Development 127, 4345-4360 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV1587

A role for GATA5 in *Xenopus* endoderm specification

Heike Weber, Carole E. Symes, Maggie E. Walmsley, Adam R. F. Rodaway and Roger K. Patient*,‡

Developmental Biology Research Centre, The Randall Institute, King's College London, 26-29 Drury Lane, London WC2B 5RL, UK

*Present address: Institute of Genetics, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, UK

‡Author for correspondence (e-mail: roger.patient@nottingham.ac.uk)

Accepted 4 August; published on WWW 26 September 2000

SUMMARY

The endoderm gives rise to the gut and tissues that develop as outgrowths of the gut tube, including the lungs, liver and pancreas. Here we show that GATA5, a zinc-finger transcription factor, is expressed in the yolk-rich vegetal cells of *Xenopus* embryos from the early gastrula stage onwards, when these cells become committed to form endoderm. At mid-gastrula stages, GATA5 is restricted to the sub-blastoporal endoderm and is the first molecular marker for this subset of endodermal cells so far identified. We show that GATA4 and GATA5 are potent inducers of endodermal marker genes in animal cap assays, while other GATA factors induce these genes only weakly, if at all. When injected into the dorsal marginal zone, GATA5 respecifies prospective mesoderm towards an endodermal fate, thereby disrupting the convergence and extension movements normally undergone by the dorsal mesoderm. The resulting phenotype is very similar to those seen after injection of dominant negative versions of the FGF-receptor or the T-box transcription factor, Xbra and can be rescued by eFGF. The ability of GATA5 to respecify ectodermal and mesodermal cells towards endoderm suggests an important role for GATA5 in the formation of this germlayer. In animal cap assays, GATA5 is induced by concentrations of activin above those known to induce dorsal mesoderm and heart, in an FGF-independent manner. These data indicate that the emerging view for endodermal induction in general, namely that it is specified by high levels of TGF-β in the absence of FGF signalling, is specifically true for sub-blastoporal endoderm.

Key words: Endoderm, *Xenopus*, GATA transcription factors

INTRODUCTION

Of the three germ layers that emerge during gastrulation, the endoderm gives rise to the lining of the lungs and the digestive tract as well as associated organs, like liver and pancreas. These organs are elaborated from the definitive gut tube, which in turn is an elaboration of a primitive gut tube called the archenteron. In *Xenopus*, at the early gastrula stage, three morphologically distinct populations of endodermal cells can be distinguished that contribute to the lining of the archenteron (Keller, 1975, 1976). The bottle cells, which are already invaginated at the dorsal side of the embryo, will form the lining of the anterior tip of the archenteron, giving rise to pharyngeal endoderm and foregut (Keller, 1981; Hardin and Keller 1988). It has been suggested that during gastrulation, these cells migrate together with the dorsoanterior mesoderm across the roof of the blastocoel, forming the leading edge of the involuting mesendoderm. The supra-blastoporal endoderm, a layer of cells superficial to the mesoderm at the beginning of gastrulation, will form the roof of the archenteron. On the dorsal side of the embryo, the supra-blastoporal endoderm participates in the strong convergence and extension movements, undergone by the overlying axial mesoderm after involution. The big yolk-rich cells of the vegetal pole, the sub-blastoporal endoderm, will form the floor of the archenteron. These cells were previously thought to be moved passively into the interior of the embryo. However, recent results establish vegetal pole cells as the main driving force for the internalisation of the mesendoderm at the beginning of gastrulation (Winklbauer and Schurfeld 1999). When gastrulation proceeds, lateral and ventral bottle cells and supra-blastoporal endoderm invaginate in the same manner as the dorsal cells and give rise to the sides of the archenteron.

Vegetal pole cells become committed to form endoderm by the early gastrula, when they can no longer contribute to other germ layers, if transplanted into the blastocoel of a host embryo (Heasman et al., 1984; Wylie et al., 1987). It is of significant interest to understand the molecular basis of this commitment and the regulatory mechanisms involved in its establishment. Therefore early molecular markers, which are restricted to the endoderm, are an important tool. IFABP (intestinal fatty acid binding protein), Xlhbox8 and the 4G6 antigen are endodermal differentiation markers later in development (Shi and Hayes, 1994; Gamer and Wright 1995; Jones et al., 1993), but the expression patterns of these genes before tailbud stages are unknown. Endodermin, a protease inhibitor, is expressed throughout the endoderm, but expression becomes endoderm-specific only in hatched larvae (stage 30; Sasai et al., 1996). Sox17α, Sox17β and *Mixer* are transcription factors that are

specifically expressed in the endoderm from the late blastula/early gastrula stages onwards (Hudson et al., 1997; Henry and Melton, 1998). A similar expression pattern was recently described for Xenf1, a nuclear protein of unknown function (Nakatani et al, 2000). While Sox17α and β are uniformly distributed, *Mixer* is predominantly expressed at the endoderm/mesoderm boundary, an expression pattern very similar to the related factors *Mix.1* and *Milk* at mid-gastrula stages (Lemaire et al., 1998; Ecochard et al., 1998). Bix1-4 are expressed in endoderm and mesoderm throughout gastrulation (Tada et al., 1998; Casey et al., 1999).

All endoderm specific genes can be induced by activin or Vg1 in *Xenopus* animal cap assays, suggesting an important role for members of the transforming growth factor β (TGFβ) family in endodermal specification (Gamer and Wright, 1995; Henry et al., 1996; Rosa, 1989; Sasai et al., 1996; Hudson et al., 1997; Henry and Melton, 1998; Ecochard et al., 1998). In addition, a dominant negative activin receptor (Hemmati-Brivanlou and Melton, 1992) and a mutant Vg1 ligand inhibit endogenous endodermal marker gene expression in vegetal pole explants (Gamer and Wright, 1995; Henry et al., 1996; Joseph and Melton, 1998), and a constitutively active TGFβ receptor type I subunit (TARAM-A*) converts zebrafish blastomeres to an endodermal fate (Peyrieras et al., 1998). Recent data in zebrafish and *Xenopus* suggest that the endogenous TGFβ molecule might be a be a nodal-related protein. In zebrafish doubly mutant for the nodal-related genes *squint* and *cyclops*, endoderm (and mesoderm) differentiation is disrupted (Sampath et al., 1998; Rebagliati et al., 1998; Feldman et al., 1998; Rodaway et al., 1999; Alexander and Stainier, 1999). In *Xenopus* the nodal-related proteins Xnr1, 2 and 4 are expressed in vegetal pole cells from the late blastula onwards, and all three have been shown to induce endodermal markers in animal cap assays (Yasuo et al., 1999; Clements et al., 1999). Another TGFβ molecule with endoderm-inducing abilities is derriere (Sun et al., 1999). Besides members of the TGFβ family, the T-box transcription factor VegT has been implicated in endodermal specification. VegT mRNA is localised in the vegetal cortex of the *Xenopus* egg and early embryo and depletion of the maternal message, using antisense oligonucleotides, disrupts endodermal differentiation (Zhang et al., 1998). Recent results demonstrate that VegT generates endoderm cell-autonomously and by generating TGFβ signals that reinforce endodermal differentiation (Yasuo et al., 1999; Clements et al., 1999). Endodermal markers can be rescued in VegT-depleted embryos by Bix.4 (Casey et al., 1999). Another maternal protein with endoderm-inducing activity is Bicaudal-C, a putative RNA-binding molecule (Wessely and De Robertis, 2000).

GATA factors are zinc-finger transcription factors that bind to a consensus DNA sequence, T/A(GATA)A/G, from which they derive their name. So far six family members have been characterised in vertebrates, which fall into two subfamilies. The members of the first subfamily, GATA1, 2 and 3, have been implicated in the specification of blood cell lineages, ventral mesoderm and non-neural ectoderm (Pandolfi et al., 1995; Pevny et al., 1991; Tsai et al., 1994; Sykes et al., 1998; Read et al., 1998). Members of the second subfamily, GATA4, 5 and 6, are expressed in endodermal derivatives and the heart, an expression pattern that is highly conserved in all vertebrate species so far analysed (Jiang and Evans, 1996; Gove et al., 1997; Laverriere et al., 1994; Morrisey et al., 1997; Tamura et al., 1993; Huggon et al., 1997).

In *Drosophila* and *Caenorhabditis elegans*, GATA homologues have been shown to be essential for endodermal specification (Reuter, 1994; Zhu et al., 1997; Shoichet et al, 2000). In vertebrates, the roles of GATA4, 5 and 6 in endodermal specification are less clear. In the mouse, expression of GATA4 and GATA6 is seen in the visceral and parietal endoderm of primitive streak embryos (Morrisey et al., 1997). Embryoid bodies derived from GATA4$^{-/-}$ or GATA6$^{-/-}$ embryonic stem cells lack visceral endoderm differentiation, demonstrating an important role for GATA4 and 6 in vitro (Morrisey et al., 1998; Soudais et al., 1995). However, GATA4$^{-/-}$ embryos form morphologically normal visceral endoderm, which expresses elevated levels of GATA6, suggesting that in vivo GATA6 may compensate for GATA4 function (Molkentin et al., 1997; Kuo et al., 1997). In GATA6$^{-/-}$ embryos, either partial loss of visceral endoderm (Koutsourakis et al., 1999) or intact visceral endoderm lacking the expression of endodermal differentiation markers (Morrisey et al., 1998) have been described. Recent in vivo footprinting studies have shown that GATA-binding sites are occupied on a silent gene in endoderm that has the potential to be activated solely in this germ layer (Zaret, 1999). In *Xenopus*, mRNAs for GATA4, 5 and 6 are abundant at the early gastrula stage (Jiang and Evans, 1996) and dissection analysis reveals GATA5 expression in vegetal pole cells (Kelley et al., 1993 – note that GATA5 was named GATA4 in this study), which are fated to form endoderm (Dale and Slack, 1987).

In this study, we present a detailed analysis of GATA5 expression during blastula and gastrula stages, when the endoderm becomes specified (Wylie et al., 1987). We show that GATA5 is restricted to the sub-blastoporal endoderm during mid-gastrula stages, making it a useful marker to study the regulatory mechanisms that are involved in the specification of this type of endoderm. We further demonstrate that GATA4 and 5, but not GATA6, are potent inducers of early endodermal marker genes in animal cap assays, and change the fate of prospective ectoderm and mesoderm towards endoderm. This suggests an important role for these factors in establishment or maintenance of endodermal specification during development.

MATERIALS AND METHODS

Expression constructs, RNA synthesis and in vitro translation

Expression constructs for GATA1 and GATA6 have previously been described (Gove et al., 1997). pSP64T-XGATA2a was constructed by inserting a 1.3 kb *Bam*H1 fragment of GATA2 (Zon et al., 1991) into *Bgl* II sites of pSP64T (Krieg and Melton, 1984). An *Eco*RI site within the GATA2 open reading frame was disrupted to allow linearisation with *Eco*RI before SP6 transcription. pβUT-XGATA3 was constructed by inserting a 1.9 kb *Spe*I/*Hin*cII fragment of GATA3 (Zon et al., 1991) into *Xba*I/*Hin*cII sites of pβUT-2 (Sykes et al., 1998). For RNA synthesis, the plasmid was *Eco*RI-linearised and T3-transcribed. pβUT-XGATA5 was constructed by amplifying GATA5 cDNA (Jiang and Evans, 1996) using GGCTCTAGAGTAGCACCGGATC-ATGTAC (forward) and CCGCTCGAGAGGCAAGTGCCAGCGCG (reverse) primers to create *Xba*I and *Xho*I sites, which were used to insert the fragment into corresponding sites of pβUT-2. Linearisation

and transcription were performed as described earlier for pβUT-XGATA3. PT7TS-XGATA4 was constructed by inserting an *Eco*RI/*Bsp*EI fragment (filled in) of GATA4 (Jiang and Evans, 1996) into *Eco*RI and *Eco*RV sites of pT7TS (R1+). For RNA synthesis the plasmid was *Sma*I-linearised and T7-transcribed. Plasmids for eFGF, BMP4, XFD, d50, dnXAR and XMyf5 RNA synthesis were published previously (Isaacs et al., 1994; Neave et al., 1997; Amaya et al., 1991; Rodaway et al., 1999; Hopwood et al., 1991). RNA for microinjection was synthesised using T3, SP6 or T7 RNA polymerase megascript kits (Ambion), according to the manufacturer's instructions with cap analogue added to a final concentration of 0.5 mM. The transcripts were isopropanol precipitated twice, to remove unincorporated nucleotides more efficiently. Transcripts were translated in vitro using a rabbit reticulocyte lysate kit (Promega) following the manufacturer's instructions. ^{35}S-Methionine was included in the reaction and translation products were visualised after SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) by autoradiography.

Embryo manipulations

Embryos were obtained and cultured as described previously (Gove et al., 1997) and staged according to Nieuwkoop and Faber (1967). Capped mRNA was injected in a volume of 4 nl per blastomere. Animal caps were dissected at stage 9 and cultured in 1 × MBS (Gurdon and Wickens, 1983) in petri dishes coated with 1% agarose. For activin treatment (human recombinant activin A; kindly provided by Dr Yuzuru Eto, Central Research Laboratory, Ajinomoto Co., Kawasaki, Japan), 0.1% BSA was added. Induction of GATA5 by activin was only observed when large animal caps were dissected (diameter about 0.8 mm). Explants of this size elongated strongly when treated with 10 or 100 ng/ml activin. Thus, the induction of GATA5 as well as the elongation of animal caps might be favoured by molecules located closer to the marginal zone.

In situ hybridisation

Whole-mount in situ hybridisation was performed as previously described (Bertwistle et al., 1996) using BM Purple (Boehringer) as the alkaline phosphatase substrate. As a lineage tracer 250 pg RNA coding for nuclear localised β-galactosidase (Smith and Harland, 1991) was injected and activity was detected as in Griffin et al. (1995). 80 μm vibratome sections were cut as previously described (Gove et al., 1997).

For in situ hybridisations on sections, embryos were fixed according to the whole-mount protocol and embedded in 98% Histoplast Wax/2% Beeswax after washing them for 20 minutes in ethanol twice, 30 minutes in xylene twice, 30 minutes and 1 hour in the melted wax at 60°C. 20 μm paraffin sections were mounted on APES (3-Aminopropyltriethoxy-silane)-coated slides and in situ hybridisations were performed on sections according to a protocol previously described for mouse whole-mount in situ hybridisations (Wilkinson, 1992). The protocol was modified to omit the RNAse digestion, to block nonspecific antibody binding with 2% blocking reagent in maleic acid buffer and to develop the signal using BM-Purple (Boehringer Mannheim). Incubations were performed in coplin jars except for the proteinase K digestion, refixation, prehybridisation, hybridisation, antibody incubation and colour-reaction, which were performed on the slide in a humidified chamber. During the hybridisation steps and colour-reaction the slides were covered with parafilm to prevent condensation. The antisense probes have been previously described: cerberus (Bouwmeester et al., 1996); goosecoid (Sykes et al., 1998); Sox17α (Hudson et al., 1997); GATA5 (Jiang and Evans, 1996); chordin (Sasai et al., 1994); Xbra (Smith et al., 1991); cardiac actin (Mohun et al., 1988). The GATA5 containing vector was cut with *Sma*I to obtain a full-length probe. Specificity versus GATA4 and GATA6 was demonstrated by comparison of expression patterns: all three genes have unique expression patterns at gastrula, neurula and larval stages (data not shown).

RT-PCR analysis

Embryos or explants were snap frozen in dry ice in a minimum volume of buffer and stored at –70°C. RNA from 5 embryos or 15 animal caps was extracted as described by Sambrook et al. (1989) in 400 μl homogenisation buffer, containing 10 μg tRNA carrier, omitting the LiCl precipitation step. RNA was resuspended in 50 μl water containing 10 μg tRNA carrier, and genomic DNA was digested in a volume of 100 μl using 1 unit RQ1 DNAase (Promega) in 1 × transcription buffer (Promega), 10 mM DTT, in the presence of 40 units RNasin (Promega) for 1 hour at 37°C. RNA was purified by phenol/chloroform and choroform extractions, and ethanol precipitation. For reverse transcription, 1.5 μg RNA was denatured at 65°C for 3 minutes and cooled on ice. Reverse transcription reactions (20 μl) contained a final volume of 1mM dNTPs, 2.5 mM DTT, 200 ng/μl random 10mer primers, 20 units RNasin and 5 units M-MLV RT in 1 × RT buffer (Gibco BRL). After incubation for 90 minutes at 42°C, the reactions were diluted to 50 μl and terminated by boiling for 5 minutes. RT reactions were linear, as using twice the amount of RNA resulted in a twofold increased signal after amplification (data not shown). PCR reactions in a 12.5 μl volume contained 1-3 μl RT reaction and a final concentration of 1 μM primers, 1.5 μM $MgCl_2$, 0.2 mM dNTPs, 0.25 μCi α^{32}P-dCTP and 0.625 units Goldstar Taq polymerase (Perkin Elmer) in 1 × Goldstar buffer. PCRs were performed in Gene Amp PCR system 2400 (Perkin Elmer). After a 15 minutes initial denaturation, PCR conditions were 30 seconds at 94°C, 1 minute at 55°C and 1 minute at 72°C for all used primers. Ornithine decarboxylase (ODC) was amplified in the same tube as the tested markers, by adding ODC primers after the relevant number of cycles. Samples were resolved on 6% polyacrylamide gels, monitored by autoradiography and quantified by phosphorimager analysis.

Primers and cycle numbers were GATA5 (Jiang and Evans, 1996) 25 cycles; Sox17α (Hudson et al., 1997) 22-25 cycles; HNF1β (Hudson et al., 1997) 29 cycles; Xbra (Henry et al., 1996) 25 cycles; chordin AACTGCCAGTGGATGGT (forward) GGCAGGATTTA-GAGTTGCTTC (reverse) 22 cycles; epidermal cytokeratin (Henry et al., 1996) 23 cycles; Xlhbox8 (Hudson et al., 1997) 27 cycles; IFABP (Hudson et al., 1997) 29 cycles; MyoD (Rupp and Weintraub, 1991, positions 662-952) 27 cycles; ODC 5′-TGTTGCCTCATCTTTC-ACCC (forward), 5′-CAAGTTCCATTCCGCTCTCC (reverse) 19 cycles.

RESULTS

GATA5 is expressed in prospective endoderm during gastrulation and in ventrolateral domains of the embryo at the early tailbud stage

Low amounts of GATA5 mRNA are detectable in the *Xenopus* egg, and transcript levels increase dramatically during gastrulation (Jiang and Evans, 1996). However, analysis of the expression pattern of GATA5 has so far been restricted to later stages, focussing on the role of GATA5 in heart differentiation (Kelley et al., 1993; Jiang and Evans, 1996). To define the expression pattern of GATA5 during blastula and gastrula stages, in situ hybridisation on whole mounts as well as on paraffin sections was performed. At the blastula stage (st. 9), no GATA5-specific staining in vegetal pole cells is obvious by whole-mount in situ hybridisation or on sections (Fig. 1A,B). In the early gastrula (st. 10.25), the first superficial staining is visible in embryos viewed from the vegetal pole in yolk-rich cells close to the dorsal blastopore lip (Fig. 1C, arrow). In situ hybridisation on sections of this stage reveal expression of GATA5 throughout the yolk-rich cells of the inner cell mass, with gradually stronger staining towards the floor of the

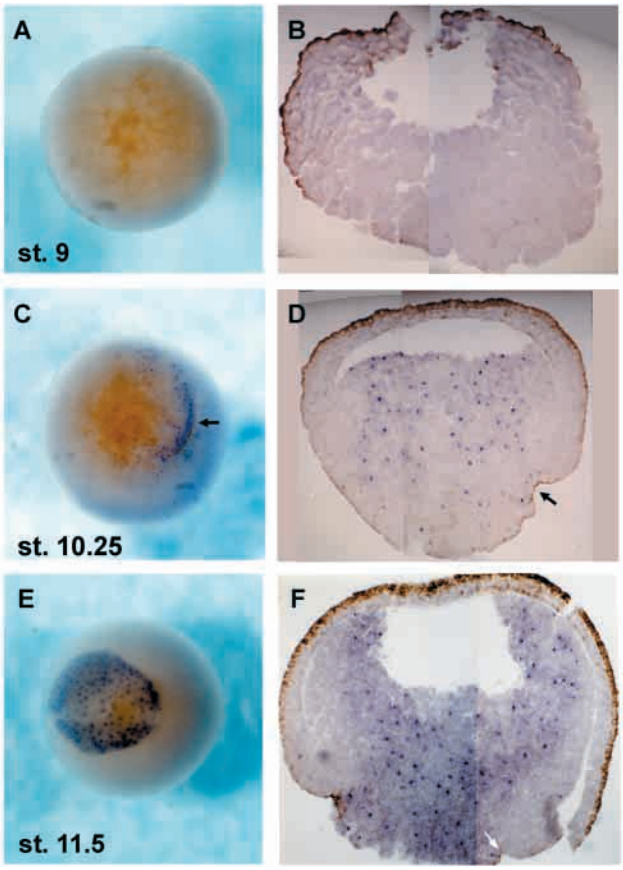

Fig. 1. GATA5 is expressed in prospective endoderm during *Xenopus* gastrulation. Whole-mount in situ hybridisations of embryos viewed from the vegetal pole (A,C,E) and in situ hybridisations on sections of corresponding stages (B,D,F), using a GATA5-specific probe. No GATA5-specific staining is obvious in blastula stage (st. 9) embryos (A,B). At the early gastrula stage (st. 10.25), staining is seen in bottle cells and in the sub-blastoporal endoderm (C,D). In the advanced gastrula (st. 11.5), GATA5 accumulates in the sub-blastoporal endoderm and appears to be excluded from the involuting mesendoderm at the dorsal and ventral side of the embryo (E,F). Arrows in C,D,F mark the dorsal blastoporal lip.

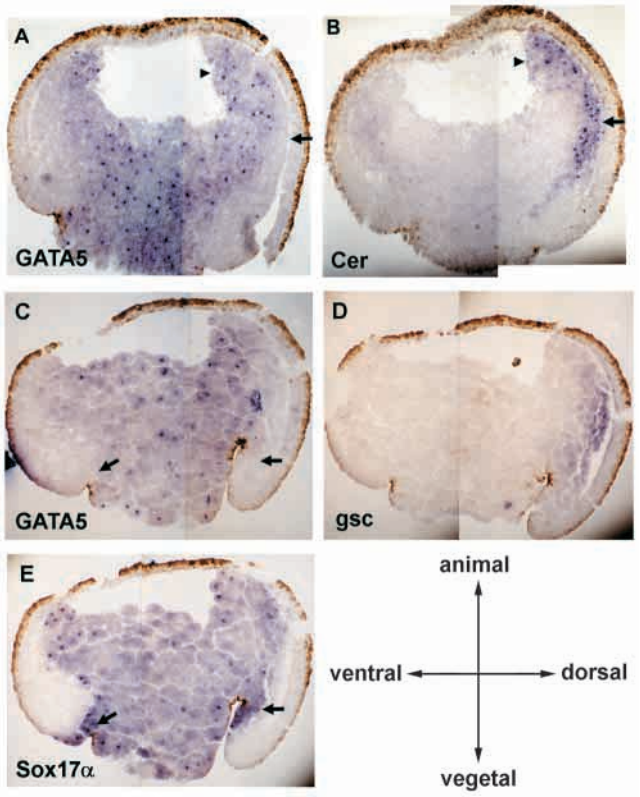

Fig. 2. GATA5 is restricted to the sub-blastoporal endoderm at mid-gastrula stages. In situ hybridisation of serial sections of stage 11.5 embryos using GATA5 (A) and cerberus (B) or stage 11 embryos using GATA5- (C), goosecoid- (D) and Sox17α- (E) specific probes. GATA5 overlaps with cerberus in the dorsoanterior yolk-rich cells (arrowheads), but not in more posterior located smaller cells, which appear to represent the leading edge of the involuting mesendoderm (arrows in A,B). GATA5 is excluded from prechordal plate mesendoderm stained by goosecoid and the supra-blastoporal endoderm, which is strongly stained by Sox17α (arrows in C,E).

blastocoel (Fig. 1D). The dorsal bottle cells, which are invaginated at this stage, also express GATA5 (Fig. 1D, arrow). As gastrulation proceeds, GATA5 message accumulates and is seen throughout the yolk-plug of embryos viewed from the vegetal pole and throughout the inner cell mass on sections (Fig. 1E,F), but appears to be excluded from the dorsally (to the right of the arrow) and ventrally involuting mesendoderm.

To further define the population of GATA5-expressing cells, serial sections of mid-gastrula embryos (st. 11.5) were stained for GATA5 and various endoderm- or mesoderm-specific markers. At this stage, expression of cerberus, a marker for dorsoanterior mesendoderm (Bouwmeester et al., 1996) overlapped with GATA5 in the most anterior dorsal yolk-rich cells (compare Fig. 2A with 2B, arrowheads). These cells, which are part of the sub-blastoporal endoderm, originate from the floor of the blastocoel and move ahead of the anterior margin of the involuting mesendoderm (Keller, 1976). They come to lie close to the liver primordium during later development. A population of more-posterior cerberus-expressing cells, located close to Brachet's cleft, did not express GATA5 (compare Fig. 2A with 2B, arrows). These cells can be easily distinguished from the sub-blastoporal endoderm because of their smaller size, and they appear to represent the leading edge of the involuting mesendoderm, giving rise to prechordal plate (dorsal) and heart mesoderm (dorsolateral), and to pharyngeal and head endoderm (Keller, 1976). In agreement with this, GATA5 does not overlap with the main expression domain of goosecoid, a marker for prospective prechordal plate (Cho et al., 1991) compare Fig. 2C with 2D). GATA5 is also excluded from more posterior mesoderm stained by chordin (Sasai et al., 1994) and Xbra (Smith et al., 1991) (data not shown) and from the supra-blastoporal endoderm, which is strongly stained by Sox17α (arrows in Fig. 2E, compare with C), a panendodermal marker gene (Hudson et al., 1997). Thus, expression of GATA5 at the mid-gastrula stage is restricted to the sub-blastoporal endoderm, which is characterised by big, yolk-rich cells (Keller, 1991), but is not seen in the supra-blastoporal endoderm or the anterior involuting mesendoderm.

During neurulation (st. 15) strong GATA5 expression was seen in the ventral anterior endoderm, underlying the floor of

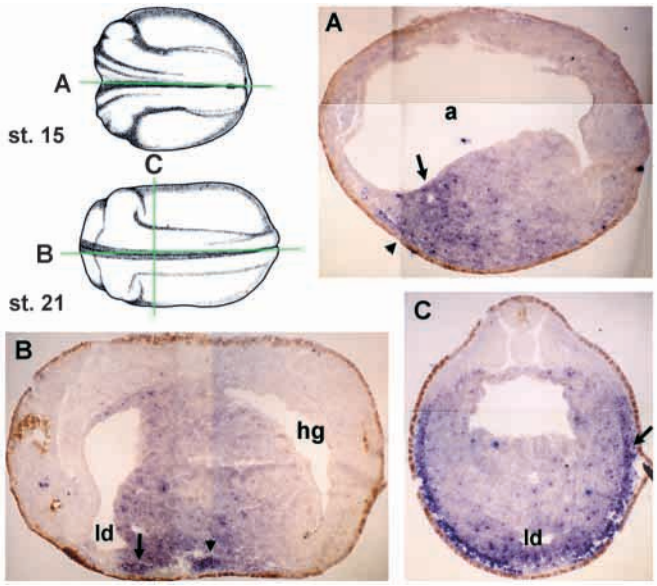

Fig. 3. In neurula and tailbud stages GATA5 is expressed in endoderm and ventrolateral mesoderm. Sagittal sections of a neurula embryo (A) demonstrating expression of GATA5 in ventral anterior endoderm (arrow) and ventral mesoderm in the prospective heart field area (arrowhead) and a tailbud embryo (B), showing expression of GATA5 in the endoderm, heart mesoderm (arrow) and blood islands (arrowhead). Anterior is to the left in A and B. Transverse section of a tailbud embryo in the region of the liver diverticulum (C), showing GATA5 specific staining in endoderm and lateral plate mesoderm (arrow). a, archenteron; hg, hindgut; ld, liver diverticulum.

the archenteron (Fig. 3A, arrow), reflecting the expression of GATA5 in the dorsoanterior yolk-rich cells during gastrulation. This expression pattern was maintained at the early tailbud stage (st. 21, Fig. 3B), when strongest staining became obvious in the region of the liver diverticulum (ld), and became progressively weaker in dorsal and posterior directions. However, no endodermal staining was found in the head region, underlying cement gland, forebrain or prechordal plate. This probably reflects the lack of GATA5 expression in the leading edge of the involuting mesendoderm at mid-gastrula stages. In neurula and tailbud stages, GATA5 expression was also obvious in ventral and lateral mesoderm, in the heart field (Fig. 3A, arrowhead and Fig. 3B, arrow), the ventral blood islands (Fig. 3B, arrowhead) and the lateral plate mesoderm (Fig. 3C, arrow). However, GATA5 remained excluded from dorsal mesoderm, such as muscle and notochord and from neural tissue. This expression pattern is similar to GATA2 (Walmsley et al., 1994) and GATA6 (Gove et al., 1997) at equivalent stages.

Ectopic expression of GATA5 in animal caps induces endodermal marker genes

GATA factors have been shown to act as transcriptional activators (Jiang and Evans, 1996), and GATA5 stimulates reporter gene transcription in animal cap assays (data not shown). Thus, the expression of GATA5 in prospective endoderm suggests a functional role for GATA5 in the activation of endoderm-specific gene transcription. To test this possibility, GATA5 mRNA was injected into the animal pole of fertilised eggs, which is fated to form ectodermal derivatives during normal development. At the blastula stage animal caps were dissected, cultured until larval stages (st. 31) and expression of endodermin was analysed by in situ hybridisation. At this stage of development, expression of endodermin is restricted to the endoderm and is therefore not normally present in animal caps (Sasai et al., 1996). Injection of GATA5 mRNA clearly induced ectopic endodermin in isolated animal caps (Fig. 4A), which was not seen when similar concentrations of GATA2 (Fig. 4B) or the muscle-specific transcription factor XMyf5 (Fig. 4C) were injected. Myf5 has been shown to induce muscle specific genes, when misexpressed (Hopwood et al., 1991). These results suggest that GATA5 is sufficient to activate endoderm-specific gene transcription in prospective ectoderm.

The majority of intact embryos derived from these animal cap injections were characterised by a concentration of ectopic pigment located at the ventral side of the body (Fig. 4D, arrow; Fig. 4E; Table 1) and defects in eye differentiation (Fig. 4E; Table 1). Neither phenotype was seen when equal concentrations of GATA2 or Myf5 mRNAs were injected (Fig. 4D,E; Table 1). As both defects are complex and not directly linked to endodermal specification, β-galactosidase mRNA was coinjected as a lineage tracer to determine if, in these experiments, GATA5 is respecifying injected cells towards an endodermal fate. In control embryos injected in the animal pole with β-galactosidase mRNA alone, β-galactosidase staining was predominantly seen in the head ectoderm, including the eyes, and in somites (Fig. 4F, top). However, when GATA5

Table 1. Phenotypes obtained after ectopic expression of GATA5 in prospective ectoderm or mesoderm

Site of injection	RNA	pg injected/ blastomere	*n*	Ectopic pigment (%)	Eye defects (%)	Open blastopore (%)	wt (%)
AC	Myf5	100	30	0	0	0	100
	GATA2	80-100	63	0	0	0	100
	GATA5	80-100	49	84	63	0	16
DMZ	Myf5	100	17	0	0	0	100
	GATA2	50	55	0	0	5	95
		100	82	0	0	16	84
	GATA5	25	19	0	0	53	47
		50	51	0	0	86	14
		100	56	0	0	96	4

The indicated amounts of RNA were injected either into the animal pole of fertilised eggs (AC) or into two dorsal blastomeres of four-cell embryos (DMZ). Embryos injected with GATA5 mRNA in the animal pole are characterised by ectopic pigment concentrations and eye defects and often by both phenotypes. Injection of GATA5, but not Myf5, and only to a lesser extend of GATA2a in the dorsal marginal zone leads to embryos with an open blastopore as the result of disrupted convergence and extension movements.

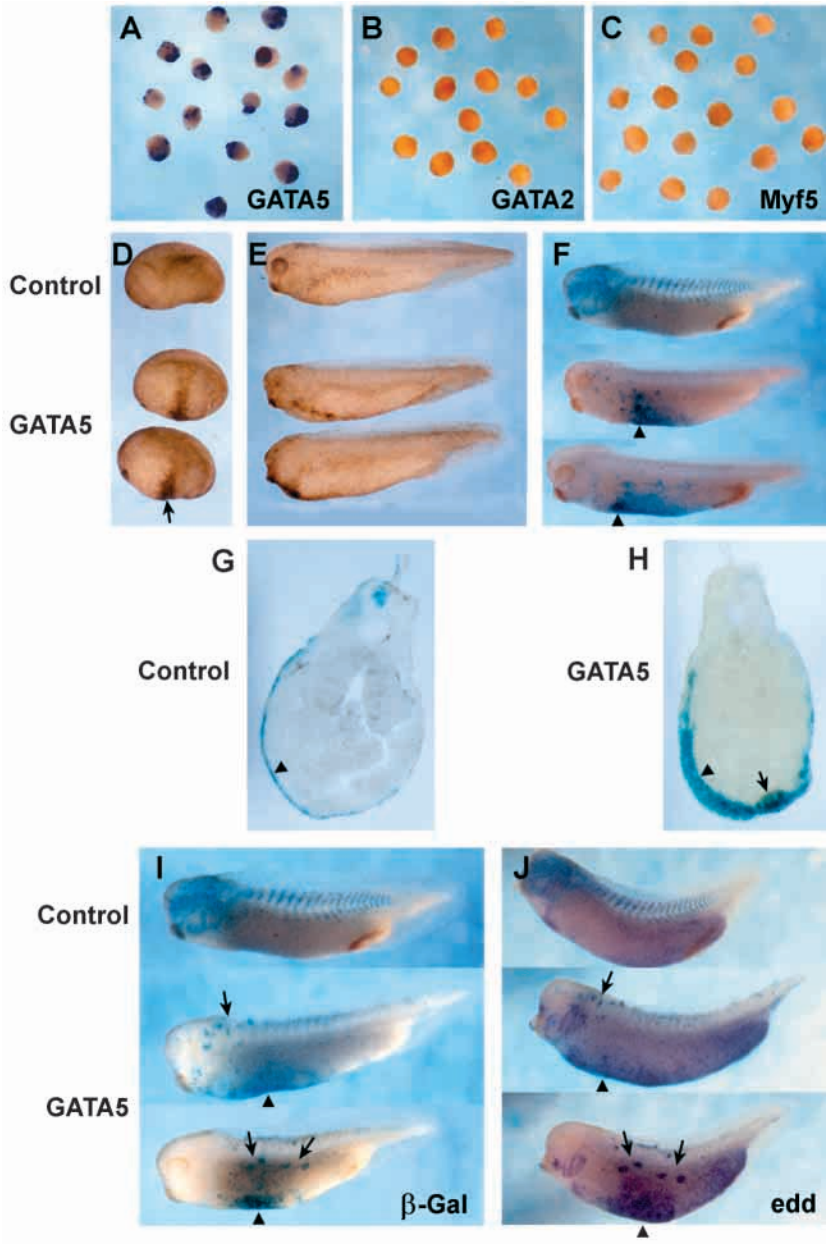

Fig. 4. GATA5 respecifies prospective ectoderm towards endoderm. Whole-mount in situ hybridisation to endodermin of GATA5- (A), GATA2a- (B) or Myf5- (C) injected animal caps at stage 31 equivalent. Injection of GATA5 mRNA induces patches of ectopic endodermin. (D,E) GATA2a (top row) or GATA5 (bottom rows) injected embryos at stage 22, showing GATA5 induced ectopic pigment (D, arrow) or stage 36 embryos showing GATA5 induced loss of eyes (E). Fertilised eggs were injected in the animal pole with 100 pg Myf5 or GATA2a RNAs or 50 pg GATA5 RNA. β-Galactosidase-stained larvae at stage 34 (F) injected in the animal pole with RNA coding for nuclear localised β-galactosidase on its own (top row) or coinjected with 50 pg GATA5 RNA (bottom rows). Arrowheads mark the areas with strongest β-galactosidase staining in GATA5-injected embryos. Vibratome cross sections of β-galactosidase-stained control (G) or GATA5-injected embryos (H) through the midgut region (arrowheads in F). β-Galactosidase staining is restricted to the ectoderm in control embryos and covers several cell layers in GATA5-injected embryos (arrowheads in G and H). Ectopic pigment is marked with an arrow in H. (I,J) Control (top row) or GATA5-injected embryos (bottom rows) at stage 34 after β-galactosidase staining (I,J) and in situ hybridisation with an endodermin specific probe (J). In control embryos, *lacZ*-positive (turquoise) and endodermin-expressing cells (purple) do not overlap. In GATA5-injected embryos, many *lacZ*-positive cells in the ventral region (arrowheads) and in isolated patches (arrows) express endodermin.

mRNA was coinjected with the lineage tracer, the location of β-galactosidase-expressing cells changed dramatically, with staining now mainly obvious in ventral regions of the embryo (Fig. 4F, bottom two embryos). Strongest β-galactosidase staining was typically found just posterior to the foregut (arrowheads). Cross-sections through this region of control embryos show β-galactosidase staining restricted to the ectodermal cell sheet that covers the embryo (Fig. 4G, arrowhead). In contrast, cross-sections through this region of GATA5-injected embryos show β-galactosidase staining in several cell layers (Fig. 4H, arrowhead) and include ectopic pigmented cells (arrow). Thus, the ectopic pigment at the ventral body side of GATA5-injected embryos appears to be due to the migration of pigmented animal pole cells to this region.

To analyse whether GATA5-injected cells were being respecified towards endoderm, β-galactosidase stained embryos (Fig. 4I) were hybridised in situ with a probe for the pan-endodermal marker, endodermin (Fig. 4J). In control embryos, β-galactosidase staining (turquoise, Fig. 4I ,J) and endodermin staining (purple, Fig. 4J) did not overlap. In contrast, in GATA5-injected embryos, strong endodermin staining was obvious in β-galactosidase positive cells at the ventral body side (compare Fig. 4I with 4J, arrowheads), as well as in patches of β-galactosidase-positive cells in other regions of the embryo (Fig. 4I,J, arrows). These results demonstrate that GATA5 is sufficient to activate endoderm-specific gene transcription in prospective ectoderm and to change the fate of these cells within the embryo. However, although the fate of the injected cells has been altered in the direction of endoderm, the conversion appears to be incomplete because the majority of injected cells did not contribute to the endodermal cell mass.

As GATA5 expression is restricted to the sub-blastoporal endoderm during gastrulation, we wondered whether GATA5

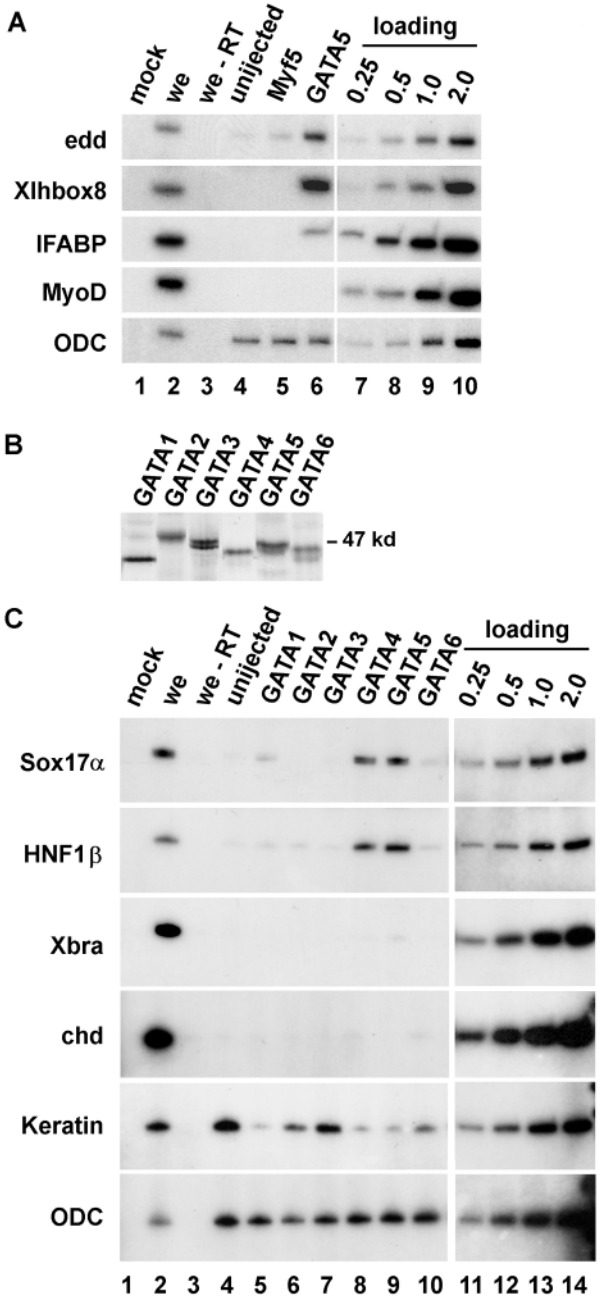

Fig. 5. GATA4 and GATA5 induce endodermal marker genes in animal cap explants. (A) RT-PCR of whole embryos (we), uninjected or with 50 pg Myf5 or GATA5 injected animal caps at stage 33/34, using primers for the indicated genes. GATA5 induces Xlhbox8 and IFABP. To assess for genomic contamination an RT reaction without reverse transcriptase was performed on whole embryo RNA (we-RT) and general contamination was assessed by taking a mock tube through RNA preparation, RT-reaction and PCR. ODC served as a loading control and sample titrations were performed to ensure that signals were in the linear range ('loading'). (B) In vitro translation of GATA1 – 6 mRNAs in reticulolysate lysate, demonstrating that all GATA RNAs translate with similar efficiencies. (C) RT-PCR of whole embryos (we), uninjected (lane 4) or with 50 pg of GATA1, 2a, 3, 4, 5 or 6 RNAs injected (lane 5-10) animal caps at stage 11 using primers for the indicated genes. GATA4 and 5 clearly induce Sox17α and HNF1β and downregulate cytokeratin. Controls were as for part A.

specifically induced only a subset of endodermal differentiation markers. Two regionally restricted endodermal differentiation markers identified in *Xenopus* are Xlhbox8, which is expressed in pancreas and duodenum (Wright et al, 1988), and IFABP, which is restricted to the intestine (Shi and Hayes, 1994). To investigate whether these markers could be activated by GATA5, GATA5-injected animal caps were cultured until larval stages (stage 33/34) and analysed for marker gene expression by RT-PCR. In these experiments GATA5 clearly induced endodermin (Fig. 5A, lane 6), confirming the induction seen by in situ hybridisation (Fig. 4A). In addition, GATA5 further efficiently activated Xlhbox8 (Fig. 5A, lane 6). In three independent experiments, the induction of Xlhbox8, relative to ODC levels, clearly exceeded the Xlhbox8 signal seen in whole embryos at the same stage (compare lanes 2 and 6). The induction of IFABP by GATA5 was more variable, ranging from a weak induction in comparison with the signal in the whole embryo (Fig. 5A, compare lanes 2 and 6) to a strong induction seen in other experiments (not shown). The variability of IFABP activation might be due to the timepoint when animal caps were collected. While expression of Xlhbox8 is clearly established at stage 33/34 (Wright et al, 1988), expression of IFABP is only just starting (Shi and Hayes, 1994), and therefore small differences in timing between experiments might have a large impact. Myf5, which translated with similar efficiency as GATA5 and was injected as a control, did not induce any endodermal differentiation markers (Fig. 5A, lane 5). To assess mesodermal induction, MyoD expression was analysed in GATA5-injected animal caps. While the MyoD signal in whole embryos is of similar strength as the signal seen for endodermal markers (Fig. 5A, lane 2), no induction by GATA5 is obvious (lane 6). All signals were within the linear range (lanes 7-10). Thus, GATA5 induces pancreas as well as intestine-specific gene transcription, suggesting that the sub-blastoporal endoderm contributes to both of these tissues. A recent fate map agrees with this conclusion (Chalmers and Slack, 2000).

Xlhbox8 and IFABP are differentiation markers late in development. To analyse whether GATA5 or other members of the GATA family induce early endoderm-specific genes, mRNAs coding for GATA1-6 were injected into animal caps and expression of endo-, meso- and ectodermal markers was analysed by RT-PCR at the mid-gastrula stage equivalent (st. 11). All GATA factor RNAs were shown to translate with similar efficiencies in vitro (Fig. 5B) and equal amounts of each mRNA were injected. GATA4, 5 and 6 belong to the same subfamily of GATA factors and are expressed in the endoderm during gastrulation (J. Broadbent, N. Holder and R. K. P., unpublished) and in later development (Jiang and Evans, 1996). GATA2 is expressed in the endoderm at the neurula stage (Walmsley et al., 1994) and GATA3 is found in branchial arch endoderm and gut in larval stages (E. M. Read and R. K. P., unpublished). GATA1 has so far not been detected in endodermal derivatives and served therefore as a control.

During normal development Sox17α and HNF1β are restricted to the endoderm in gastrula stage embryos (Hudson et al., 1997; Demartis et al., 1994). Injection of GATA4 or GATA5 mRNAs into animal caps clearly induced ectopic Sox17α and HNF1β (Fig. 5C, lanes 8 and 9). Induction of these markers by either GATA factor was highly reproducible and, relative to ODC levels, of the same order of magnitude as the

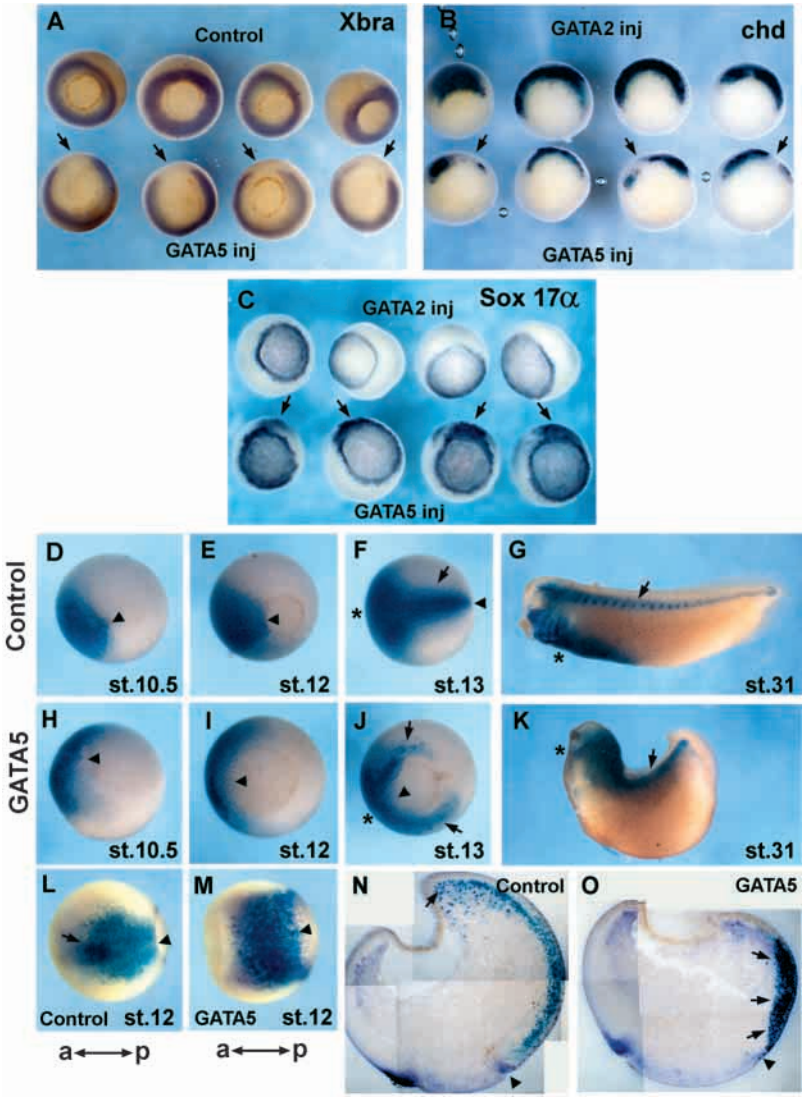

Fig. 6. GATA5 respecifies prospective mesoderm towards endoderm. Whole-mount in situ hybridisations of control embryos (A-C top row, D-G,L) or GATA5 injected embryos (A-C bottom row, H-K, M) at stage 11 (A-C) or the stages indicated (D-M) using the indicated probes. Embryos were injected with either 50 pg GATA2a or 50 pg GATA5 RNA per blastomere as indicated in the figure and described in the text. Expression of Xbra (A) and chordin (B) is reduced in GATA5 injected embryos (arrows), while Sox17α is ectopically induced (C, arrows). Embryos injected at the four-cell stage into two dorsal blastomeres with RNA coding for nuclear β-galactosidase on its own (D-G) or coinjected with 50 pg GATA5 RNA (H-K) were stained for β-galactosidase at the indicated stages. Dorsal injection of GATA5 disrupts convergence and extension movements. The dorsal blastopore is marked by arrowheads, axial mesoderm by arrows and anterior mesendoderm by asterisks. (L,M) Whole mount in situ hybridisations of β-galactosidase stained embryos in E and I, respectively, with a Sox17α specific probe. (N,O) Vibratome cross sections of stage 12 embryos shown in L and M, respectively. In control embryos the majority of *lacZ*-positive cells (turquoise) do not express Sox17α (purple), while in GATA5-injected embryos *lacZ*-positive cells also express Sox17α (arrows in O; cytoplasm as well as nuclei now blue). Arrowheads mark the dorsal blastopore lip and the arrows in L,N indicate the Sox17α positive dorsoanterior endoderm. Embryos in A-E,H-J are viewed from the vegetal pole. Anterior is towards the left in the other whole mount panels.

signal in the whole embryo (Fig. 5C, compare lane 2 with lanes 8 and 9). The activation of endodermal gene expression by other GATA factors was, in comparison to GATA4 and GATA5, only weak and never exceeded the weak induction seen by GATA1 (Fig. 5C, lane 5). As GATA1 has so far not been implicated in endodermal differentiation, these effects might be due to cross-reactivity between different GATA factors. It is noteworthy that GATA6, although it shares a similar expression pattern with GATA4 and 5, did not significantly induce Sox17α or HNF1β (Fig. 5C, lane 10). Xbra, a marker for posterior mesoderm (Smith et al., 1991), and chordin, a marker for dorsal mesoderm at the stage analysed (Sasai et al., 1994), were not significantly induced by any GATA factor. Expression of cytokeratin, a marker for epidermis (Jones et al., 1989), was strongly reduced in explants injected with mRNA for either GATA1, 4, 5 or 6. While in the case of GATA4 and 5 this is probably due to the conversion of ectoderm towards endoderm, the reason for the downregulation of cytokeratin by GATA1 and 6 is still unclear. GATA2 and 3, both of which are expressed in the ectoderm during gastrulation, did not reduce keratin expression, when uneven ODC levels are taken into account (Fig. 5C, lanes 6 and 7, compare with lane 4). The above results demonstrate that GATA4 and 5, on the basis of the analysed markers, are sufficient to convert ectoderm towards endoderm. This suggests an important role for either factor in the establishment of an endodermal cell fate during normal development.

Ectopic expression of GATA5 in the marginal zone downregulates mesodermal and induces endodermal marker genes

In *Xenopus* embryos, mesoderm derives from the equatorial cells of the marginal zone. To analyse whether GATA5 can change the fate of presumptive mesoderm towards endoderm, GATA5 mRNA was injected into the marginal zone of early cleavage embryos and whole-mount in situ hybridisations for mesodermal and endodermal marker genes were performed. Injection of GATA5 mRNA into one blastomere of eight-cell embryos reduced the expression of Xbra at the site of injection irrespective of whether dorsal or ventral blastomeres were targeted (Fig. 6A arrows, compare top and bottom line). To confirm this apparent suppression of mesoderm formation by

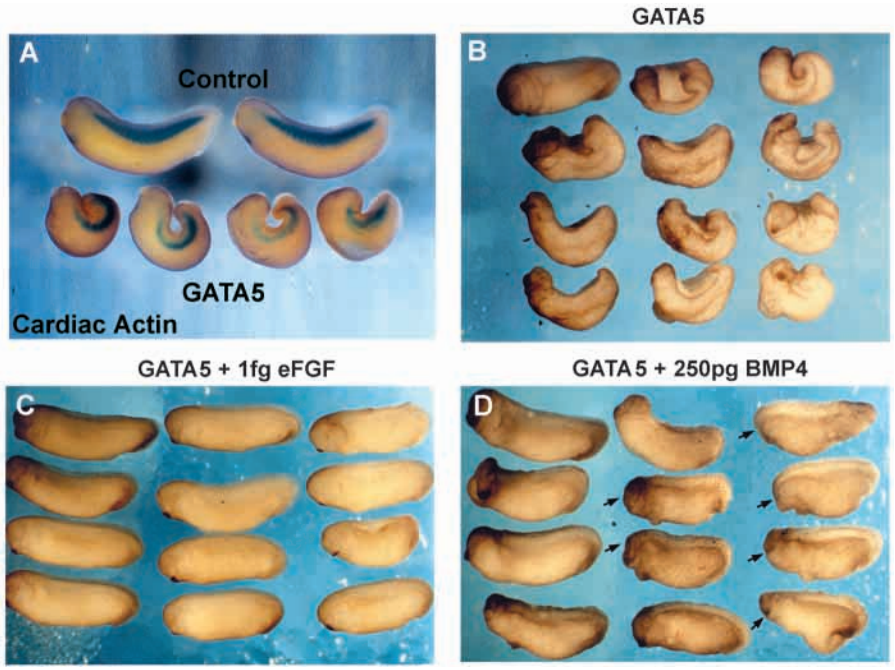

Fig. 7. The GATA5 induced open blastopore phenotype can be rescued by eFGF or BMP4. Embryos were injected into the marginal zone of two dorsal blastomeres at the 4-cell stage with 25 pg GATA5 RNA per blastomere (bottom row in A; panel B), 25 pg GATA5 and 1 fg eFGF (C), or 25 pg GATA5 and 250 pg BMP4 (D) and cultured until stage 30. Embryos in the bottom row of A and in B, except for the top left embryo in B, are characterised by an open blastopore. 1 fg eFGF rescues to the wild type phenoptype (C) and embryos rescued with 250 pg BMP4 are either wild type or ventralised (D), as assessed by the loss of head structures (arrows). ((A) In situ hybridisation with a cardiac actin specific probe at stage 30 demonstrates the reduced somitic tissue in GATA5-injected embryos compared to control embryos).

GATA5, expression of chordin, a gene expressed in dorsal mesoderm (Sasai et al., 1994), was analysed. Embryos were injected at the four-cell stage into two dorsal (chordin) blastomeres. GATA2, which did not induce endodermal markers in animal caps (Fig. 4B; Fig. 5C, lane 6), was injected as a control. Expression of chordin was reduced (Fig. 6B, arrows) in GATA5-injected embryos compared with control embryos. Suppression of chordin due to ventralisation by GATA2 was only seen when significantly higher concentrations of GATA2 were injected (Sykes et al., 1998). In the same batch of embryos, ectopic Sox17α staining was obvious in the marginal zone of GATA5-injected embryos, but not of GATA2-injected control embryos (Fig. 6C, arrows), suggesting that GATA5 alters the identity of mesoderm towards endoderm.

In intact embryos dorsally injected with GATA5 mRNA the blastopore failed to close and, in comparison to control embryos, a reduced amount of somitic mesoderm (Fig. 7A) and neural tissue (not shown) differentiated on either side of the open blastopore. This phenotype was frequently seen even when low amounts of GATA5 mRNA were injected (25 pg per blastomere), but not after injection of Myf5 and only rarely when equal amounts of GATA2 mRNA were injected (Table 1). Staining of control embryos, which were injected in the dorsal marginal zone with β-galactosidase RNA, demonstrates the convergence and extension movements undergone by the dorsal mesoderm during gastrulation (Fig. 6D-G). These movements result in the progressive closure of the blastopore (arrowhead), which is completed at the end of gastrulation, when β-galactosidase staining is obvious in prospective axial mesoderm (Fig. 6F, arrow) and anterior mesendoderm (Fig. 6F, asterisk). In contrast, ectopic expression of GATA5 in the dorsal marginal zone disrupts convergence and extension movements and the blastopore fails to close (Fig. 6H-K). The injected cells stay close to the dorsal blastopore lip (arrowhead) and spread laterally around the open blastopore as gastrulation proceeds (Fig. 6I,J). They will form the head (Fig. 6J,K, asterisks) and axial structures (Fig. 6J,K, arrows), respectively. To analyse whether the GATA5-induced phenotype is due to the respecification of injected cells towards endoderm, β-galactosidase-stained embryos were analysed for Sox17α expression by whole-mount in situ hybridisation, and vibratome sections of these embryos were made. In control embryos, *lacZ*-positive cells extend from the dorsal blastopore lip (Fig. 6L,N, arrowhead) to the most anterior endoderm (arrow). The majority of *lacZ*-positive cells (turquoise) do not express Sox17α (purple) and overlap appears to be restricted to the most anterior endoderm (Fig. 6L, arrow). In contrast, in GATA5-injected embryos, *lacZ*-positive cells extend less in the anterior/posterior direction but spread laterally round the blastopore lip (Fig. 6M,O). The majority of *lacZ*-positive cells express Sox17α, resulting in overlap of turquoise and purple colours (Fig. 6M,O, arrows. (Note that in contrast to β-galactosidase, Sox17α staining is non-nuclear, therefore in cells expressing both, the nuclear localisation of β-galactosidase staining is no longer obvious). These results suggest that in GATA5-injected embryos the failure of the blastopore to close is due to the respecification of mesoderm towards endoderm.

The GATA5-induced open blastopore phenotype can be rescued by eFGF and BMP4

A phenotype very similar to the one obtained after ectopic expression of GATA5 in the marginal zone is seen in embryos expressing either a dominant negative fibroblast growth factor (FGF)-receptor (XFD; Amaya et al., 1991; Isaacs et al., 1994) or a dominant interfering Xbra-construct (Xbra-En^{R}; Conlon and Smith, 1999) and has been described as the consequence of mesoderm disruption in these studies. The XFD-induced failure of the blastopore to close can be rescued by overexpression of the wild-type receptor, which restores the FGF-signalling pathway (Amaya et al., 1991). We therefore analysed whether the phenotype caused by GATA5 injection

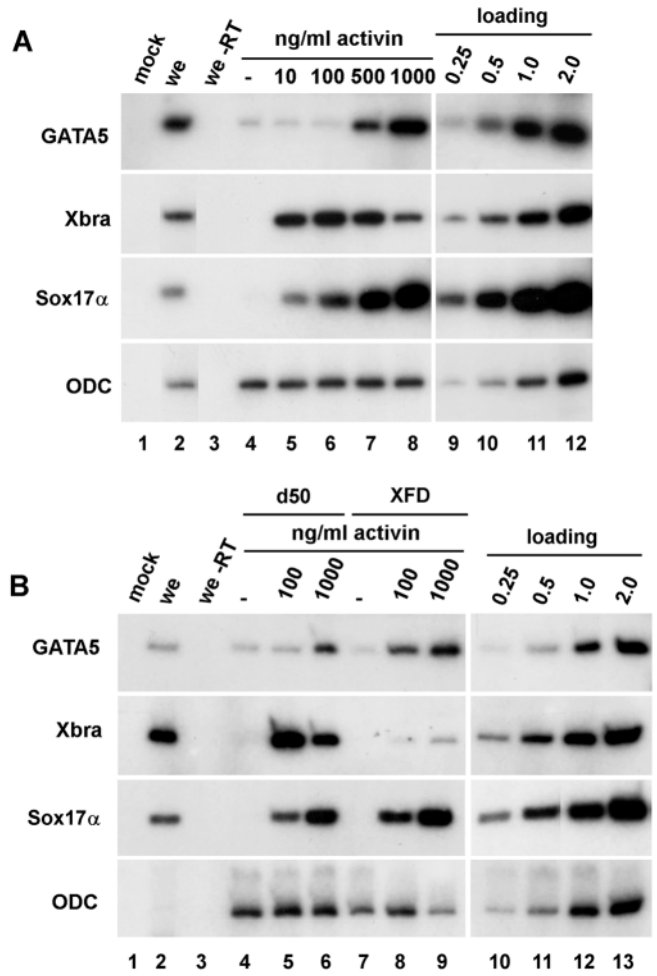

Fig. 8. High concentrations of activin induce GATA5 in an FGF independent manner. (A) RT-PCR of animal caps at stage 11.5 equivalent incubated with the indicated concentrations of activin. (B) Stage 11.5 animal caps injected with RNAs for a control receptor (d50) or a dominant negative FGF-receptor (XFD) before treatment with the indicated concentrations of activin. 500 pg d50 or XFD RNAs were injected into the animal pole of each blastomere of two-cell embryos. Controls were as described in the legend to Fig.5A.

can be rescued by co-injection of eFGF. We also analysed rescue by bone morphogenic protein 4 (BMP4), since this protein, as well as FGF, has been shown to induce mesoderm in animal cap assays (Isaacs et al., 1994; Jones et al., 1996). Sonic hedgehog (SHH), a signalling molecule that has so far not been implicated in mesoderm induction (Smith, 1994), was injected as a control. The eFGF RNA was found to be extremely potent in mesoderm induction assays and thus very low levels were injected. Co-injection of GATA5 with 0.5 fg eFGF decreased the number of embryos with an open blastopore only slightly, from 86% to 66% (Table 2). However, when 1 fg eFGF mRNA was coinjected, the phenotype was clearly rescued, reducing the number of affected embryos from 86% to 13%. Embryos, which were rescued with these low levels of eFGF mRNA, looked phenotypically normal (Fig. 7C). Co-injection of 250 pg BMP4 mRNA rescued with the same efficiency, from 84% embryos with an open blastopore when only GATA5 was injected to 15% in the presence of BMP4 (Table 2). However, the rescued embryos were often ventralised, as assessed by the loss of anterior structures (Fig. 7D, arrows), to a degree comparable with that seen when these levels of BMP4 were injected in the absence of GATA5 (Table 2). Nevertheless, a moderate rescue was also observed with lower BMP4 levels (50 pg; Table 2), which did not ventralise, when injected alone. This indicates that the ventralisation by BMP4 is not necessary for the rescue. Co-injection of SHH did not rescue (Table 2), although the injected levels were sufficient to perturb muscle differentiation in parallel experiments (Blagden et al., 1997 and not shown). Thus, the re-establishment of mesodermal gene expression in the dorsal marginal zone by eFGF and BMP4 appears to be sufficient to rescue the GATA5-induced gastrulation defects, possibly by converting endoderm to mesoderm, by turning Xbra back on or by creating conditions that disturb the ability of GATA5 to induce endoderm in the first place.

In a more recent report the open blastopore phenotype was observed after radial expression of an antimorphic goosecoid-construct (MT-gsc; Ferreiro et al., 1998). Analysing gastrulation movements by time-lapse video microscopy and the elongation of MT-gsc-expressing isolated dorsal marginal zones (DMZs), the authors suggest that this phenotype is caused by the disruption of dorsal extension movements, which is consistent with our results (Fig. 6). MT-gsc-expressing DMZs show reduced differentiation of dorsal mesoderm and although ventral markers are induced in RT-PCR assays, these explants resemble undifferentiated endoderm by morphological appearance (Ferreiro et al., 1998). The same phenotype was also induced by ectopic expression of *Otx2*, a gene expressed in anterior endoderm and neural tissue (Pannese et al., 1995) and expression of Xbra-En^{R} in animal caps is sufficient not only to induce *Otx2* but also endodermin. Xbra and FGF maintain each other in an autoregulatory loop (Isaacs et al., 1994; Schulte-Merker and Smith 1995), and the absence of FGF-signalling in animal caps results in an ectopic and increased induction of GATA5 by activin (see below). We therefore wondered whether the absence of FGF-signalling in the marginal zone was sufficient to induce endoderm, thereby inhibiting convergence and extension movements and, as a consequence, the closure of the blastopore. However, embryos, which express the dominant negative FGF receptor XFD in the dorsal marginal zone, did not ectopically express either GATA5 or Sox17α at gastrula stages, although they were later characterised by a failure of the blastopore to close (not shown). Thus, the absence of FGF signalling is not sufficient to induce endoderm in the marginal zone. Furthermore the open blastopore phenotype appears to be generally correlated with mesoderm disruption, which only in some cases (GATA5 and possibly MT-gsc) is obtained by conversion of mesoderm towards endoderm.

GATA5 is induced by high concentrations of activin in an FGF-independent manner

Members of the TGFβ family have been implicated in the induction of endoderm as well as mesoderm. As GATA5 is restricted to the sub-blastoporal endoderm at mid-gastrula stages (Fig. 1), we used it as an early molecular marker to study the induction of this subset of endodermal cells. Animal caps were dissected at the late blastula stage, treated with a

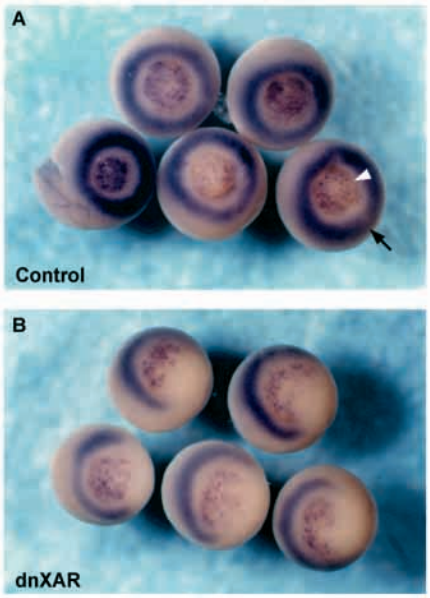

Fig. 9. Injection of a dominant negative activin receptor (dnXAR) inhibits GATA5 expression. Double in situ hybridisation for Xbra (arrow in A) and GATA5 (arrowhead in A) on stage 11.5 embryos. These were injected with either 1 ng β-galactosidase (A) or 1 ng dnXAR (B) RNA vegetal of the marginal zone into one blastomere of two-cell embryos.

concentration range of activin and analysed for GATA5 expression by RT-PCR at mid-gastrula stage equivalent (st. 11.5). Low concentrations of activin (0.1-1 ng/ml) have been shown to induce ventral mesoderm and muscle, while medium concentrations (10-100 ng/ml) induce dorsal mesoderm and heart tissue in animal cap assays (Asashima, 1994). As histologically defined endoderm is predominantly induced by high activin levels (Tiedemann, 1990), we tested concentrations up to 1000 ng/ml. Medium activin levels (10 and 100 ng/ml) efficiently induced the expression of Xbra but not of GATA5 (Fig. 8A, lanes 5 and 6). However, higher activin levels significantly induced GATA5 and, within the concentration range tested, induction was strongest at 1000 ng/ml activin, the concentration at which Xbra induction declined. The absence of GATA5 in animal caps treated with up to 100 ng/ml activin and the induction of GATA5 by 1000 ng/ml activin, accompanied by reduced Xbra expression, was highly reproducible in replicate experiments. These data suggest that the specification of sub-blastoporal endoderm requires levels of activin above those known to induce dorsal mesoderm.

In contrast to GATA5, Sox17α was induced by 10 and 100 ng/ml activin, along with the induction of Xbra (Fig. 8A, lanes 5 and 6). This could reflect the expression of Sox17α in the supra-blastoporal endoderm, which because of its location in close vicinity to the mesoderm may be induced in a similar manner. However, at higher activin concentrations (500 and 1000ng/ml), while Xbra expression was reduced, Sox17α induction increased strongly (Fig. 8A, lanes 7 and 8), which may reflect the expression of Sox17α in sub-blastoporal endoderm.

FGF signalling has been shown to be essential for mesoderm differentiation and to counteract the specification of endoderm (Cornell and Kimelman, 1994; Labonne and Whitman, 1994; Cornell et al., 1995). To test this for the sub-blastoporal endoderm, animal caps were injected with the dominant negative FGF-receptor XFD (Amaya et al., 1991) or a mutated inactive FGF-receptor, d50, as a control; injected caps were treated with activin and analysed for the expression of GATA5 and Xbra at mid-gastrula stage equivalent. While induction of Xbra was strongly reduced in the presence of XFD compared to d50 injected control explants, expression of GATA5 was not negatively affected and, taking uneven ODC levels into account, was even stronger when FGF-signalling was disrupted (Fig. 8B, compare lanes 6 and 9). Furthermore, XFD-injection led reproducibly to induction of GATA5 at lower activin levels (100ng/ml) than in control injected animal caps (Fig. 8B, compare lanes 5 and 8). Induction of Sox17α by activin was also increased in the absence of FGF-signalling, irrespective of whether the explants were treated with 100 or 1000 ng/ml activin. Thus, the induction of GATA5 and Sox17α by activin does not require FGF signalling. Since, in contrast to the animal cap, FGF signalling is absent from the vegetal pole, which gives rise to endoderm during normal development, this suggests that in vivo lower concentrations of TGF-β molecules might be required for the induction of endoderm than in the animal cap assay.

Our experiments show that ectopic activin can induce GATA5, but not whether TGFβ signalling is essential for the induction of the endogenous gene. To investigate this, we analysed whether the vegetal expression of GATA5 can be inhibited by a dominant negative activin receptor (dnXAR, gift of D. Mahony and J. B. Gurdon; Rodaway et al., 1999). RNA for dnXAR was injected vegetal of the equator into one blastomere of 2 cell embryos. At the mid-gastrula stage

Table 2. The GATA5-induced 'open blastopore' phenotype can be rescued by the mesoderm inducers eFGF and BMP4, but not by sonic hedgehog (SHH)

RNA	*n*	Open blastopore (%)	Wild type/ventralised (%)
25 pg GATA5	63	86	14
25 pg GATA5+0.5 fgeFGF	76	66	34
25 pg GATA5+1.0 fg eFGF	57	13	87
25 pg GATA5	44	84	16
25 pg GATA5+50 pg BMP4	28	43	54/3
25 pg GATA5+250 pg BMP4	40	15	55/30
50 pg BMP4	27	0	89/11
250 pg BMP4	32	3	47/50
25 pg GATA5	50	78	22
25 pg GATA5+250 pg SHH	73	72	28
25 pg GATA5+400 pg SHH	18	72	28

expression of GATA5 and Xbra was analysed by whole-mount in situ hybridisation. Overexpression of a dominant negative activin receptor has been shown to block Xbra expression (Hemmati-Brivanlou and Melton, 1992). Therefore, the downregulation of Xbra on one side of the embryo identifies the injected side as well as confirming the activity of the dominant negative receptor. While in β-galactosidase injected control embryos, Xbra is detected in a ring around the blastopore (Fig. 9, arrow) and GATA5 throughout the yolk plug (arrowhead), dnXAR blocks the expression of Xbra as well as GATA5 in the injected half. These results demonstrate that TGFβ signalling is necessary for the induction of GATA5 and confirm a role for TGFβs in the specification of the sub-blastoporal endoderm.

DISCUSSION

Expression pattern of GATA5

Significant levels of GATA5 mRNA are detectable in the vegetal pole of *Xenopus* embryos from the early gastrula stage onwards. By this stage vegetal pole cells have become committed to form endoderm (Heasman et al., 1984; Wylie et al., 1987). Several transcription factors, which are expressed in the endoderm around this stage, have recently been implicated in endodermal specification. Sox17α and Sox17β, transcription factors containing an HMGbox DNA-binding domain, are specifically expressed in the endoderm between late blastula and larval stages (Hudson et al., 1997; Henry and Melton, 1998). *Mix.1* (Rosa, 1989; Lemaire et al., 1998), *Milk* (Ecochard et al., 1998), *Mixer* (Henry and Melton, 1998), *Mix.3*, *Mix.4* (Mead et al., 1998) and the more diverged *Bix1* (Tada et al., 1998) contain *Mix*-related homeodomains. As far as their expression patterns are known, these factors are expressed in a short temporal window between the late blastula/early gastrula stage and the end of gastrulation. However, at the early stages of their expression most of these genes are found in the mesoderm as well as in the endoderm. While *Bix1* is maintained in both germlayers, *Mix.1, Milk* and *Mixer* are excluded from the Xbra expressing mesoderm at mid-gastrula stages. Expression of *Mix.1*, *Milk* and *Mixer* appears to be strongest at the mesoderm/endoderm boundary, suggesting a functional role for these genes in the establishment of this boundary during gastrulation.

GATA factors represent a new class of transcriptional activators involved in early endoderm specification. They are characterised by two C-X_2-C-X_{17}-C-X_2-C zinc fingers, a DNA-binding motif most related to those found in members of the steroid/thyroid hormone receptor superfamily (Evans and Felsenfeld, 1989). In contrast to the previously characterised endoderm or endo/mesoderm specific genes, GATA5 is restricted to the sub-blastoporal endoderm at mid-gastrula stages and is to our knowledge the first early molecular marker for this subset of endoderm. Endodermal expression of GATA5 is maintained throughout larval development and in the adult organism (Kelley et al., 1993).

The characterisation of an increasing number of transcription factors that are expressed in the endoderm at the time of its specification, raises the question of how these factors are functionally related. So far it has been shown that *Mixer* is expressed later than Sox17α and Sox17β, and is required for the maintenance but not initial induction of these genes (Henry and Melton, 1998). Mix.1, Sox17α and Sox17β are activated cell-autonomously at about the mid-blastula transition (MBT) while the later expression of Mixer and GATA4, like that of GATA5 shown here, involves TGFβ signalling (Yasuo and Lemaire, 1999; Clements et al., 1999).

During neurula stages GATA5 becomes detectable in ventral mesoderm and at the early tailbud stage expression is obvious in the heart field, the ventral blood islands and lateral plate mesoderm. Mesodermal cells are clearly distinguishable from the yolk-rich sub-blastoporal endoderm by their smaller cell size, and in gastrula stage embryos we see no evidence for GATA5 expression in the mesoderm. The endoderm has been shown to play an essential role in specification and morphogenesis of the heart in various organisms (reviewed in Nascone and Mercola, 1996; Jacobson and Sater, 1988). Furthermore, the inability of GATA4$^{-/-}$ mouse embryos to form a heart tube is due to the absence of GATA4 in the endoderm (Narita et al., 1997). The role of the endoderm in blood-cell differentiation is less well understood. However, the visceral endoderm in the mouse also appears to have a function in the formation and organisation of yolk sac blood islands and vasculature. GATA4$^{-/-}$ embryoid bodies, which lack visceral endoderm, do not form blood islands and vessels, although in chimeric embryos GATA4$^{-/-}$ ES cells contribute to these structures when juxtaposed to wild type visceral endoderm (Bielinska et al., 1996). These data on GATA4 show that the differentiation of GATA-expressing mesoderm (blood islands and heart) is dependent on GATA expression in the endoderm. GATA5 expression in the endoderm as well as in lateral plate, heart and blood islands suggests that both GATA4 and GATA5 may be involved in the inductive relationships between these two germ layers.

Ectopic expression of GATA factors

Ectopic expression of GATA4 or 5 in prospective ectoderm induces the endodermal markers Sox17α and HNF1β and downregulates expression of cytokeratin, thus changing the specification of ectodermal cells towards endoderm. Evidence supporting a role for GATA4 in *Xenopus* endoderm formation has very recently been reported (Shoichet et al, 2000). In addition, studies in the zebrafish, using ectopic expression and analysis of the GATA5 mutant, *faust*, also support a role for GATA5 in endoderm formation (Reiter et al, 1999; J. F. Reiter and D. Y. R. Stainier, unpublished). GATA6 belongs to the same subfamily of GATA factors and shares an overlapping expression pattern with GATA4 and 5 (Jiang and Evans 1996; Gove et al., 1997; J. Broadbent, N. Holder and R. K. P., unpublished). However, although expression of cytokeratin is significantly downregulated by GATA6 in animal caps, Sox17α and HNF1β are not induced. Approximately 450 bp upstream of the generally accepted translation start site of GATA6 (MYQ), human and mouse GATA6 contain an alternative translation initiation site (MALT; Brewer at al., 1999). The predicted amino acid sequences of the mouse and human genes in this region are highly homologous (82% identity), and both translational start sites can be used in vitro. The *Xenopus* GATA6 sequence upstream of MYQ is not yet available, but it seems likely that *Xenopus* GATA6 also contains an alternative translation start site upstream of MYQ. In order to analyse whether the additional N-terminal amino acids change the

endoderm-inducing abilities of GATA6, RNAs coding for the long and short versions of human GATA6 were tested for their endoderm-inducing abilities in animal cap assays. While both versions downregulated keratin, GATA6 did not significantly induce Sox17α or HNF1β (data not shown), in agreement with our data obtained with *Xenopus* GATA6. We conclude that GATA6 has a function in endoderm development that is distinct from GATA4 and 5.

During the differentiation of the *Xenopus* heart, GATA6 expression levels progressively decrease from neurula stages onwards and artificial maintenance of GATA6 in prospective heart blocks induction of the late differentiation markers cardiac actin and myosin light chain (Gove et al., 1997). In the chick intestinal epithelium, GATA6 is restricted to the proliferating progenitor cells that are located in the crypt of the villus, while GATA4 and 5 are predominantly found in differentiating cells that migrate towards the tip of the villus (Gao et al., 1998). These cells co-express the late intestinal differentiation marker IFABP. While GATA4 and 5 significantly activate IFABP reporter constructs (five- to ninefold), activation by GATA6 is weak (two to threefold) and might reflect cross-reactivity between different GATA factors, since the expression patterns of GATA6 and IFABP in the intestinal epithelium are mutually exclusive. Thus, despite the overlapping expression pattern of GATA4, 5 and 6 in gastrula stage endoderm, GATA6 might have a function associated with the progenitor state of these cells, while GATA4 and 5 drive endoderm-specific differentiation.

Induction of GATA5

In animal cap assays, GATA5 is reproducibly induced only by levels of activin above those known to induce dorsal mesoderm, while Sox17α can be induced at medium as well as high activin concentrations. In these experiments animal caps were analysed at mid-gastrula stage equivalent, when expression of GATA5 is restricted to the yolk-rich sub-blastoporal endoderm, while Sox17α is found in a broader domain, including the supra-blastoporal endoderm and anterior involuting mesendoderm. In contrast to the sub-blastoporal endoderm, these endodermal cell types originate from the marginal zone and their induction might therefore be more similar to the induction of the mesoderm.

Mesoderm is induced in the marginal zone by a signal emanating from the underlying vegetal pole (Nieuwkoop, 1969). We show in animal cap assays that the induction of GATA5 is strongest at high levels of activin (1 μg/ml), when the induction of Xbra significantly declines. Furthermore, the activin-mediated induction of GATA5, in contrast to the induction of Xbra, does not require FGF signalling. These data support the hypothesis that high concentrations of TGFβ molecules, in combination with the absence of FGF signalling, specify endoderm in the vegetal hemisphere of the embryo, while lower TGFβ levels, complemented by FGF signalling are required for mesoderm differentiation in the marginal zone (Cornell et al., 1995). An important contribution to our understanding of how germlayers are generated at the onset of transcription has recently been obtained by the depletion of the vegetally localised, maternal VegT RNA (Zhang et al., 1998). VegT depletion not only results in the absence of endodermal differentiation, but also in a shift of the fate map towards the vegetal pole. In these embryos mesoderm is formed from vegetal pole cells and ectoderm from the vegetal pole and marginal zone. As the disruption of TGFβ signalling using a dominant negative TGFβ receptor results in a similar phenotype, in which mesoderm is lost in the marginal zone and mesodermal and ectodermal markers are ectopically expressed in the vegetal pole (Hemmati-Brivanlou and Melton, 1992; Henry et al., 1996), Kimelman and Griffin (1998) put forward the following model: vegetal pole cells contain a weak mesoderm-inducing factor of unknown identity and VegT. At the onset of transcription, the maternal VegT promotes endodermal fate and activates high levels of TGFβ signalling, two effects that may be related. This results in the induction of mesoderm in the overlying marginal zone. In the absence of VegT the low level maternal signal induces mesoderm in the vegetal pole. It has recently been shown that VegT induces endoderm cell autonomously and generates TGFβ family signals that reinforce endodermal differentiation and are entirely responsible for the mesoderm inducing activities of VegT. The induced TGFβ molecules include derriere, Xnr1, 2 and 4 and activin B (Clements et al., 1999; Yasuo and Lemaire, 1999) and derriere as well as the nodal-related factors rescue mesoderm formation in VegT depleted embryos (Kofron et al, 1999). Our results clearly establish endodermal differentiation in vegetal pole cells as the result of high TGFβ levels, on the basis of strongest induction of GATA5 as well as of Sox17α being seen by the highest activin levels tested, accompanied by declining Xbra induction. Recently also nodal-related factors and derriere have been shown to induce endoderm predominantly at high concentrations (Clements et al., 1999; Sun et al, 1999). Once Sox17α and GATA5 are induced, they may maintain each other (this paper and H. Woodland personal communication) and disruption of this maintenance loop by a Sox17engrailed construct results in ectopic expression of mesodermal markers in the vegetal pole cells (Hudson et al., 1997), as earlier described for the disruption of TGFβ signalling and maternal VegT. Consistent with this model, the inhibition of FGF signalling in the marginal zone is not sufficient to induce endoderm (data not shown), as TGFβ concentrations in the marginal zone are not high enough.

In zebrafish, high concentrations of the constitutively active activin receptor, Alk4, induce GATA5 in the injected cells, while brachyury (ntl) is induced in a ring around the injected cells, with a small area of overlap (Rodaway et al., 1999). While the endogenous expression of GATA5 is not affected by expression of the dominant negative FGF receptor XFD, ntl expression is mostly abolished, with the exception of one row of ntl-expressing cells closest to the yolk cell. These experimental data are highly consistent with our results in *Xenopus*, suggesting that the regulation of GATA5 expression is conserved in both organisms. In zebrafish, at the blastula stage, expression of GATA5 and brachyury (ntl) is initially activated in the same cells, in the three to four cell layers closest to the yolk cell. Prior to gastrulation ntl expression extends further in the animal direction and during gastrulation the expression domains of GATA5 and ntl separate. Both genes are induced by a signal originating from the yolk cell. The data obtained by Alk4 and XFD expression suggest that GATA5 as well as the ntl expression closest to the yolk cell are induced by TGFβ signalling, with nodal-related proteins being the best candidates, while the ntl expression in the other cell layers is induced and maintained by a relay mechanism via FGF. In

contrast to the situation in zebrafish, the expression of GATA5 and Xbra in *Xenopus* is exclusive at all analysed stages. There are two possible explanations for these differences. Prior to gastrulation, when overlap of GATA5 and ntl is obvious in zebrafish, expression of GATA5, which is clearly detectable by RT-PCR analysis at this stage (Jiang and Evans, 1996), is too weak to be detectable by in situ hybridisation. Therefore an initial overlap of GATA5 and Xbra is also possible in *Xenopus*. Alternatively, in zebrafish, GATA5 is induced by a signal from the yolk cell in cells adjacent to the yolk cell. The yolk cell itself is not thought to contribute to the embryo proper. In contrast, in *Xenopus*, GATA5 is expressed in the vegetal pole cells, which induce mesoderm and endoderm in the marginal zone, but themselves form part of the endoderm. This could account for differences in the expression patterns of GATA5 in *Xenopus* and zebrafish.

We thank Todd Evans for the kind gift of GATA4 and GATA5 cDNAs prior to publication, Yuzuru Eto for the gift of human recombinant activin, Hugh Woodland for Sox17 primer sequences prior to publication, Jeremy Reiter and Didier Stainier for exchange of results prior to publication, and members of our laboratory for valuable contributions during the course of this work. Special thanks go to Claire McNulty for help with in situ hybridsations on sections and Joanne Broadbent for discussing results. This work was supported by the EU (H. W.), the Wellcome Trust (A. R. and H. W.) and the MRC (C. S and M. W.).

REFERENCES

Alexander, J. and Stainier, D. Y. R. (1999). A molecular pathway leading to endoderm formation in zebrafish. *Curr. Biol.* **9**, 1147-1157

Amaya, E., Musci, T. J. and Kirschner, M. W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos. *Cell* **66**, 257-270.

Asashima, M. (1994). Mesoderm induction during early amphibian development. *Dev. Growth Differ.* **36**, 343-355.

Bertwistle, D., Walmsley, M. E., Read, E. M., Pizzey, J. A. and Patient, R. K. (1996). GATA factors and the origins of adult and embryonic blood in Xenopus: responses to retinoic acid. *Mech. Dev.* **57**, 199-214.

Bielinska, M., Narita, N., Heikinheimo, M., Porter, S. B. and Wilson, D. B. (1996). Erythropoiesis and vasculogenesis in embryoid bodies lacking visceral yolk sac endoderm. *Blood* **88**, 3720-3730.

Blagden, C. S., Currie, P. D., Ingham, P. W. and Hughes, S. M. (1997). Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog. *Genes and Development* **11**, 2163-2175.

Bouwmeester, T., Kim, S.-H., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382**, 595-601.

Brewer, A., Gove, C., Davies, A., McNulty, C., Barrow, D., Koutsourakis, M., Farzaneh, F., Pizzey, J., Bomford, A. and Patient, R. (1999) The human and mouse GATA-6 genes utilise two promoters and two initiation codons. *Journal of Biological Chemistry* **274**, 38004-38016

Casey, E. S., Tada, M., Fairclough, L., Wylie, C. C., Heasman, J. and Smith, J. C. (1999). Bix4 is activated directly by VegT and mediates endoderm formation in *Xenopus* development. *Development* **126**, 4193-4200.

Chalmers, A. D. and Slack, J. M. W. (2000) The Xenopus tadpole gut: fate maps and morphogenetic movements. *Development* **127**, 381-392.

Cho, K. W. Y., Blumberg, B., Steinbeisser, H. and De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene *goosecoid*. *Cell* **647**, 1111-1120.

Clements, D., Friday, R. V. and Woodland, H. R. (1999). Mode of action of VegT in mesoderm and endoderm formation. *Development* **126**, 4093-4911

Conlon, F. L. and Smith, J. C. (1999) Interference with brachyury function inhibits convergent extension, causes apoptosis, and reveals seperate requirements in the FGF and activin signalling pathways. *Dev. Biol.* **211**, 85-100

Cornell, R. A. and Kimelman, D. (1994). Activin mediated mesoderm induction requires FGF. *Development* **120**, 453-462.

Cornell, R. A., Musci, T. J. and Kimelman, D. (1995) FGF is a prospective competence factor for early activin-type signals in *Xenopus* mesoderm induction. *Development* **121**, 2429-2437.

Dale, L. and Slack, J. M. W. (1987). Fate map for the 32-cell stage of *Xenopus laevis*. *Development* **99**, 527-21.

Demartis, A., Maffei, M., Vignali, R., Barsacchi, G. and De Simone, V. (1994). Cloning and developmental expression of LFB3/HNF1β transcription factor in Xenopus laevis. *Mech. Dev.* **47**, 19-28.

Ecochard, V., Cayrol, C., Rey, S., Foulquier, F., Cailliol, D., Lemaire, P. and Duprat, A. M. (1998). A novel Xenopus Mix-like gene milk involved in the control of endomesodermal fates. *Development* **125**, 2577-2585.

Evans, T. and Felsenfeld, G. (1989). The erythroid-specific transcription factor Eryf1: a new finger protein. *Cell* **58**, 877-885.

Feldman, B., Gates, M. A., Egan, E. S., Dougan, S. T., Rennebeck, G., Sirotkin, H. I., Schler, A. F. and Talbot, W. S. (1998). Zebrafish organiser development and germ layer formation require nodal-related signals. *Nature* **395**, 181-185.

Ferreiro, B., Artinger, M., Cho, K. W. J. and Niehrs, C. (1998). Antimorphic goosecoids. *Development* **125**, 1347-1359.

Gamer, L. W. and Wright, C. V. E. (1995). Autonomous endodermal determination in *Xenopus:* regulation of expression of the pancreatic gene *XlHbox 8*. *Dev. Biol.* **171**, 240-251.

Gao, X., Sedgwick, T., Shi, Y.-B. and Evans, T. (1998). Distinct functions are implicated for the GATA-4,-5,-6 transcription factors in the regulation of intestine epithelial cell differentiation. *Mol. Cell. Biol.* **18**, 2901-2911.

Gove, C., Walmsley, M., Nijjar, S., Bertwistle, D., Guille, M., Partington, G., Bomford, A. and Patient, R. (1997). Over-expression of GATA-6 in *Xenopus* embryos blocks differentiation of heart precursors. *EMBO J.* **16**, 355-368.

Gurdon, J. B. and Wickens, M. P. (1983). The use of Xenopus oocytes for the expression of cloned genes. *Methods Enzymol.* **101**, 30-386.

Hardin, J. and Keller, R. E. (1988). The behaviour and function of bottle cells during gastrulation of Xenopus laevis. *Development* **103**, 211-230.

Heasman, J., Wylie, C. C., Hausen, P. and Smith, J. C. (1984). Fates and states of determination of single vegetal pole blastomeres of *X. laevis*. *Cell* **37**, 185-194.

Hemmati-Brivanlou, A. and Melton, D. A. (1992). A truncated activin receptor inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. *Nature* **359**, 609-614.

Henry, G. L., Brivanlou, I. H., Kessler, D. S., Hemmati-Brivanlou, A. and Melton, D. (1996). TGF-β signals and a prepattern in *Xenopus laevis* endodermal development. *Development* **122**, 1007-1015.

Henry, G. L. and Melton, D. A. (1998). Mixer, a homeobox gene required for endoderm development. *Science* **281**, 91-96.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1991). Xenopus Myf-5 marks early muscle cells and can activate muscle genes ectopically in earl embryos. *Development* **111**, 551-560.

Hudson, C., Clements, D., Friday, R. V., Stott, D. and Woodland, H. R. (1997). Xsox17α and β Mediate Endoderm Formation in Xenopus. *Cell* **91**, 397-405.

Huggon, C. I., Davies, A., Gove, C., Moscoso, G., Moniz, C., Foss, Y., Farzaneh, F. and Towner, P. (1997). Molecular cloning of human GATA-6 DNA binding protein, high levels of expression in heart and gut. *Biochim. Biophys. Acta* **1353**, 98-102.

Isaacs, H. V., Pownall, M. E. and Slack, J. M. W. (1994). eFGF regulates Xbra expression during Xenopus gastrulation. *EMBO J.* **13**, 4469-4481.

Jacobson, A. G. and Sater, A. K. (1988). Features of embryonic induction. *Development* **104**, 341-359.

Jiang, Y. and Evans, T. (1996). The *Xenopus* GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during emryogenesis. *Dev. Biol.* **174**, 258-270.

Jones, E. A., Snape, A. M. and Sargent, T. D. (1989). Transcriptional regulation of a Xenopus embryonic keratin gene. *Development* **106**, 399-405.

Jones, E., Abel, M. and Woodland, H. (1993). The possible role of mesodermal growth-factors in the formation of endoderm in *Xenopus laevis*. *Roux Arch.* **202**, 233-239.

Jones, C. M., Dale, L., Hogan, B. L. M., Wright, C. V. E. and Smith, J. C. (1996). Bone morphogenetic protein-4 (BMP-4) acts during gastrula stages to cause ventralization of *Xenopus* embryos. *Development* **122**, 1545-1554.

Joseph, E. M. and Melton, D. A. (1998). Mutant Vg-1 ligands disrupt endoderm and mesoderm formation in Xenopus embryos. *Development* **125**, 2677-2685.

Keller, R. E. (1975). Vital dye mapping of the gastrula and neurula of Xenopus laevis. Prospective areas and morphogenetic movements of the superficial layer. *Dev. Biol.* **42**, 222-241.

Keller, R. E. (1976). Vital dye mapping of the gastrula and neurula of *Xenopus laevis* II. Prospective areas and morphogenetic movements of the deep layer. *Dev. Biol.* **51**, 118-137.

Keller, R. E. (1981). An experimental analysis of the role of bottle cells and the deep marginal zone in gastrulation of Xenopus laevis. *J. Exp. Zool.* **216**, 81-101.

Keller, R. E. (1991). Early embryonic development of *Xenopus laevis*. *Methods Cell Biol.* **36**, 61-113.

Kelley, C., Blumberg, H., Zon, L. I. and Evans, T. (1993). GATA-4 is a novel transcription factor expressed in endocardium of the developing heart. *Development* **118**, 817-827.

Kimelman, D. and Griffin, K. J. P. (1998). Mesoderm induction: a postmodern view. *Cell* **94**, 419-421.

Kofron, M., Demel, T., Xanthos, J., Lohr, J., Sun, B., Sive, H., Osada, S., Wright, C., Wylie, C. and Heasman, J. (1999) Mesodermal induction in Xenopus is a zygotic event regulated by VegT via TFG-β growth factors. *Development* **126**, 5759-5770

Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R. and Grosveld, F. (1999). The transcription factor GATA6 is essential for early extraembryonic development. *Development* **126**, 723-732.

Krieg, P. A. and Melton, D. A. (1984). Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucleic Acids Res.* **12**, 7057-7070.

Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C. and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev.* **11**, 1048-1060.

Labonne, C. and Whitman, M. (1994). Mesoderm induction by activin requires FGF mediated intracellular signals. *Development* **120**, 463-472.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**,680-685.

Laverriere, A. C., MacNiell, C., Mueller, C., Poelmann, R. E., Burch, J. B. E. and Evans, T. (1994). GATA-4/5/6: a subfamily of three transcription factors transcribed in the developing heart and gut. *J. Biol. Chem.* **269**, 23177-23184.

Lemaire, P., Darras, S., Caillol, D. and Kodjabachian, L. (1998). A role for the vegetally expressed Xenopus gene, Mix.1 in endoderm formation and in the restriction of mesoderm to the marginal zone. *Development* **125**, 2371-2380

Mead, P. E., Zhou, Y., Lustig, K. D., Huber, T. L., Kirschner, M. W. and Zon, L. I. (1998). Cloning of Mix-related homeodomain proteins using fast retrieval of gel shift activities, (FROGS), a technique for the isolation of DNA binding proteins. *Proc. Natl. Acad. Sci. USA* **95**, 11251-11256.

Mohun, T. J., Garett, N., Stutz, F. and Spohr, G. (1988) A third striated muscle actin gene is expressed during early development in the amphibian Xenopus laevis. *J. Mol. Biol.* 202, 67-76.

Molkentin, J. D., Lin, Q., Duncan, S. A. and Olson, E. N. (1997). Requirement ot the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev.* **11**, 1061-1072.

Morrisey, E. E., Ip, H. S., Tang, Z., Lu, M. M. and Parmacek, M. S. (1997). GATA-5: a transcriptional activator expressed in a novel temporally and spatially restricted pattern during embryonic development. *Dev. Biol.* **183**, 21-36.

Morrisey, E. E., Tang, Z., Sigrist, K., Lu, M. M. Jiang, F. Ip, H. S. and Parmacek, M. S. (1998). GATA6 regulates HNF4 and is required dor differentiation of visceral endoderm in the mouse embryo. *Genes Dev.* **12**, 3579-3590.

Nakatani, J., Mizuseki, K., Tsuda, H., Nakanishi, S. and Sasai, Y. (2000) Xenopus Xenf: an early endodermal nuclear factor that is regulated in a pathway distinct from Sox17 and Mix-related gene pathways. *Mech. Dev.* **91**, 81-89.

Narita, N., Bielinska, M. and Wilson, D. B. (1997). Wild-type endoderm abrogates the ventral developmental defects associated with GATA-4 deficiency in the mouse. *Dev. Biol.* **189**, 270-274.

Nascone, N. and Mercola, M. (1996). Endoderm and cardiogenesis. *Trends Cardiovasc. Med.* **6**, 211-216.

Neave, B., Holder, N. and Patient, R. K. (1997). A graded response to BMP-4 spatially coordinates patterning of the mesoderm and ectoderm in zebrafish. *Mech. Dev.* **62,** 183-195

Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table of Xenopus laevis (Daudin)*. Amsterdam: North Holland.

Nieuwkoop, P. D. (1969). The formation of mesoderm in Urodelean amphibians. I. Induction by the endoderm. *Wilhelm Roux's Arch. Entwicklungsmech. Org.* **162**, 341-373.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G. and Boncinelli, E. (1995) The Xenopus homologue of Otx2 is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* **121**, 707-720.

Pandolfi, P. P., Roth, M. E., Karis, A., Leonard, M. W., Dzierzak, E., Grosveld, F. G., Engel, J. D. and Lindenbaum, M. H. (1995). Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. *Nat. Genet.* **11**, 40-44.

Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S., D'Agati, V., Orkin, S. H. and Constantini, F. (1991). Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* **349**, 257-260.

Peyrieras, N., Strahle, U. and Rosa, F. (1998). Conversion of zebrafish blastomeres to an endodermal fate by TGF-β related signalling. *Curr. Biol.* **8**, 783-786.

Read, E. M., Rodaway, A. R. F., Neave, B., Brandon, N., Holder, N., Patient, R. K. and Walmsley, M. E. (1998). Evidence for non-axial A/P patterning in Xenopus and zebrafish pregastrula embryos. *Int. J. Dev. Biol.* **42**, 763-774.

Rebagliati, M. R., Toyama, R., Haffter, P. and Dawid, I. B. (1998). cyclops encodes a nodal related factor involved in midline signalling. *Proc. Natl. Acad. Sci. USA* **95**, 9932-9937.

Reiter, J. F., Alexander, J., Rodaway, A., Yelon, D., Patient, R., Holder, N. and Stainier, D. Y. R. (1999) Gata5 is required for the development of the heart and endoderm in zebrafish. *Genes Dev.* **13**, 2983-2995.

Reuter, R. (1994). The gene *serpent* has homeotic properties and specifies endoderm versus ectoderm within the *Drosophila* gut. *Development* **120**, 1123-1135.

Rodaway, A. R. F., Takeda, H., Koshida, S., Price, B. M. J., Smith, J. C., Patient, R. K. and Holder, N. (1999) Induction of the mesendoderm in the zebrafish germ ring by yolk cell derived TGF-β family signals and discrimination of mesoderm and endoderm by FGF. *Development* **126**, 3067-3078

Rosa, F. M. (1989). *Mix.1*, a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of *Xenopus* embryos. *Cell* **57**, 965-974.

Rupp, R. A. W and Weintraub, H. (1991). Ubiquitous MyoD transcription at the midblastula transition precedes induction-dependent MyoD expression in presumptive mesoderm of *X. laevis*. *Cell* **65**, 927-937

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sampath, K., Rubinstein, A. L., Cheng, A. M. S., Liang, J. O., Fekany, K., Solnica-Krezel, L., Korzh, V., Halpern, M. E. and Wright, C. V. E. (1998). Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature* **395**, 185-189.

Sasai, Y., Lu, B., Steinbesser, H., Geissert, D., Gont, L. K. and De Robertis, E. M. (1994). Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.

Sasai, Y., Lu, B., Piccolo, S. and De Robertis, E. M. (1996). Endoderm induction by the organizer-secreted factors chordin and noggin in *Xenopus* animal caps. *EMBO J.* **15**, 4547-4555.

Schulte-Merker, S. and Smith, J. C. (1999). Mesoderm formation in response to brachyury requires FGF signalling. *Curr. Biol.* **5**,62-67

Shi, Y.-B. and Hayes, W. P. (1994). Thyroid hormone-dependent regulation of the intestinal fatty acid-binding protein gene during amphibian morphogenesis. *Dev. Biol.* **161**, 48-58.

Shoichet, S. A., Malik, T. H., Rothman, J. H. and Shivdasani, R. A. (2000) Action of the Caenorhabditis elgans GATA factor END-1 in Xenopus suggests that similar mechanisms initiate endoderm development in ecdysozoa and vertebrates. *Proc. Natl. Acad. Sci. USA* **97**, 4076-4081.

Smith, J. C. (1994). Hedgehog, the floorplate, and the zone of polarizing activity. *Cell* **76**, 193-196.

Smith, W. C. and Harland, R. M. (1991) Injected Xwnt-8 RNA acts early in Xenopus embryos to promote formation of a vegetal dorsalizing center. *Cell* **67**, 753-765.

Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D. and Herrmann,

B. G. (1991). Expression of the Xenopus homologue of Brachyury(T) is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.

Soudais, C., Bielinska, M., Heikinheimo, M., MacArthur, C. A., Narita, N., Saffitz, J. E., Simon, M. C., Leiden, J. M. and Wilson, D. B. (1995). Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts viceral endoderm differentiation in vitro. *Development* **121**, 3877-3888.

Sun, B. I., Bush, S. M., Collins-Racie, L. A., LaVallie, E. R., DiBlasio-Smith, E. A., Wolfman, N. M., McCoy, J. M. and Sive, H. L. (1999) derriere: a TGF-β family member required for posterior development in *Xenopus*. *Development* **126**, 1467-1482.

Sykes, T. G., Rodaway, A. R. F., Walmsley, M. E. and Patient, R. K. (1998). Suppression of GATA factor activity causes axis duplication in *Xenopus*. *Development* **125**, 4595-4605.

Tada, M., Casey, E. S., Fairclough, L. and Smith, J. C. (1998). Bix1, a direct target of Xenopus T-box genes, causes formation of ventral mesoderm and endoderm. *Development* **125**, 3997-4006.

Tamura, S., Wang, X.-H., Maedea, M. and Futai, M. (1993). Gastric DNA-binding proteins recognise upstream sequence motifs of parietal cell-specific genes. *Proc. Natl. Acad. Sci. USA* **90**, 10876-10880.

Tiedemann, H. (1990). Cellular and molecular aspects of embryonic induction. *Zool. Sci.* **7**, 171-186.

Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W. and Orkin, S. H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221-226.

Walmsley, M. E., Guille, M. J., Bertwistle, D., Smith, J. C., Pizzey, J. A. and Patient, R. K. (1994). Negative control of *Xenopus* GATA-2 by activin and noggin with eventual expression in precursors of the ventral blood islands. *Development* **120**, 2519-2529.

Wessely, O. and De Robertis, E. M. (2000) The Xenopus homologue of Bicaudal-C is a localized maternal mRNA that can induce endoderm formation. *Development* **127**, 2053-2062.

Winklbauer, R. and Schurfeld, M. (1999). Vegetal rotation, a new gastrulation movement involved in the internalization of the mesoderm and endoderm in *Xenopus*. *Development* **126**, 3703-3713

Wilkinson, D. (1992). Whole mount in situ hybridisation of vertebrate embryos. *In situ hybridisation: a practical approach*. IRL Press. 75-84.

Wright, C. V. E, Schnegelsberg, P. and De Robertis E. M. (1988). XlHbox 8: a novel homeo protein restricted to a narrow band of endoderm. *Development* **104**, 787-794

Wylie, C. C., Snape, A., Heasman, J. and Smith, J. C. (1987). Vegetal pole cells and commitment to form endoderm in *Xenopus laevis*. *Dev. Biol.* **119**, 496-502.

Yasuo, H. and Lemaire, P. (1999). A two-step model for the fate determination of presumptive endodermal blastomeres in *Xenopus* embryos. *Curr. Biol.* **9**,869-879

Zaret, K. (1999). Developmental competence of the gut endoderm: Genetic potentiation by GATA and HNF3/fork head proteins. *Dev. Biol.* **209**, 1-10

Zhang, J., Houston, D., King, M. L., Payne, C., Wylie, C. C. and Heasman, J. (1998). The role of maternal VegT in establishing the primary germ layers in Xenopus embryos. *Cell* **94**, 513-524.

Zhu, J., Hill, R. J., Heid, P. J., Fukuyama, M., Sugimoto, A., Priess, J. R. and Rothman, J. H. (1997). end-1 encodes an apparent GATA factor that specifies the endoderm precursor in Caenorhabditis elegans embryos. *Genes Dev.* **11**, 2883-2896.

Zon, L. I., Mather, C., Burgess, S., Bolce, M., Harland, R. M. and Orkin, S. H. (1991). Expression of GATA binding proteins during embryonic development in *Xenopus laevis*. *Proc. Natl. Acad. Sci. USA* **88**, 10642-10646.