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Mezzo, a *paired-like* homeobox protein is an immediate target of Nodal signalling and regulates endoderm specification in zebrafish

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SUMMARY

Endoderm specification in zebrafish is mediated by the zygotic transcription factors Bon/Mixer, Faust/Gata5, Casanova and Sox17, whose expression is induced by Nodal signalling. Bon/Mixer and Gata5 require Casanova in order to promote endoderm formation and all three factors act upstream of *sox17*, but it is not clear whether Casanova acts downstream of or in parallel to Bon/Mixer and Gata5. An additional factor induced at the margin of the blastoderm by Nodal signalling is thought to be required to induce *casanova* expression. We show that Mezzo, a novel *paired-like* homeobox protein, may be this missing transcription factor. The homeobox of Mezzo is mostly related to the homeodomain of the Mix-like and Mixer homeoproteins, but Mezzo is distinct from Bon/Mixer, the product of the *bonnie and clyde* gene. Like *bon/mixer*, *mezzo* is expressed transiently in mesendoderm precursors. By analysing the expression of *mezzo* in various mutants of Nodal signalling, we show that its expression strictly depends on a functional Nodal signalling pathway. By expressing a constitutively active Nodal receptor in the presence of translation inhibitors, we further demonstrate that *mezzo*, *bonnie and clyde*, and *casanova* are all immediate early targets of Nodal signalling, while *sox17* requires post-MBT protein synthesis in order to be induced. Overexpression of *mezzo* mRNA can induce ectopic expression of *casanova* and *sox17* and can also turn on the pan mesodermal marker gene *ntl*. We show that the function of *mezzo* is redundant with that of *bonnie and clyde* and that *mezzo* RNA can partially rescue *bonnie and clyde* mutants. Injection of antisense Morpholino oligonucleotides targeted against *mezzo* into *bonnie and clyde* mutant embryos abolishes all *sox17* expression and aggravates their mutant phenotype. These results highlight the complexity of the transcriptional network operating during endoderm formation. They place *mezzo* as a new transcription factor with unique properties acting in parallel with *bonnie and clyde*, *faust* and *casanova* in the Nodal signalling pathway that controls specification of mesoderm and endoderm in zebrafish.

Key words: *mezzo*, Homeobox, Nodal signalling, Endoderm, Mesoderm, *sox17*, *ntl*, *casanova*, *faust*, *mixer*, Zebrafish

INTRODUCTION

In zebrafish, endoderm is induced at the vegetal margin of the blastoderm by secreted TGFβ molecules of the Nodal family (Rodaway et al., 1999; Warga and Nusslein-Volhard, 1999; Stainier, 2002). Recent genetic studies have identified a number of loci involved in endoderm formation and epistatic analyses have helped to order them in a molecular pathway (Alexander and Stainier, 1999; Kikuchi et al., 2000; Dickmeis et al., 2001; Reiter et al., 2001; Aoki et al., 2002a). In the current model, the endoderm specification pathway is initiated by activation of serine/threonine kinase receptors, which bind Nodal-type TGFβ ligands. Two Nodal ligands encoded by the *cyclops* and *squint* genes are implicated in this first step (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998). *cyclops* and *squint* are expressed in the vegetal marginal region of the blastoderm where endoderm precursors have been mapped. Single mutants lacking the function of either gene still have endoderm but *cyclops;squint* double mutant lack all endoderm and have little mesoderm demonstrating a functional redundancy between these two factors. The activities of these two Nodal ligands can be inhibited by Antivin, a potent endogenous antagonist of Nodal factors that is induced in a feedback mechanism of the signalling pathway. Overexpression of low doses of Antivin suppresses endoderm formation while higher doses also affect the mesoderm (Thisse and Thisse, 1999; Thisse et al., 2000). TARAM-A, a zebrafish type I TGFβ receptor structurally related to Alk4, is a candidate receptor for Nodal ligands (Renucci et al., 1996; Peyrieras et al., 1998; Aoki et al., 2002b). Endoderm formation also requires the activity of Oep-Crypto proteins which are transmembrane proteins acting as co-receptors for Nodal factors (Zhang et al., 1998). Embryos lacking maternal and zygotic *oep* transcripts (*MZoep*) resemble *cyclops;squint* double mutants and lack all endoderm (Gritsman et al., 1999). Signal transduction from Nodal receptors requires the maternally expressed Smad2 factor, which translocates to the nucleus after phosphorylation (Muller

et al., 1999; Dick et al., 2000). Once in the nucleus, Smad2 factors bind to co-factors such as FoxH1/Fast1 through a Smad interacting motif (SIM) (Germain et al., 2000; Randall et al., 2002). Maternal and zygotic *schmalspur* mutants (*MZsur*), which have a disrupted *foxH1/fast1* gene are cyclopic and lack prechordal mesoderm but surprisingly they still have endoderm (Pogoda et al., 2000; Sirotkin et al., 2000). The phenotype of *MZsur* mutant embryos, which is less severe than that of *cyclops;squint* or *MZoep* mutants demonstrates that in zebrafish, factors with overlapping function can mediate the early response to Nodal.

In addition to maternal ligands, receptors and transducers, several zygotic transcription factors induced by Nodal signalling have been identified as key regulators of endoderm development. These include the Mix-like homeobox protein Bon/Mixer; the product of the *bonnie and clyde* gene (*bon/mixer*); the zinc finger-containing factor Gata5, which is encoded by the *faust* gene; and the Sox-related gene Casanova. *mixer* was originally described in *Xenopus* as a gene able to activate *sox17* gene expression in presumptive ectodermal cells when overexpressed (Henry and Melton, 1998). The C-terminal region of *Xenopus* Mixer contains a SIM domain required for interaction with phosphorylated Smads suggesting that Mixer, like FoxH1/Fast1 can cooperate with Smads to transduce Nodal signals. Unlike *mixer* in *Xenopus*, *bon* transcripts in zebrafish are broadly expressed in the marginal region, which contains mesodermal as well as endodermal precursors. *bonnie and clyde* mutant embryos display a reduced number of cells expressing endodermal markers during gastrulation and later lack a functional gut tube. Therefore, despite the broad early expression of *bon/mixer* in presumptive mesoderm and endoderm, only endodermal tissues are affected in *bon* mutants. *faust* mutants, which have a disrupted *gata5* gene, also have a reduced number of *sox17*-positive cells and are affected in endoderm formation, although the *faust* mutant phenotype is weaker than the *bon* mutant phenotype. Mutant embryos that lack the product of the *casanova* gene (*cas*) have the most severe defects regarding endoderm formation and completely lack *sox17* expression during gastrulation. Ectopic expression of *faust/gata5*, *bon/mixer* or *casanova* can upregulate *sox17* expression in mutants with an attenuated Nodal signalling pathway (*Zoep*), demonstrating that *bon*, *faust* and *cas* are upstream regulators of *sox17*. However, only *casanova* mRNA can induce *sox17* in mutants with a completely inactive Nodal signalling pathway (*MZoep*) (Kikuchi et al., 2001; Aoki et al., 2002a). The levels of *bon/mixer* or *faust* transcripts are normal in a *casanova* mutant background (Alexander et al., 1999; Reiter et al., 2001). By contrast, expression of *casanova* is greatly reduced in *bon* or *faust* mutants embryos (Kikuchi et al., 2001; Aoki et al., 2002a). In addition, TARAM-A, *bon* and *faust* all require the function of *casanova* in order to turn on *sox17*. Based on this set of observations it has been hypothesised that Bon and Faust/Gata5 act either upstream of or in parallel with Casanova, and that Casanova is the most critical regulator of *sox17*.

Several lines of evidence suggest that in addition to *bon/mixer* and *faust/gata5*, other unidentified factors induced by Nodal signalling are involved in the regulation of *casanova* expression (Alexander et al., 1999; Kikuchi et al., 2000; Kikuchi et al., 2001; Reiter et al., 2001; Aoki et al., 2002a). First, *bon* or *faust* single mutants as well as *bon;faust* double mutants embryos still have residual expression of *casanova* and *sox17*. Moreover, overexpression of *faust* or *bon* alone or in combination is not sufficient to cause ectopic expression of *casanova* outside the margin and does not restore significant *sox17* expression in *cyclops;squint* mutants. This suggests that another factor normally present at the margin is required to induce *sox17* and is lost in Nodal mutants (Kikuchi et al., 2000; Kikuchi et al., 2001). Finally, neither *bon* nor *faust* mRNAs are able to induce *sox17* or *casanova* in *MZoep* embryos. All these observations have led Alexander et al. and Aoki et al. to postulate the existence of an unknown factor induced by Nodal signals at the margin of the blastoderm, which would act at the same level as *bon* and *faust*, and would be a positive regulator of *casanova* expression.

We report the identification of a novel *paired-like* homeobox protein named Mezzo, which may be the missing factor described above. Mezzo is related to the Mix-like/Mixer homeoproteins and like *bon/mixer*, *mezzo* is expressed transiently during zebrafish development. Moreover, we show that *mezzo* transcripts are restricted to mesendoderm precursors during gastrulation and can be induced by activation of the Nodal/TARAM-A signalling pathway. By analysing the expression of *mezzo* in various mutants with attenuated or completely defective Nodal signalling, we show that activation and maintenance of this gene strictly depend on a functional Nodal signalling pathway. By expressing a constitutively active form of the TARAM-A receptor in the presence of translation inhibitors we have further demonstrated that *mezzo*, *bon/mixer* and *casanova* are all immediate early targets of Nodal signalling, while *sox17* is not. Overexpression of *mezzo* mRNA can induce ectopic expression of *casanova* and *sox17* and can also turn on the pan mesodermal marker gene *ntl*. We show that the function of *mezzo* is redundant with that of *mixer*. *mezzo* mRNA can partially rescue *bon* mutants, and when the function of *mezzo* and *bon/mixer* is inhibited, embryos develop without any *sox17* expression and fail to form prechordal plate mesoderm, a phenotype that suggests a further reduction of Nodal signalling.

These results place *mezzo* as a new transcription factor with unique properties acting in parallel with *bonnie and clyde*, *faust* and *casanova* in the Nodal signalling pathway controlling specification of mesoderm and endoderm in zebrafish.

MATERIALS AND METHODS

Isolation of zebrafish *mezzo* and plasmid constructions

mezzo was identified in a PCR-based screen for new homeobox genes expressed early during zebrafish development. DNA fragments corresponding to conserved regions of *paired-like* type homeoproteins were amplified from gastrula stage cDNA by RT-PCR using degenerate oligonucleotides (Faucourt et al., 2001). Most of the clones obtained in this screen corresponded to previously described sequences such as *bon/mixer*, *pitx2*, *mtx1* and *mtx2*. However, we also isolated a 117 bp cDNA fragment with only partial sequence homology to the homeobox of the Mix-Mixer family. This fragment was subsequently used as probe to screen a zebrafish gastrula cDNA library constructed in Uni-Zap XR (T. Lepage, unpublished). Out of the 12 independent clones isolated, one appeared to contain the largest insert and was entirely sequenced on both strands. The sequence of this 4075 bp cDNA was further confirmed by partial sequencing of

the remaining clones and by sequencing genomic DNA. The Accession Number for the *mezzo* sequence is AF466189.

Zebrafish strains, embryo manipulation, cycloheximide treatments

Adult zebrafish were maintained at 28.5°C using standard procedures (Westerfield, 1994). Wild-type embryos were collected by natural spawning from the AB strain. Mutant embryos were obtained by intercrossing heterozygous carrier fish identified by random crossing. We used the following mutant alleles: *one-eyed pinhead*, oep^{m134} (Schier et al., 1997), $cyclops^{b16}$ (Hatta et al., 1991), $squint^{cz35}$ (Feldman et al., 1998), $faust^{tm236a}$ (Chen et al., 1996), *bonnie and* $clyde^{m425}$ (Kikuchi et al., 2000), $casanova^{ta56}$ (Chen et al., 1996), *no* $tail^{c41b}$ (Odenthal et al., 1996) and $schmalspur^{m768}$ (Pogoda et al., 2000).

Note that oep^{m134} which causes a truncation in the C-terminal hydrophobic domain of the protein, is not a null mutation.

cyclops;squint double mutant carriers were identified by backcross in the progeny of a cross between identified *cyclops* and identified *squint* fish. As expected, one sixteenth of the embryos produced by intercrossing *cyc;sqt* carriers displayed the typical double mutant phenotype (Feldman et al., 1998). Adult *MZoep* and *MZsquint* fish were a generous gift of Frederic Rosa and adult *MZsur* fish were kindly provided by Dirk Meyer. bon^{m425}embryos were genotyped following the procedure published by Kikuchi et al. (Kikuchi et al., 2000).

In protein synthesis inhibition experiments, cycloheximide was added to dechorionated embryos at the 64- to 128-cell stage at a final concentration of 50 µg/ml. Cleavage in the treated embryos was usually arrested before the 512-cell stage, an indication of the effectiveness of the translation inhibitor.

RNA and oligonucleotides microinjection

For overexpression studies, the coding sequence of *mezzo* was amplified by PCR using the Pfu DNA polymerase and inserted at the *Cla*I-*Xba*I sites of pCS2+ (Turner and Weintraub, 1994) to generate *pCS2-mezzo*. Capped mRNA was synthesised from a template linearized with Asp718 using the SP6 mMessage mMachine kit (Ambion). Capped RNA encoding Antivin, TARAM-A*, GFP and NLS β-Gal were synthesised as described (Peyrieras et al., 1998; Thisse and Thisse, 1999; Faucourt et al., 2001). After synthesis, all the capped RNAs were purified on Sephadex G50 columns and quantitated by spectrophotometry.

In control experiments where morpholino oligonucleotides were co-injected with RNA containing the 5′ UTR region of *mezzo*, both reagents were mixed together and co-injected.

In situ hybridisation

In situ hybridisation was performed following a protocol adapted from Harland (Harland, 1991) with antisense RNA probes and staged embryos. All the riboprobes were used following published protocols.

Radiation hybrid mapping

mezzo was mapped on the LN54 radiation hybrid panel (Hukriede et al., 2001; Hukriede et al., 1999) using the primers 5′ CAGCAACCAATCCCGATTTA 3′ and 5′ CAGAGCTTCCTCC-AAACTGC 3′.

RESULTS

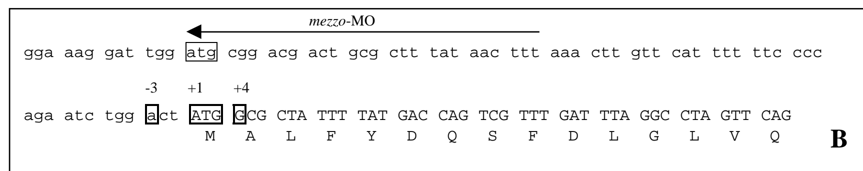
Isolation of zebrafish *mezzo* and structure of the *mezzo* transcripts

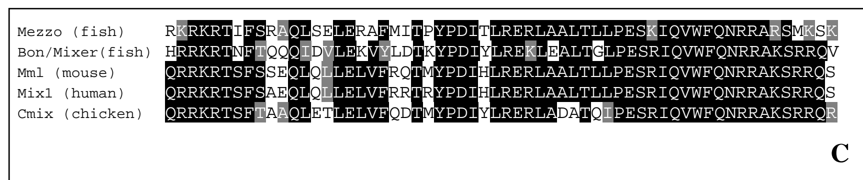
Using degenerate oligonucleotides against conserved segments of homeobox genes of the *paired-like* family, we isolated a DNA fragment corresponding to a novel sequence. A full-length cDNA was subsequently isolated by screening a cDNA library (Fig. 1). Comparison of the sequence of this cDNA with sequences present in GenBank revealed significant similarity with several transcripts encoding *paired-like* homeobox proteins (Fig. 1A). However, sequence comparisons did not reveal with certainty a clear orthologue of this transcript (see below), which, outside the coding sequence, did not share significant homology with any other mRNA. This suggested that this cDNA represented a new zebrafish gene and we named it *mezzo* (for mesendoderm homeobox).

mezzo encodes a homeodomain protein related to the Sebox and Mixer homeoproteins

Analysis of the *mezzo* transcript sequence revealed an 879 bp single long open reading frame (ORF) (Fig. 1). Starting at the second ATG (see legend of Fig. 1B for a justification of the choice of the initiator codon), the open reading frame is predicted to encode a protein of 293 amino acids. A comparison of the predicted Mezzo protein sequence with other protein sequences present in the PIR NBRF and Swiss Prot databases revealed significant but modest similarities with several other *paired-like* homeoproteins. The highest similarity was found with the human Sebox (E: $7e^{-15}$) and mouse OG9 (E: $4e^{-13}$) proteins (Cinquanta et al., 2000). An alignment of these three proteins is shown in Fig. 1A. This alignment shows that the human and mouse proteins are more related one to the other (63% identities) than to Mezzo (32% identities). Moreover, the Mezzo protein (293 amino acids) is significantly longer than the human Sebox (221 amino acids) and mouse OG9 proteins (190 amino acids). Outside the homeodomain, Mezzo and Sebox share limited sequence homology, sequence conservation being found only in the last 25 amino acids. This low conservation between two vertebrate sequences indicates that these genes, while clearly related, are probably not orthologues. When the comparison was limited to the homeodomain region, the highest scores were obtained with the Mix-like (Mml) protein from mouse (Pearce and Evans, 1999) and the Mix1 protein from *Xenopus* (68% identity) (Rosa, 1989) (Fig. 1C). Slightly lower scores were obtained for the CMIX factor from chick (62% identity) (Peale et al., 1998; Stein et al., 1998), the zebrafish Mixer/Bonnie and Clyde protein (58% identity) (Alexander et al., 1999), and the mouse Sebox/OG9 (57% identity). The C-terminal region of Mezzo does not contain any sequence resembling the acidic domain or the PPNK core sequence present in the Smad interacting motif (SIM) of some Mix-like factors (Germain et al., 2000). Mezzo therefore represents a divergent member of the Mix-like subclass of homeoproteins. The C-terminal region of Mezzo is rich in proline (11%) and serine (13%), two amino acids frequently found in the activator domains of transcription factors.

Southern analysis revealed that the *mezzo* gene is very likely to be a single copy gene (data not shown), while analysis of genomic DNA showed that the *mezzo* locus encompasses about 10 kb of sequence in zebrafish and consists of four exons and three introns. The intron-exon organisation of the *mezzo* gene is depicted in Fig. 1D. As for many members of the *paired-like* class of homeobox genes, the homeodomain of Mezzo is encoded by two exons separated by an intron in position 44 of the homeodomain (Duboule, 1994). The *mezzo* gene was mapped using the radiation hybrid mapping method using the





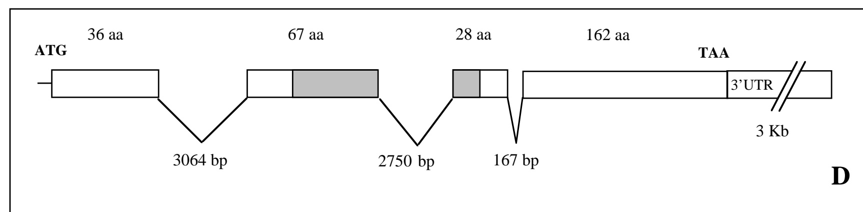


Fig. 1. *mezzo* encodes a *paired-like* homeobox transcription factor related to the Mix-like family. (A) Sequence alignment between zebrafish Mezzo, mouse Sebox and human Sebox. The homeodomain is overlined. Identical amino acids are printed on a black background, similar residues are printed on a grey background. (B) Nucleotide and deduced amino acid sequence of the 5′ end of the *mezzo* transcript. The likely potential translation initiation codon is boxed (lower) and the sequence targeted by the morpholino oligonucleotide used in this study is shown. Note that the 5′ end of the cDNA contains another in frame potential initiation codon TGGATGC (top). However, the sequence surrounding this ATG fits poorly with the consensus sequence defined by Kozak ACCATGG. The second potential initiator codon (lower): ACTATGG located 57 bp 3′ from the first in the ORF, is in good agreement with Kozak's rules with the most critical residues –3 and +4 being conserved. Furthermore, attempts to isolate longer cDNA clones by rescreening the library with a probe derived from the 5′ end of the cDNA gave only clones that started at about the same position as the cDNA described above. Mapping of the 5′end of the *mezzo* transcript by primer extension revealed that the 5′ end of the *mezzo* mRNA is located a just a few base pairs from the 5′ end of the *mezzo* cDNA we isolated. Finally, in the course of overexpression studies, we observed that only the protein initiated at this second ATG has a biological activity (data not shown). (C) Comparison of the Mezzo, Bon/Mixer, Mml, Mix-1 and C-Mix homeodomains. (D) Schematic representation of the *mezzo* gene structure. The homeodomain is shaded and the position of three introns is indicated.

LN54 mapping pannel (Hukriede et al., 1999). The *mezzo* locus resides on linkage group 5, 0.4 cR from *coe2*.

mezzo expression is restricted to the mesendoderm precursors during gastrulation

The spatial expression of *mezzo* was analysed by in situ hybridisation on staged embryos. *mezzo* transcripts were first detected by this technique at sphere stage (4 hours) in a small group of cells at the margin of the blastoderm (Fig. 2A,F). As epiboly started, the expression domain of *mezzo* extended over the whole circumference of the margin of the blastoderm (Fig. 2B,G). Expression of *mezzo* was restricted to the blastoderm and no expression was detected in the yolk syncytial layer. A high magnification view of embryos labelled at 50% epiboly shows that the expression domain of *mezzo* extends over six rows of cells, that is over a region that includes the precursors of mesoderm and endoderm (Fig. 2C,H) (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999). At the shield stage, *mezzo* transcripts are also expressed in the invaginating axial mesendoderm (Fig. 2D,I). After the shield stage, the abundance of the *mezzo* transcripts declined abruptly, only a weak expression is detected in embryos at 60% epiboly and no expression at 70% epiboly or later (Fig. 2E,J). The *mezzo* gene is therefore expressed zygotically during zebrafish development. The early and transient nature of *mezzo* expression was confirmed by northern blot and RT-PCR and no transcripts were detected in RNA extracted from 4-day-old embryos or from adult fish (data not shown).

The temporal and spatial expression profiles of *mezzo* are similar to those of *bon/mixer* (Fig. 2K-O) (Alexander et al., 1999; Kikuchi et al., 2000), which has been shown to be regulated by Nodal signalling.

mezzo expression is regulated by a TARAM-A/antivin signalling pathway

To determine if *mezzo* expression is dependent on the Nodal signalling pathway, we made use of TARAM-A*, a constitutively activated form of a type I TGFβ receptor presumed to be a receptor for the Nodal factors (Renucci et al., 1996; Peyrieras et al., 1998; Aoki et al., 2002b). We injected mRNA encoding TARAM-A* into one blastomere of embryos at the eight-cell stage together with mRNA encoding a nuclear β-Gal as a lineage tracer. Injected embryos were allowed to develop up to the 50% epiboly then were fixed in order to

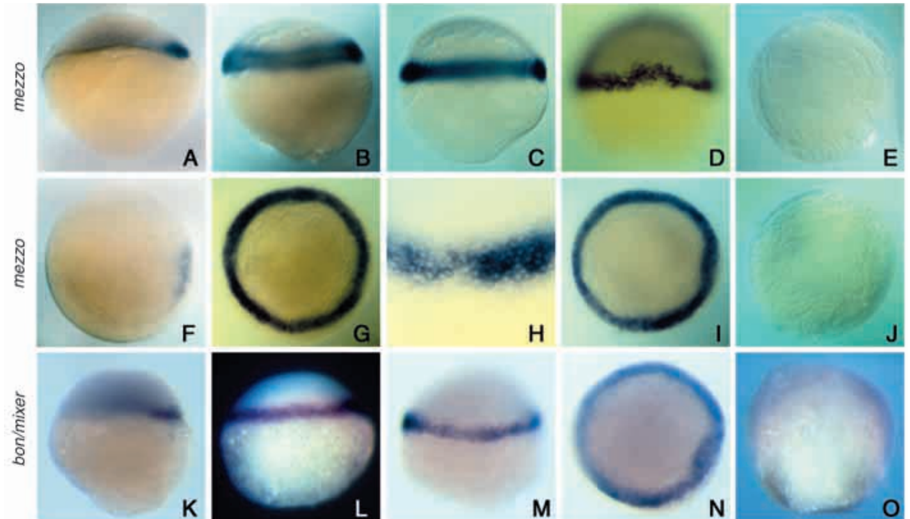


Fig. 2. Analysis of *mezzo* expression in wild-type embryos and comparison with *bon/mixer*. (A,F,K) Sphere stage (4 hpf), (B,G,L) 30% epiboly (4.7 hpf), (C,H,M) 50% epiboly (5.3 hpf), (D,I,N) shield stage (6 hours), (E,J,O) 70% epiboly (8 hpf). (A-E,K-M,O) Side views. (G,I,J,N) Views from the animal pole. (F,D) Dorsal views. *mezzo* (A,F) and *bon/mixer* (K) start to be expressed in a small group of cells on the dorsal side at the sphere stage. At 30% epiboly, expression of *mezzo* (B,G) and *mixer* (L) has extended over the whole marginal region. A close examination of *mezzo* expression at 50% epiboly (H) shows that *mezzo* transcripts are present in about six rows of cells at the margin of the blastoderm. No expression is detected in the YSL (C,H,M). At the shield stage, *mezzo* and *mixer* continue to be expressed throughout the marginal region and in the dorsal shield (D,I,N). At 70% epiboly, *mezzo* and *mixer* have been turned off and transcripts are no longer detected in the embryo (E,J,O).

analyse the expression of *mezzo*. In addition to the normal expression of *mezzo* at the margin of the blastoderm, *mezzo* expression was strongly induced in the clones of cells inheriting *TARAM-A** RNA (Fig. 3B). To inhibit the Nodal signalling pathway, we overexpressed *antivin*, a potent antagonist of Nodal and Activin in zebrafish (Bisgrove et al., 1999; Thisse and Thisse, 1999). Injection of mRNA encoding Antivin into embryos at the one- to two-cell stage drastically reduced the level of *mezzo* expression at the onset of gastrulation (Fig. 3C). These experiments suggest that *mezzo,* like the other genes acting early in endoderm specification, is regulated by a TGF-β signalling pathway.

Analysis of *mezzo* expression in mutants of the Nodal signalling pathway

To confirm the results above indicating that the Nodal signalling pathway regulates expression of the *mezzo* gene, we examined *mezzo* expression in various mutants with attenuated or completely defective Nodal signalling (Fig. 4; Table 1). We first analysed the level of *mezzo* transcripts in embryos carrying mutations in the *cyclops*, *squint* and *oep* genes, which act early in the Nodal pathway. In *cyclops* mutants embryos, *mezzo* expression was indistinguishable from control embryos (data not shown). By contrast, in *squint* mutant embryos, the expression domain of *mezzo* was thinner along the animal-vegetal axis and displayed a gap (Fig. 4C,I). A similar gap in the expression domain of *mezzo* was also observed in embryos lacking zygotic transcripts of *oep* (Fig. 4B,H). Double labelling experiments using a probe for the *chordin* gene, which is still expressed in the dorsal marginal zone in absence of Nodal signalling (Gritsman et al., 1999) showed that this gap corresponds to the dorsal side of the margin where the *squint* ligand is expressed (data not shown). We then looked at *mezzo* expression in *cyc;sqt* double mutant embryos. In the progeny of crosses between double heterozygotes *cyc;sqt*, we found that 21% of the embryos showed a dorsal gap in the expression domain of *mezzo*, while 8% had a barely detectable expression (Fig. 4D,J). From the frequency of occurrence of these phenotypic classes, we infer that embryos displaying a dorsal gap are the single *squint* mutants (3/16th) while embryos

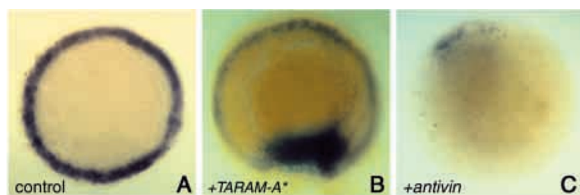


Fig. 3. Expression of *mezzo* is regulated by a TGFβ/TARAM-A signalling pathway. Animal pole views of embryos at 50% epiboly showing mezzo in situ hybridisation (purple). (A) Control uninjected embryo. (B) Embryo injected at the eight-cell stage into one blastomere with 40 pg of *TARAM-A** and with 100 pg of mRNA encoding a nuclear β-Gal (blue colour) as a lineage tracer. *TARAM-A** induces confluent patches of *mezzo*-expressing cells. (C) Expression of *mezzo* transcripts was barely detectable in embryos injected with 50 pg of *antivin* mRNA.

Table 1. Nodal-related signals are required for normal *mezzo* expression

Mutant background	*n*	Embryos showing normal expression of *mezzo*	Embryos showing altered expression of *mezzo*
one eyed pinhead	173	80%	20%
squint	70	76%	24%
cyclops	105	100%	0%
cyclops;squint	184	71%	7.6% (without expression) 21% (dorsal gap)
casanova	149	100%	0%
faust	134	100%	0%
bonnie and clyde	72	100%	0%
bon;faust	294	93%	7%
MZschmalspur	117	0%	100%

Embryos from heterozygous or homozygous intercrosses of the genotype indicated were analysed at shield stage for the expression of *mezzo*. Examples of altered expression are shown in Fig. 4.

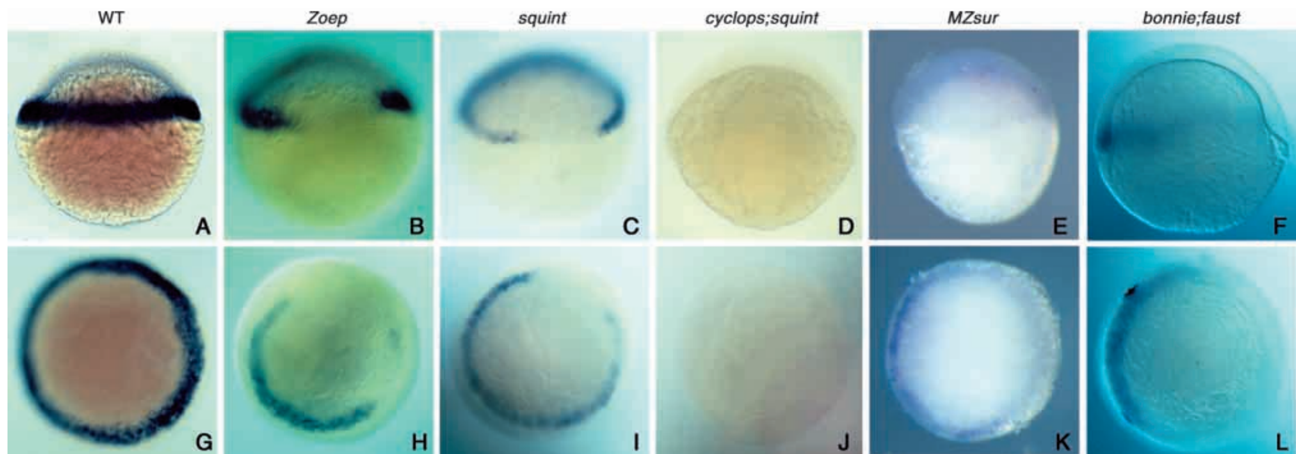


Fig. 4. Analyses of *mezzo* expression in various mutants of the Nodal signalling pathway. All the embryos were fixed at the shield stage except those in B,H (40% epiboly). (A-F) Side views, (G-L) animal pole views. (A,G) Control wild-type embryos. Expression of *mezzo* was lost on the dorsal side in *Zoep* (B,H) and *squint* (C,I) mutant embryos. Expression of *mezzo* was abolished in *cyc;sqt* double mutants (D,J) and variably affected in *MZsu*r embryos. The variable expression of *mezzo* in *MZsur* correlated with the expressivity of the *schmalspur* phenotype. The embryos shown in E,K derived from a cross producing severely affected embryos. Expression of *mezzo* is greatly diminished in two thirds of the marginal region in *bon;faust* double mutants (F,L).

displaying largely absent *mezzo* expression are the double mutants (1/16th). Similarly, we found that *mezzo* expression was variably affected in *schmalspur* (*MZsur*), the variation correlating with the strength of the *MZsur* phenotype (Fig. 4E,K). Two maternal genes implicated in Nodal signalling (*oep* and *schmalspur*) are thus critically required for normal expression of *mezzo*.

To determine if the zygotic genes acting more downstream in the signalling pathway are similarly required, we analysed the expression of *mezzo* in embryos carrying mutations in the *bonnie and clyde*, *faust*, *casanova* and *ntl* genes. In all these single mutant backgrounds, expression of *mezzo* appeared to be normal, suggesting that *mezzo* is not a downstream target of these zygotic transcription factors. Expression of *mezzo* in *faust;cas* double mutant embryos was also indistinguishable from that of control embryos, further indicating that *cas* does not participate in the regulation of *mezzo* expression. By contrast, we found that 6% of the embryos derived from a cross between *bon;faust* double heterozygotes displayed a significant reduction of *mezzo* expression over half of the circumference of the blastoderm (Fig. 4F,L). This result indicates that, while *bon* and *faust* are not individually required for *mezzo* expression, removing the function of *bon* and *faust* does significantly reduce *mezzo* expression.

Taken together, our analyses of *mezzo* expression in mutant embryos suggest that transcription of the *mezzo* gene may be activated by maternal transcription factors in response to Nodal signalling and that Bon/Mixer and GATA5 are subsequently required for sustained expression of this gene.

mezzo, *bon/mixer* and *casanova* are immediate early targets of nodal signalling

Analysis of the temporal expression of *mezzo*, *bon/mixer*, *casanova* or *sox17* shows that all four genes start to be expressed approximately at the same time, *sox17* appearing slightly after the others (Alexander and Stainier, 1999; Rodaway et al., 1999; Kikuchi et al., 2000; Reiter et al., 2001; Aoki et al., 2002b). Epistasis experiments have revealed that *sox17* probably acts downstream of *bon/mixer*, *faust* and *casanova* during endoderm formation; however, it is not clear whether *casanova* acts downstream of or in parallel to *bon/mixer* and *faust* (Alexander and Stainier, 1999; Reiter et al., 2001; Aoki et al., 2002a; Stainier, 2002). We tested whether *mezzo*, *bon/mixer*, *sox17* and *casanova* are transcribed in immediate response to Nodal signalling or if their transcription requires protein synthesis. To achieve this, we injected *TARAM-A** into one- to four-cell stage embryos, then treated the injected embryos with the protein synthesis inhibitor cycloheximide (CHX) at the 64- to 128-cell-stage and analysed expression of the different genes by in situ hybridisation at the dome stage. As a control in this experiment, we measured the expression of *no tail* (*ntl*), which has already been shown to be an immediate early target gene of Nodal signalling in zebrafish (Vogel and Gerster, 1999). Injection of *TARAM-A** induced very robust expression of *sox17*, *ntl*, *mezzo*, *bon/mixer* and *casanova* in large patches of cells (Fig. 5C,H,M,R, data obtained with *mixer* are similar to those obtained with *mezzo* and are not shown here). By contrast, the response of these different genes after *TARAM-A** injection in the presence of translation inhibitors was quite different. Ectopic expression of *sox17* was largely suppressed by CHX as expected for a gene acting downstream of *casanova*, *bon/mixer* and *faust* (Fig. 5D,E). By contrast, *mezzo* (Fig. 5N,O), *casanova* (Fig. 5S,T) and *bon/mixer* transcripts were clearly detectable after inhibition of protein synthesis and all three genes behaved in the same manner as the immediate target gene *ntl* (Fig. 5I,J).

From this experiment, we conclude that transcription of *mezzo*, *bon/mixer* and *casanova* in response to Nodal signalling is probably activated directly by maternal factors. By contrast, *sox17* expression is completely dependent on zygotic factors, in agreement with the previous finding that Casanova is necessary for *sox17* expression.

Overexpression of *mezzo* induces ectopic expression of *casanova, sox17* and *no tail*

To test the role of *mezzo* in germ layer specification, we

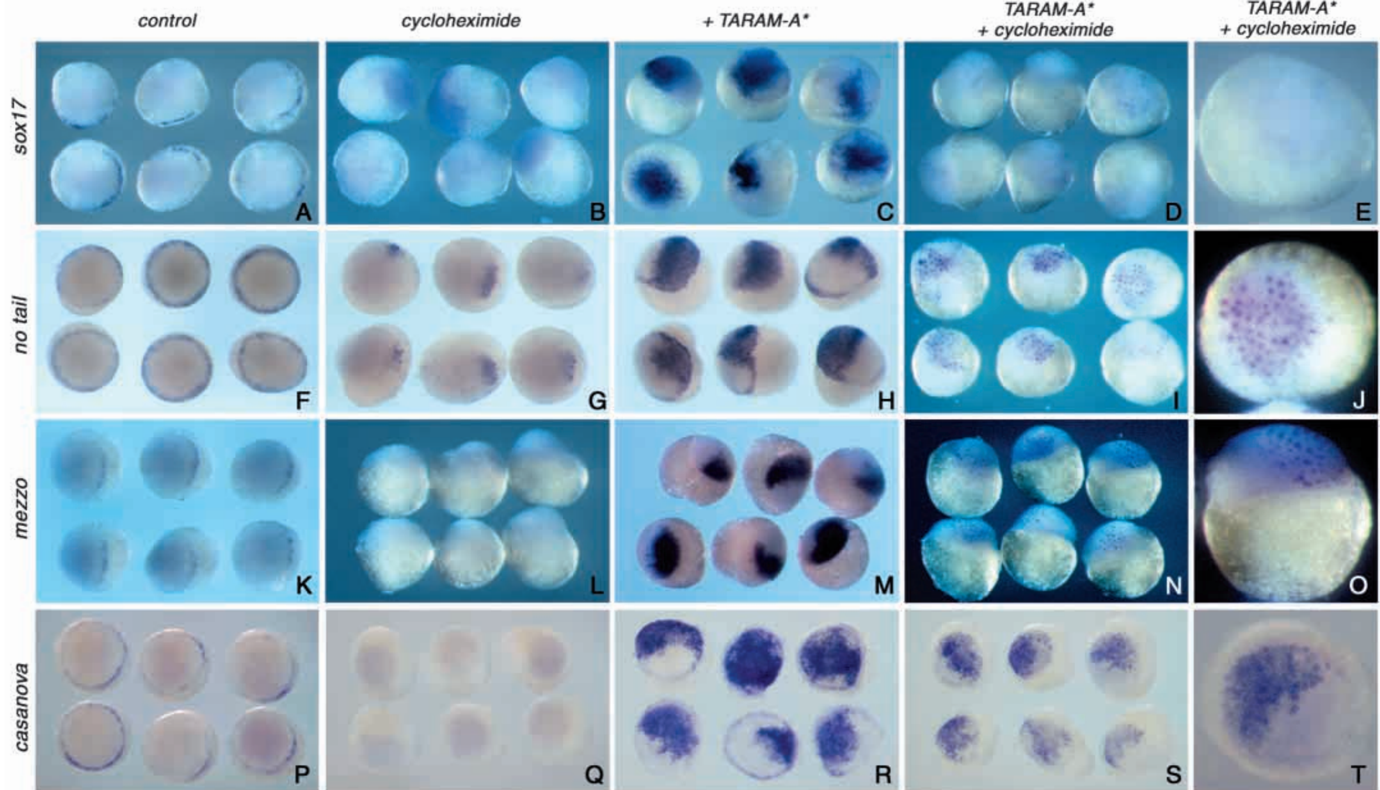


Fig. 5. *mezzo*, *casanova*, *bon/mixer* and *ntl* are immediate early targets of Nodal signalling, while *sox17* requires post MBT protein synthesis. Embryos at the one- to four-cell stage were microinjected with 50 pg of TARAM-A* mRNA, treated continuously with 50 μg/ml of cycloheximide, starting at the 64- to 128-cell stage and analysed at dome-30% epiboly stage for the expression of *sox17* (A-E), *ntl* (F-J), *mezzo* (K-O) and *casanova* (P-T). (A,F,K,P) Untreated and uninjected control embryos. (B,G,L,Q) Uninjected control embryos treated with cycloheximide. (C,H,M,R) Control untreated embryos injected with TARAM-A*. (D,E,I,J,N,O,S,T), Embryos injected with TARAM-A* and treated with cycloheximide. Although treatment with cycloheximide prevents induction of *sox17*, some ectopic *mezzo*, *ntl*, *bon/mixer* and *cas* expression is clearly visible in absence of protein synthesis.

overexpressed it in early zebrafish embryos. Synthetic mRNAs encoding Mezzo or GFP were microinjected into cleavage stage embryos at the two- to 16-cell stage and the effects were analysed at the morphological and molecular level. Embryos injected with 100 pg of GFP mRNA or with low doses (1-5 pg) of *mezzo* mRNA developed just like control uninjected embryos. Developmental defects were observed with higher doses of *mezzo* (50-100 pg) and included reduced convergence and extension movements of the dorsal mesoderm during gastrulation, leading to later defects in neural tube closure. Overexpression of *mezzo* was also associated with defects in patterning of the axial mesoderm, as evidenced by the presence of U-shaped somites and kinked notochords. More rarely, outgrowths of tissues often located in the trunk and tail regions, were also observed but head development was largely normal (data not shown).

To determine whether the observed morphological defects were the consequence of earlier defects in formation of the mesoderm and endoderm, we examined the expression of early endodermal and mesodermal marker genes in the injected embryos. We injected synthetic mRNA encoding Mezzo into eight- or 16-cell stage embryos and analysed at shield stage the expression of *sox17* and *casanova* (Fig. 6). We found that overexpression of *mezzo* mRNA induced strong ectopic expression of *sox17* and *casanova* across the animal hemisphere (Fig. 6B,D,E,F). The animal cells ectopically expressing *sox17* or *casanova* did not seem to contribute to the hypoblast but remained in a superficial position in the epiblast. Similar observations have been reported in previous studies on *casanova* and *bon* (David and Rosa, 2001; Kikuchi et al., 2001; Aoki et al., 2002a). The ability of *mezzo* RNA to induce ectopic expression of endodermal markers in presumptive ectodermal cells is remarkable as *bon/mixer* RNA is apparently not able to induce *sox17* expression outside the margin (Alexander and Stainier, 1999; Kikuchi et al., 2000).

To test the ability of *mezzo* to promote the expression of endodermal marker genes in embryos in which the Nodal signalling pathway is completely inactive, we microinjected *mezzo* mRNA into embryos derived from *MZoep* mothers. In the absence of maternal and zygotic *oep* transcripts, cells are unable to respond to Nodal signals and the expression of endodermal markers genes is abolished (Fig. 6C) (Alexander et al., 1999; Gritsman et al., 1999; Kikuchi et al., 2000). By contrast, all the injected embryos clearly showed ectopic expression of *sox17* (Fig. 6G), indicating that *mezzo* mRNA can partially rescue *sox17* expression in *MZoep* embryos.

Casanova has previously been shown to be largely required for the induction of *sox17* by *faust/gata5* and strictly required for the induction of *sox17* by *bon/mixer* (Alexander and Stainier, 1999; Reiter et al., 2001; Aoki et al., 2002a). We

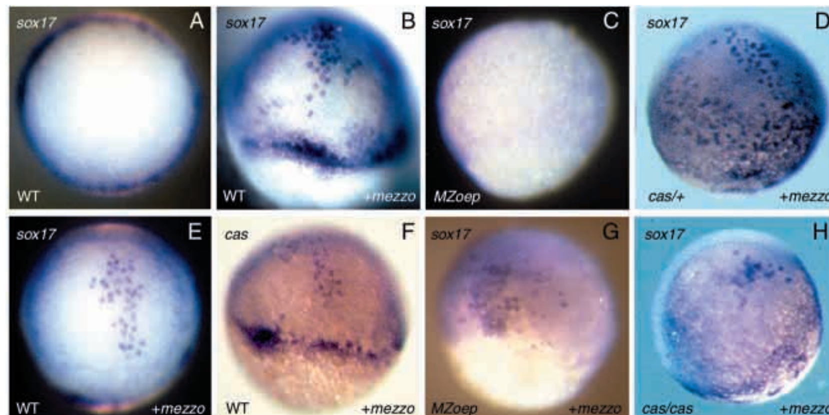


Fig. 6. Induction of endodermal marker genes induced by ectopic *mezzo* in wild-type embryos, *MZoep* mutants and *casanova* mutants. (A,E) animal pole views. (B-D,F-H) Side views. Embryos were injected at the 2- to 16-cell stage with 40 pg of GFP (A) or 40 pg of *mezzo* mRNA and examined at the shield (A,B,E,F) or at 80% epiboly (C,D,G,H) for the expression of *sox17* (B,C,D,E,G,H) or *casanova* (F). Ectopic *mezzo* induces robust expression of *sox17* (B,D,E) or *casanova* (F). These cells were typically large and scattered like the endogenous endoderm precursors; however, most of those cells remained in the epiblast. Overexpression of *mezzo* rescues *sox17*-expressing cells in *MZoep* (G) and weakly induces ectopic expression of *sox17* in *casanova* (H).

therefore tested the ability of *mezzo* mRNA to induce expression of *sox17* in absence of the *casanova* gene product. Out of 56 injected embryos, 12 (21%) were identified as homozygous *cas* embryos by the lack of endogenous *sox17* expression in the blastoderm and the upregulation of this gene in the YSL. Six out of the 12 homozygous embryos showed weak ectopic expression of *sox17* in small patches of cells, while the other six showed no expression. (Fig. 6D,H). Overexpression of *mezzo*, like overexpression of *faust/gata5,* can thus activate low levels of *sox17* expression in complete absence of *casanova* gene function. Nevertheless, in the normal embryo, *sox17* expression is abolished in the absence of the *casanova* gene product. This confirms that Casanova is the most crucial activator of *sox17*. It also suggests that, although the *sox17* gene may receive some direct input from Mezzo and Gata5, the main role of Mezzo, Faust/Gata*5* and Bon/Mixer is probably to maintain *casanova* transcription.

Taken together, these results strongly suggest that *mezzo* is indeed a mediator of the Nodal signalling pathway that cooperates with *bon/mixer* and *casanova* during endoderm specification.

We next examined whether *mezzo* could promote the expression of other downstream targets of the Nodal signalling pathway, and particularly of mesodermal marker genes such as *ntl*. We injected *mezzo* mRNA into one blastomere of embryos at the eight-cell stage, at doses which induced ectopic expression of endodermal markers. Out of 163 embryos injected with *mezzo* mRNA, 88 (54%) showed an expansion of the *ntl* territory compared with control uninjected embryos (Fig. 7A,B). The remaining fraction of the injected embryos, however, did not display ectopic *ntl* expression but instead showed an interruption in the ring of marginal *ntl* expression. Co-injection of β-gal RNA as a lineage tracer revealed that when targeted at the margin, high doses of *mezzo* mRNA could suppress *ntl* expression in a cell autonomous manner (Fig. 7G). To determine if mesodermal markers were repressed as a consequence of upregulation of endodermal genes, we performed double in situ hybridisation to detect both *casanova* and *ntl* after injection of *mezzo* mRNA. We found that, out of 75 embryos injected into one blastomere at the eight cell stage, 25 showed marginal clones of cells in which *casanova* was overexpressed while *ntl* expression was simultaneously repressed (Fig. 7C,H). This result suggests that ectopic expression of *mezzo* could repress expression of *ntl* at the margin by inducing *casanova*, consistent with previous data (Aoki et al., 2002a).

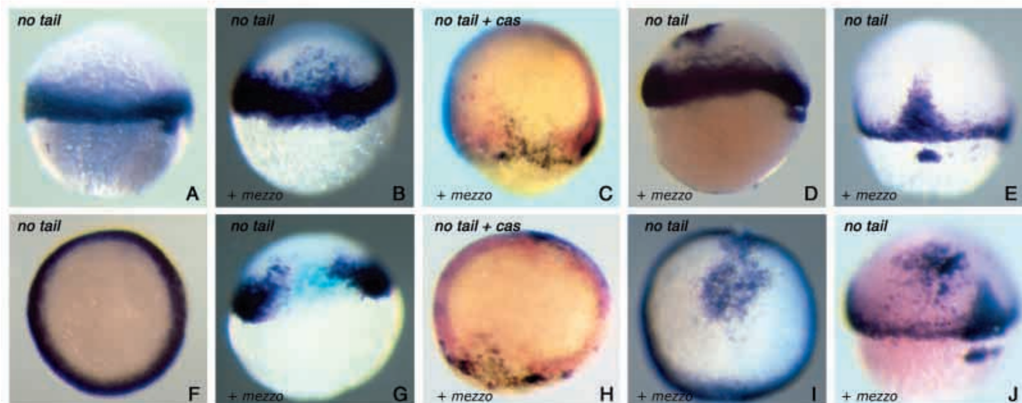


Fig. 7. Effects of *mezzo* mRNA injection on *ntl* expression. Overexpression of *mezzo* induces ectopic expression of *ntl* in the animal hemisphere and repress endogenous *ntl* at the margin. (A,B,C,D,E,G,J) Side views and (F,H,I) animal pole views of embryos at the shield stage (A-D,F-I) or 60% epiboly (E,J). (A,E,F) Control uninjected embryos. (B,G) Embryos injected at the eight-cell stage with 40 pg of *mezzo* plus β-Gal RNA. (B) Expansion of the territory expressing *ntl* caused by injection of *mezzo* mRNA in the non marginal region. A close examination of these embryos showed the presence of the lineage label outside the margin. (G) Repression *ntl* expression caused by injection of *mezzo* in the marginal region. Note the presence of the lineage label in the sector of cells that do not express *ntl*. (C,H) Two-colour in situ hybridisation showing induction of *casanova* (brown) and repression of *ntl* (red) after overexpression of *mezzo* in the marginal region. (D,I,J) Injection of *mezzo* mRNA at the same concentration as above into one central blastomere of embryos at the 16-cell stage induced patches of *ntl* expression in the animal hemisphere.

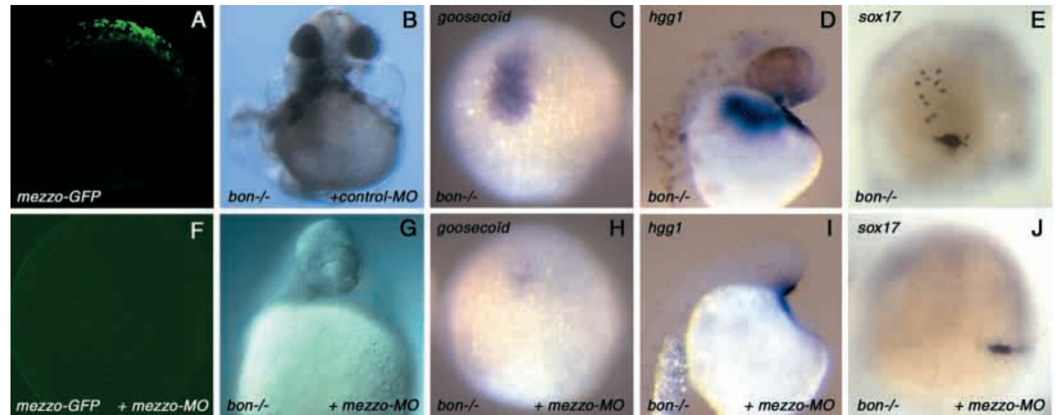


Fig. 8. Injection of an antisense morpholino oligonucleotide directed against *mezzo* in *bon* mutants. (A) Control embryo injected with 100 pg of an mRNA encoding a fusion protein between the 5′ leader sequence of *mezzo* and the sequence coding for the first 43 amino acids fused to the coding sequence of the green fluorescent protein to make *mezzo*-GFP mRNA. (F) Embryo injected with 100 pg of *mezzo*-GFP mRNA and with 12 ng the antisense morpholino directed towards the 5′ end of *mezzo* (*mezzo*-MO). All the embryos injected with *mezzo*-GFP mRNA were brightly fluorescent when examined at the shield stage (A). In embryos co-injected with *mezzo*-GFP and 12 ng of *mezzo*-MO, the fluorescence was abolished (F). Note that the camera gain in this image was much higher than for the image in A. Using the same settings as A, the image would be black. (B) *bon* mutants injected with an unrelated morpholino directed against the sea urchin hatching enzyme mRNA used as a negative control. (G) *bon* mutant embryo injected with 12 ng of antisense morpholino targeted against *mezzo*. (C,H) Expression of the prechordal plate marker *goosecoid* in control uninjected (C) and in morpholino injected (H) *bon* mutant embryos at 80% epiboly. (D,I) Expression of the hatching gland marker *hgg1* in control uninjected (D) and in morpholino injected (I) *bon* mutants. (E,J) Residual *sox17* expression in homozygous *bon* mutants at 80% epiboly (E), and absence of *sox17*-expressing cells in *bon* mutants injected with the morpholino (J). To unambiguously identify homozygous *bon* mutants, their genotype was determined by PCR (Kikuchi et al., 2001).

Similarly, we found that the expression of *ntl* in the injected embryos at 80% epiboly and during somitogenesis was severely perturbed by *mezzo* overexpression, leading either to a complete absence of the *ntl* in part of the marginal region (30%) or to a patchy and broadened marginal and axial expression of *ntl* (54%) (data not shown). These observations suggest that the developmental defects caused by overexpression of *mezzo* are the consequences of early patterning defects.

Remarkably, when similar doses of *mezzo* mRNA were injected into one internal blastomere of embryos at the 16-cell stage, ectopic *ntl* expression was clearly observed near the animal pole in about half of the injected embryos (*n*=65) (Fig. 7D,I,J). Thus, *mezzo* can induce mesodermal markers in presumptive ectodermal cells. This property distinguishes *mezzo* from *bon/mixer*, which appears to lack mesoderm inducing activity (Alexander et al., 1999) (M. P. and T. L., unpublished).

Inhibition of *mezzo* function using antisense Morpholino oligonucleotides

The experiments described above have shown that *mezzo* is expressed at the right time and at the right place to be a downstream mediator of Nodal signalling. Moreover, the results from ectopic expression of *mezzo* strongly suggest that this gene is involved in specification of the fate of mesodermal and endodermal cells. To determine if *mezzo* function is required for specification of mesendodermal cells, we used a loss-of-function approach using antisense morpholino oligonucleotides (Summerton and Weller, 1997). To first test the effectiveness of this approach in the case of *mezzo*, we constructed an artificial mRNA where the 5′ leader sequence of *mezzo* and the sequence coding for the first 43 amino acids were fused to the coding sequence of the green fluorescent protein to make (*mezzo*-GFP mRNA). This *mezzo*-GFP mRNA was injected in embryos alone or mixed together with the antisense morpholino directed towards the 5′ end of *mezzo* (*mezzo*-MO). An unrelated morpholino was also injected at various concentrations as a negative control. All the embryos injected with *mezzo*-GFP mRNA were brightly fluorescent when examined at the shield stage (Fig. 8A) (*n*>50). By contrast, embryos co-injected with *mezzo*-GFP and 12 ng of

Table 2. Effects of the injection of an antisense morpholino oligonucleotide against *mezzo* (*mezzo-MO*) in several mutants of the Nodal signalling pathway

	Uninjected			Embryos injected with 24 ng of *mezzo-MO*		
Mutant background	Number of embryos with mutant phenotype	*n*	Percentage of embryos with mutant phenotype	Number of embryos with mutant phenotype	*n*	Percentage of embryos with mutant phenotype
one eyed pinhead	12	50	24	11	43	26
squint	3	43	7	19	96	20
MZsquint	15	25	60	33	33	100
faust	7	35	20	9	40	23
casanova	13	62	21	6	26	23

mezzo-MO increases the penetrance of *squint* and *MZsquint*.

mezzo-MO did not show any fluorescence (*n*>100) when examined at the same stage indicating that the morpholino oligonucleotides efficiently reduced translation of the *mezzo*-GFP mRNA (Fig. 8F). We then microinjected this oligonucleotide at various concentrations into wild-type embryos. Even at high doses (up to 12 ng), injection of *mezzo*-MO did not perturb development of the injected embryos and the expression of marker genes such as *ntl* and *sox17* was normal in these embryos (data not shown).

The lack of effects of *mezzo*-MO suggested that in wild-type embryos, another factor was able to compensate for the absence of *mezzo* function. To address this possibility, we tested the effects of inhibiting *mezzo* function in embryos lacking the activity of various genes acting in the Nodal pathway (Table 2). We first injected *mezzo*-MO into embryos derived from heterozygous *squint* parents. The *squint* mutation is incompletely penetrant and typically 5-15% of the progeny of *squint* parents manifests a cyclopic phenotype at 24 hours. We selected pairs of fish in which the penetrance of the mutation was reproducibly low (near 5%). Injection of *mezzo*-MO in the progeny of such fish caused a significant increase (near 20%) in the percentage of embryos displaying a cyclopic phenotype (Table 2). A similar experiment was performed using *MZsquint* embryos and, again, a significant increase (from 60% to 100%) in the penetrance of the phenotype was observed after injection of *mezzo*-MO. We then extended this analysis to the *oep* mutant and to mutants in downstream targets of the Nodal signalling pathway such as *bon*, *faust* and *cas*. Injection of *mezzo*-MO in *Zoep*, *faust* and *cas* mutant backgrounds increased neither the percentage of mutant embryos nor the severity of the phenotype. By contrast, injection of *mezzo*-MO in the progeny of heterozygous *bon* parents produced a striking result. About 13% of the injected embryos showed a cyclopia very similar to that caused by inactivation of *squint* or *oep* (*n*=262) and most of these cyclopic embryos also displayed a cardiac phenotype suggesting that only *bon* homozygous embryos were affected. We confirmed that the cyclopic embryos were homozygous *bon* mutants by determining their genotype by PCR (Kikuchi et al., 2000). Injection of a control antisense MO did not perturb development of the *bon* embryos demonstrating that this phenotype was specific to the *mezzo*-MO (Fig. 8B).

In the case of *cyclops*, *squint* or *oep* mutants, cyclopia has been correlated with the early loss of *goosecoid* (Stachel et al., 1993; Thisse et al., 1994) and later of *hgg1* (Vogel and Gerster, 2000), two genes expressed in the prechordal mesoderm cells during and at the end of gastrulation. We analysed the expression of these markers in embryos derived from *bon*/+ parents injected with *mezzo*-MO. Expression of *goosecoid* appeared normal in most of the injected embryos when examined at the shield stage; however, in about 20% of these embryos, expression of *goosecoid* was drastically reduced or lost at 70% epiboly (Fig. 8C,H; *n*=36) in good agreement with the morphological observations. Similarly, expression of *hgg1*, a late marker of prechordal mesoderm, was strongly reduced in the cyclopic embryos at 24 hours (Fig. 8D,I). These results confirm that the cyclopia caused by the lack of both Bon and Mezzo proteins is associated with the loss of prechordal mesoderm precursors during gastrulation.

We also analysed *sox17* expression in *bon* mutants injected or not with *mezzo*-MO. In homozygous *bon*^{m425} embryos, expression of *sox17* is reduced but not abolished (Kikuchi et al., 2000). Typically, about 20 *sox17*-expressing cells are still present, suggesting that additional factors are involved in regulating *sox17* expression (Fig. 8E). Inhibition of *mezzo* function in *bon* mutant embryos eliminated all residual *sox17* expression (Fig. 8J; *n*=25/82). This results shows that the activities of Mixer and Mezzo are both required for specification of the normal number of *sox17*-expressing cells.

In summary, inhibition of *mezzo* function using an antisense oligonucleotide increases the penetrance of the *squint* and *MZsquint* mutations and increases the severity of the phenotype caused by the lack of *bon/mixer*, leading to defects in endoderm and prechordal plate development similar to those observed in the *oep*, *cyclops* and *squint* mutants.

Overexpression of *mezzo* partially rescues the phenotype of *bon* mutants

To test if overexpression of *mezzo* could compensate for the lack of Mixer in *bon* mutants, we microinjected *mezzo* mRNA into *bon* mutant embryos. *bon* mutant embryos have a drastic reduction of *casanova* and *sox17* expression during gastrulation and, typically, 25% of the embryos later develop with two well separated hearts primordia because of improper specification of the endoderm (Kikuchi et al., 2000) (Fig. 9B,E,H; Table 3). By contrast, when *mezzo* mRNA was microinjected into the progeny of *bon*/+ parents, the percentage of embryos showing a *cardia bifida* was consistently around 15% and some embryos displayed a morphologically abnormal but single heart chamber (Fig. 9C). This suggested that injection of *mezzo* mRNA had allowed some mutant embryos to develop with a single heart. In one experiment, out of 77 injected embryos, only 12 embryos displayed a typical *cardia bifida* at 35 hours (15%). Out of the 65 remaining embryos, 15 had a single but abnormal heart. We genotyped those 15 embryos and found that six (7.8%) were in fact homozygous *bon* mutants. The other nine embryos were heterozygous bon/+ in which *mezzo* overexpression had probably caused abnormal heart morphogenesis.

We conclude that overexpression of *mezzo* can partially rescue heart morphogenesis in *bon* homozygous embryos. Because the *cardia bifida* phenotype of *bon* mutants is a

Table 3. Overexpression of *mezzo* increases the number of *sox17*-expressing cells and partially rescues heart morphogenesis in *bonnie and clyde* mutants

	Average number of *sox17*-expressing cells			Phenotype		
Injected RNA	Normal/increased	1-25	25-100	Wild type	*cardia bifida*	Single abnormal heart
None	46	13 (22%)	0	64	23 (26%)	1 (1%)
mezzo	65	9 (10%)	12 (14%)	191	41 (15%)	48 (17%)

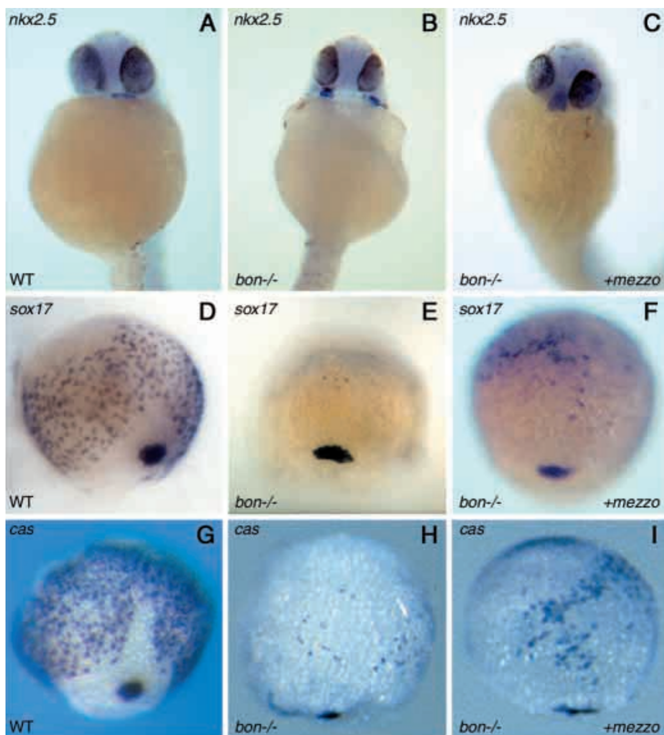


Fig. 9. Overexpression of *mezzo* partially rescues heart morphogenesis in *bon* mutants and increases the number of *sox17*- and *cas*-expressing cells. (A-C) Rescue of heart morphogenesis after injection of *mezzo* mRNA into *bon* mutant embryos monitored by expression of *nkx2.5* at 30 hours. The failure of heart primordia to migrate and fuse in the midline in *bon* mutants (B) was partially restored by overexpression of *mezzo* (C). (D-I) Dorsal views of embryos at 80% epiboly, showing expression of endodermal markers in wild-type embryos (D,G), *bon* mutant embryos (E,H) and *bon* mutant embryos injected with *mezzo* mRNA (F,I). Embryos were injected with 20-40 pg of *mezzo* mRNA a the one- to four-cell stage and examined for expression of *sox17* (F) or *casanova* (I). The number of *sox17*- or *casanova*-positive cells is significantly increased in homozygous *bon* mutants injected with *mezzo* mRNA (F,I) compared with control uninjected mutant embryos (E,H). *bon* mutant embryos were genotyped by PCR.

consequence of abnormal specification of the endodermal cells, the partial rescue of cardiac fusion caused by *mezzo* suggests that ectopic *mezzo* may have also partially rescued endodermal gene expression in these embryos. Analysis of *sox17* and *casanova* expression in homozygous *bon* mutants injected with *mezzo* mRNA confirmed that *mezzo* partially rescued the expression of these two endodermal marker genes in *bon* mutant embryos (Fig. 9F,I). Taken together, these results suggest that the function of Mezzo is partially redundant with the function of Bon/Mixer during endoderm specification.

DISCUSSION

Mezzo as a downstream mediator of the Nodal signalling pathway

Endoderm specification in zebrafish is mediated by the transcription factors Bon/Mixer, Faust/Gata5 and Casanova, whose expression is induced by Nodal signalling. The involvement of other factors had been anticipated from previous work showing that the activities of *bon/mixer* and *faust/gata5* are not sufficient to restore endoderm formation in absence of Nodal signalling (Alexander and Stainier, 1999; Kikuchi et al., 2000; Dickmeis et al., 2001; Reiter et al., 2001; Aoki et al., 2002a). This study describes the characterisation, expression pattern and functional analysis of a novel zebrafish gene that fulfils several of criteria expected for such a factor.

mezzo encodes a homeobox transcription factor with sequence similarities to the human Sebox and to the Mix-like and Mixer homeoproteins. The mouse Sebox gene was characterised recently as a *paired-like* homeobox gene not closely related to any other homeobox gene. In mouse, Sebox is expressed in maturing oocytes as well as in epidermis, brain and liver in newborn and adult mice. By contrast, we found that zebrafish *mezzo* is expressed early and transiently in mesoderm and endoderm precursors. The expression pattern of *mezzo* is much more similar to the expression pattern of the early zygotic Bon/Mixer and Mix/Bix genes, which have been shown to participate in the processes of germ layer specification in zebrafish and *Xenopus*. The significance of the sequence similarities between Sebox and *mezzo* genes is unclear.

By injecting mRNA encoding TARAM-A* and Antivin we showed that *mezzo*, like *mixer* and the *mix-like* genes, is regulated by TGFβ signals. Furthermore, we showed that *mezzo* expression is reduced in *squint* and *oep* mutants, and completely lost in *cyc;sqt* double mutants. Finally, we found that the activity of the maternal transcription factor Schmalspur/FoxH1 is required for the normal expression of *mezzo*, although we do not know whether this factor is required for initiation or maintenance of *mezzo* expression.

An important finding was obtained by analysing the effects of translational inhibitors on the expression of *mezzo* and of *bon/mixer*, *casanova* and *sox17* after overexpression of TARAM-A*. We were able to show that the transcriptional activation of *mezzo*, *bon/mixer* and *casanova* in response to activation of the Nodal pathway occurs in absence of post-MBT protein synthesis. By contrast, activation of *sox17* was found to require zygotic factors, consistent with the previous finding that *sox17* acts downstream of *casanova* in the process of mesendoderm specification in zebrafish. These results suggest that *mezzo*, *bon/mixer* and *casanova* are, like *ntl*, immediate downstream targets of maternal factors activated by Nodal signalling.

By analysing *mezzo* expression in *bon;faust* double mutants, we also uncovered a requirement for zygotic transcription factors for the maintenance of *mezzo* expression. The picture emerging from these experiments is that *mezzo* expression is regulated by maternal factors activated by Nodal signals but that accumulation of *mezzo* transcripts at the beginning of gastrulation also requires an interplay between zygotic Bon/Mixer and Faust/Gata5 transcription factors.

Role of *mezzo* in endoderm determination

Recently, Alexander and Stainier (Alexander and Stainier, 1999) proposed a model of endoderm formation based on overexpression experiments and mutant analysis. In this model, they postulated the existence of a putative zygotic gene they named X which would be expressed at the margin under the

control of Nodal signals and would be required in parallel with *mixer* and *gata5* for endoderm formation. The arguments for postulating the existence of additional factor are the following: first, although in reduced number compared with controls, *sox17*-expressing cells are still present in *bon* or *faust* mutants, as well as in *bon;faust* double mutants. Second, co-injection of *bon/mixer* and *gata5* RNAs in *MZoep* mutant embryos does not restore the normal number of cells expressing *sox17*, indicating that additional factors induced by Nodal signalling are required for this rescue to occur. Third, overexpression of *mixer* and/or *gata5* in normal embryos does not activate *sox17* expression outside the territory of the margin, suggesting that a co-factor present only in cells of the margin restricts spatially the activity of *bon* and/or *gata5*. Aoki and colleagues reached the same conclusion by testing the ability of *bon/mixer* to induce *casanova* in Nodal-deficient embryos (Aoki et al., 2002a). Mezzo is a good candidate for such a factor: it is expressed early and transiently in the same cells as *mixer* and *gata5* and its expression is directly controlled by Nodal signalling. Most importantly, ectopic expression of *mezzo* induces ectopic expression of the endodermal markers *sox17* and *cas* even near the animal pole. The ability of Mezzo to induce expression of endodermal markers in presumptive ectodermal cells is remarkable compared with the inductive ability of Mixer, which is restricted to the marginal zone (Alexander and Stainier, 1999; Kikuchi et al., 2000).

Other early zygotic factors, in addition to Mezzo, might participate with Mixer and Gata5 in endoderm specification. One candidate is the homeobox-containing gene *pitx2*. Like *gata5*, *pitx2* is expressed transiently in the four vegetal most rows of cells of the margin at the late blastula stage (Faucourt et al., 2001). Moreover, the early expression of *pitx2* is strictly dependent on a functional Nodal signalling pathway as is the late expression in the left lateral plate mesoderm during determination of left/right asymmetries (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998; Campione et al., 1999). In *Xenopus*, inhibition of *pitx2* function using a chimeric Engrailed-Pitx2 fusion suppresses *sox17* expression in presumptive endoderm and blocks the response of cells to Nodal signalling. However, overexpression of *pitx2* in zebrafish does not promote expression of endodermal marker genes but instead promotes prechordal plate markers genes (Essner et al., 2000; Faucourt et al., 2001) (M. P. and T. L., unpublished). Other factors that might participate in the regulation of *casanova* might be orthologues of the Bix homeobox genes which in *Xenopus* are transcribed in an immediate-early response to Nodal signalling (Tada et al., 1998; Ecochard et al., 1998).

Role of *mezzo* in mesoderm specification

The processes of endoderm and mesoderm specification are intimately linked in vertebrates and both germ layers require a functional Nodal signalling pathway (Dale, 1999; Kimelman and Griffin, 2000; Whitman, 2001). The *bon/mixer* and *milk/Bix* genes have been mainly studied in the context of endoderm specification, although they may also participate in mesoderm formation as the *Bix* genes are capable of inducing ventral mesoderm at low doses and *goosecoid* at high doses (Tada et al., 1998). Our study suggests a requirement for Mezzo and perhaps *bon/mixer* in both development of endoderm and mesoderm. The main evidence supporting this conclusion is that *mezzo*, in addition to its ability to promote endodermal gene expression, is also an inducer of the pan mesodermal gene marker *ntl*. These observations are consistent with the spatial and temporal expression pattern of *mezzo*. *mezzo* transcripts are present in the first six rows of cells at the margin of the blastoderm, a region that has been shown to contain progenitors of both mesodermal and endodermal lineages. Interestingly, the Bix genes in *Xenopus* are first expressed in both mesoderm and endoderm before becoming restricted to endoderm during gastrulation (Ecochard et al., 1998). An additional indication of roles for *mezzo* and *bon/mixer* in mesoderm formation is that inhibition of *mezzo* function in *bon* mutants interferes with *goosecoid* expression and causes a reduction of prechordal plate mesoderm.

In contrast with the inducing activity of *mezzo* outside the margin of the blastoderm, we have shown that this homeobox gene can repress *ntl* expression when overexpressed in marginal cells and that this repression is associated with upregulation of *casanova*. Many studies in zebrafish and in *Xenopus* have documented mutual repressive effects of mesoderm and endoderm determination factors, including Mix1 (Lemaire et al., 1998; Latinkic and Smith, 1999), Milk/Bix (Ecochard et al., 1998; Tada et al., 1998), Pitx2 (Faucourt et al., 2001), Sox-17 (Engleka et al., 2001) and Casanova (Aoki et al., 2002a). As we have shown that *mezzo* is an inducer of endoderm determination genes such as *casanova* and *sox17*, it is likely that high doses of *mezzo* lead to repression of *ntl* in part by inducing *casanova*.

Functional redundancy between *mezzo* and *bon/mixer* and relation between *mezzo*, *mixer* and *cas*

Several lines of evidence support the idea that *mezzo* and *bon/mixer* are functionally redundant factors. First, *mezzo* and *bon/mixer* are expressed at the same time in the same cells. Second, the homeodomains of both genes are structurally related. Third, while *bon* mutants still express *sox17* in a few

Fig. 10. Model of endoderm formation in zebrafish incorporating the results of our study [modified from Alexander et al. (Alexander et al, 1999), Reiter et al. (Reiter et al., 2001) and Aoki et al. (Aoki et al., 2002)]. Nodal ligands, together with TARAM-A and Oep, induce the expression of *mezzo*, *bon/mixer*, *faust/gata5* and *casanova* in a direct manner. Maintenance of *mezzo* expression requires the activity of *bon* and *faust* (broken lines) but not *cas*. Other cross-regulatory interactions are not known. *mezzo*, *bon/mixer* and *faust/gata5* cooperate to induce *sox17* by establishing a stable expression of *casanova*, which is the main determinant of *sox17* expression.

cells, injection of *mezzo*-MO into *bon* mutants abolishes this residual *sox17* expression and results in a phenocopy of the *cyclops* mutant phenotype. By contrast, injection of *mezzo*-MO into *cyclops*, *cas* or *faust* mutant backgrounds does not cause such a phenotype. Most importantly, injection of *mezzo* RNA into *bon* mutants partially restores expression of endodermal gene markers and rescues heart morphogenesis, which is disrupted in the absence of functional *bon/mixer* gene product.

Based on our various observations, we would like to integrate *mezzo* into a model of gene regulation involved in endoderm specification (Fig. 10) inspired by those of Stainier et al. (Stainier et al., 2002) and Aoki et al. (Aoki et al., 2002a). In this model, *mezzo* stands as an immediate-early target of Nodal/TARAM-A/Oep signalling, together with *bon/mixer* and *faust/gata5*. *casanova*, which we have also shown to be an immediate-early target of Nodal, occupies the same position but requires imputs from Mezzo, Bon/Mixer and Faust to assure continual expression. Finally, in agreement with previous proposals, this model shows that *casanova* is a crucial node of this network and the most important transcriptional regulator of *sox17*.

In conclusion, we have identified a novel transcription factor acting in the gene network that regulates specification of the mesendoderm in zebrafish. The function of *mezzo* is partially redundant with the function of *bon/mixer* but differs in that *mezzo* may also participate in mesoderm formation. Future studies should aim at understanding how these different transcription factors interact and what is the molecular basis for the segregation of the endodermal and mesodermal germ layers from common precursors.

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