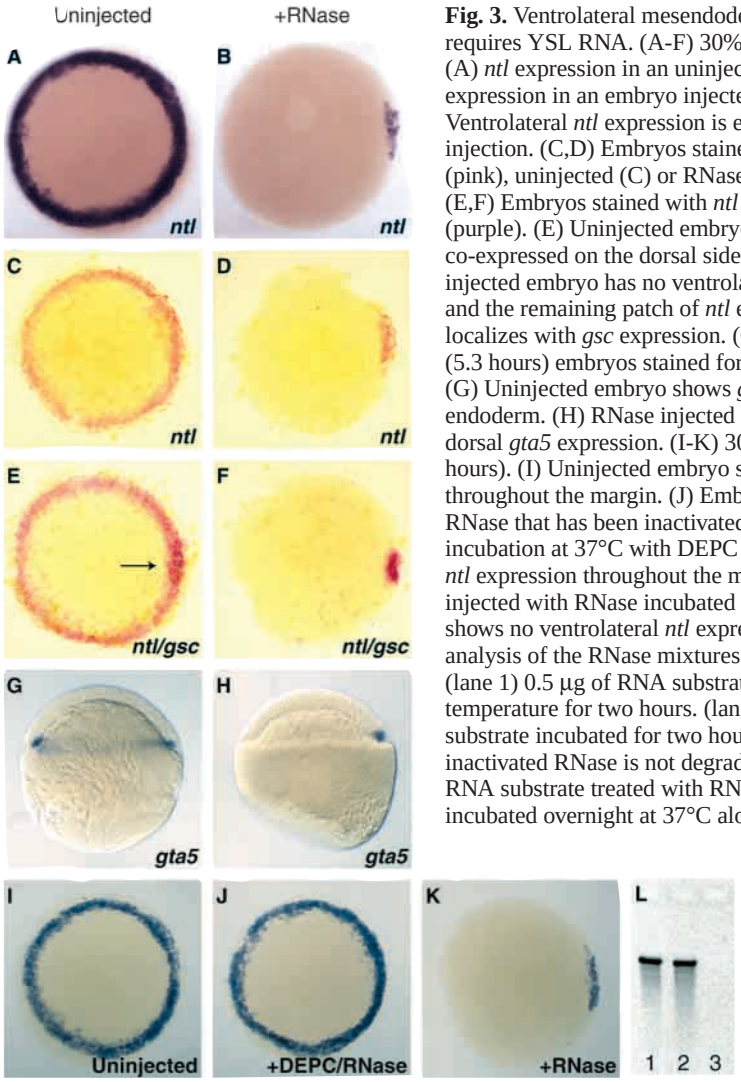

Fig. 3. Ventrolateral mesendoderm induction requires YSL RNA. (A-F) 30% epiboly (4.7 hours). (A) *ntl* expression in an uninjected embryo. (B) *ntl* expression in an embryo injected with RNase. Ventrolateral *ntl* expression is eliminated by RNase injection. (C,D) Embryos stained with *ntl* alone (pink), uninjected (C) or RNase injected (D). (E,F) Embryos stained with *ntl* (pink) and *gsc* (purple). (E) Uninjected embryo shows *ntl* and *gsc* co-expressed on the dorsal side (arrow). (F) RNase injected embryo has no ventrolateral *ntl* expression, and the remaining patch of *ntl* expression co-localizes with *gsc* expression. (G,H) 50% epiboly (5.3 hours) embryos stained for *gta5* expression. (G) Uninjected embryo shows *gta5* throughout the endoderm. (H) RNase injected embryo shows only dorsal *gta5* expression. (I-K) 30% epiboly (4.7 hours). (I) Uninjected embryo shows *ntl* expression throughout the margin. (J) Embryo injected with RNase that has been inactivated by overnight incubation at 37°C with DEPC also shows normal *ntl* expression throughout the margin. (K) Embryo injected with RNase incubated at 37°C overnight shows no ventrolateral *ntl* expression. (L) In vitro analysis of the RNase mixtures injected in I-K. (lane 1) 0.5 μg of RNA substrate incubated at room temperature for two hours. (lane 2) 0.5μg RNA substrate incubated for two hours with DEPC-inactivated RNase is not degraded. (lane 3) 0.5μg RNA substrate treated with RNase that was incubated overnight at 37°C alone is completely degraded. (A-F, I-K) Animal pole views. (G,H) Side view with dorsal to the right, when distinguishable.

Ventrolateral mesendoderm induction requires a YSL RNA between 1K (3 hours) and sphere stages (4 hours)

We examined the effects of RNase injection at the 1K cell stage (3 hours) on the expression of the pan-mesodermal marker *no tail* (*ntl*; Schulte-Merker et al., 1992) and the endodermal marker *gata5* (*gta5*; Rodaway et al., 1999) and found that *ntl* expression at 30% epiboly (4.7 hours) was almost completely absent, except for a patch of cells on the dorsal side (Fig. 3A,B). Double-label in situ hybridization with the dorsal mesodermal marker *goosecoid* (*gsc*; Stachel et al., 1993) confirmed that ventrolateral *ntl* expression was completely abolished, leaving only a dorsal region where *gsc* and *ntl* were co-expressed (Fig. 3C-F). Dorsal expression of *ntl* could not be eliminated, even upon injection of ten times as much RNase (data not shown). Examination of the endodermal marker, *gta5*, in RNase injected embryos at 50% epiboly (5.3 hours) demonstrated that ventrolateral endoderm also requires signals from the YSL (Fig. 3G,H). These results demonstrate that induction of ventrolateral mesendoderm requires signals from the YSL.

In order to establish that the elimination of ventro-lateral *ntl* expression was a specific effect of RNase activity, we repeated the injections with inactivated RNase. Since we were concerned that any RNase inhibitor we would add to the RNase could have its own effects when injected into embryos, we inactivated the RNase using a volatile protein modifier, diethyl pyrocarbonate (DEPC). Overnight treatment of RNase with DEPC at 37°C resulted in the inactivation of RNase, measured in vitro on an RNA substrate (Fig. 3L). Treatment of the RNA substrate with RNase that had been incubated at 37°C overnight without DEPC resulted in its complete degradation (Fig. 3L, lane 3). Moreover, while fresh DEPC results in the degradation of the RNA substrate (data not shown) the DEPC

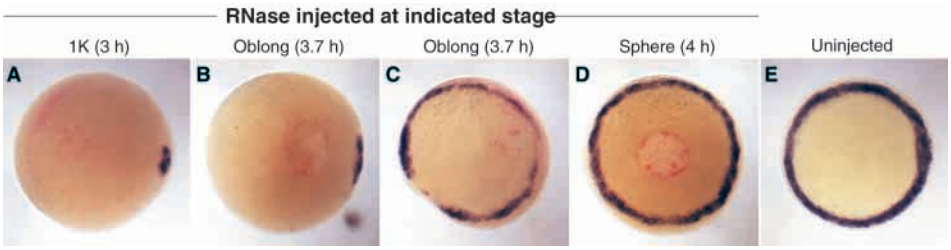

Fig. 4. Ventrolateral mesoderm induction requires YSL RNA between the 1K cell (3 hours) and sphere stages (4 hours). (A-E) Expression of *ntl* at 50% epiboly (5.3 hours) in (E) uninjected and (A-D) RNase injected embryos. (A) Embryo injected with RNase at the 1K cell stage (3 hours) shows no ventrolateral *ntl* expression. (B,C) Embryos injected with RNase at oblong stage (3.7 hours) include embryos that have (B) no ventrolateral *ntl* expression and those in which (C) some ventro-lateral *ntl* expression is retained. (D) Embryo injected with RNase at sphere stage (4 hours) looks the same as (E) an uninjected embryo. (B,D) Pink color in the center of the embryo is a portion of rhodamine-labeled dextran that has not diffused from the site of injection. All views are from the animal pole.

incubated overnight with RNase had no effect on the RNA substrate (Fig. 3L, lane 2). We found that DEPC-inactivated RNase had no effect on *ntl* expression when injected into embryos (Fig. 3J). These results demonstrated that the elimination of ventrolateral *ntl* expression is a result of RNase activity.

Using RNase injections, we have shown that ventrolateral mesendoderm induction requires a signal from the YSL. To determine when this RNA is present in the YSL, we injected embryos with RNase at different times and analyzed the effects on *ntl* expression. Embryos were injected with RNase at the 1K cell (3 hours), oblong (3.7 hours) and sphere (4 hours) stages, and examined at 50% epiboly (5.3 hours) for *ntl* expression (Fig. 4A-E). Embryos injected at the oblong stage (3.7 hours) showed a reduced effect compared with those injected at 1K (Fig. 4C). Embryos injected at sphere stage (4 hours) showed normal *ntl* expression (Fig. 4D,E), demonstrating that an RNA in the YSL is required between 1K cell (3 hours) and sphere stage (4 hours).

Ventrolateral expression of *nodals* requires YSL signal

Since RNase injections into the YSL eliminated ventrolateral expression of the endodermal marker *gta5*, we wondered if this would be accompanied by a reduction in the expression of the *nodal*-related factors in the ventrolateral region. We observed that expression of *sqt* (Erter et al., 1998; Rebagliati et al., 1998) and *cyc* (Rebagliati et al., 1998; Sampath et al., 1998) was eliminated by RNase injection at the 1K cell stage (3 hours) in the ventrolateral marginal regions, just as *ntl* was (Fig. 5A-D). Dorsal expression of both *sqt* and *cyc* remained in RNase injected embryos, as was observed for *ntl*, *gsc* and *gta5* in RNase injected embryos (Fig. 3). These results demonstrate that the ventrolateral expression of the *nodal*-related factors is also dependent upon RNAs in the YSL.

Stabilized β-catenin can induce dorsal mesoderm independently of the YSL

The previous experiments established a role for the YSL in induction of ventrolateral mesoderm, but clearly demonstrated that dorsal mesoderm is regulated differently. Since *boz* has been shown to be required for dorsal mesoderm induction (Fekany et al., 1999), we examined its expression in RNase injected embryos. We observed that *boz* was expressed in the dorsal blastomeres of RNase injected embryos, and that this expression persisted past the point at which *boz* expression in uninjected siblings has been restricted to the dorsal YSL (Fig. 6I,J). As described earlier (Fig. 1E), *boz* expression was not observed in the YSL of RNase injected embryos. These results suggest that induction of *boz* in the dorsal blastomeres is independent of YSL signals, although the normal temporal regulation of *boz* requires YSL signals.

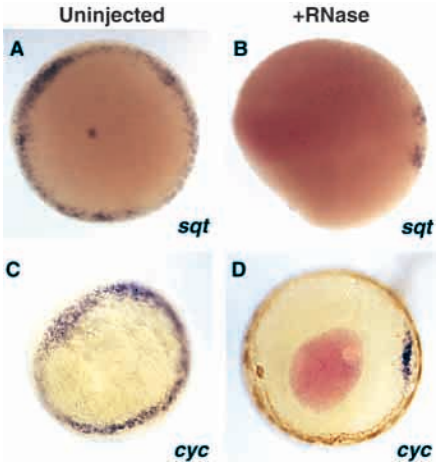

Fig. 5. Ventrolateral expression of the *nodal*-related genes requires a YSL signal. Expression of (A,B) *sqt* and (C,D) *cyc* at 30% epiboly (4.7 hr). (A) *sqt* expression in an uninjected embryo. (B) RNase injected embryo displays lack of ventrolateral *sqt* expression. (C) *cyc* expression in an uninjected embryos. (D) RNase injected embryos show no ventrolateral *cyc* expression. Brown color in the YSL is biotin-avidin peroxidase staining of co-injected rhodamine-labeled biotinylated dextran. All views are from the animal pole.

In *Xenopus*, stabilization of β-catenin has been shown to be required for the induction of dorsal mesoderm (reviewed in Moon and Kimelman, 1998). In zebrafish embryos, β-catenin accumulates in the dorsal YSL and dorsal blastomeres (Schneider et al., 1996). We hypothesized that the presence of β-catenin in the dorsal blastomeres would allow these cells to activate dorsal mesoderm independently of the dorsal YSL. In order to test this, we treated embryos with LiCl, which has been shown to result in ectopic stabilization of β-catenin throughout the embryo (Schneider et al., 1996) by inhibiting GSK-3β (Hedgepeth et al., 1997; Klein and Melton, 1996; Stambolic et al., 1996). We expected that if ectopically stabilized β-catenin did not require the YSL to induce dorsal mesoderm, embryos treated with LiCl and injected with RNase would still express ectopic dorsal mesodermal markers. As shown previously, LiCl-treated embryos ectopically express the dorsal mesodermal marker *gsc* throughout the margin (Fig. 6C; Stachel et al., 1993).

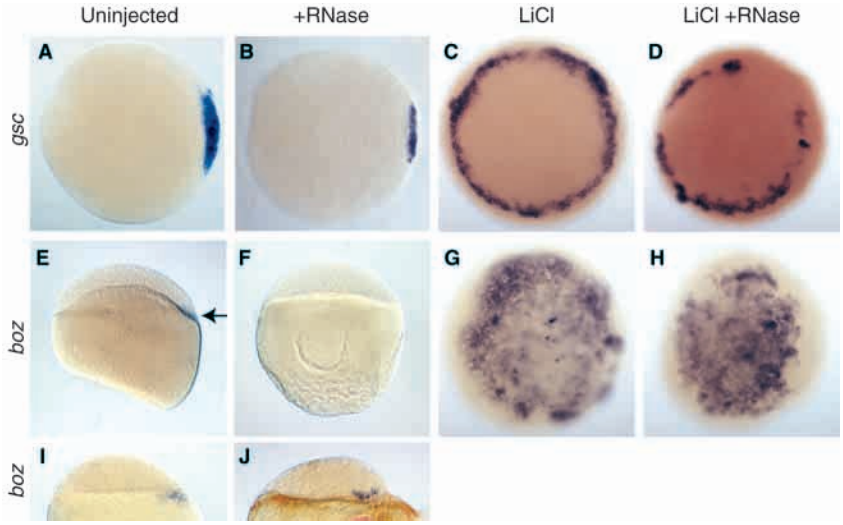

Fig. 6. Stabilized β-catenin in dorsal blastomeres can induce dorsal mesoderm independently of YSL signals. (A-H) Embryos shown are at 30% epiboly (4.7 hours). (A) *gsc* expression in an untreated embryo. (B) RNase injected embryo retains *gsc* expression. (C) LiCl treated embryo shows ectopic *gsc* expression at the margin. (D) LiCl treated and RNase injected embryo shows ectopic *gsc* expression at the margin. (E) Untreated embryo shows *boz* expression in the dorsal YSL. (F) RNase injected embryo has no *boz* in the YSL. (G) LiCl treated embryo shows ectopic *boz* expression throughout the blastoderm. (H) LiCl treated, RNase injected embryo still shows ectopic *boz* expression throughout the blastoderm. (I) *boz* expression in an embryo at sphere stage (4 hours) is restricted to the YSL. (J) RNase injected embryo retains *boz* in the dorsal blastomeres. (A-D, G,H) Animal pole views, (E,F,I,J) Side view, dorsal to the right, when distinguishable.

We observed that this ectopic expression is largely resistant to injection of RNase (Fig. 6D), just as dorsal expression of *gsc* is resistant in embryos not treated with LiCl (Fig. 6B).

We also examined the expression of *boz* in LiCl and RNase injected embryos. As previously described, *boz* is restricted to the dorsal YSL at 30% epiboly (4.7 hours; Fig. 6E; Koos and Ho, 1998; Yamanaka et al., 1998). In RNase injected embryos, YSL expression of *boz* at 30% epiboly (4.7 hours) was eliminated (Fig. 6F). At the same stage in LiCl-treated embryos, *boz* was ectopically expressed throughout the blastoderm and not just restricted to the margin as was seen for *gsc* (Fig. 6 compare G to C). LiCl-treated embryos, which were also injected with RNase, still show ectopic *boz* expression throughout the blastoderm at 30% epiboly (Fig. 6H), demonstrating that LiCl-dependent expression of *boz* does not require YSL signals. As *boz* is required for the formation of the dorsal mesoderm downstream of β-catenin (Fekany et al., 1999), this agrees with our observations that induction of dorsal mesoderm can occur independently of YSL signals and we conclude that this is mediated by stabilized β-catenin.

DISCUSSION

While transplantation experiments have shown that the YSL is sufficient to induce mesoderm and endoderm (Mizuno, 1996; Ober and Schulte-Merker, 1999; Rodaway et al., 1999) whether or not the YSL was required for germ layer formation was not known. As genes encoding key regulatory factors known to be expressed in the YSL are also expressed in the marginal blastomeres, mutants in those genes could not resolve the issue of whether gene expression in the YSL is required, as mutants abolished their activities in both the YSL and marginal blastomeres. Therefore, it was necessary to devise a technique to eliminate gene expression specifically in the YSL in order to demonstrate whether or not it was required. We have accomplished this by injecting RNase into the YSL, and have demonstrated that the YSL is required for ventrolateral mesendoderm induction and the expression of *nodal*-related factors within the marginal blastomeres, while dorsal mesoderm induction can occur independently of the YSL.

RNase injections

In order to address whether or not the YSL is required for germ layer formation, we wished to eliminate the activity of the YSL. Dissection of the blastoderm away from the yolk cell in order to separate it from yolk cell signals was not considered a viable option. The dissection process typically results in loss or damage of the most marginal cells, which are the site of *sqt* and *cyc* expression (Erter et al., 1998; Feldman et al., 1998; Gritsman et al., 2000; Rebagliati et al., 1998). This would probably compromise the ability of the explanted blastoderm to induce mesendodermal genes. Therefore, we wanted to eliminate the capacity of the YSL to signal without removing the blastoderm. As we hypothesized that the inducing signal would be zygotic, based on studies in *Xenopus*, we originally sought to inhibit transcription in the YSL specifically. Since commonly available transcriptional inhibitors are membrane permeable, we chose to inject RNase. As proteins can not diffuse into the blastomeres after the YSL forms (Kimmel and Law, 1985), injection of RNase protein into the YSL would specifically degrade YSL RNAs. Although we have performed injections with RNase A and RNase T1, most of our injections were performed using a DNase-free RNase mix. We found that the RNase must be injected with a carrier to obtain consistent results, and we used biotinylated dextrans as carriers, since this afforded us the option of visualizing the extent of RNase diffusion using biotin-avidin peroxidase staining. We have demonstrated that RNase injections into the yolk cell at the 1K cell stage (3 hours) are limited to the YSL, do not affect ubiquitously expressed genes, and we present data that indicate proteins in the YSL are unaffected. These experiments indicate that injection of RNase to the yolk cell after the YSL forms is an effective technique for eliminating RNA transcripts in the YSL specifically.

A role for YSL RNAs in epiboly

We have presented evidence that suggests that injection of RNase into the YSL eliminates RNAs without affecting the stability of proteins. Consistent with studies that suggest a role for the YSL in epiboly, we see the same phenotype in our RNase-injected embryos as in embryos treated with UV or nocodazole, which disrupt microtubules: epiboly is slowed,

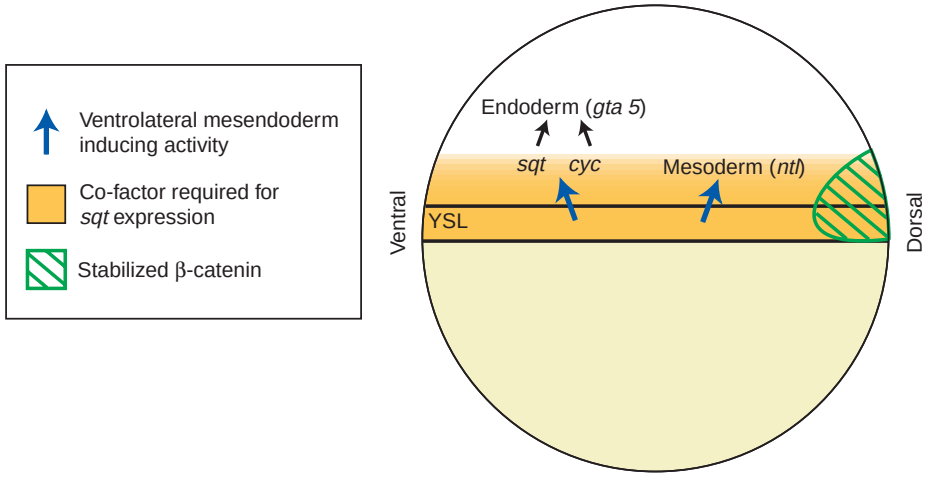

Fig. 7. Model for mesendoderm induction. Ventrolateral mesendoderm induction requires a signal from the YSL (blue arrow), which is present as at least one RNA between the 1K cell stage (3 hours) and sphere stage (4 hours) to separately activate *sqt* and *cyc*, and *ntl*. Sqt and Cyc, in turn, are required to induce ventro-lateral endoderm. Dorsal mesendoderm inducing factors are activated in the dorsal blastomeres and YSL as a result of stabilized β-catenin in both places (green hatched region). β-catenin requires a co-factor (yellow) that is restricted to the YSL and marginal blastomeres in order to induce *sqt* expression, but not *boz* (see text for details).

and the blastoderm of RNase injected embryos constricts at the margin and lifts off of the yolk cell when control embryos are past 50% epiboly (5.3 hours) (Strahle and Jesuthasan, 1993). The manner in which the blastoderm lifts off of the yolk cell was proposed to be due to both constrictive forces in the margin of the blastoderm, which normally facilitates blastopore closure, and a weakening of the yolk cytoskeleton (Strahle and Jesuthasan, 1993). We observe that this process occurs more rapidly in our RNase injected embryos, where the blastoderm lifts off the yolk cell by the time uninjected siblings reach 60-70% epiboly (6.7 hours). We suggest that this is because cytoskeletal elements that are turned over after RNase injection cannot be replaced due to the presence of RNase. This results in a weaker cytoskeleton in RNase-injected embryos than in embryos treated with UV or nocodazole, in which the cytoskeleton can recover to some degree after treatment (Strahle and Jesuthasan, 1993). The similarity between the results with RNase injection, and UV and nocodazole treatment suggests that the effects of RNase injection are limited to the YSL, and that the blastoderm itself can properly undergo morphogenetic processes that are independent of the YSL. In addition, our data indicate that there is one or more RNA in the YSL that are essential for epiboly.

RNA in the YSL is required for ventrolateral mesoderm induction

We have shown that expression of mesendodermal markers and inducing genes requires at least one RNA in the YSL between the 1K cell stage (3 hours) and sphere stage (4 hours). A significant exception is dorsal mesendoderm, discussed below. Moreover, the RNase-sensitive activity induces the expression of *sqt* and *cyc*, which are required for endoderm induction (Rodaway et al., 1999). Although *sqt;cyc* double mutants show defects in mesoderm formation during gastrulation, *sqt* and *cyc* are not required for ventrolateral mesoderm induction (Feldman et al., 1998). Therefore, the activity must separately induce the expression of *ntl*, either directly or through an unknown pathway (Fig. 7). Whether or not the RNase sensitive activity is zygotic or maternal is not known, as the injected RNase would eliminate both.

Based on a number of investigations, likely candidates for the RNase-sensitive inducing activity belong to the transforming growth factor β (TGFβ) family of signaling molecules. Overexpression of the TGFβ family member *antivin* (Thisse and Thisse, 1999), which has been shown to antagonize TGFβ signaling, abolishes mesendoderm induction. Additionally, overexpression of the TGFβ signaling molecule *activin*, results in the induction of mesodermal genes (Schulte-Merker et al., 1992).

We do not consider *activin* a likely candidate for our mesendoderm-inducing activity, since a study using an *activin* cleavage mutant, which must interact with newly translated activin ligand in order to function as a dominant negative, had no effect on mesoderm induction in medaka (Wittbrodt and Rosa, 1994). Since our RNase injection experiments indicated that RNA in the YSL is required for mesoderm induction, the activin cleavage mutant described above should have also eliminated ventrolateral mesoderm if *activin* transcripts were the targets of the RNase.

We can also exclude *nodal*-related signals as candidates for the factor. Although *sqt* and *cyc* are required for endoderm induction (Rodaway et al., 1999), *cyc* is not expressed in the YSL, and *sqt* is only expressed in the dorsal YSL between the 1K cell stage (3 hours) and sphere stage (4 hours). Our data suggest that the RNase sensitive mesendoderm-inducing activity is expressed as RNA throughout the YSL during these stages. In addition, maternal and zygotic mutants in *one-eyed pinhead*, which is required for nodal signaling, still induce ventrolateral mesoderm (Gritsman et al., 1999), as do *sqt;cyc* double mutants (Feldman et al., 1998). This indicates that the *nodal*-related genes do not play a role in the initial induction of ventrolateral mesoderm (Feldman et al., 1998; Gritsman et al., 1999).

In summary, our data demonstrate that there is an unidentified RNA required for mesendoderm induction that is expressed specifically in the YSL at the start of zygotic transcription (Fig. 7).

Dorsal mesoderm induction

Our data demonstrated that dorsal mesendoderm is induced through a different pathway from ventrolateral mesendoderm. We found that dorsal mesendoderm was not eliminated in RNase injected embryos, and that dorsal mesoderm could be induced by LiCl treatment in the absence of a functional YSL. Although we can not rule out the possibility that the effects of LiCl are due in part to changes in phosphoinositide

metabolism (Ault et al., 1996), since LiCl treatment results in the ectopic stabilization of β-catenin in zebrafish embryos (Schneider et al., 1996a) and since stablized β-catenin has been shown in *Xenopus* embryos to activate dorsal genes (reviewed in Moon and Kimelman, 1998), we conclude that the stabilization of β-catenin in the dorsal blastomeres makes them independent of signaling from the YSL. Our results correlate with a number of experiments that contribute to a model in which dorsalizing factors, responsible for stabilizing β-catenin, are localized to the vegetal pole of the oocyte and moved up to the future dorsal side via microtubule transport prior to the 16-cell stage (1.5 hours) (Jesuthasan and Stahle, 1996; Ober and Schulte-Merker, 1999; Yamaha et al., 1998). We believe that these dorsalizing factors are delivered to a region that includes both the dorsal YSL and dorsal blastomeres, thus accounting for the ability of the YSL to induce dorsal mesodermal genes in transplantation experiments (Mizuno, 1996; Ober and Schulte-Merker, 1999; Rodaway et al., 1999), and of the blastomeres to induce dorsal mesodermal genes independently of the YSL (our results, Fig. 7). Consistent with this interpretation, ectopic *gsc* expression was frequently less robust in LiCl-treated and RNase-injected embryos when compared with embryos treated with LiCl alone. This suggests that stabilization of β-catenin in the dorsal YSL also plays a non-autonomous role in the induction of dorsal mesoderm (Fig. 6, compare D with C)

One caveat to our interpretation is that there may be a signal from the dorsal YSL that acts too early for our RNase injections to interrupt. We do not believe this is the case, as we inject RNase as soon as the YSL is formed and have shown that injection of RNase eliminates most of the RNA in the YSL within 10 minutes. In addition, nuclear localized β-catenin has been observed in marginal and non-marginal blastomeres at the 1K cell stage (3 hours) (S. Dougan, A. Schier and W. Talbot, personal communication). These observations suggest that the dorsal blastomeres are capable of activating dorsal mesodermal genes independently of signals from the YSL via stabilized β-catenin.

The expression of all mesendodermal markers examined, *sqt*, *cyc*, *ntl* and *gta5*, was shown to be regulated differently on the dorsal side than in the ventrolateral margin. Our data suggest that dorsal expression of these genes can be induced downstream of stabilized β-catenin. This has been previously demonstrated for *boz* (Fekany et al., 1999; Yamanaka et al., 1998), *sqt* (Shimizu et al., 2000) and *ntl* (Kelly et al., 1995), and our data suggest it is also true for dorsal *cyc* and *gta5* expression.

Regulation of dorsal mesodermal genes requires the YSL

In addition to playing a cooperative role with the dorsal blastomeres to induce dorsal mesoderm, our data suggest that the YSL is required to regulate dorsal mesoderm formation. Although the dorsal blastomeres can induce the expression of *boz* independently of the YSL, in the absence of YSL RNAs the expression pattern of *boz* is altered such that it is still expressed in the dorsal blastomeres when uninjected siblings have restricted their expression of *boz* to the dorsal YSL (Fig. 6I,J). This result suggests that dorsal YSL activity regulates *boz* expression by inhibiting *boz* in the dorsal blastomeres during epiboly.

Evidence for a localized co-factor involved in dorsal mesoderm induction

While stabilization of β-catenin is required in order to induce dorsal mesoderm, it is not sufficient to do so (Schneider et al., 1996b). It has been previously shown in *Xenopus* that treatment with LiCl results in ectopic stabilization of β-catenin throughout the embryo, but that dorsal mesodermal markers are restricted to the equatorial regions where the mesodermal layer is normally formed (Schneider et al., 1996b). Similarly, in zebrafish we observed that LiCl-treated embryos show *gsc* (Fig. 6C), *ntl* and *sqt* (data not shown) expression only at the margin even though *boz* expression is induced throughout the blastoderm (Fig. 6G; Yamanaka et al., 1998). Both *sqt* and *boz* have been shown to be downstream of β-catenin (Shimizu et al., 2000), which suggests that a co-factor required for *sqt* expression is responsible for restricting dorsal mesoderm induction to the margin. We suggest that both the YSL and marginal blastomeres contain a co-factor required for expression of *sqt*, but not *boz* (Fig. 7).

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