Development 133, 2189-2200 (2006) doi:10.1242/dev.02387

Zebrafish endoderm formation is regulated by combinatorial Nodal, FGF and BMP signalling

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In the zebrafish embryo, the mesoderm and endoderm originate from common precursors and segregate during gastrulation by mechanisms that are largely unknown. Understanding how the signalling pathways that regulate endoderm and mesoderm formation interact is crucial to understanding how the germ layers are established. Here, we have analysed how the FGF and BMP pathways interact with Nodal signalling during the process of endoderm formation. We found that activation of the FGF/ERK pathway disrupts endoderm formation in the embryo and antagonizes the ability of an activated form of Tar/Acvr1b to induce endoderm at the animal pole. By contrast, inhibition of FGF signalling increases the number of endodermal precursors and potentiates the ability of Tar*/Acvr1b to induce endoderm at the animal pole. Using a pharmacological inhibitor of the FGF receptor, we show that reducing FGF signalling partially rescues the deficit of endoderm precursors in *bon* mutant embryos. Furthermore, we found that overexpression of BMPs compromises endoderm formation, suggesting that formation of endoderm precursors is negatively regulated by BMPs on the ventral side. We show that simultaneous inhibition of the FGF/Ras and BMP pathways results in a dramatic increase in the number of endoderm precursors. Taken together, these data strongly suggest that BMP and FGF-ERK pathways cooperate to restrict the number of endodermal progenitors induced in response to Nodal signalling. Finally, we investigated the molecular basis for the FGF-MAPK-dependent repression of endoderm formation. We found that FGF/ERK signalling causes phosphorylation of Casanova/Sox32, an important regulator of endoderm determination, and provide evidence that this phosphorylation attenuates its ability to induce *sox17*. These results identify a molecular mechanism whereby FGF attenuates Nodal-induced endodermal transcription factors and highlight a potential mechanism whereby mesoderm and endoderm fates could segregate from each other.

KEY WORDS: Endoderm, Zebrafish, FGF, BMP, MAP kinase, Mesoderm, Casanova, Bon, ERK

INTRODUCTION

In the zebrafish embryo, precursors of the ectoderm derive from the animal pole region, while precursors of the endoderm and mesoderm originate from partially overlapping territories near the equatorial region, or margin, of the embryo (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999). The endodermal progenitors arise from the first four rows of marginal cells, while mesodermal precursors arise from the entire marginal region (Kikuchi et al., 2004). Fate-mapping experiments have shown that when single cells located near the margin are labelled at the late blastula stage, their progeny frequently populate both germ layers (Warga and Nusslein-Volhard, 1999). Therefore, in the most vegetal rows of cells, mesodermal and endodermal fates are intermingled and both germ layers share common mesendodermal precursors. The molecular mechanisms that allow segregation of these two germ layers are poorly understood.

In zebrafish, as in other vertebrates, signalling by secreted TGFβ factors of the Nodal family is crucial for the formation of the mesoderm and endoderm (Schier, 2003). The *nodal*-related genes *cyclops* and *squint* are expressed in the first two rows of cells and are potent inducers of endoderm and mesoderm when overexpressed (Erter et al., 1998; Feldman et al., 1998; Gritsman et al., 2000; Peyrieras et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998). *cyclop;squint* double mutants or mutants lacking the maternal and zygotic contribution of *oep* (*MZoep*), which encodes a Nodal co-receptor, lack all endoderm and have little or no mesoderm (Agathon et al., 2003; Gritsman et al., 1999; Zhang et al., 1998).

Several lines of evidence suggest that in addition to being required for endoderm and mesoderm formation, differential Nodal signalling may also be involved in the separation of these two germ layers. First, many studies have documented that high levels of Nodal signalling promote endoderm formation and expression of endodermal determination genes, while lower levels of Nodal signalling promote mesoderm formation and expression of mesodermal genes such as *brachyury* (Alexander et al., 1999; Alexander and Stainier, 1999; Clements et al., 1999; Erter et al., 1998; Faucourt et al., 2001; Gritsman et al., 2000; Henry et al., 1996; Jones et al., 1995; Piccolo et al., 1999; Rodaway et al., 1999; Sun et al., 1999; Yasuo and Lemaire, 1999). Second, overexpression of low doses of *lefty1*, which encodes a potent endogenous antagonist of Nodal, suppresses endoderm formation while higher doses also affect the mesoderm (Thisse et al., 2000; Thisse and Thisse, 1999). Similarly, in zygotic *oep* mutants that have reduced Nodal signalling, the endoderm is absent while the mesoderm is modestly affected (Schier et al., 1997; Strahle et al., 1997). By contrast, *MZoep* embryos resemble *cyclops;squint* double mutants and lack all endoderm and most mesoderm (Agathon et al., 2003; Gritsman et al., 1999; Zhang et al., 1998). Finally, it has been demonstrated that Squint acts as a morphogen capable of inducing different cell fates and that it does not require a relay mechanism (Chen and Schier, 2001; Le Good et al., 2005).

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Accepted 31 March 2006

The endodermal determination program initiated by Nodal signalling requires the maternally expressed Smad2 factor (Dick et al., 2000; Gaio et al., 1999) and eomesodermin (Bjornson et al., 2005) as well as several zygotic transcription factors such as the Mix-like homeobox proteins Bon; the product of the *bonnie and clyde* gene (*bon)* (Alexander et al., 1999; Trinh et al., 2003) and Mezzo (Poulain and Lepage, 2002). Mix-like proteins act in parallel with the zinc-finger-containing factor Gata5, which is encoded by the *faust* gene to induce the *sox*-related gene *casanova/sox32* (Kikuchi et al., 2004; Reiter et al., 1999). *casanova/sox32* mutants do not express the endodermal marker *sox17*, and lack endodermal precursors and organs derived from the gut tube (Alexander et al., 1999; Alexander and Stainier, 1999; Aoki et al., 2002a; Dickmeis et al., 2001; Kikuchi et al., 2001; Kikuchi et al., 2004; Reiter et al., 1999; Reiter et al., 2001; Sakaguchi et al., 2001). In the absence of Casanova activity, cells that normally contribute to the endoderm change their fate and differentiate into mesodermal cells. Therefore, Casanova is a key transcription factor in the endoderm determination network and is required for the expression of *sox17*.

In addition to Nodal, the FGF and BMP signalling pathways have been shown to play crucial roles in formation and patterning of mesoderm and endoderm in vertebrates. Basic FGF was first identified as a mesoderm-inducing factor that promotes ventral mesoderm formation (Kimelman, 1991). Then, studies in *Xenopus* and zebrafish demonstrated that FGF is required for posterior mesoderm formation through the maintenance of the expression of Tbox transcription factors such as No Tail and Tbx-16 (Bottcher and Niehrs, 2005; Griffin et al., 1995a; Griffin and Kimelman, 2003; Schulte-Merker and Smith, 1995). Because overexpression of a dominant-negative FGF receptor blocks the dorsal mesoderm inducing activity of Activin, FGF ligands have been proposed to act as competence factors needed by presumptive mesodermal cells to respond to TGF-β signals (Cornell et al., 1995; Zhao et al., 2003). In zebrafish, FGF signals may relay the action of TGFβ ligands over long distances, allowing activation of the pan-mesodermal marker *brachyury* in cells distant from the Nodal source (Reiter et al., 1999). Consistent with these observations, FGF is required downstream of Nodal signalling to induce the co-receptor Oep in cells distant from the source of Nodal, a mechanism that contributes to the amplification and propagation of Nodal signals (Mathieu et al., 2004). Thus, the FGF pathway might be involved in the initiation of, as well as in the maintenance of, mesodermal populations. Finally, studies in zebrafish have shown in addition to its effects on anteroposterior patterning of the mesoderm, FGF signalling controls patterning along the DV axis by repressing the expression of BMPs (Fürthauer et al., 2004). Although a large body of evidence supports the role of FGF in mesoderm formation and patterning, the implication of FGF signalling in formation of the endoderm has received considerably less attention and the results obtained are unclear. In *Xenopus*, studies with vegetal pole explants by Henry et al. showed that FGF is required for expression of the pancreatic marker *pdx1* but not for expression of the intestinal marker intestinal fatty acid binding protein (IFABP) (Henry et al., 1996). By contrast, Gamer and Wright (Gamer and Wright, 1995) found that bFGF is a potent inhibitor of *pdx1* expression in vegetal pole explants. A third study concluded that overexpression of eFGF inhibits expression of *mixer*, while inhibition of FGF signalling in animal caps induces the ectopic expression of the key endodermal regulator *mixer* and of the gene marker *endodermin* (Cha et al., 2004). These observations suggest that in *Xenopus*, FGF signalling may antagonize endoderm specification. However, nothing is known about the role of FGF signalling in endoderm specification in other vertebrates.

Similarly, the roles of BMPs in patterning of the mesoderm and ectoderm are well documented but only a few studies focused on the role of BMPs on endoderm formation. Sasai et al. (Sasai et al., 1996) reported that, in *Xenopus*, overexpression of *noggin* or *chordin* induces endoderm in animal caps. Furthermore, they showed that endoderm induction by *chordin* is strongly potentiated by inhibition of FGF signalling and counteracted by activation of FGF signalling. Taken together these observations suggest that both BMP and FGF signalling antagonize endoderm formation in *Xenopus* but the molecular mechanism responsible for this antagonism is not known and these observations have not been extended to other vertebrates.

Here, we have attempted to unravel the interactions between the Nodal, FGF and BMP pathways during formation of the endoderm in zebrafish. We show that both the FGF/MAPK and the BMP pathways antagonize endoderm formation in response to Nodal signals. We have found that activation of the FGF/MAPK pathway by overexpression of FGF ligands or constitutively active versions of Ras or ERK caused a severe reduction in the number of endodermal precursors in the whole embryo and antagonized the ability of Tar/Acvr1b to induce endoderm at the animal pole. By contrast, the triple inhibition of Fgf8, Fgf17b and Fgf24 caused a strong increase in the number of endodermal precursors while inhibition of FGF/ERK signalling potentiated the ability of Tar*/Acvr1b to induce endoderm at the animal pole. Furthermore, we found that overexpression of BMPs also inhibits endoderm formation and that simultaneous inhibition of the FGF/Ras and BMP pathways causes formation of an excess of endoderm in the embryo. Furthermore, we provide evidence that FGF/ERK signalling results in phosphorylation of Casanova and that this phosphorylation attenuates its activity. These results suggest that the FGF and BMP pathways counteract the Nodal signalling pathway and limit formation of endoderm. Importantly, they identify Casanova as a key factor at the crossroads between signalling pathways and highlight a potential molecular mechanism that may help explain the separation of the mesoderm and endoderm.

MATERIALS AND METHODS

Zebrafish strains, embryo manipulation, inhibitor 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 inter-crossing heterozygous carrier fish identified by random crossing. We used the *bonnie and clyde*^{m425} mutant allele (Kikuchi et al., 2000). Embryos were genotyped following the procedure published by Kikuchi et al. (Kikuchi et al., 2000). To inhibit FGFR activity, embryos were treated with SU5402 (Mohammadi et al., 1997), at 15 μM after 1000 cell stage at 28.5°C in the dark. This concentration gave the most consistent results but some variability was observed in the effects of the drug, depending of the batch of inhibitor, the cell line and the time of addition of the drug. SU5402 activity was monitored by its ability to inhibit MAPK activity and *sprouty4* expression (see Fig. S1 in the supplementary material).

RNA and oligonucleotides microinjection

Constructs for microinjection of Fgf8 (Fürthauer et al., 1997), DN-Fgfr1 and *noggin1* (Furthauer et al., 1999), DN-Ras and CA-Ras (Whitman and Melton, 1992), Erk2* (Emrick et al., 2001), *tar*/acvr1b* (Peyrieras et al., 1998), and BMP2b, BMP4 and BMP7 have been previously described (Schmid et al., 2000). All capped mRNA were synthesised from templates linearised with Asp718 using the SP6 mMessage mMachine kit (Ambion). After synthesis, capped RNAs were purified on Sephadex G50 columns and quantitated by spectrophotometry. Injections were performed either in the yolk at the one-cell stage or in the animal blastomere at the 16- to 128-cell stages. The Fgf8, Fgf17b and Fgf24 morpholino antisense oligonucleotides were injected at respectively 0.2, 0.125 and 0.5 μM. At these doses, the Fgf24 morpholino phenocopied the *ikarus* mutation that disrupts the *fgf24*

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gene (Fischer et al., 2003), while the Fgf8 morpholino phenocopied the *acerebellar* mutation, which disrupts the *fgf8* gene. When co-injected, these oligos caused severe defects in the posterior region of the embryo, which is known to require FGF signalling. The sequence of the morpholinos used are: MO Fgf8, 5′-GAGTCTCATGTTTATAGCCTCAGTA-3′; MO Fgf24: 5′-GACGGCAGAACAGACATCTTGGTCA-3′; MO Fgf17b, 5′-AGTGTTCAATATCCAGGGCTCTCCT-3′. *noggin1* mRNA was injected at 25 μg/ml.

Whole-mount in situ hybridisation

In situ hybridisation was performed following a protocol adapted from Thisse et al. (Thisse et al., 2004) with antisense RNA probes and staged embryos. In some cases, the lineage tracer (FLDX) was detected after in situ hybridisation using an anti-fluorescein antibody coupled to alkaline phosphatase and Fast Red as substrate. The probes used in this publication have been described previously: *sox17* (Alexander and Stainier, 1999), *sprouty4* (Fürthauer et al., 2001), *nkx2.5* (Chen and Fishman, 1996) and *foxi1* (Fürthauer et al., 2004). All probes were synthesized with the T7 RNA polymerase after linearization by *Not*I.

To count the number of cells expressing *sox17*, the embryos were photographed under different angles so that the whole surface was covered. Four to six different pictures were taken by progressively rotating the embryo and landmarks were used to delimit nonoverlapping areas. Alternatively, flat preparations of the blastoderm were made after removing the yolk platelets (see Fig. S2 in the supplementary material).

Immunochemistry and western blot

Capped mRNA of FLAG-tagged *casanova/sox32* (100 ng/μl) were injected into embryos at the two-cell stage. When the embryos reached 30% epiboly (5 hpf), the chorion was removed with pronase and the yolk was removed using an ‘eye hair knife’ in an agarose chamber in MBS 1× (Modified Barth Saline Buffer) supplemented with gentamycin (Peng, 1991; Sagerstrom et al., 1996). Fifteen blastoderms were pooled, allowed to recover for 20 minutes after dissection in MBS 1× and then lysed in SDS sample buffer. Proteins were fractioned by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to PVDF membranes. The replicate was blocked for 1 hour in 1% BSA, BRBT (Tris-HCl 10 mM, NaCl 150 mM, EDTA 1 mM, pH 7.5, Tween 0.01%) and incubated overnight at 4°C with a monoclonal antibody [α-Casanova-Phospho at 1/100 or α-Flag M2 (Kodak) at 1/1000]. After washing in BRBT, the membrane was incubated with the secondary anti body at 1/10000 (anti-Rabbit HRP or anti-mouse HRP conjugated antibodies, Amersham). Bound antibodies were revealed by ECL western blotting detection reagent (Pierce). The α-Casanova-Phospho antibody was made in rabbit by Eurogentec and was purified by affinity chromatography using HPLC (BioRad system). Immunostaining was performed essentially as described (Shinya et al., 2001).

RNA extraction and reverse transcription-polymerase chain reaction

Total RNA from staged embryos was extracted by the method of Chomczynski (Chomczynski, 1987). For RT-PCR, cDNA synthesis and PCR were performed as described by Sagerström (Sagerström, 1996). Primers pairs for histone H4 were synthesized according to previous published reports. Other primer pairs used were derived from the ORF of each protein: Zsox-17 forward, 5′-ACGAGGTGGAGTTTGAGCAC; Zsox-17 reverse, 5′-GGCTGCTCTAAAAGCTGCTG (amplified fragment 465 bp); Casanova forward, 5′-CAGCATTCTGTCCAGCAGAG; Casanova reverse, 5′-CAAAATCAGCAGCAATCTGG (amplified fragment 480 bp). Cycling parameters were as follows: initial denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute 30 seconds. Thirty cycles were used for ease of comparison. Each experiment was repeated three times using one or three whole embryos.

Site-directed mutagenesis and construction of expression plasmids

To make *pCS2 cas-S47A* construct, the AGC codon encoding serine in position 47 of *pCS2 cas* was mutated GCC (alanine) by splicing PCR using the following oligonucleotides: Casanova-S47A Fw, 5′-TCGGGCCCATTAGCCCCGGTGTCTGTC-3′; Casanova-S47A Rev; 5′-GACAGACACCGGGGCTAATGGGCCCGA-3′. The exchange was verified by sequencing. *pCS2 cas-WT-Flag* and *pCS2 cas-S47A-Flag* were constructed as follows. A *Cla*I-*Eco*RI fragment containing Casanova was PCR amplified with the following oligonucleotides (restriction sites sequences underlined and ATG in bold): Fw-Casanova-ClaI-ATG, 5′-CCCATCGAT**ATG**TATCTCGACCGGATG-3′; Casanova-*Eco*RI-Rev, 5′-CTTGAATTCCCTTTTTGCTGTGGTCCAA-3′ and cloned into the pCS2-Flag Vector digested with *Eco*RI and *Cla*I.

RESULTS

FGF/ERK signalling represses endoderm formation

To test the role of FGF signalling on endoderm formation, embryos were injected at the one- or two-cell stage with synthetic Fgf8 mRNA and analysed by in situ hybridization for the expression of *sox17*, as a read out of endoderm specification. Injection of *fgf8* RNA drastically reduced the number of *sox17*-expressing cells. In the most severe cases, only a few residual *sox17* progenitors were present in the injected embryos (Fig. 1C). To test if Fgf8 overexpression interfered with Nodal expression, we examined the expression of *lefty1*, which is a target of Nodal signalling (Thisse and Thisse, 1999). Expression of *lefty1* was not affected in the embryos overexpressing Fgf8 (data not shown), indicating that the loss of endodermal precursors caused by Fgf8 was not a consequence of interfering with Nodal expression. Similarly, overexpression of an activated form of Ras (CA-RAS) (Whitman and Melton, 1992) or of an activated form of human ERK2* (Emrick et al., 2001) strongly reduced the number of *sox17*-expressing cells (Fig. 1B,D). As FGFs are expressed (Draper et al., 2003; Fürthauer et al., 2004) and MAPK activity is detected in the endomesodermal

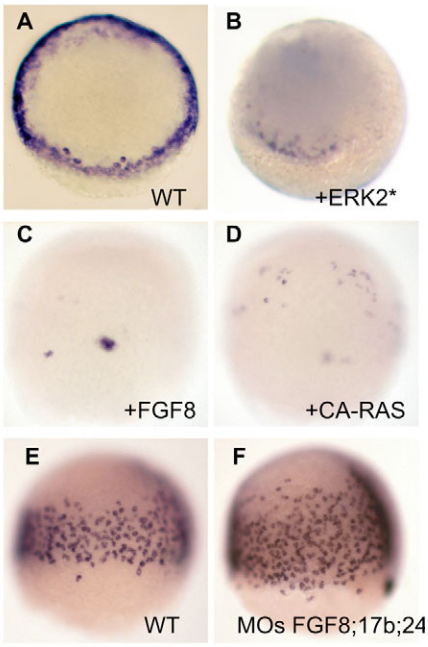

Fig. 1. FGF/ERK signalling antagonises endoderm formation. (**A-F**) Expression of *sox17* at 50% epiboly (A,B) or 80% epiboly (C-F). Injection of ERK2* RNA at 50 ng/μl (B), Fgf8 ligands RNA at 10 ng/μl (C) or RNA encoding constitutively activated form of Ras at 5 ng/μl (D) at the one-cell stage inhibits endoderm specification. (E,F) The triple morpholino knockdown of Fgf8, Fgf17b and Fgf24 causes overproduction of endoderm precursors. (A,B) Animal pole views; (C,D) dorsal views; (E,F) lateral views.

territory during gastrulation (Fig. S1 in the supplementary material), FGFs are good candidates for endogenous factors that negatively regulate formation of the endodermal precursors. To test this hypothesis we attempted to block FGF signalling using morpholinos. Injection of morpholinos directed against either Fgf8 or Fgf17b or Fgf24 mRNA alone did not affect significantly the number of endoderm precursors. By contrast, the triple knockdown of Fgf8, Fgf17b and Fgf24 resulted in a large increase in the number of endodermal precursors that formed (Fig. 1E,F; see Table 3). Taken together, these results show that endogenous FGF signals restrict endoderm formation during gastrulation.

FGF/ERK signalling antagonizes the ability of Tar*/Acvr1b to induce endoderm at the animal pole

To document the repressive effect of FGF/MAPK signalling on endoderm formation and circumvent cell-migration dependent effects, we used the ability of Nodal signals to induce endoderm at the animal pole as a model to examine the consequences of activating or inhibiting the FGF pathway. We used a constitutively activated form of Tar*/Acvr1b, a type I receptor for the Nodal factors, to activate Nodal signalling (Aoki et al., 2002b; Peyrieras et al., 1998; Renucci et al., 1996). To block FGF signalling, we used a dominant-negative form of FGFR1 (DN-FGFR1) (Fürthauer et al., 2004). In agreement with the known role of FGF in mesoderm formation, injection of the dominant-negative form of FGFR1 potently inhibited endogenous mesodermal expression (data not shown). To block FGF signalling, we also used the pharmacological agent SU5402, a specific inhibitor of FGFR function (Mohammadi et al., 1997).

Injection of RNA encoding Tar*/Acvr1b at the animal pole induced ectopic expression of the endodermal marker *sox17* (55%, *n*=40, Fig. 2E) and of the mesodermal marker ZFIN *CB187* (50%, *n*=14, Fig. 2B) (see Thisse et al., 2001 at http://zfin.org). In agreement with previous studies (Mathieu et al., 2004), we found that inhibition of FGF signalling reduced the ability of Tar*/Acvr1b to induce expression of the mesodermal marker *CB187* (6.9%, *n*=28, Fig. 2C). By contrast, the frequency of *sox17* induction by *tar*/acvr1b* was moderately enhanced (75.5%, *n*=49, Fig. 2F). Similarly, whereas injection of very low doses 1-2 pg of *tar*/acvr1b* caused the ectopic expression of *sox17* in about 32% of the embryos, treatment with SU5402 following the injection increased this percentage to 54% (Table 1). RT-PCR assays confirmed that the level of *casanova/sox32* and *sox17* transcripts in response to *tar*/acvr1b* overexpression was increased following treatment with SU5402 (Fig. 2G). By contrast, when mRNA encoding an activated Erk2* was co-injected with *tar*/acvr1b*, the number of embryos expressing *sox17* ectopically was decreased to 23% (Table 1) and the size of the clones showing ectopic expression of the marker was strongly reduced (Fig. 2H,I). Taken together, these results show that the FGF/ERK pathway negatively regulates the ability of ectodermal cells to be converted into endoderm in response to *tar*/acvr1b* overexpression.

Inhibition of FGF/MAPK signalling partially rescues the phenotype of *bonnie and clyde* mutants

The gain-of-function experiments and the experiments based on treatments with inhibitors strongly suggest that FGF-ERK signals antagonize endoderm formation during normal development. This raised the possibility that inhibiting FGF-ERK signals could rescue mutants displaying a reduced number of endoderm precursors.

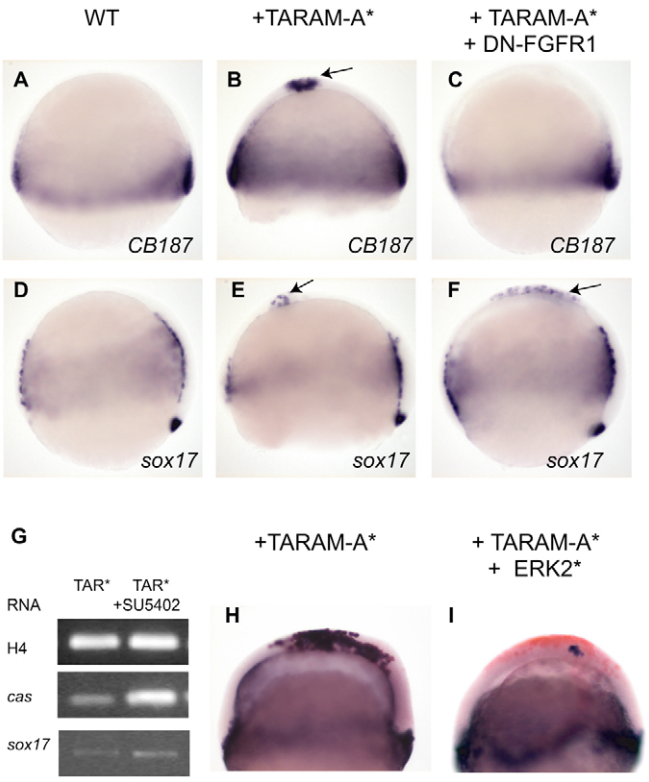

Fig. 2. FGF/ERK activity antagonizes the ability of *tar*/acvr1b* to induce endoderm. Expression of *CB187* (**A-C**) and *sox17* (**D-F**) in embryos at 80% epiboly. (A,D) Wild type. (B,C,E,F) *tar*/acvr1b* RNA at 200 ng/μl was injected alone or in combination with DN-FGFR1 RNA at 500 ng/μl in one animal pole blastomere at the 64-cell stage embryo. Injection of *tar*/acvr1b* RNA alone is able to induce ectopic *CB187* (B) and *sox17* (E) expression at the animal pole (arrows). Co-injection with DN-FGFR1 abolishes the ability of *tar*/acvr1b* to induce *CB187* (C), whereas DN-FGFR1 slightly increases the ability of *tar*/acvr1b* to induce *sox17* (F). (**G**) RT-PCR analysis of three whole embryos injected with *tar*/acvr1b* RNA at 25 ng/μl. FGF signal inhibition by treatment with 15 μM of SU5402 after the 1000-cell stage enhances *cas* and *sox17* responses. (**H**,**I**) *tar*/acvr1b* RNA at 1 ng/μl was injected alone or in combination with ERK2* RNA at 50 ng/μl into one blastomere of a 16-cell stage embryo. In both cases a lineage tracer (FLDX) was co-injected and later detected by immunostaining and *sox17* expression was detected. (H) The clone of cells ectopically expressing *sox17* in H is congruent with the territory containing the lineage tracer. (I) The size of the clone of cells ectopically expressing *sox17* is greatly decreased in the presence of ERK*. (A-F,H,I) Lateral views.

Indeed, we found that inhibiting FGF signalling partially rescues the deficit of endodermal precursors of *bon* mutants (Kikuchi et al., 2000), which have a reduced expression of *casanova/sox32* (Aoki et al., 2002a). Although the average number of *sox17*-expressing cells in homozygous *bon* mutants at 75% epiboly is around 11, this number increased more than threefold following application of SU5402 at 15 μM at the blastula stage (Fig. 3A-C; Table 2) and endodermal precursors could be detected even on the ventral side of the embryos. In addition, treatment with SU5402 partially rescued the cardia bifida phenotype caused by the defect in endoderm formation in this mutant (Fig. 3D-F, Table 2). However, in most cases, the rescued hearts beat at a very low frequency and did not differentiate properly into an atrium and a ventricle but remain as an elongated tube. We found that a combination of morpholinos against

Table 1. FGF/ERK activity antagonizes the ability of Tar*Acvr1b to induce endoderm at the animal pole

RNA injected at the 16-cell stage	TAR* (1 ng/μl)	TAR* (1 ng/μl) + SU5402	TAR* (1 ng/μl) + Erk2*	TAR* (200 ng/μl)	TAR* (200 ng/μl) + DN-FGFR1
Embryos showing ectopic *sox17* expression (%)	32	54	23	55	75.5
Total number of embryos	152	85	83	40	49

Table 2. Inhibition of the FGF pathway increases the number of *sox17*-expressing cells and partially rescues heart morphogenesis in *bonnie and clyde* mutants

	bon$^{-/-}$	*bon*$^{-/-}$ + SU5402	bon$^{-/-}$ + Fgf8 and Fgf24 MOs
Average number of cells expressing *sox17* per embryo at about 80% epiboly	11 (*n*=12)	37 (*n*=13)	Not determined
Average number of *sox17*-expressing cells at about 90% epiboly	22 (*n*=38)	Not determined	28 (*n*=25)
Percentage of embryos with cardia bifida at 30 hpf	19 (*n*=62)	9 (*n*=403)	2 (*n*=86)

Fgf8 and Fgf24 also rescued the cardia bifida of *bon* mutants, while causing only a modest increase in the number of endodermal precursors present at 80% epiboly (Table 2). Thus, inhibition of FGF signalling is able to compensate for the reduced activity of the molecular cascade leading to endoderm formation. We then attempted to rescue the lack of endoderm of embryos injected with antisense morpholino oligonucleotides directed against *casanova/sox32* (Dickmeis et al., 2001). Treatment with SU5402, did not rescue endoderm formation in these embryos (not shown). These results suggest that blocking the FGF-MAPK pathway cannot compensate for the absence of Casanova but can attenuate the endodermal deficiency of mutants with a reduced expression of *casanova/sox32*.

BMP and FGF signalling cooperate to restrict endoderm formation

Taken together, our results so far indicate that FGF signalling is involved in regulating endoderm formation. However, in the course of experiments in which we tried to interfere with MAPK signalling at the level of Ras, we found that overexpression of RNA encoding a dominant-negative form of Ras (DN-Ras) (Whitman and Melton, 1992) did not, result in an overall excess of endoderm (354/477, *n*=88/93) (compare Fig. 5A,E with 5C,G). The number of endodermal cells was slightly increased dorsally (Fig. 5C), but in fact, fewer *sox17*-expressing cells were present in lateral and ventral regions (Fig. 5G). This observation suggests that, in addition to FGF signalling, the extent of endoderm formation is also regulated by signals emanating from the ventral side of the embryo. As BMPs are known to be essential for the specification of ventral cell fates (Kishimoto et al., 1997; Schmid et al., 2000), we investigated whether their activity modulates endoderm formation. The gene encoding the transcription factor Foxi1 (Nissen et al., 2003) displays a BMP-dependent expression in the prospective epidermis and can therefore be considered as a molecular readout of ongoing BMP signalling. In the ventral region of wild-type embryos, the expression domains of *foxi1* and *sox17* are strikingly complementary (Fig. 4A-C). Moreover, when FGF signalling is blocked using DN-Ras, DN-FGFR1 or SU5402, the expression of the *bmp* genes and their target *foxi1* expands dorsally (Fig. 4D-I) (Fürthauer et al., 2004). In these FGF-signalling inhibited embryos, *sox17* expression becomes restricted to the residual dorsal BMP-free zone that does not express *foxi1*, consistent with a potential role of BMPs in restricting endoderm formation. In support of this hypothesis, co-injection of the three BMP ligands at the ventral margin created a local gap in *sox17* expression (97.3%, *n*=38, Fig. 4K). Injection of the same molecules at the one-cell stage produced a range of effects going from a loss of endoderm on the ventral side (Fig. 4L, *n*=14/53) to an almost complete loss of endoderm precursors all around the embryo (not shown; *n*=39/53). We therefore investigated whether BMP signalling is responsible for the inhibition of endoderm formation in the ventrolateral region of FGF-depleted embryos. In agreement with a previous study, we found that the number of endodermal progenitors was not increased in zebrafish embryos mutant for *bmp2b* or *bmp7* (Tiso et al., 2002) (data not shown). Similarly, inhibition of BMP signalling with *noggin* caused only a slight increase in the number of endodermal precursors that formed (Fig. 5B,F, *n*=84/84 and Table 3). However, we found that simultaneously inhibiting MAPK signalling and BMP signalling caused a massive excess of endodermal precursors to form all around the embryo (Fig. 5D-H, *n*=70/88, Table 3). By counting individual *sox17*-positive cells in embryos at 75% epiboly co-injected with *noggin* and DN-Ras we found that the number of

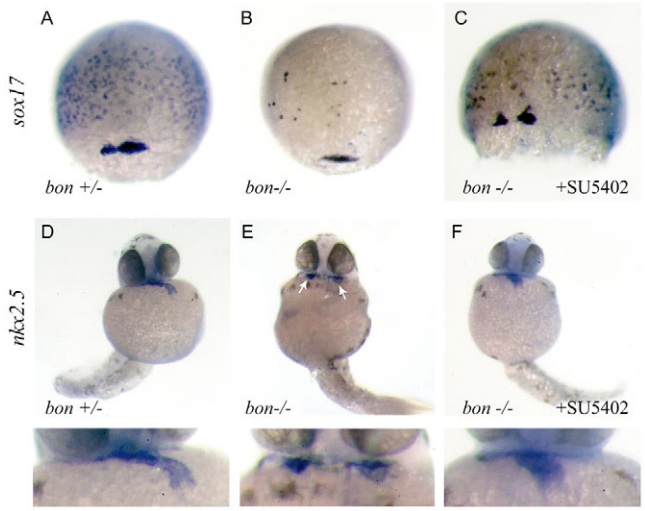

Fig. 3. Inhibition of the FGF/MAPK pathway rescues the phenotype of *bon* mutants. (**A-C**) Dorsal views of embryos at 80% epiboly showing expression of *sox17* in wild-type embryos (A), *bon* mutant embryos (B) and *bon* mutant embryos treated with SU5402 at 15 μM after 1000-cell stage (C). (**D-F**) Partial rescue of heart morphogenesis following SU5402 treatment 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 was partially restored by inhibition of FGF pathway (F). The genotype of the rescued embryos was verified by RFLP.

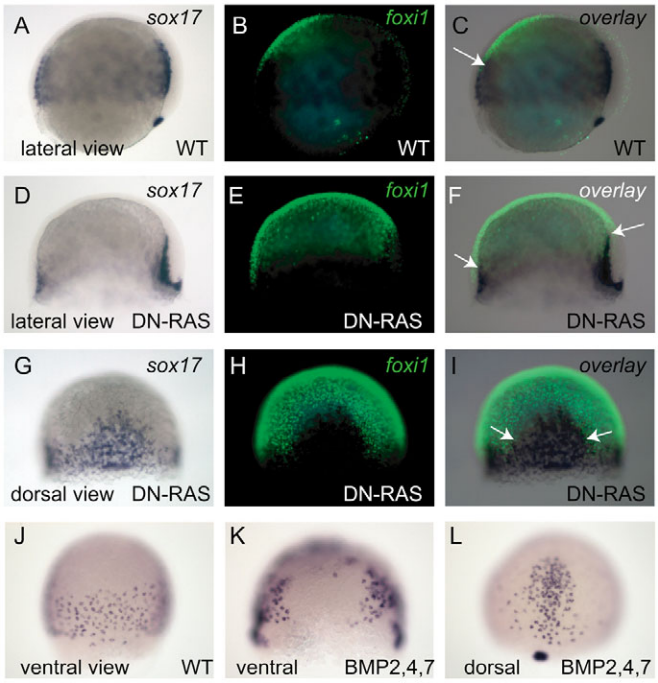

Fig. 4. BMP signalling restricts ventral endoderm formation. *sox17* (in blue) and *foxi1* (green) expression at 70% epiboly in wild type (**A-C**) and in embryos injected with *DN-Ras* RNA (300 ng/μl) at the one-cell stage (**D-I**). Arrows in C,F,I indicate the boundary between the epidermis and endoderm. (**J-L**) *sox17* expression at 75% epiboly in wild-type embryo (J); in an embryo injected into one ventral blastomere at the 16-cell stage with a mixture of RNAs encoding Bmp2b, Bmp4 and Bmp7 (5 ng/μl, 5 ng/μl and 40 ng/μl) (K); and in an embryo injected at the one- or two-cell stage with a mixture of Bmp2b, Bmp4 and Bmp7 RNAs (2.5 ng/μl, 2.5 ng/μl and 20 ng/μl) (L). (A-F) Lateral views; (G-I,L) dorsal views; (J,K) ventral views.

endodermal precursors was increased by 50% (652/437, n=15, Table 3). Furthermore, the quadruple inhibition of Fgf8, Fgf17b, Fgf24 using morpholinos and of BMP signalling with *noggin* mRNA resulted in an even higher number of endodermal precursors compared with wild type (1000/437, n=6). These data suggest that FGF signals acting all around the margin cooperate with BMP signals that act ventrally to limit the number of endoderm precursors during normal development.

Casanova protein is phosphorylated by MAPK in vivo

To better understand the molecular basis of the repressive effect of FGF/MAPK signalling on endoderm formation, we looked for putative consensus ERK phosphorylation sites in the transcription factors that act downstream of Nodal signals. Among the transcription factors regulated by Nodal signalling, the Smads have been shown to stand at the intersection of TGFβ and MAPK signalling pathways. All the Smads contain a cluster of ERK consensus sites in the linker domain and MAP kinase-dependent phosphorylation of these sites has been shown to downregulate their activity (Kretzschmar et al., 1997; Pera et al., 2003) (reviewed by Massagué, 2003). We therefore examined whether the repressive effect of FGF/ERK on specification of endoderm precursors was mediated by phosphorylation of Smad2 or Smad3. To test this idea, we overexpressed Smad2 and Smad3 constructs with the serines of the four putative phosphorylation sites in the Smad linker domain mutated to alanine (Kretzschmar et al., 1999). These mutant versions of Smads were not able to rescue the loss of endodermal precursors following overexpression of Fgf8 (not shown). Moreover, we found that the expression of *lefty1*, which is regulated by Nodal signalling, is unchanged in Fgf8-injected embryos, indicating that Fgf8 does not inhibit directly the Smads. Taken together, these observations suggested that the repressive effect of FGF/ERK on endoderm formation occurred downstream or in parallel of the Smads. Therefore, we focused on Casanova/Sox32, which is an important factor in the endoderm determination cascade acting downstream of Smads. We noticed that Casanova contains a single ERK consensus phosphorylation site PL**S**P (Gonzalez et al., 1991), which is conserved in zebrafish *sox17*, while the mouse and human Sox17 proteins contain a related sequence SL**S**P (Fig. 6A). In order to test whether this putative site is phosphorylated in vivo, we generated antibodies against a 15 amino acids peptide spanning the phosphorylated serine (Fig. 6A). Antibodies recognizing the phosphorylated peptide were purified by affinity chromatography. We were not able to detect the endogenous Casanova protein using these antibodies, probably because of the very low level of expression of this gene. However, these antibodies recognized a single protein of 37 kDa, the predicted molecular weight of Casanova, in protein extracts from embryos overexpressing *casanova/sox32* (Fig. 6B). These antibodies were specific for the phosphorylated form of Casanova as they did not recognize a protein band following injection of *cas-S47A* mRNA, which contains a mutated phosphorylation site in which serine 47 is replaced with alanine (Fig. 6B). As expected,

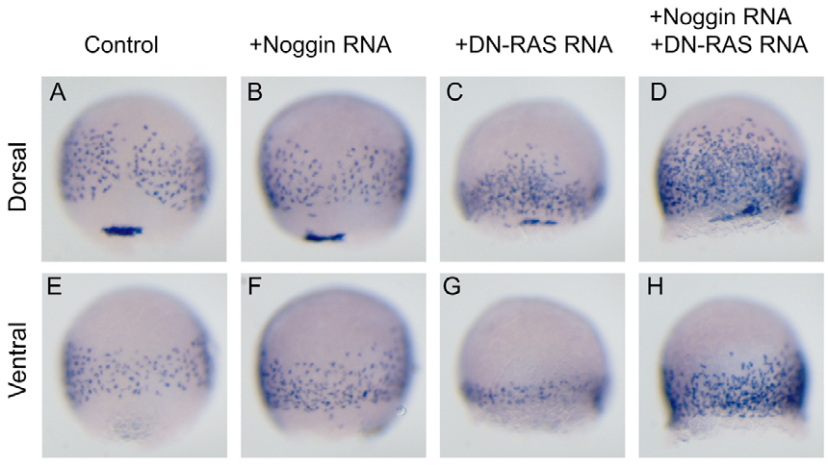

Fig. 5. Double inhibition of the FGF and BMP pathways causes formation of an excess of endoderm precursors all around the embryo. (**A-H**) *sox17* expression at 80% epiboly in wild-type embryos (A,E) or embryos injected at the one-cell stage with *noggin* RNA at 25 ng/μl (B,F), *DN-Ras* RNA at 300 ng/μl (C,G), or co-injected with *noggin* and *DN-Ras* RNA (D,H). Although inhibition of the BMP (B,F) or FGF/MAPK (C,G) pathways alone have modest effects on endoderm formation, blocking both pathways dramatically increases the number of endoderm precursors. (A-D) Dorsal views; (E,H) ventral views.

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Table 3. Double inhibition of the FGF and BMP pathways causes formation of an excess of endoderm precursors at 75% epiboly

RNA injected at the one-cell stage	Wild type	Noggin	DN Ras	DN Ras + Noggin	Fgf8, Fgf17b and Fgf24 MOs	Fgf8, Fgf17b and Fgf24 MOs + Noggin
Average number of *sox17*-positive cells per embryo	437±47	498±89	354±64	652±87	802±63	1000±172
Total number of embryos	14	10	15	15	6	6

the level of exogenous phosphorylated Casanova was markedly increased following co-injection with ERK2* (Fig. 6C). Similarly, the phosphorylated form of Casanova was no longer detected when Casanova was co-expressed with zebrafish MKP3, which encodes a zebrafish MAPK phosphatase (Fig. 6D). Treatment with SU5402 resulted in a reduction of the level of phosphorylated Casanova protein in embryos injected with *cas* mRNA (Fig. 6E). By contrast, the MAPK P38 inhibitor SB205580 did not have any effect on the level of phosphorylated Casanova protein (Fig. 6F). These results suggest that Casanova protein is a target of ERK in vivo and that the FGF pathway is largely responsible for the phosphorylation of Casanova protein.

Phosphorylation of Casanova/Sox32 attenuates its activity

We next examined whether phosphorylation of Casanova modifies its activity. First, we compared the ability of low doses of *cas-WT* or *cas-S47A* to induce ectopic *sox17* expression. When 20 pg of *cas-WT RNA* was injected, it caused ectopic expression of *sox17* in 35% of the embryos (n=381, Fig. 7J, Table 4). When the same dose of mutated *cas-S47A* was injected, the percentage of embryos displaying *sox17* was slightly increased to 47% (n=214). Furthermore, in situ hybridisation signals were consistently stronger in the *cas-S47A* than in the *cas-WT*-injected embryos (Fig. 7A,B, see Fig. S3A-C in the supplementary material). These results suggest that the presence of the ERK target site has a negative effect on the ability of Casanova to induce *sox17* expression.

We then tested the effect of activating or inhibiting FGF-ERK signalling on the ability of ectopic wild-type or mutated Casanova to induce *sox17* expression at the animal pole. Injection of Fgf8 (Fig. 7C), a constitutively active Ras (Fig. 7E) or an activated version of ERK (Fig. 7G; see Fig. S3D,E in the supplementary material) strongly decreased the ability of Casanova to induce *sox17* (Table 4). Importantly, activating the MAPK pathway with these reagents had only modest effects on the ability of the mutated form of Casanova to induce ectopic expression of *sox17* at the animal pole (Fig. 7D,F,H). This result indicates that the ERK consensus phosphorylation site is required for the attenuation of Casanova activity in response to FGF pathway activation. In agreement with this conclusion, inhibition of the FGF-ERK pathway by treatment with SU5402, or by co-injection with zebrafish MKP3, potentiated the ability of exogenous Casanova to induce *sox17* (Table 4, Fig. 7J). Taken together, these results suggest that FGF-ERK activity antagonises endoderm formation by acting at the level of transcription factors induced by Nodal signalling through direct phosphorylation of Casanova.

DISCUSSION

We have investigated how the Nodal, FGF and BMP pathways interact during the process of endoderm formation in the zebrafish embryo. We found a double repressive effect of the FGF and BMP pathways on induction of endodermal precursors by Nodal signalling. When either of these pathways is

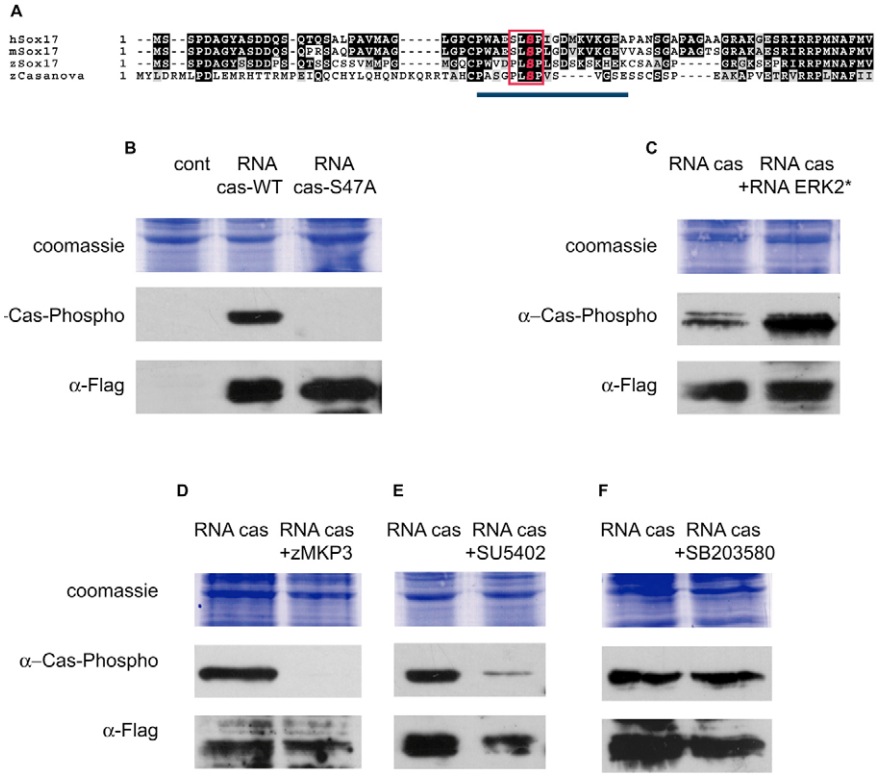

Fig. 6. Casanova protein is phosphorylated by MAPK in vivo. (**A**) Putative conserved MAPK phosphorylation sites (red box) in Casanova and in Sox17 from human, mouse and zebrafish. The blue line corresponds to the 15 amino acid peptide used to generate antibodies ('α Casanova-Phospho' antibody). (**B-F**) Western blot of protein extracts of embryos at 30% epiboly (15 embryos per lane). Embryos were injected with *cas-flag* RNA at 100 ng/μl into one blastomere at the two-cell stage. Zebrafish *mkp3* RNA (50 ng/μl) (D) or SB203580 (200 μM) (F) were co-injected with *cas-flag* RNA, while 15 μM SU5402 was added to the medium after the injection (E). Overexpressed Casanova proteins are Flag tagged and detected by a mouse anti-Flag antibody. (B) The 'α Casanova-Phospho' antibody recognised the product of ectopically expressed Casanova RNA (lane 2). Product of *casanova/sox32-S47A* form RNA is not recognised by the antibodies directed against the phosphorylated consensus site (lane 3). (C-E) The level of phosphorylated overexpressed Casanova increases following overexpression of an activated form of ERK (C, compare lane 1 with 2) but decreases following inhibition of FGF-MAPK signalling (D,E). It is not affected by the P38-MAPK inhibitor that has been shown to work in zebrafish (F) (Fujii et al., 2000).

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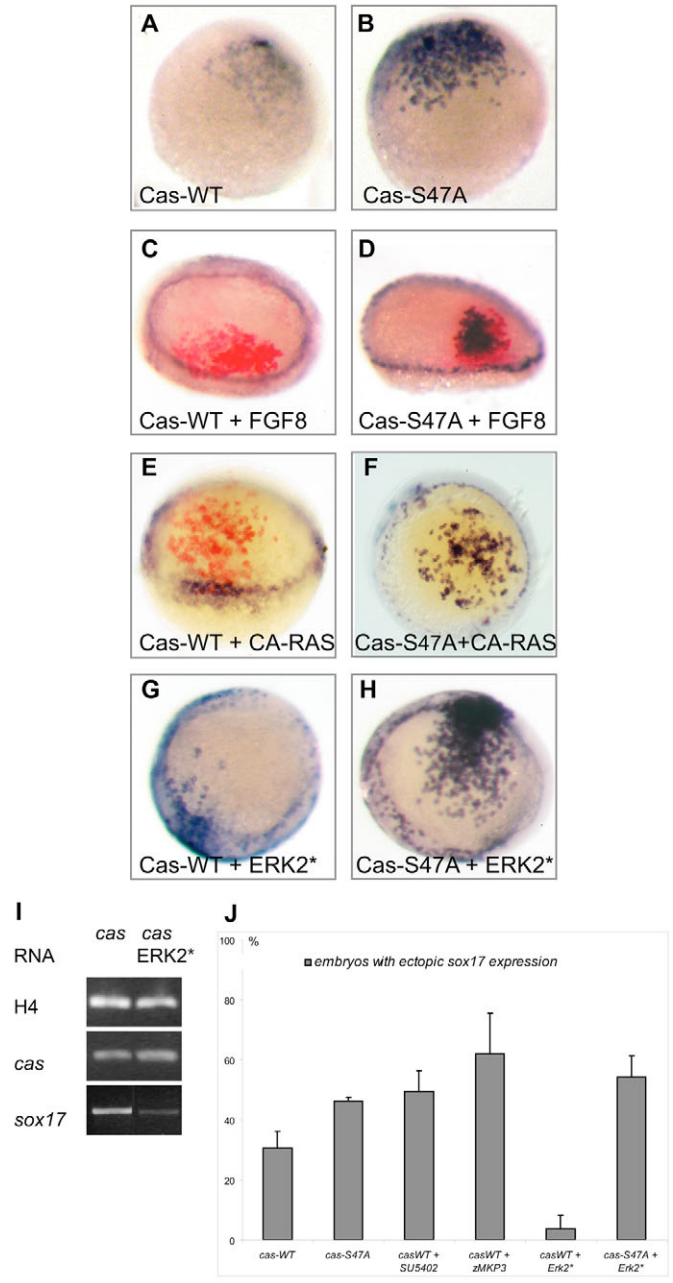

Fig. 7. Casanova activity is negatively regulated by the FGF-ERK signalling pathway. Expression of *sox17* (blue) in embryos at 30% epiboly (**A**,**B**) or 50% epiboly (**C-H**). In C-E, FLDX used as lineage marker was revealed by immunochemistry (red labelling). Embryos injected at the 16-cell stage with RNA encoding Casanova-WT (A) or Casanova-S47A (B), each at 10 ng/μl (see also Fig. S2 in the supplementary material). Mutation of the putative phosphorylation site slightly increases Casanova activity. Co-injection with *fgf8* RNA at 10 ng/μl (C), CA-Ras RNA at 5 ng/μl (E) or ERK* RNA at 50 ng/μl (G) inhibits the ability of Casanova-WT to induce *sox17* (see Fig. S3 in the supplementary material). However Casanova-S47A is insensitive to Fgf8 (D), CA-Ras (F) or ERK* (H) overexpression. (**I**) RT-PCR analysis from three embryos injected with *cas* RNA at 10 ng/μl alone or together with ERK2* RNA at 50 ng/μl. (**J**) Differential activity of Casanova WT and Casanova S47A, based on the percentage of embryos ectopically expressing *sox17* (see Table 4). The activity of exogenous Casanova-WT was compared with the activity of Casanova-S47A, treated or not with MAPK, FGF inhibitors or an activated form of Erk2.

overactivated, the number of endodermal precursors formed is severely reduced. By contrast, when the FGF pathway is blocked, embryonic cells are more easily induced to form endoderm by activation of Nodal signalling. Moreover, we showed that inhibition of FGF signalling partially rescued the deficit of endoderm of *bonnie and clyde* mutants, and that the simultaneous inhibition of both the FGF and BMP pathways results in an excess of endodermal precursors. Finally, we identified a molecular mechanism that explains the repressive action of FGF on endoderm formation. We show that FGF signals control the number of endodermal precursors formed via phosphorylation of the transcription factor Casanova.

FGF signalling and endoderm formation

Although a large number of studies have implicated FGF signalling in the control of mesoderm formation and patterning, only a few studies, mainly in *Xenopus*, have addressed the role of FGF signalling in formation of the endodermal germ layer. In *Xenopus*, numerous observations indicate a repressive role of FGF signalling on endoderm formation. Gamer and Wright (Gamer and Wright, 1995) found that bFGF is a potent inhibitor of the expression of the pancreatic marker *pdx1*. Bouwmeester et al. (Bouwmeester et al., 1996) found that co-injection of XFD, a dominant-negative version of Fgfr1, potentiates the ability of *chordin* to induce expression of endodermal markers in animal caps assays. Finally, a recent study showed that overexpression of eFGF inhibits expression of *mixer* and that inhibition of FGF signalling in animal caps induces the ectopic expression of *mixer* and *endodermin* (Cha et al., 2004). Taken together, these studies suggest that in *Xenopus* FGF signalling antagonizes the molecular pathways that control endoderm specification.

To investigate whether FGF and Nodal signalling interact during endoderm formation in zebrafish, we ectopically expressed FGF signals. Overexpression of FGFs, as well as overexpression of activated versions of the FGF receptor, activated forms of Ras and the MAP kinase ERK, all strongly interfered with formation of the endoderm. Reciprocally, inhibition of FGF signalling with antisense morpholinos oligonucleotides caused a dramatic increase in the number of endodermal precursors. By contrast, interfering with the MAPK pathway using DN-Ras only increased the number of endoderm precursors present on the dorsal side as well as the number of endoderm precursors induced by ectopic activation of the Nodal pathway at the animal pole. Finally, we showed that treatments with SU5402 or injection of morpholinos against Fgf8 and Fgf24 partially rescued the deficit of endoderm precursors and the associated cardia bifida phenotype of the *bonnie and clyde* mutant, which have a reduced expression of *casanova/sox32*. This finding is consistent with the results of David and Rosa (David and Rosa, 2001), who showed that grafted wild-type endodermal cells rescue the *cardia bifida* defects of *casanova/sox32* mutants. Therefore, attenuation of FGF signalling partially compensates for the lack of a downstream effector of Nodal probably by increasing the activity of Casanova. This provides a further indication that in the intact embryo, endoderm formation in response to Nodal signalling is negatively regulated by FGF.

Studies with zebrafish mutants and experiments with inhibitors had previously shown that FGF and Nodal signalling cooperate during mesoderm formation (Griffin and Kimelman, 2003; Griffin et al., 1995b; Mathieu et al., 2004). Our results show that during endoderm formation there is a strong antagonism between the FGF and the Nodal signalling pathways.

Table 4. FGF/ERK signalling negatively regulates the activity of Casanova at the animal pole

RNA injected at the 16-cell stage	Casanova-WT	Casanova-S47A	Casanova-WT + SU5402	Casanova-WT + Mkp3	Casanova-WT + Erk2*	Casanova-S47A + Erk2*
% Embryos showing ectopic *sox17* expression	30	46	49	64	3	54
Total number of embryos	381	214	182	205	167	118

Endoderm formation and BMP signalling

Previous studies had suggested that, in *Xenopus*, endoderm formation is regulated negatively by signals emanating from the ventral side. In particular, Sasai et al. reported that overexpression of *noggin* or *chordin* induces endoderm in animal caps and that this effect is strongly potentiated by inhibition of FGF signalling (Sasai et al., 1996). However, Tiso et al. found that, in zebrafish, the number of endoderm precursors is neither affected by overexpression of BMP nor reduced in the *swirl* mutant which has a disrupted *bmp2* gene (Tiso et al., 2002). By contrast, we found that overexpression of a cocktail of *bmp2*, *bmp4* and *bmp7* potently affects the number of endoderm precursors and that overexpression of *noggin* increases, although only slightly, the number of endodermal precursors. More strikingly, we found that while inhibition of the BMP signalling pathway with *noggin* mRNA results in only a modest increase in the number of endodermal precursors, when *noggin* and DN-Ras are co-injected, they cause formation of a massive excess of endodermal precursors. Finally, the largest number of endodermal precursors was observed following simultaneous inhibition of BMP signalling with *noggin* and of FGF signalling with morpholinos, further implicating BMP signals in restricting endoderm formation. Taken together, these experiments reveal a previously unknown role for BMP signals in repressing endoderm formation. Therefore, our results show that in addition to being positively regulated by signals from the TGFβ family, formation of the endoderm is negatively regulated by a combination of FGF and BMP signals (Fig. 8).

Repression of endoderm formation by FGF or BMP may not be mediated by phosphorylation or competition for Smad2/3

Phosphorylation of Smad in their inter-linker region has been implicated in modulating the response to Tgfβ signals (Massagué, 2003; Pera et al., 2003). In *Xenopus* gastrulae, it has been shown that MAP kinase-dependent phosphorylation of Smad2 inhibits its translocation into the nucleus. As a consequence, animal blastomeres loose their competence to respond to Tgfβ and differentiate into ectoderm instead of mesoderm (Grimm and Gurdon, 2002). Consequences of Smad2 phosphorylation are not clear as different studies gave opposite results. One report has shown that in human cells, activation of Ras caused the exclusion of Smad2 and Smad3 from the nucleus (Kretzschmar et al., 1999). These data contrast with an earlier report in which Erk2-dependant phosphorylation of Smad2 correlated with an increase in the nuclear localisation and activity of Smad2 (de Caestecker et al., 1998). Our finding that a Nodal target gene such as *lefty1* is expressed normally following overexpression of Fgf8 suggests that, in zebrafish, the antagonism between endoderm formation and FGF/ERK signalling does not rely on an inhibitory phosphorylation of Smads. The inability of a Smad2 mutant than can not be phosphorylated by ERK to rescue endoderm formation in FGF overexpressing embryos reinforces this conclusion.

Although the inhibitory effect of FGF signalling on endoderm formation can be explained by an inhibitory phosphorylation of Casanova, the molecular mechanism responsible for the inhibitory action of BMP is not presently known. Both the Nodal signalling pathway and the BMP signalling pathway share a common downstream component, Smad4. It is therefore tempting to speculate that the inhibitory action of BMP on endoderm formation may be caused by a competition between Smad1/Smad5, which are

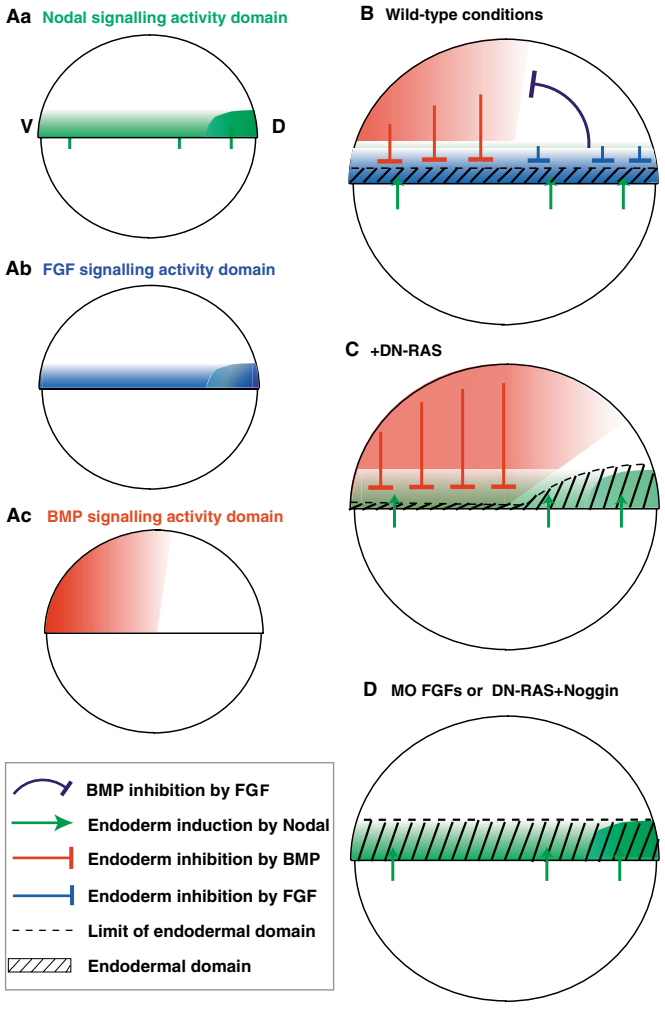

Fig. 8. Schematic representation of the repressive effects of the BMP and FGF pathways on endoderm formation. (**A**) Wild-type context: (a-c) schematic representation of signalling activities of Nodal, FGF and BMP signalling. This representation is speculative and based on the potential range of signals and the expression pattern and range of antagonists (Schier and Talbot, 2005). (**B**) Formation of the endoderm is negatively regulated by a combination of FGF and BMP signals. (**C**) DN-Ras overexpressing embryos: inhibition of MAPK signalling promotes endoderm formation on the dorsal side but causes a loss of endoderm precursors on the ventral side owing to increased expression of the BMPs. (**D**) Triple inhibition of FGF signalling with morpholinos or combined inhibition of Ras and BMP signalling with DN-Ras + *noggin*: simultaneous removal of the BMP and FGF dependent inhibitions promotes endoderm formation all around the embryo.

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activated by BMP signalling, and Smad2/Smad3, which are activated by Nodal signalling for binding to Smad4 (Candia et al., 1997). However, we found that overexpression of Smad4 was not able to rescue the loss of *sox17* expression in BMP-overexpressing embryos (not shown), therefore, a competition at the level of Smad proteins does not seem to be the cause of the inhibitory action of BMPs on endoderm formation.

Casanova/Sox32 as a transcription factor at the crossroad of the FGF and Nodal signalling pathways

We have uncovered a potential molecular mechanism whereby FGF signalling acts to attenuate endoderm formation by identifying Casanova, a Nodal target gene, as a major target of this repression. First, we found that overexpressed Casanova is phosphorylated and that the level of phosphorylation is correlated with activation or inhibition of this pathway. Second, we showed that activation of FGF/ERK attenuates the ability of Casanova to induce *sox17* when ectopically expressed at the animal pole, while inhibition of FGF/ERK makes Casanova a more potent inducer of endoderm. Finally, we showed that although the ability of wild type Casanova to induce endoderm at the animal pole is antagonized by overexpression of FGF or activation of the ERK pathway, a mutated phosphorylation-insensitive form of Casanova is no longer subject to this inhibition. Therefore, these data support the hypothesis that Casanova is at the crossroads of the Nodal and the MAPK signalling pathways, and that the antagonism between these pathways is caused, at least in part, by phosphorylation of this transcription factor. Nevertheless, interactions between these pathways could occur at other levels. Fgf8 or CA-Ras overexpression decrease the expression of *sox17* and of *casanova/sox32*. This suggests that Casanova may not be the only target of this repressive mechanism. In addition to Casanova, MAPK consensus sites are present in factors acting at the level of, or downstream of, Casanova, such as Eomesodermin, Gata5 and Sox17. Future experiments should address whether Eomesodermin, Gata5 and Sox17 are also involved in the crosstalk between the MAPK and Nodal signalling pathways. Alternatively, FGF may additionally affect the expression of *casanova/sox32* by compromising the activity of upstream regulators in the cascade. For example, the homeobox proteins Bon and Mezzo act upstream of Casanova and are both able to bind purified Smad2 (Germain et al., 2000) (M.P. and T.L., unpublished). Phosphorylation of Smad2/3 may affect their interaction with Mixer and Mezzo.

In zebrafish, the endodermal and mesodermal precursors originate from a common endomesodermal territory and both require Nodal signalling. The molecular mechanisms that allow these two cell fates to segregate during gastrulation are not well understood. It was previously known that factors promoting endoderm formation, such as Casanova or Mezzo repress mesoderm formation when overexpressed (Aoki et al., 2002a; Kikuchi et al., 2001; Poulain and Lepage, 2002). Our finding that endogenous FGF signals strongly antagonize endoderm formation by downregulating Casanova shows that a reciprocal negative interaction exists between factors that promote mesoderm formation and transcription factors required for endoderm formation. This mutual antagonism may therefore help to understand how mesodermal- or endodermal-specific gene regulatory networks are established in the precursors of these two germ layers, allowing different cell fates to be segregated.

In conclusion, we have shown that the FGF and BMP signals antagonize endoderm formation by Nodal factors. Furthermore, we have shown that Casanova is subject to an inhibitory phosphorylation in response to FGF signalling and therefore stands at the intersection between the FGF and Nodal signalling pathways. This phosphorylation may represent a general mechanism whereby FGF attenuates Nodal-induced endodermal transcription factors, and therefore these results may help to understand how mesoderm and endoderm segregate from each other.

We thank F. Rosa for the *tar*/acvr1b*, *nkx2.5* and *casanova/sox32* plasmids, and for the *casanova/sox32* morpholino oligonucleotides. We thank N. G. Ahn for sending us ERK2* plasmid, J. Massagué for Smad2 and Smad3 constructs, and M. Whitman for CA-Ras and DN-Ras plasmids. We thank D. Meyer for the *bonnie and clyde* strain. We thank our colleagues at the Marine Station of Villefranche for help and support, and Lydia Besnardeau for excellent technical help. We thank Clare Hudson and Hitoyoshi Yasuo for fruitful discussions and careful reading of the manuscript. We particularly thank Jean-Phillipe Chambon for helping us with HPLC chromatography and western blotting. We thank Laurent Gilleta for taking care of the zebrafish. This work was supported by the CNRS, by the Asssociation pour la Recherche contre le Cancer, by the Ligue Nationale Contre le Cancer, by the Ministère de la Recherche and by the National Institute of Health. M.F. was supported by CNRS and HFSP.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/11/2189/DC1

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