Function and regulation of *FoxF1* during *Xenopus* gut development

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Summary

Development of the visceral mesoderm is a critical process in the organogenesis of the gut. Elucidation of function and regulation of genes involved in the development of visceral mesoderm is therefore essential for an understanding of gut organogenesis. One of the genes specifically expressed in the lateral plate mesoderm, and later in its derivative, the visceral mesoderm, is the Fox gene *FoxF1*. Its function is critical for *Xenopus* gut development, and embryos injected with *FoxF1* morpholino display abnormal gut development. In the absence of *FoxF1* function, the lateral plate mesoderm, and later the visceral mesoderm, does not proliferate and differentiate properly. Region- and stage-specific markers of visceral mesoderm differentiation, such as *Xbap* and α-smooth muscle actin, are not activated. The gut does not elongate and coil. These experiments provide support for the function of *FoxF1* in the development of visceral mesoderm and the organogenesis of the gut. At the molecular level, *FoxF1* is a downstream target of BMP4 signaling. BMP4 can activate *FoxF1* transcription in animal caps and overexpression of *FoxF1* can rescue twinning phenotypes, which results from the elimination of BMP4 signaling. The cis-regulatory elements of *FoxF1* are located within a 2 kb DNA fragment upstream of the coding region. These sequences can drive correct temporal-spatial expression of a GFP reporter gene in transgenic *Xenopus* tadpoles. These sequences represent a unique tool, which can be used to specifically alter gene expression in the lateral plate mesoderm.

Key words: BMP4, *FoxF1*, Forkhead, Gut, Lateral plate mesoderm, Morpholino, Visceral mesoderm

Introduction

Development of the gut requires a coordinated proliferation and differentiation of the visceral mesoderm and gut endoderm. These two processes are regulated by several key transcription factors and signaling pathways (Karlsson et al., 2000; Montgomery et al., 1999). Since visceral mesoderm and endoderm are continuously involved in reciprocal interactions, altered proliferation or differentiation of either of these tissues can have serious consequences with regards to the formation of the gut. While most of the gut is formed from endoderm, mesoderm has a critical role in gut development, as it provides signals necessary for region-specific differentiation of the gut and the epithelial differentiation of the endoderm (Haffen et al., 1987; Immergluck et al., 1990; Kedinger et al., 1986, 1998; Panganiban et al., 1990; Rawdon and Andrew, 1993). Later, as smooth muscle, mesoderm is largely responsible for the physical integrity and motility of the gut. During embryogenesis, the visceral mesoderm develops from the lateral plate mesoderm that is located between the ectoderm and endoderm in the lateral region of the embryo. While many genes have been isolated that are involved in development of the dorsal mesoderm, only a few are specifically expressed in the lateral plate mesoderm. One of them is the Fox gene *FoxF1* (El-Hodiri et al., 2001; Koster et al., 1999). Fox genes encode transcription factors that play important roles in embryonic pattern formation and tissue-specific gene expression (Brownell et al., 2000; Carlsson and Mahlapuu, 2002; Dirksen and Jamrich, 1992, 1995; Hatini et al., 1994; Kaufmann and Knochel, 1996; Kenyon et al., 1999; Martinez et al., 1997). They are also known as forkhead and winged-helix genes. They encode proteins that contain a highly conserved 110 amino acid DNA binding domain (Lai et al., 1990; Weigel and Jackle, 1990). They are expressed in tissues derived from all three germ layers. In *Xenopus* mesoderm, the expression of Fox genes displays distinct differences along the dorsoventral axis, which suggests that they are involved in specification of dorsoventral properties of the mesoderm (El-Hodiri et al., 2001). Several Fox genes are expressed in the dorsal mesoderm of *Xenopus*, but only *FoxF1* is expressed in the lateral plate mesoderm and its derivative, the visceral mesoderm (El-Hodiri et al., 2001; Koster et al., 1999).

Xenopus FoxF1 (El-Hodiri et al., 2001; Koster et al., 1999) is the ortholog of the human *FREAC-1* (Clevidence et al., 1993; Hellqvist et al., 1996; Larsson et al., 1995), the murine and chick *HFH-8* (Funayama et al., 1999; Peterson et al., 1997) and the *Drosophila biniou* (Zaffran et al., 2001). Because of relatively recent genome duplication in *X. laevis*, most *Xenopus* genes are present in two copies per haploid genome. These two copies are usually labeled as ‘a’ and ‘b’. Typically, the ‘a’ and ‘b’ genes have the same expression pattern and function. Since previously published studies show that *FoxF1a* and *FoxF1b* have the same expression pattern (El-Hodiri et al., 2001; Koster et al., 1999), we will treat them as the same. However, for the

sake of accuracy, we should mention that these studies were performed using *FoxF1b*.

Xenopus FoxF1 is first activated during gastrulation in the presumptive ventro-lateral mesoderm. During neurulation, *FoxF1* expression becomes restricted to the lateral plate mesoderm (El-Hodiri et al., 2001; Koster et al., 1999). This expression is very similar to the expression of its murine ortholog *HFH-8* (*Foxf1*) (Peterson et al., 1997) with some species-specific differences. While both of the genes are expressed in the lateral plate mesoderm and later in the visceral mesoderm, the murine *Foxf1* is intensely transcribed in the extra-embryonic mesoderm of allantois, amnion and yolk sack, structures that do not exist in *Xenopus* embryos. Interestingly, even the *Drosophila* gene *biniou* displays a highly conserved expression in the visceral mesoderm. The unique expression pattern of FoxF genes in the lateral plate/visceral mesoderm makes them important targets for evaluation of function in order to shed light on their role in gut development. In vertebrates, the function of *Foxf1* has been investigated only in mice. A targeted elimination of *Foxf1* resulted in defects in mesodermal differentiation and incomplete separation of splanchnic and somatic mesoderm (Mahlapuu et al., 2001b). Analysis of *Foxf1* function in older mouse embryos was hampered by the fact that the *Foxf1* deficient embryos have severe defects in extra-embryonic structures, and these animals die of apoxia by embryonic day (E) 10.

In invertebrates, the function of *FoxF* has been investigated in *Drosophila*. The *Drosophila* FoxF gene *biniou* (*bin*) is activated in the trunk visceral mesoderm primordia and has a role during specification and differentiation of the trunk visceral mesoderm. Its function is essential for the differentiation of the splanchnic mesoderm into midgut musculature (Zaffran et al., 2001), suggesting that the FoxF gene family might have an important, evolutionarily conserved, function in the development of the lateral plate/visceral mesoderm.

To gain a better understanding of the function of FoxF genes in vertebrates, we have decided to monitor the effects of *FoxF1* absence on gut development in *X. laevis*. We argued that in this developmental system the interactions with the mother's circulatory system are not required and therefore the analysis of *FoxF1* function is not going to be hampered by early deaths of embryos. *Xenopus* embryos are a very good system for studying gut development, as these embryos are easily accessible at all developmental stages and the gut development has been described in precise detail (Chalmers and Slack, 1998, 2000). In our study, we used a morpholino oligonucleotide-based approach to interfere with *FoxF1* function. Morpholinos are modified oligonucleotides that can inhibit translation of the target mRNA (Heasman et al., 2000). They are resistant to degradation and therefore are present in embryos for several days after injection. Since injections of morpholinos provide substantial reduction of the targeted gene product, this type of interference is often called a gene 'knockdown'. Using this approach, we found that *FoxF1* is critical for the proliferation and differentiation of the lateral plate/visceral mesoderm.

Xenopus FoxF1 is a target of BMP4 signaling and its function is required for normal activation of *Xbap* and α-smooth muscle actin. The cis-regulatory elements of *FoxF1* required for BMP4-mediated activation are located within 2 kb, upstream of the 5′ coding region. These sequences are able to direct expression of a GFP reporter cassette into the lateral plate mesoderm of transgenic tadpoles. For the first time, the identification of these elements provides a tool to specifically manipulate gene expression only in the mesodermal component of the gut.

Materials and methods

mRNAs and oligonucleotides for microinjection of embryos

The complete coding sequence of *FoxF1* with or without 99 base pairs of 5′ UTR was amplified by PCR and cloned into *Cla*I/*Xho*I sites of the expression plasmid pCS2+C-MT to generate Myc-tagged UTR *FoxF1* and *FoxF1*. Capped mRNA for injection was synthesized by in-vitro transcription using mMessage mMachine kit (Ambion). Two *FoxF1* morpholino oligonucleotides and a standard control morpholino were synthesized by Gene-Tools: *FoxF1*Mo (5′-AGCTGAAGAAAATAGGGAAAGCTGA-3′) and a second *FoxF1* morpholino (5′-AGGATAAGAGCCCCTCAGTCTGCAC-3′). Injection of embryos was carried out as previously described (El-Hodiri et al., 1997). Developmental stages of embryos were determined according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

Constructs for transgenesis

A 2 kb *Eco*RI-*Eco*RI DNA fragment upstream of the transcription start site of *FoxF1* was screened out from the *Xenopus* genomic library. For the GFP reporter construct, this fragment was subcloned into pBS-GFP (Zhang et al., 2003) to generate pBS-FoxF1-GFP. For the *lacZ* reporter construct, a *Bam*HI-*Not*I fragment containing nuclear localization sequence (NLS) and β-galactosidase (*lacZ*) from pCS2+nβgal was subcloned into pBluescriptIISK and then the 2 kb *Eco*RI-*Eco*RI *FoxF1* 5′ fragment was subcloned into the *Eco*RI site to generate pBS-FoxF1-NLS-βgal. DNA for transgenesis was prepared by digestion with *Sac*II-*Pvu*I for pBS-FoxF1-GFP and *Not*I-*Hin*dIII for pBS-FoxF1-NLS-βgal.

Western blotting

For in-vitro transcription/translation of *FoxF1*, we used the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. SDS-PAGE was performed using a 10% polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell), and detected with the monoclonal mouse anti-myc antibody 9E10 (Invitrogen) using the ECL Western Blotting Detection Reagents and Analysis System (Amersham Biosciences).

In-situ hybridization, immunostaining, β-galactosidase staining and histology

Whole-mount in-situ hybridization was performed as described by Harland (Harland, 1991). Digoxigenin-labeled probes were generated from the following plasmids: a *FoxF1* clone containing 99 base pairs of 5′ UTR and the complete coding sequence in the pCRII-TOPO vector was linearized with *Xba*I and transcribed with SP6 RNA polymerase; *Xbap* (Newman and Krieg, 1998). Immunostaining of paraffin sections and whole embryos was performed as previously described (El-Hodiri et al., 1997). Primary and secondary antibodies were used at the following dilutions: 12/101, 1:200 (Developmental Studies Hybridoma Bank); α-smooth muscle actin, 1:400 (Sigma); HRP-conjugated sheep anti-mouse IgG, 1:200 (Sigma). Embryos were dehydrated in ethanol, embedded in paraffin wax and 12 μm sections were cut. Sections were de-waxed in xylene and mounted with Permount (Fisher). Nuclei were stained with 2 μg/ml Hoechst 33342 (Sigma). β-galactosidase activity was detected as described by Turner and Weintraub (1994).

TUNEL and BrdU incorporation assays

Apoptotic cells were identified by whole-mount TUNEL staining

following a protocol by Zhang et al. (Zhang et al., 2003). BrdU incorporation was performed as previously described (Hardcastle et al., 2000) with some modifications on the injection sites: 10 nl of 5-bromo-deoxyuridine (BrdU) (Roche) were injected into both sides of the neural plate and lateral plate of stage 19 embryos.

RNA isolation and RT-PCR assay

Preparation of total RNA from animal caps using TRIzol reagent (Invitrogen) was carried out according to the manufacturer's instructions. RT-PCR was performed by using the following primers and cycling condition: *FoxF1* (55°C, 30 cycles; forward, 5′-AACCCTCT-GTCCTCCAGCCT; reverse, 5′-GGTTAGTGCAATGACTAACTTC), Xbra (55°C, 30 cycles; U: 5′-GGATCGTTATCACCTCTG; D: 5′-GTGTAGTCT GTAGCAGCA), EF-1α (55°C, 26 cycles U: 5′-CA-GATTGGTGCTGGATATGC; R: 5′-ACTGCCTTGATGACTCC-TAG).

Transgenesis

Transgenic *Xenopus laevis* embryos were generated by restriction enzyme-mediated integration (REMI) as described (Amaya and Kroll, 1999; Kroll and Amaya, 1996) with some modification (Zhang et al., 2003).

Results

FoxF1 function is required for normal gut development

Xenopus FoxF1 is initially activated during gastrulation, in the lateral and ventral mesoderm (Koster et al., 1999). During neurulation *FoxF1* transcripts are present in the lateral plate mesoderm and in the neural crest-derived structures of the head and branchial arches (Fig. 1A). At this stage the most intense transcription of *FoxF1* is in the anterior-dorsal region of the lateral plate mesoderm. At stage 30 abundant transcription of *FoxF1* can be also detected in the most ventral region of the embryo, with the notable exception of the heart (Fig. 1B-D) (El-Hodiri et al., 2001; Koster et al., 1999). When the gut starts to retract, the lateral plate, which is initially a homogeneous mesenchymal structure, splits into the splanchnic mesoderm surrounding the gut and the somatic mesoderm that lines the body cavity. Concomitant with this subdivision, expression of *FoxF1* becomes progressively restricted to the splanchnic mesoderm (Fig. 1E,F).

To investigate the effects of elimination of *FoxF1* protein on lateral plate mesoderm development, we injected 8-cell stage embryos into the ventral vegetal blastomeres with morpholinos that inhibit translation of *FoxF1*mRNA (Fig. 2A). Using this knockdown strategy, we found that inhibition of *FoxF1* function dramatically affected development of embryos in a dose-dependent manner. At high concentrations, the frog embryos developed normally during gastrulation and neurulation, but they lysed on the ventral side around stage 30 (not shown). Using concentrations of morpholinos that do not cause lysis of the ventral body cavity, we found that tadpoles showed specific abnormalities of gut development. The most obvious abnormality was the lack of gut elongation and coiling (Fig. 2C). Coiling of the gut is a highly reproducible process that proceeds according to a well-characterized pattern and is used in *Xenopus* as a staging criterion for tadpoles (Chalmers and Slack, 1998; Chalmers and Slack, 2000; Nieuwkoop and Faber, 1967). The lack of gut elongation and coiling present in knockdown tadpoles was not due to a delay, as tadpoles two days later still did not display any coiling (Fig. 2E). This lack

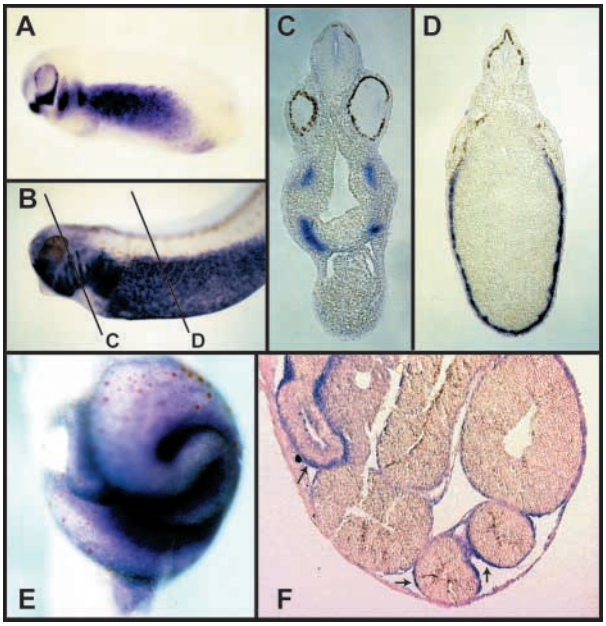

Fig. 1. Expression of *FoxF1* during *Xenopus* development. (A,B) Embryos are shown with anterior to the left and dorsal to the top. (A) Whole-mount in-situ hybridization of a *FoxF1* probe to a stage-25 embryo. *FoxF1* expression is present in the neural crest-derived structures of the head and in the lateral plate mesoderm. At stage 30 the expression of *FoxF1* intensifies and is also present in the ventral mesoderm (B). Letters with lines in (B) indicate the position of sections in (C,D). (C) Section through the head, branchial arches, and heart regions shows the lack of *FoxF1* expression in the heart. (D) Section through the mid-trunk region of the embryo shows expression in the lateral plate mesoderm. (E) Ventral view of whole-mount in-situ hybridization of a stage-45 embryo shows *FoxF1* expression in the gut. (F) Transverse section through the embryo shown in (E) demonstrates that *FoxF1* transcripts are present in the splanchnic mesoderm (arrow).

of elongation and coiling of the gut was specific to the action of *FoxF1* morpholinos, as demonstrated by several control experiments. To start with, injection of control morpholinos provided by the company did not lead to any malformations in these tadpoles (Fig. 2F). Furthermore, injection of a second set of *FoxF1* morpholinos, which were targeted against a different region of the *FoxF1* RNA, resulted in a similar abnormal phenotype (not shown). While these experiments demonstrate a certain degree of specificity, the most stringent test of morpholino specificity is the ability of the targeted RNA to rescue the mutant phenotype. However, for the RNA to be able to rescue the abnormal phenotype in the presence of morpholinos, this RNA needs to be mutated in the morpholino-binding site. This is necessary because otherwise the injected morpholinos would inhibit the translation of the injected RNA, making such a rescue experiment virtually impossible. For this reason, we deleted 25 bp in the morpholino-recognition site of the *FoxF1* RNA. As a result of this deletion, the mutated *FoxF1* RNA lacked the 5′UTR region recognized by the morpholino and therefore its translation took place even in the presence of the *FoxF1* morpholino. When this RNA was co-injected with the *FoxF1* morpholino, a large proportion of the injected embryos was rescued and showed normal gut elongation and

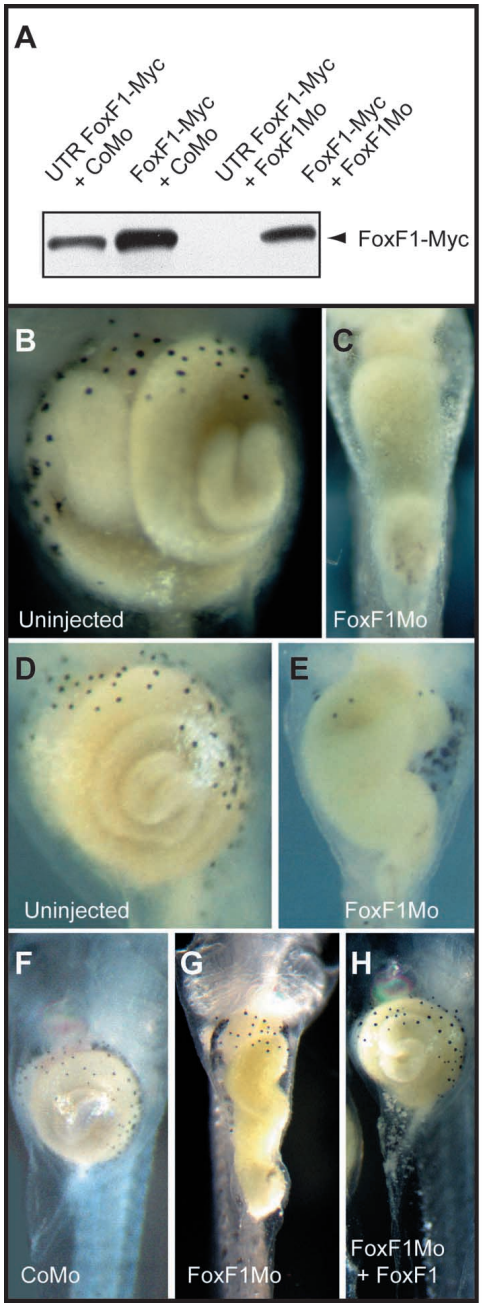

Fig. 2. Inhibition of *FoxF1* function results in impaired gut morphogenesis. (A) Western blot analysis of *FoxF1* protein tagged with a Myc epitope at the C-terminus using anti-Myc antibody. Translation of UTR *FoxF1*-Myc was blocked in the presence of *FoxF1* morpholino (*FoxF1*Mo), but not by standard control morpholino (CoMo). Translation of *FoxF1*-Myc RNA lacking the 5′ UTR sequences was not inhibited by either CoMo or *FoxF1*Mo. (B) The ventral view of 5-day-old (stage 45/46) uninjected embryo and embryo injected with *FoxF1*Mo (2.2 pmol) into two ventral blastomeres at the 8-cell stage (C). Embryos injected with *FoxF1*Mo display gut elongation and looping defects. (D) The ventral view of 7-day-old (stage 46/47) uninjected and *FoxF1*Mo-injected embryos (E), showing that knockdown embryos still do not display normal gut morphogenesis. (F) The ventral view of stage 46/47 embryos injected with CoMo (2.2 pmol) showing normal gut morphogenesis. *FoxF1*Mo (2.2 pmol) injected embryos with mutant gut (G), can be rescued by co-injection of *FoxF1* RNA (1.25 ng) (H).

coiling (Fig. 2H). The percentage of normal embryos increases from 15.2% in morpholino injections to 85.2% in RNA rescue experiments. These experiments demonstrate that the abnormal gut development is due to the reduction of *FoxF1* function.

FoxF1 is required for differentiation of the gut mesoderm

A detailed morphological and molecular analysis revealed the reasons for the abnormal development of the gut in *FoxF1* knockdown embryos. As expected on the basis of *FoxF1* expression, the primary defects in gut organogenesis reside in the abnormal development of the lateral plate/visceral mesoderm. Since normal gut development requires a coordinated proliferation and differentiation of the gut mesoderm and endoderm, abnormalities in these processes in either of the two layers can lead to abnormal gut development. During normal development, the visceral mesoderm develops into the smooth muscle that surrounds and supports the gut endoderm. The visceral mesoderm changes from a tissue with a relatively weak cell adhesion to a tissue with high cohesiveness. This increase in cohesiveness is especially critical in *Xenopus*, as in tadpoles prior to metamorphosis the smooth muscle forms only a single layer. Consequently it is very susceptible to perturbations. Abnormalities in the development of smooth muscle can be detected at the morphological level or by following expression of genes that are activated during the transition from visceral mesoderm to smooth muscle. One of the genes diagnostic of correct transition from relatively undifferentiated visceral mesoderm to the functional adult-type smooth muscle is the α-smooth muscle actin. We analyzed expression of this gene in knockdown tadpoles by immunocytochemistry using antibodies against α-smooth muscle actin. Fig. 3A shows the typical distribution of α-smooth muscle actin in stage 43/44 control morpholino-injected tadpoles. α-smooth muscle actin is present in a layer of cells surrounding the gut. This layer can be also visualized with Hoechst dye as it contains morphologically distinct, concentrically aligned, slightly elongated nuclei (Fig. 3B). This is the morphology characteristic of the developing smooth muscle. In knockdown tadpoles, the mesodermal layer does not stain with the α-smooth muscle actin antibodies (Fig. 3C) and when stained with Hoechst dye, the nuclei appear highly disorganized and the cells have a more of an undifferentiated, mesenchymal appearance (Fig. 3D,F). In many places this layer loses its integrity and the yolky endodermal cells of the gut spill into the body cavity (Fig. 3C,E).

The α-smooth muscle actin is not the only gene that presents altered expression in *FoxF1* knockdown embryos. Fig. 4 shows the expression of *Xenopus bagpipe* (*Xbap*), which is a marker of differentiation of the anterior gut mesoderm. In wild-type embryos, *Xbap* is activated several hours after *FoxF1* (Newman et al., 1997; Newman and Krieg, 1999), making it a potential target of this gene. While *Xbap* is expressed on both sides of the anterior gut mesoderm of the embryos injected on one side with the control morpholino (Fig. 4A,B,I), in embryos that were injected on one side with *FoxF1* morpholino *Xbap* expression is absent on the injected side (Fig. 4C,D,J). This indicates that *FoxF1* activity is required for *Xbap* expression in the anterior gut mesoderm. To make sure that the lateral plate mesoderm formed in these embryos, we confirmed its presence

by in-situ hybridization. The lack of suitable molecular markers for the development of the lateral plate mesoderm presented a problem, but fortunately *FoxF1* itself can be used as a marker of lateral plate mesoderm development. This is because in knockdown embryos morpholinos inhibit *FoxF1* translation, but they do not inhibit the transcription of this gene. Fig. 4E,F,K show the distribution of *FoxF1* mRNA in control morpholino-injected embryos. When embryos injected on one side with *FoxF1* morpholino were hybridized with a *FoxF1* probe, *FoxF1* mRNA was detected on both sides of the knockdown tadpoles (Fig. 4G,H,L). This demonstrates that *FoxF1* function is not necessary for the formation of the lateral plate mesoderm and that the lack of the *Xbap* expression in knockdown tadpoles is not due to the lack of lateral plate mesoderm.

While the in-situ hybridization with *FoxF1* probe demonstrated that the lateral plate mesoderm was formed in the absence of *FoxF1* function, it also revealed that there was a reduced amount of it on the morpholino-injected side of tadpoles. This raised the possibility that in addition to differentiation, the proliferation or the apoptosis of lateral plate mesoderm was affected by ablation of *FoxF1* function.

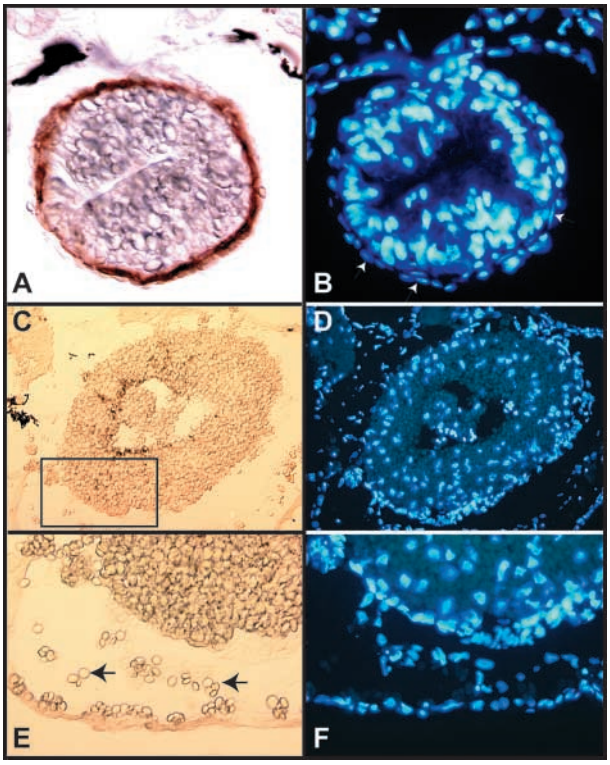

Fig. 3. *FoxF1* is required for smooth muscle differentiation in the gut. Immunostaining with antibodies against α-smooth muscle actin on transverse sections through the hindgut of CoMo (A) and *FoxF1* knockdown (C) embryos at stage 43/44. α-smooth muscle actin is expressed in the smooth muscle layer surrounding the gut endoderm (A) but absent in the knockdown embryo (C). (B,D) Nuclear staining by Hoechst dye on the same sections shows a layer of smooth muscle cells with elongated nuclei (arrows) surrounding the gut of CoMo-injected embryos (B), but highly disorganized gut mesoderm in knockdown embryos (D). (E) Close-up view of the area boxed in (C), visualizing the endodermal yolk platelets (arrows) in the visceral cavity.

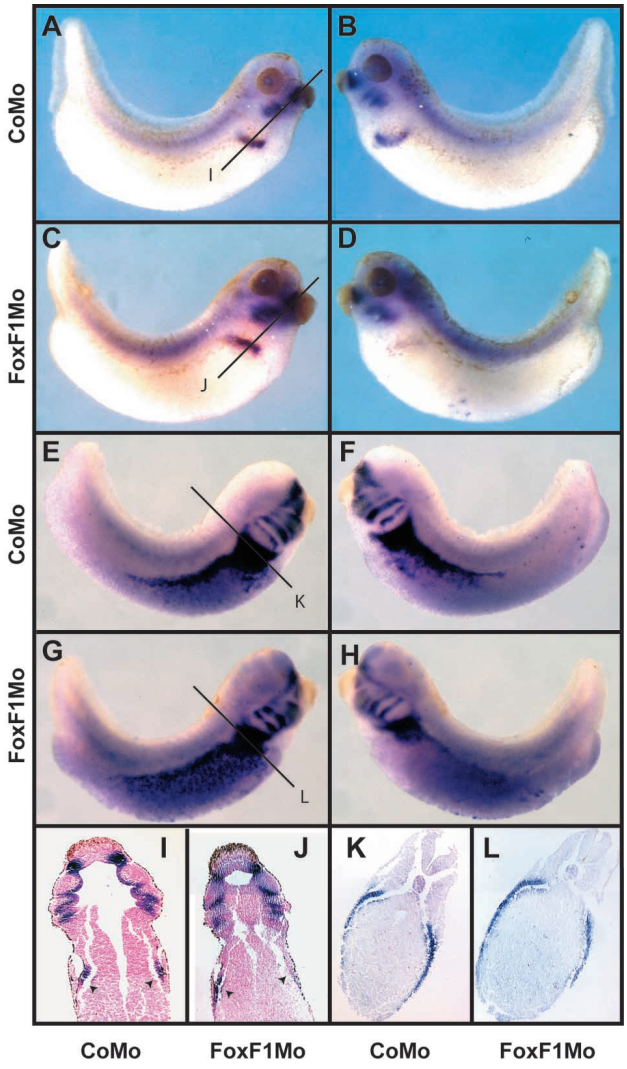

Fig. 4. Abnormal expression of *Xbap* in *FoxF1* knockdown embryos. (A,B) Whole-mount in-situ hybridization showing *Xbap* expression on both sides of a CoMo-injected stage 35/36 embryo. A line with a letter in (A) indicates the position of the section in (I). (C,D) Whole-mount in-situ hybridization of *Xbap* RNA to a *FoxF1*Mo-injected stage 35/36 embryo. *Xbap* expression is present on the uninjected side (C) but is absent on the injected side (D). A line with a letter in (C) indicates the position of the section in (J). (E,F) Whole-mount in-situ hybridization showing *FoxF1* expression on both sides of a CoMo-injected stage 35/36 embryo. A line with a letter in (E) indicates the position of the section in (K). (G,H) Whole-mount in-situ hybridization of *FoxF1* RNA to a *FoxF1*Mo-injected stage 35/36 embryo. *FoxF1* expression is present on both sides of the embryo. A line with a letter in (G) indicates the position of the section in (L). (I) A section through the embryo in (A) shows that *Xbap* is expressed in the CoMo-injected (shown as right) side as well as on the uninjected (left). (J) A section through the embryo in (C) shows that *Xbap* is expressed on the uninjected (left) side of a *FoxF1*Mo-injected embryo but not on the injected (right) side of a tadpole. (K) A section through an embryo hybridized with *FoxF1* RNA shows that the lateral plate mesoderm is present on the CoMo-injected (right) side as well as on the uninjected side (left). (L) A section through an embryo hybridized with *FoxF1* RNA shows that the lateral plate mesoderm is present on the *FoxF1Mo*-injected (right) side as well as on the uninjected side (left). Arrows point to the anterior lateral plate mesoderm.

FoxF1 is required for normal proliferation of the lateral plate mesoderm

While the abnormal degree of differentiation alone could explain the lack of cohesiveness of the smooth muscle layer, a reduced cell proliferation or increase in cell death in the developing lateral plate mesoderm could be other contributing factors. Alteration in either of these processes could lead to a reduced number of mesodermal cells during smooth muscle formation. To evaluate whether cell proliferation was affected in *FoxF1* knockdown experiments, we used BrdU incorporation to visualize proliferating cells in these embryos. While cell proliferation was high in the lateral plate mesoderm of control morpholino-injected embryos (Fig. 5A,C), it was strongly reduced in *FoxF1* knockdown embryos (Fig. 5B,D). Counting of BrdU-positive cells revealed that the lateral plate mesoderm of *FoxF1* knockdown tadpoles contained about 40% less BrdU-positive cells (Fig. 5E). At the same time the cell proliferation in the neuroectoderm was not affected. This demonstrates a significant reduction of cell proliferation in the lateral plate mesoderm of *FoxF1* morpholino-injected embryos.

To evaluate whether cell death was affected in *FoxF1* knockdown experiments, we used TUNEL assays on stage 28 embryos. We chose this stage because some embryos lyse around stage 28-30, which might be indicative of increased cell death. However, the TUNEL analysis did not show higher cell death in the lateral plate mesoderm of the *FoxF1* morpholino-injected embryos (Fig. 5G) than in the control morpholino-injected tadpoles (Fig. 5F). These observations show that in addition to having a role in the differentiation of the visceral mesoderm, the function of the *FoxF1* is also required for the proliferation of cells of the lateral plate mesoderm. A combinatory effect on these two processes leads to a reduced number of partially differentiated cells. These cells are unable to form a layer of smooth muscle that would properly support the gut endoderm.

Regulation of *FoxF1* during *Xenopus* development

Dorsoventral properties of the mesoderm depend on antagonistic interactions between dorsalizing and ventralizing signals (Heasman, 1997; Sive, 1993). The major ventralizing signal in *Xenopus* embryos is the signaling molecule BMP4. Since *FoxF1* is predominantly expressed in the ventral mesoderm, BMP4 might an upstream regulator of *FoxF1*. This possibility is further strengthened by the observation in *Drosophila* that *biniou* is regulated by *dpp*, the *Drosophila* homolog of BMP4 (Zaffran et al., 2001). We performed a series of experiments in order to address this possibility. First, we injected BMP4 RNA into 8-cell stage *Xenopus* embryos. At early blastula stage we cut animal caps and cultured them until

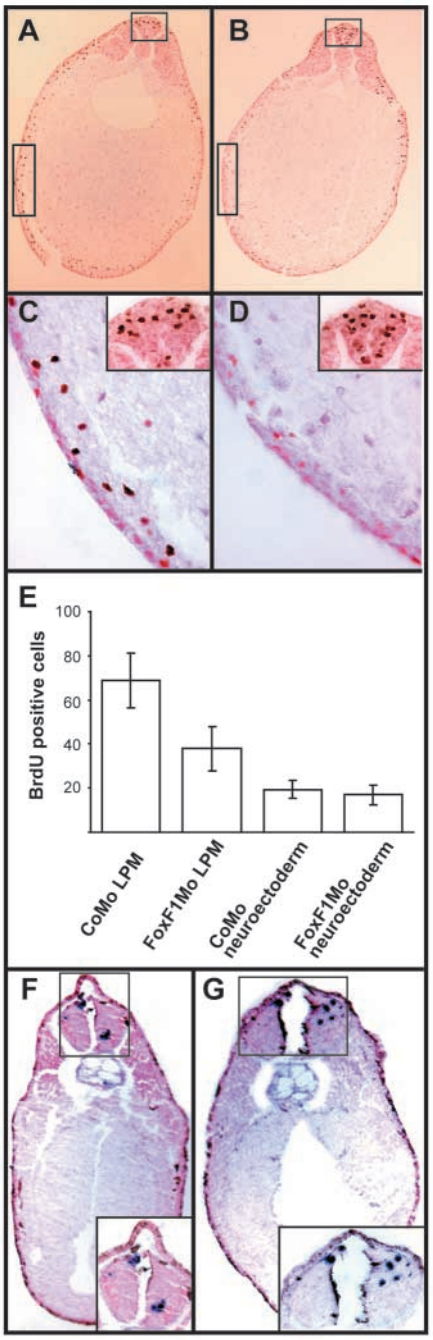

Fig. 5. A lower rate of lateral plate mesoderm proliferation in *FoxF1* knockdown embryos. (A-D) Cell proliferation as visualized by BrdU incorporation (brown nuclear staining). Transverse sections through the midtrunk of stage-20 embryos injected with CoMo (A) or *FoxF1*Mo (B) show drastically reduced cell proliferation in the lateral plate mesoderm of *FoxF1* knockdown embryos. The boxed areas are magnified in (C,D). (C) Higher magnification of the boxed areas in (A), showing BrdU-positive cells in the lateral plate mesoderm and in the neuroectoderm (inset) of a CoMo-injected embryo. (D) Higher magnification of the boxed areas in (B), showing a lack of BrdU-positive cells in the lateral plate mesoderm but a normal number of BrdU-positive cells in the neuroectoderm (inset) of a *FoxF1*Mo-injected embryo. (E) A column chart showing the numbers of BrdU-positive nuclei in the lateral plate mesoderm and neuroectoderm of CoMo- and *FoxF1*Mo-injected embryos at midtrunk level (averages±s.e.m.). Nuclei were counted on 10 sections derived from 5 control embryos and 20 sections from 11 *FoxF1* knockdown embryos in two independent experiments. The difference in proliferation rate between control and knockdown lateral plate mesoderm is statistically significant (P=5.9 × 10^{-6} in a two-tailed t-test). LPM: lateral plate mesoderm. (F,G) TUNEL assay on stage-28 embryos injected with CoMo (F) and *FoxF1*Mo (G). Apoptotic cells (blue staining, inset) are mostly located in the neuroectoderm. No significant differences were observed between embryos injected with CoMo and *FoxF1*Mo.

sibling embryos reached the late gastrula stage. We used RT-PCR to determine whether these caps expressed *FoxF1* RNA. As can be seen in Fig. 6A, the BMP4 RNA-injected caps expressed *FoxF1* mRNA, while the control, uninjected caps did not express *FoxF1* mRNA. This shows that BMP4 alone can activate *FoxF1* transcription in uncommitted ectoderm.

In the second experiment, we took advantage of the fact that overexpression of BMP4 gives rise to embryos with a ventralized phenotype (Dale et al., 1992; Suzuki et al., 1997). Therefore, if *FoxF1* is a target of BMP4, its gene product might ventralize embryos. To test this hypothesis, we injected *FoxF1* mRNA into one or both dorsal blastomeres of 4-cell stage embryos. These blastomeres normally form dorsal structures. We found that embryos injected with the *FoxF1* RNA displayed a strongly ventralized phenotype (Fig. 6D). These embryos had markedly reduced dorsal structures on the injected side and, as the staining with muscle-specific antibodies demonstrates, they had only few, poorly differentiated somites (Fig. 6F). Therefore, the effects of *FoxF1* RNA injection mimicked the effects of BMP4 RNA injection, suggesting that *FoxF1* is a target and a mediator of BMP4 signaling.

In the third experiment, we examined the ability of *FoxF1* RNA to rescue dorsalized embryos that suffer from a lack of BMP4 signaling. It has been demonstrated previously that embryos injected with the dominant negative BMP4 receptor (DNBR) into the ventral blastomeres of 8-cell stage embryos lost most of their ventral structures. This is because DNBR eliminates BMP4 signaling; and as a result, the ventral mesoderm converts to dorsal mesoderm, leading to the formation of a secondary axis (Suzuki et al., 1994). If *FoxF1* is a downstream target of BMP4 and mediates BMP4 function, a co-injection of *FoxF1* RNA with DNBR RNA should lead to a rescue of the dorsalized phenotype and to the elimination of the secondary axis. As can be seen in Fig. 6G,H, this was indeed the case. Injection of DNBR RNA led to formation of the secondary axis in 95.8% (69/72) of embryos (Fig. 6G). By contrast, when *FoxF1* RNA was co-injected with the DNBR RNA, only 4.4% (4/90) of embryos formed a secondary axis (Fig. 6H). This provides further evidence that *FoxF1* is a downstream mediator of BMP4 signaling.

Several papers demonstrated induction of mesodermal markers in *Xenopus* animal caps by BMP4. For example, Jones et al. (Jones et al., 1992) and Suzuki et al. (Suzuki et al., 1997) showed that BMP4 can induce transcription of mesoderm-specific genes such as Brachyury (*Xbra*). Since our experiments confirmed these findings (Fig. 6A), we examined the possibility that the activation of *FoxF1* might be mediated by *Xbra*. *Xbra* has a partially overlapping pattern of expression during early *Xenopus* development and could be a potential mediator of BMP4 signaling in *FoxF1* activation. Indeed, our experiments showed that *Xbra* could activate *FoxF1* in animal caps (Fig. 6B), opening the possibility that *FoxF1* activation by BMP4 is indirect.

Furthermore, since the lack of gut coiling in *Xenopus* embryos injected with *FoxF1* morpholinos strongly resembles the phenotype of embryos injected with RNA encoding the

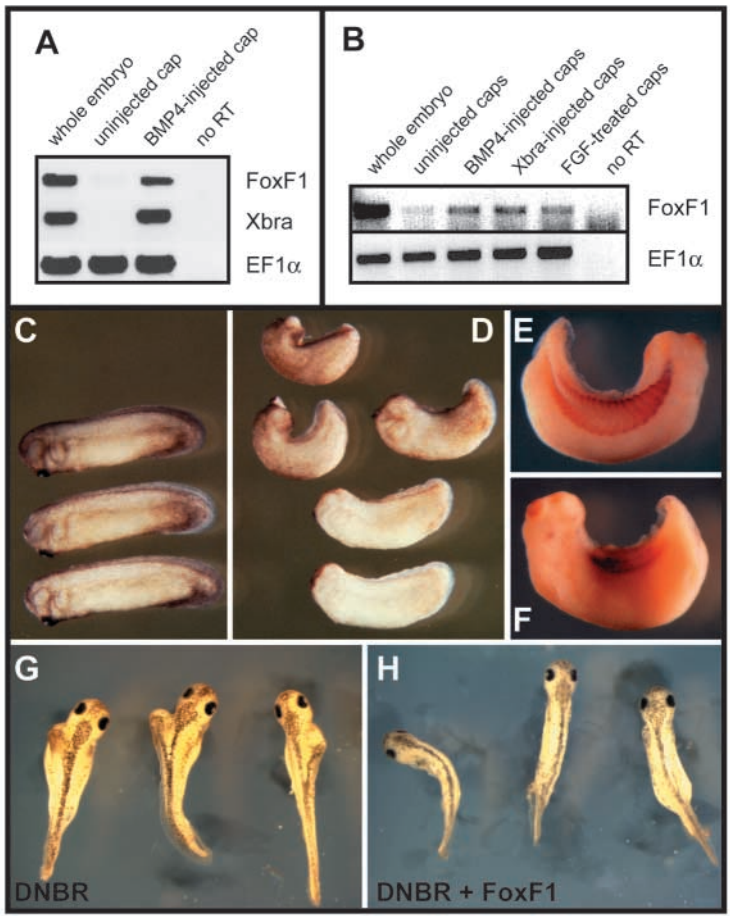

Fig. 6. *FoxF1* is a target and a mediator of BMP4 signaling. (A) RT-PCR analysis of RNA isolated from animal caps injected with BMP4 RNA. Animal caps from embryos injected with BMP4 RNA (1.5 ng) into the animal blastomeres or uninjected embryos were dissected at stage 8 and collected when siblings reached stage 12.5. Xbra was used as a positive control for BMP4 induction and EF1α as a loading control. Lane 1 – RT-PCR on RNA from a whole embryo. Lane 2 – uninjected cap. Lane 3 – BMP4 injected cap. Lane 4 – no RT (no enzyme, RNA from the whole embryo). (B) RT-PCR analysis of RNA isolated from animal caps injected with BMP4 or *Xbra* RNA, or treated with FGF protein. Animal caps from embryos injected with BMP4 (1.5 ng) or *Xbra* (2.5 ng) RNA into the animal blastomeres or uninjected embryos were dissected at stage 8. Caps were collected when siblings reached stage 12.5 and assayed by RT-PCR. For FGF experiments, a set of uninjected caps was dissected at stage 8 and treated with 200 ng/ml bFGF for 1 hour. (C-F) Effects of *FoxF1* RNA injection on the morphology of *Xenopus* embryos. Two dorsal blastomeres at the 4-cell stage were injected with *FoxF1* RNA (0.4-1 ng), and phenotypes were analyzed at stage 28-30. The injected embryos (D) show different degrees of ventralization, while their siblings (C) display normal morphology. (E,F) The right (E) and left (F) side of a stage-30 embryo injected in the left side with *FoxF1* RNA immunostained with 12/101 antibodies that recognize somatic mesoderm. The left side of the embryo shows a significant reduction of this marker. (G,H) *FoxF1* RNA can rescue axis duplication caused by the injection of dominant-negative BMP receptor (DNBR). (G) Embryos injected with DNBR RNA (1.5 ng) showing axis duplications. (H) Embryos injected with DNBR and *FoxF1* RNA (1.5 ng, 1.25 ng) demonstrate that *FoxF1* RNA can rescue the DNBR phenotype.

dominant negative FGF receptor 1 (Saint-Jeannet et al., 1994), we examined whether FGF can also activate this gene. As can be seen in Fig. 6B, *FoxF1* could be activated in animal caps by FGF as well. All these experiments suggest that the regulation of *FoxF1* expression is complex and that there might be more than one way of activating this gene. In order to shed more light on the regulation of FoxF1, we decided to identify the cis-regulatory sequences that are responsible for the correct temporal-spatial expression of this gene.

FoxF1 regulatory elements are located in the 5′ upstream region of the gene

Since the *FoxF1* gene must contain regulatory sequences that direct gene expression into the lateral plate mesoderm, we used transgenic frog embryos to identify the segment of *FoxF1* DNA that is responsible for the temporal-spatial regulation of *FoxF1* expression. For this purpose, we isolated a genomic clone of *FoxF1* and, using GFP or LacZ as a reporter, we delineated the sequences that can direct gene expression into the lateral plate mesoderm. We found that a 2 kb DNA fragment upstream of the 5′ end of the transcription start site of *FoxF1* can direct GFP expression into the lateral plate mesoderm of *Xenopus* tadpoles (Fig. 7A-C). When performing these experiments, we found that while the monitoring of the GFP fluorescence was informative, it was difficult to evaluate GFP expression in sections as the autofluorescence of the yolk made the evaluation of GFP fluorescence unreliable. For this reason, we performed whole-mount in-situ hybridization on transgenic embryos with an anti-GFP digoxigenin-labeled probe. This eliminated the problem with autofluorescence and allowed visualization of GFP expression in sections (Fig. 7D,E). The levels of GFP RNA varied, but in general they were limited to the lateral plate mesoderm (Fig. 7D). In a significant portion of embryos, the expression was only on one side of the embryo (Fig. 7E). We are currently investigating whether this expression pattern is due to a delayed integration of the transgene or due to the fact that the transgene contains sequences that bias gene expression toward unilateral expression. To further refine this promoter, we also used LacZ as a reporter system. The expression pattern of LacZ was similar to GFP, but the expression was somewhat more mosaic (Fig. 7G). However, the appearance of mosaicism is enhanced by the fact that the nuclear localization signal of the LacZ reporter construct (Fig. 7F) directs the protein into the nuclei. Nevertheless, all these experiments show that the 2 kb upstream fragment of the *FoxF1* contained the necessary sequences to direct gene expression into the developing lateral plate mesoderm. Since we have shown in previous experiments that *FoxF1* might be regulated by BMP4 signaling, we investigated whether this LacZ reporter construct is responsive to BMP4 induction. As can be seen in Fig. 7I, animal caps injected with this construct and BMP4 RNA display significant expression of the LacZ reporter. This is in contrast to the animal caps injected with the construct alone (Fig. 7H). This shows that the 2 kb construct contains sequences that mediate BMP4 signaling.

Discussion

Role of FoxF genes in visceral mesoderm development

Organogenesis of the gut requires a well-orchestrated expression of genes in the developing visceral mesoderm and gut endoderm. The developmental controls that govern the coordinate proliferation and differentiation of these two tissues are not well understood. One of the genes that plays a critical role in development of the *Xenopus* lateral plate/visceral mesoderm is the Fox gene *FoxF1*. *FoxF1* is initially expressed in the lateral plate mesoderm and later in the visceral mesoderm surrounding the gut. The expression of *FoxF1* is of functional importance, as we find that interference with the translation of this gene using *FoxF1* morpholino results in

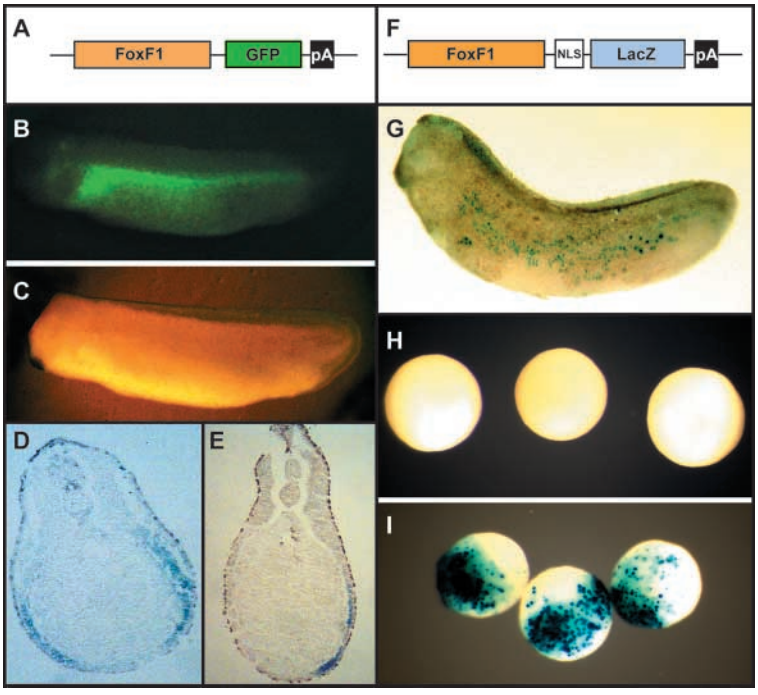

Fig. 7. 5′ upstream regulatory sequences of *FoxF1* direct gene expression into the lateral plate mesoderm and are responsive to BMP4 induction. (A) Diagram of a GFP reporter construct used to map the regulatory sequences of *FoxF1* in transgenic embryos. (B) A transgenic *Xenopus* tadpole displaying GFP fluorescence in the ventral half of the body. (C) Same embryo in transmitted light. (D) A cross-section of the tadpole hybridized with digoxigenin labeled anti-GFP probe showing expression of the GFP RNA in the lateral plate mesoderm. (E) A cross-section of the embryo hybridized with digoxigenin labeled anti-GFP probe showing expression of the GFP RNA on one side of the embryo. (F) Diagram of a LacZ reporter construct used to map the regulatory sequences of *FoxF1* in transgenic embryos. (G) A transgenic *Xenopus* embryo displaying LacZ staining in the ventral half of the body. (H) The pBS-FoxF1-NLS-LacZ reporter construct does not show any expression of LacZ in cultured animal caps from injected embryos but shows high levels of expression when co-injected with BMP4 RNA (I).

abnormal development of visceral mesoderm, which in turn leads to abnormal gut development. The phenotype of *FoxF1* knockdown embryos is concentration dependent. When a low concentration of *FoxF1* morpholino is injected into embryos, the visceral mesoderm does not undergo proper cytodifferentiation. Consequently, the gut does not elongate and coil. The underlying reason for this morphologically abnormal gut development is the lack of normal differentiation and proliferation of the lateral plate/visceral mesoderm. During normal development the gut endoderm recruits the splanchnic mesoderm to form a single layer of smooth muscle cells around the gut. In *FoxF1* morpholino-injected embryos, the lateral plate mesoderm does not proliferate properly and is not able to generate enough cells to create an intact mesodermal layer around the gut endoderm. This unfavorable situation is further exacerbated by simultaneous abnormal differentiation of the visceral mesoderm in these *FoxF1* morpholino-injected embryos. While the visceral mesoderm is present, stage-specific differentiation markers are not activated. The smooth muscle cells do not differentiate properly and the physical integrity of the gut mesoderm is compromised. This mesoderm cannot expand and as a result the gut does not elongate and coil.

At high concentrations of *FoxF1* morpholino, the visceral mesoderm surrounding the gut loses its physical integrity completely and the yolky endodermal cells spill into the body cavity. Eventually the entire ventral body cavity will lyse, which might indicate that the somatic mesoderm is also deficient in cell numbers as a result of reduced proliferation in the lateral plate mesoderm during neurulation.

When one compares *Xenopus FoxF1* with its murine ortholog, several similarities can be found. In addition to the structural conservation between these two genes, *Xenopus* and murine *Foxf1* have a similar expression pattern. In both species *Foxf1* is expressed in the lateral plate/visceral mesoderm. In the mouse, additional high levels of *Foxf1* transcription can be found in the extra-embryonic mesoderm-derived structures – amnion, allantois and yolk sack – which are not found in *Xenopus*. These two genes also show high levels of functional conservation. In both species the formation of visceral mesoderm is strongly affected by the expression of *FoxF1*. A lower rate of proliferation is present in the lateral plate mesoderm of *FoxF1* morpholino-treated *Xenopus* embryos as well as in *Foxf1*–/– mouse embryos (Mahlapuu et al., 2001b). Unfortunately, the *Foxf1*–/– mice die and degenerate before gut organogenesis begins, and therefore they do not provide us with information about the role of *Foxf1* in the murine gut development. The haploinsufficient *Foxf1*+/– mice, which presumably have 50% of the *Foxf1* activity intact, suffer from high perinatal mortality, partly caused by constriction of the esophagus, and provide information limited to foregut malformations (Mahlapuu et al., 2001a). However, the abnormalities observed in development of the allantois in *Foxf1* knockout mouse embryos are in many respects similar to those present in the development of *Xenopus* gut mesoderm. Allantois is a mesodermal structure that during normal development elongates and fuses with the chorion. In *Foxf1*–/– embryos, allantois does not differentiate and expands properly, and consequently does not reach the chorion. A proper placentation does not take place and the mutant embryos die (Mahlapuu et al., 2001b).

While *Foxf1* is the best-studied Fox gene in the development of the visceral mesoderm, a functional loss of the Fox gene *Foxl1* (*Fkh6*) has also been shown to result in detrimental effects on the development of the gastrointestinal epithelium (Kaestner et al., 1997). *Foxl1* is expressed in the visceral mesoderm and its function is required for regulating normal gastrointestinal proliferation and differentiation. *Foxl1*–/– mice show severe abnormalities of gut development including changes in the endoderm-derived gut epithelium. Therefore the likely role of *Foxl1* resides in the interaction between mesenchyme and epithelial cells. The putative *Xenopus* ortholog of *Foxl1*, *FoxL1 (XFD-8)*, has been isolated, but no expression of this gene was detected in embryonic stages (Lef et al., 1996). This might be because *FoxL1* has no function in embryonic development or because low levels of expression make the detection of its transcripts difficult.

Interestingly, the critical role of the FoxF gene family in the development of visceral mesoderm extends also to invertebrates. The *Drosophila* gene *biniou* (*FoxF*), which is the fly homolog of *FoxF1*, has an important function in the development of the *Drosophila* midgut (Zaffran et al., 2001). *bin* is activated in the *Drosophila* trunk visceral mesoderm primordia and has a critical role during specification and differentiation of the trunk visceral mesoderm. In its absence, the differentiation of the splanchnic mesoderm into midgut musculature is impaired. Taken together, all these data indicate that the FoxF family is an ancient gene family that has a conserved role in the visceral mesoderm development in vertebrates and invertebrates.

Regulation of *FoxF1*

Our experiments suggest that *Xenopus FoxF1* is a target and a mediator of BMP4 signaling. Several pieces of evidence support this conclusion. First, BMP4 RNA could induce expression of *FoxF1* in animal caps. Second, injection of *FoxF1* RNA into *Xenopus* embryos ventralized embryos, mimicking the action of BMP4. Finally, *FoxF1* RNA could rescue dorsalized embryos resulting from the injection of dominant negative BMP4 receptor. This BMP4 regulation of *FoxF1* is likely to reflect an evolutionarily conserved pathway, as *biniou* in the *Drosophila* visceral mesoderm is dependent on *dpp*, the fly homolog of BMP4 (Frasch, 1995). However, it is unlikely that the BMP4 pathway is the only avenue for regulating the expression of *FoxF1* in vertebrates. First, expression of *Foxf1* is largely unaffected in BMP4 knockout mouse embryos (Fujiwara et al., 2002). While it is possible that other BMP proteins compensate for the lack of BMP4 protein in these knockout embryos, there are several experiments suggesting that FGF signaling is also involved in the formation of the visceral mesoderm. Saint-Jeannet et al. (Saint-Jeannet et al., 1994) demonstrated that *Xenopus* embryos injected with RNA encoding the dominant-negative FGF receptor1 do not develop visceral mesoderm and do not activate α-smooth muscle actin expression. This is very similar to our observations of *FoxF1* morpholino-treated embryos. FGF signaling is also involved in the formation of the *Drosophila* visceral mesoderm, as the mesoderm-specific FGF receptor *DFR1/Heartless* is required for the formation of the visceral muscles (Shishido et al., 1997).

FoxF1 can also be activated by *Xbra*. While *Xbra* is expressed primarily in the notochord of neurula-stage

vertebrate embryos, during gastrulation it is expressed in the blastopore equivalent of bilateria. In *Xenopus* this means that the expression is surrounding the entire yolk plug (Wacker et al., 2004), making it possible that *Xbra* is involved in the regulation of *FoxF1*. Regulation of *FoxF1* by *Xbra* would not be entirely surprising, as the *Drosophila Brachyury* (*byn*) is necessary for the hindgut formation (for a review, see Lengyel and Iwaki, 2002). It is not clear at present to which degree these different pathways are used for the regulation of expression of *FoxF1* and its orthologs in different species. It is likely that the relative contribution of each pathway to the regulation of *FoxF1* will be species dependent. For instance, the evolutionary divergence of *FoxF1* regulation can be demonstrated using the example of regulatory interactions between *Drosophila bin* and *bap* and *Xenopus FoxF1* and *Xbap*. While the *Drosophila biniou* is activated by *bap*, this does not appear to be the case in *Xenopus*. This is because the timing of expression of *FoxF1/bin* and *Xbap/bap* has changed during evolution. While *Drosophila bap* is transcribed in the visceral mesoderm almost simultaneously with *bin* (Zaffran et al., 2001), *Xenopus bap* is activated several hours after *FoxF1* and therefore cannot be involved in the initiation of *FoxF1* transcription.

Further analysis of *FoxF1* regulation was facilitated by the finding that the 2 kb upstream region of this gene is responsive to BMP4 signaling. While a computer search for potential protein-binding sites did not detect any BMP4 responsive elements (BRE), we discovered a potential *Vent-2* binding site. *Vent-1/PV.1* (Gawantka et al., 1995; Ault et al., 1996) and *Vent-2* (Onichtchouk et al., 1996) proteins belong to the group of homeodomain proteins that mediate BMP4 signaling in the formation of ventral mesoderm. While this finding sheds some light on the potential regulatory mechanism of BMP4 signaling, the computer analysis of this fragment also locates a potential *Brachyury*-binding site. This site could account for the inducibility of *FoxF1* by *Xbra*. In addition, this fragment of DNA contains potential binding sites for several transcription factors that are known to be involved in gut formation. There are potential binding sites for GATA1-3, for the intestine-specific homeodomain factor CDX-1 and the mammalian caudal-related intestinal transcription factor Cdx-2 and a binding site for HNF3beta. A detailed experimental analysis of these potential protein-binding sites will have to be performed before a meaningful credibility can be assigned to any of these sites.

In summary, analysis of *FoxF1* genes in *Drosophila*, *Xenopus* and mouse leads to a unified picture in which this ancient gene was initially involved in the proliferation and differentiation of the visceral mesoderm. In the absence of its function, normal development of the visceral mesoderm cannot take place. In vertebrates, the lack of *FoxF1* leads to a variable phenotype depending on the peculiarities of the development of the given species. In mammals, where this gene is involved in the formation of extra-embryonic support structures, the lack of *FoxF1* gene product leads to early embryonic death because of the failure of these structures to provide essential support to the embryo proper. In *Xenopus* embryos, which do not have any extra-embryonic structures, embryos display abnormal formation of smooth muscles around the gut. How exactly *FoxF1* regulates proliferation and differentiation is not clear, but it is possible that it belongs to a group of proteins that are able to couple these two regulatory functions, such as the Hox and Six proteins (Del Bene et al., 2004; Luo et al., 2004).

Last, but not least, we have isolated the *FoxF1* regulatory sequences that are able to direct gene expression into the developing lateral plate mesoderm. These sequences are located in the 5′ upstream region of the gene. Isolation of sequences that specifically direct gene expression into the developing gut mesoderm will allow us specifically to alter gene expression in the gut mesoderm and monitor the morphological, physiological and molecular consequences of this altered gene expression in the gut mesoderm and endoderm. This is of extreme importance, as the lateral plate mesoderm, and later the visceral mesoderm, have an instructive role in the regionalization and proper development of the gut endoderm. This will make *Xenopus* uniquely suited for studying epithelial–mesenchymal interactions during gut development.

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