

Expression cloning of a *Xenopus* T-related gene (*Xombi*) involved in mesodermal patterning and blastopore lip formation

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SUMMARY

We have used a functional assay to identify a putative T-box transcription factor (*Xombi*) that has the ability to induce sites of invagination in the ectoderm of *Xenopus* embryos that resemble the blastopore lip. Maternal *Xombi* transcript is first localized to the oocyte's vegetal cortex and cytoplasm, early sources of mesoderm and endoderm-inducing signals. Soon after zygotic transcription begins, there is a wave of *Xombi* expression, beginning in dorsal mesoderm and then extending to lateral and ventral mesoderm, that precedes and parallels blastopore lip formation at the border between the mesoderm and endoderm. Transcripts encoding brachyury, *Xwnt8* and goosecoid colocalize with *Xombi* transcripts within the marginal zone; ectopic expression of *Xombi* induces

expression of all three mesodermal genes. In ectodermal explants, *Xombi* expression is induced by the secreted mesoderm inducers activinA, activinB, *Xnr1* and eFGF, and by brachyury, another *Xenopus* T-box containing gene. The time course and location of *Xombi* expression, its biological activities and the partial dependence of *Xombi* expression and blastopore lip formation on fibroblast growth factor (FGF) signaling suggest that *Xombi* contributes to a traveling wave of morphogenesis and differentiation during *Xenopus* gastrulation.

Key words: *Xenopus*, mesoderm, endoderm, morphogenesis, blastopore lip, invagination, bottle cells, brachyury, T-box

INTRODUCTION

In amphibians, as in many other vertebrates, both maternal and zygotic proteins are required to establish the basic body plan. During the first cell cycle in *Xenopus*, the thin cortex of the egg rotates approx. 30° relative to the dense vegetal cytoplasm, and maternal dorsal determinants become localized or activated in the region of the embryo where vegetal cortex mixes with animal cytoplasm. These maternal determinants initiate a series of cell-autonomous and inductive interactions that, by early blastula stages, lead to the formation of a group of dorsovegetal cells known as the Nieuwkoop center (Kimelman et al., 1992). Prior to the onset of zygotic transcription at the mid-blastula transition, at least two different types of presumptive mesoderm tissues have been induced at the marginal zone between the embryo's vegetal and animal hemispheres. Dorsal mesoderm has been induced by proteins secreted by the Nieuwkoop center, and ventral mesoderm has been induced everywhere else in the marginal zone by vegetal proteins that are active even in the absence of cortical rotation. In *Xenopus*, members of both the transforming growth factor beta (TGFβ) and fibroblast growth factor (FGF) families of secreted signaling proteins have been implicated in mesoderm induction (reviewed by Kimelman et al., 1992 and Slack, 1994). TGFβ family members such as activinβA and activinβB are found in the early *Xenopus* embryo, and are capable of inducing dorsal mesoderm. FGF family members such as embryonic FGF (eFGF) and basic FGF (bFGF) are also found in the early embryo; in contrast to TGFβ members, they are

only capable of inducing mesoderm of more ventral character. The primary pattern of mesodermal gene expression established during blastula stages, when the embryo forms a hollow ball of cells, is extensively refined during gastrula stages by interactions between zygotically-expressed proteins released by opposing dorsal and ventral organizing centers, and by reciprocal interactions within the rest of the mesoderm (Gerhart et al., 1991; Kimelman et al., 1992; Harland, 1994).

Gastrulation is the highly dynamic morphogenetic process by which the three germ layers, initially present on or near the surface of the embryo become repositioned, with mesoderm placed between exterior ectoderm and interior endoderm. In *Xenopus*, the movement of mesodermal and endodermal cells into the interior of the embryo occurs through a dynamic and transient morphogenetic structure known as the blastopore lip. Little is known of the molecular signals that direct the formation of the blastopore lip, although the changes in cell behavior have been well-studied (for review see Keller, 1991). The first visible sign of lip formation is the apical constriction of pigment in superficial dorso-vegetal epithelial cells located above the Nieuwkoop center, signifying the reduction of apical surface area as cells take on the typical appearance of bottle cells. At the onset of gastrulation, these dorsal bottle cells descend into the embryo, allowing anterior mesoderm to migrate into the interior of the embryo and initiating dorsal blastopore lip formation (Hardin and Keller, 1988). As gastrulation proceeds, bottle cells form laterally and ventrally, preceding a dorsal to ventral wave of blastopore lip formation, and a dorsal to ventral wave of tissue involution. By the end

of gastrulation, dorsal, lateral and ventral mesoderm has involuted through the circular blastopore lip to form the mesodermal mantle between the ectoderm and the endoderm. The mesoderm formed during this process undergoes complex morphogenetic movements that ultimately establish the elongated and bilaterally symmetric body axis. In *Xenopus*, the blastopore ultimately forms the posterior end of the embryo's digestive tract.

In the mesoderm, tissue-specific growth factors induce expression of a variety of transcription factors, which in turn induce expression of a different subset of growth factors in responding tissues. Understanding the interplay between growth factor signaling and tissue-specific transcription has been a major focus of vertebrate developmental biology. One of the better-characterized transcription factors involved in mesoderm specification is brachyury (T), a member of the T-box family of DNA-binding proteins (Smith et al., 1991). Homologs of brachyury are found in all chordates and appear to be involved in the differentiation of notochord, a dorsal mesoderm derivative, in all cases where they have been examined (Yasuo et al., 1995). In *Xenopus*, brachyury is induced by TGF β and FGF family members, is expressed during gastrula stages throughout the mesoderm as well as in cells fated to form the notochord, and appears to be part of the pathway by which ventral mesoderm is induced (Smith et al., 1991, 1995).

It is at present unclear whether mesoderm itself plays a role in directing blastopore lip formation, although mesoderm-inducing proteins such as brachyury have been reported to affect cell movements around the blastopore (Isaacs et al., 1994). Here we report the expression cloning of a new member of the T-box family of transcription factors (*Xombi*) that, unlike brachyury, induces ectopic structures that resemble the blastopore lip, as well as both ventral and dorsal mesoderm. *Xombi* is expressed prior to brachyury in a dorsal to ventral wave in the mesoderm, preceding a similar wave of blastopore lip formation at the border between the mesoderm and endoderm. In addition to promoting morphogenesis, *Xombi* may participate in a TGF β -inducible, FGF-dependent positive feedback loop involving brachyury that serves to refine mesodermal pattern early in gastrulation. In contrast to brachyury, *Xombi* is expressed maternally at high levels in the oocyte's vegetal cortex and cytoplasm, and thus may also play a role in the earliest signaling events that induce endoderm and mesoderm and establish dorsal-ventral polarity.

MATERIALS AND METHODS

Embryos and explants

Pigmented and albino *X. laevis* embryos were obtained by in vitro fertilization, dejellied and cultured at 18°-19°C in 0.1 \times Marc's modified Ringers (MMR) containing 50 μ g/ml gentamicin (Peng, 1991). Embryos were ventralized to a dorsoanterior index (DAI) of 0-1, as previously described (Kao and Danilchik, 1991), by briefly exposing (40 to 50 seconds) their vegetal surface to UV light about 30 minutes after fertilization. Embryos were dorsalized to a DAI of 9-10, as previously described (Kao and Danilchik, 1991), by treating them for 10 minutes with 0.3 M lithium chloride when they reached the 64- to 128-cell stage. Staging of embryos was carried out according to Nieuwkoop and Faber (1967).

Ectodermal explants were cut from the apex of the animal pole of

mid-blastula stage embryos (stage 8.5-9) using an electric cutting tool (Xenotek Engineering, Belleville, IL). The approx. 0.16 mm² square explants were cultured in 1 \times MBSH (pH 7.4; Peng, 1991) and changes in their developmental fate were evaluated by using reverse transcriptase-PCR (RT-PCR) to examine marker gene expression.

Expression screening of small library pools

The functional assay used to identify *Xombi* is similar to the assay used by LeMaire et al. (1995) to identify the homeobox-containing protein siamois. Library RNA was injected into the ventral side of an early embryo, and the embryo was visually scored later in development. Unlike Lemaire et al. (1995), who injected complete library RNA or RNA pools representing 100,000 clones into the vegetal region of a ventral blastomere and then scored for the formation of a secondary body axis, we injected small pools of library RNA (100 to 1000 clones) into either the vegetal or animal pole of a ventral blastomere and then scored relatively early in development for any visible change in embryo morphology, including but not limited to secondary axes. By significantly enhancing the likelihood of proteins with moderate or weak activity being detected, a small pool screening approach may allow this type of synthetic screen in *Xenopus* to reach saturation. Although it is labor intensive to prepare and screen small pools, the increased effort seems compensated for by the increased frequency with which active cDNAs are functionally identified.

A cDNA library was constructed in plasmid pCS2+ (Turner and Weintraub, 1994) from gastrula stage embryos (stage 10 to 10.5) that had been hyperdorsalized to a dorsoanterior index (DAI) of 8-9 by LiCl treatment (Lustig and Kirschner, 1995). Aliquots from the library (containing between 100 and 1000 independent transformants) were used to amplify plasmid DNA, which in turn was used to synthesize pools of GpppG-capped RNA in vitro (Wormington, 1991; Lustig et al., 1996). Approximately 2 ng of library RNA was injected into the animal or vegetal region of a single ventral blastomere of a four-cell embryo. Ventral blastomeres were distinguished from dorsal blastomeres based upon their larger size and darker pigmentation and the presence of a sperm pigment trail. Embryos were visually scored for alterations in cell fate at specified times during gastrula (stage 10-12), neurula (stage 17-19) and tailbud (stages 30-32) stages of early development. To isolate the active clone in each positive pool, the pool was subdivided into smaller pools (Lustig et al., 1996), which were again re-tested for activity. Positive pools were progressively subdivided in this manner until a single active cDNA was obtained.

The first active *Xombi* cDNA clone that we isolated (approx. 1.5 kb in length) contained the entire T-box DNA-binding domain but, as no upstream stop codons were found, appeared to lack the 5' end of the open reading frame. To clone the full-length coding sequence, we first used PCR to identify library pools that contained the 3' end of the *Xombi* gene, and then determined which of these pools contained the longest *Xombi* clone. Ninety six pools of library cDNA, each containing 1000 cDNAs, were assayed by PCR for *Xombi*. A 650 nt region within the open reading frame was amplified (forward primer: 5' TTC CAG AAG CTC AAA CTC AC 3'; reverse primer: 5' TGT GTT GGA ATG ACA TGA AG 3'). The twelve positive pools that contained *Xombi* were then re-amplified by PCR using a forward primer that hybridizes to the SP6 promoter in the plasmid and a reverse primer that hybridizes about approx. 90 nt downstream of the 5' end of the partial *Xombi* clone. Products as large as approx. 450 nt were amplified from several of the pools (of 1000 clones). Sequence analysis indicated that 50 bases at the 3' end of several of the PCR products exactly matched the 5' end of the original *Xombi* clone, suggesting that we had amplified part or all of the missing 5' sequence. Rather than amplifying the full-length *Xombi* cDNA by PCR (which can introduce unwanted mutations) and then subcloning the amplified product, we used PCR as an assay to progressively subdivide the original pool of 1000 clones to a single *Xombi* clone (Lustig and Kirschner, 1995). The new sequence extended the open reading frame 56 amino acids upstream to a potential translation start site methion-

ine with several upstream stop codons. From these results we conclude that we have isolated the complete open reading frame of the *Xombi* cDNA. When RNA synthesized from the longer *Xombi* cDNA clone is injected into embryos, it has similar activities as RNA synthesized from the partial *Xombi* cDNA that was initially isolated and characterized in this study.

RT-PCR analysis

Expression of *brachyury*, *Xwnt8*, *gooseoid* and *EF-1 α* in ectodermal explants or whole embryos was analyzed by RT-PCR, as previously described (Lustig and Kirschner, 1995; Lustig et al., 1996). The sequence of the forward *Xombi* primer was 5' TGT CAG CTA TAC TGC ATA CC 3' and the sequence of the reverse primer was 5' TGC AAT CTT CCC AAC GCA C 3'. These primers amplify an approx. 250 nt region in the 3' region of the *Xombi* gene.

In situ hybridization and histology

Single and double whole-mount in situ hybridization of albino or pigmented embryos was carried out as described (Harland, 1991) using digoxigenin-11-UTP-labeled probes (Boehringer), except for the brachyury probe which was labeled with fluorescein-12-UTP. 4-nitroblue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt, (BCIP) or magenta-phosphate were used as substrates for the color reactions. Antisense probe for *Xombi* was synthesized with T3 RNA polymerase using *EcoRI*-linearized plasmid as template. Antisense probes for brachyury (Smith et al., 1991), *gooseoid* (Blumberg et al., 1991) and *Xwnt8* (Christian et al., 1991) were synthesized as described previously. To localize *Xombi* transcripts, overstained embryos were embedded in JB-4 plastic resin and cut into 7 μ m sections.

Most images were obtained using incident light or transillumination on a Zeiss axiophot microscope using 2.5 \times or 5 \times objectives. Some images were acquired on a Wild M8 stereomicroscope. All images were captured using a 3-color video rate CCD camera controlled by Northern Exposure software (Phase 3 Imaging Systems). Brightness, contrast and color balance correction were performed using Adobe Photoshop.

RESULTS

Xombi induces blastopore lip-like structures

We have carried out a simple visual screen for genes that are capable of respecifying embryonic tissue. In order to identify genes with relatively weak activity, small pools of library RNA (representing only 100 to 1000 cDNA clones) were assayed for the ability to cause any gross change in morphogenesis or tissue specification when microinjected into one cell of a four-cell *Xenopus* embryo. Out of approx. 500 pools screened, we have thus far identified seven different genes capable of respecifying ventral tissue (Table 1). Four of these genes had been previously identified: *siamois* (Lemaire et al. 1995), *noggin* (Smith and Harland, 1992), *Xwnt8* (Christian et al., 1991; Smith and Harland, 1991) and β -*catenin* (Guger and Gumbiner, 1995). Three others were not in the GenBank database as of September, 1996: a new member of the TGF β family of nodal-related secreted proteins; a new member of the homeodomain family of DNA-binding proteins; and a new member of the T-box family of DNA-binding proteins. Because the T-box gene is more closely related to the *Drosophila* optomotor blind (*omb*)/Tbx2 subclass of T-box proteins than to the brachyury subclass, we have named it *Xenopus optomotor blind* (*Xombi*). This report will focus on the functional characterization of *Xombi*'s unusual activities

Table 1. cDNAs identified by screening small RNA pools for functional activity

	Putative gene product	No. of isolates
Known genes		
<i>noggin</i>	Secreted signaling protein	4
β - <i>catenin</i>	Adhesion/signaling protein	4
<i>siamois</i>	Homeodomain protein	3
<i>Xwnt8</i>	Secreted signaling protein	2
New genes		
<i>Xombi</i>	T-box transcription factor	2
nodal-related	Secreted signaling protein	1
homeobox-containing	Homeodomain protein	1

and expression pattern. The other two new genes will be the subjects of separate reports.

The phenotype of embryos injected ventrally with *Xombi* RNA is distinct from that of embryos injected ventrally with RNA encoding *siamois* (Lemaire et al., 1995), *noggin* (Smith and Harland, 1992), *Xwnt8* (Smith and Harland, 1991) or β -*catenin* (Guger and Gumbiner, 1995). Vegetal injection of these latter transcripts induce an ectopic Nieuwkoop center and thus generate partial or complete secondary axes containing axial tissue (e.g. notochord, somites) often indistinguishable from axial tissue in the primary axis (not shown). By contrast, vegetal or animal injection of *Xombi* RNA does not induce

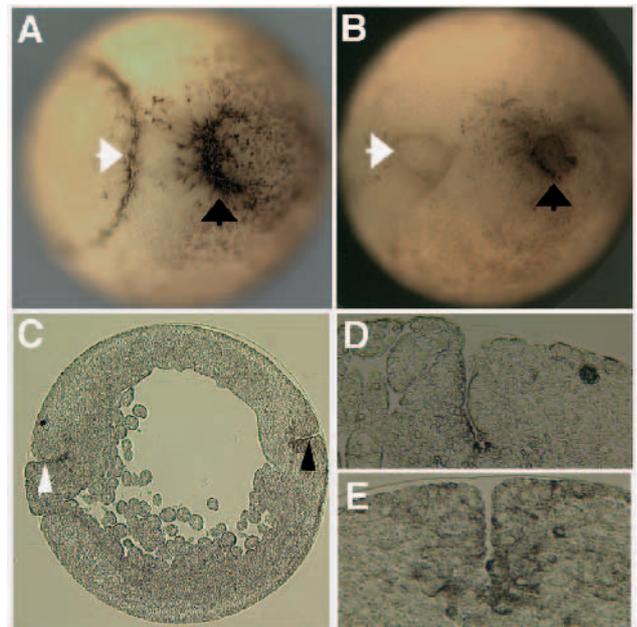


Fig. 1. Ectopic lip formation by *Xombi* in normal and ventralized embryos. *Xombi* RNA (100 pg or 300 pg) was injected at the four-cell stage of development into the animal pole region of a ventral blastomere. (A) Lateral view of a normal early gastrula-stage embryo with a slightly curved ectopic lip. (B) Lateral view of a normal late gastrula-stage embryo with an almost circular ectopic lip. At this stage, the endogenous blastopore is almost closed. (C) Sagittal section through a ventralized embryo showing both endogenous and ectopic lips. (D) A higher magnification view of the endogenous lip shown in C. (E) A higher magnification view of the ectopic lip shown in C. In A, B and C, the endogenous blastopore is indicated by a white arrowhead and the ectopic lip is indicated by a black arrowhead.

An examination of sagittal sections through ectopic blastopore lips reveals that they are often similar in appearance to endogenous blastopore lips (Fig. 1C-E). At the lowest active doses of *Xombi*, very little cell movement occurs, and the resulting invagination site is similar in appearance to the dorsal blastopore groove at stage 10+. With higher doses, extensive cell movements are observed at the ectopic site, with the invagination extending in multiple directions, with frequent internal bending (not shown).

Xombi cDNA encodes a putative protein with a T-box DNA-binding domain

Sequence analysis revealed that the 1938 nt cDNA insert had an open reading frame of 455 amino acids (Fig. 2A) with a predicted molecular mass of 52×10^3 . Comparison of the predicted protein sequence with the GenBank protein database revealed that *Xombi* is a member of the T-box family of DNA-binding proteins (Bollag et al., 1994). Members of the T-box family are highly related within a region of between 160 to 200 amino acids in length (the T-box) that is necessary and sufficient for DNA binding (Kispert and Hermann, 1993). Within its T-box, *Xombi* is most closely related (Fig. 2B) to mouse *Tbx2* (57% identity; Bollag et al., 1994), *Drosophila* *optomotor blind* (54% identity; Pflugfelder et al., 1992) and *Xenopus* *brachyury* (49% identity; Smith et al., 1991). Outside its putative DNA-binding domain, *Xombi* shows no obvious homology to other members of the gene family. The motif Ser-Pro (S-P-X-X)_n, which is found in many DNA-binding proteins (Suzuki, 1989), is found distributed throughout the putative *Xombi* protein. There is also a consensus MAP kinase phosphorylation site (P-X-S/T-P; Gonzalez et al., 1991) within the putative DNA-binding domain.

Zygotic *Xombi* expression parallels blastopore formation in gastrulating embryos

A combination of RT-PCR and whole-mount in situ hybridization analysis was used to analyze the spatial and temporal expression of *Xombi* during early *Xenopus* development. High levels of maternal transcripts are detected by RT-PCR in unfertilized eggs and cleavage-stage embryos (Fig. 3). Transcripts are found in punctate structures in the vegetal cytoplasm of stage III oocytes (Fig. 4A) and in the cortex region just under the oocyte plasma membrane (Fig. 4B). Transcripts are also evenly distributed outside the cortex, throughout the vegetal

pole (Fig. 4C). This expression pattern persists throughout the remainder of oogenesis (over weeks to months). Transcripts are found in the vegetal hemisphere and nucleus of the stage VI oocyte (Fig. 4D) and are also observed in the vegetal half of two-cell, eight-cell and 64-cell embryos (not shown).

Blastopore formation is a highly dynamic process, with the blastopore lip forming dorsally by stage 10+ to 10.25, extending laterally by stage 10.5 and extending ventrally by stages 11 to 11.5 (Nieuwkoop and Faber, 1967). The zygotic expression of *Xombi* within the marginal zone precedes blastopore lip formation at its vegetal border. Zygotic transcripts are detected very soon after the mid-blastula transition (stage 9) in the nuclei of cells located throughout the marginal zone (not shown). About 90 minutes prior to the onset of gastrulation, a high level of *Xombi* transcript accumulates in dorsal marginal zone cells (Fig. 4E). When pigment first accumulates in the bottle cells that demark the future site of blastopore lip formation (stage 10-), *Xombi* transcripts are detected dorsally and laterally, and (in most but not all embryos) ventrally as well. By the time the dorsal blastoporal groove is visible (stage 10+), *Xombi* transcripts are detected at high levels throughout the entire circumference of the marginal zone (Fig. 4F).

Although *Xombi* transcripts are detected in marginal zone cells, they are not found in the anterior aspect of the forming dorsal lip or in the bottle cells themselves (Fig. 4G). This restriction is observed as early as stage 10, when the blastopore pigment line first become visible (Fig. 4F) and is observed around the entire lateral and ventral marginal zones by mid-gastrula stages (Fig. 4H). An examination of sections of mid-gastrula stage embryos stained in whole mount confirms that *Xombi* transcripts are excluded from a small region of cells directly adjacent to the blastopore lip (Fig. 4I) as well as from the superficial involuted cells that line the archenteron and are fated to form the lining of the digestive tract. In split-open embryos, involuted and non-involuted cells can be readily distinguished and thus examined for the presence of *Xombi* transcripts. It does not appear that *Xombi* transcripts are present in invaginated bottle cells or deep cells that have undergone involution. They are only detected in superficial and deep cells of the marginal zone that have yet to involute (Fig. 4J).

Despite the fact that *brachyury* expression is maintained throughout the entire marginal zone during gastrulation, by the middle of gastrulation (stages 10.5 to 11) *Xombi* expression recedes from the dorsal lip region of the blastopore (Fig. 4G,H). This difference is apparent in mid-gastrula stage embryos that have been double stained by whole-mount in situ hybridization for *Xombi* and for *brachyury*, which at these stages is expressed in notochord-forming cells that have already involuted over the lip (Smith et al., 1991). *Xombi* transcripts and *brachyury* transcripts co-localize in the entire marginal zone (dark blue stain) with the exception of a narrow stripe of involuted dorsal cells (magenta stain) that express *brachyury* only (Fig. 4K).

Xombi expression in the post-gastrula embryo

The overall level of *Xombi* transcript in the embryo declines considerably at the end of gastrulation (Fig. 3), but *Xombi* transcripts are nevertheless detected at low levels until the tadpole stage (stage 38). During neurulation *Xombi* continues to be expressed in the ventral and lateral but not dorsal domains of the circumblastoporal collar (Fig. 4L), the region of the embryo

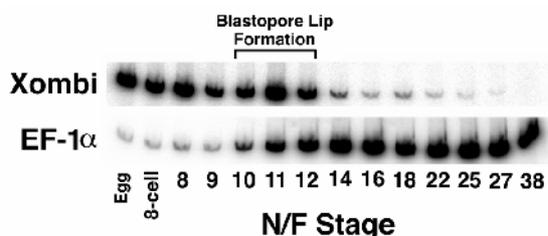
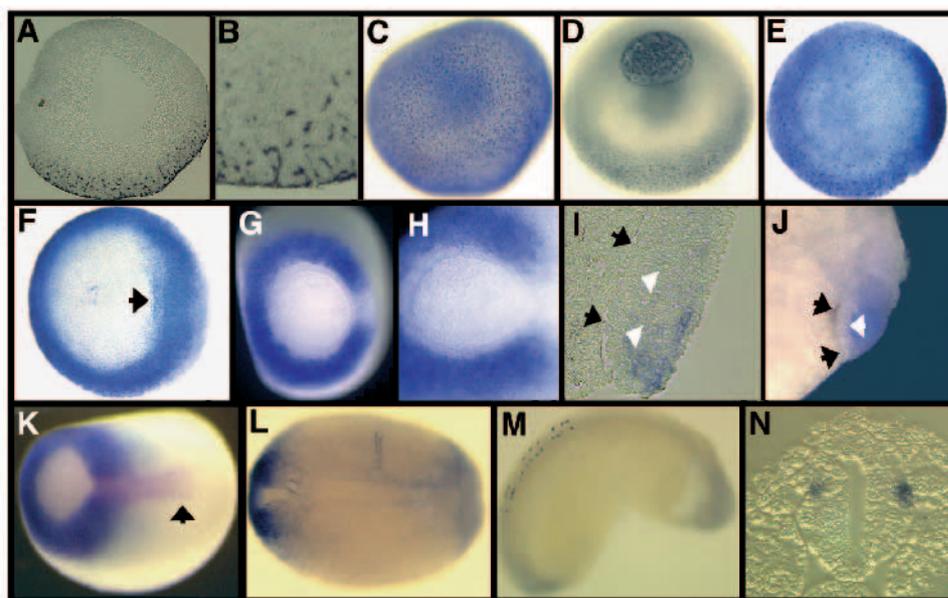


Fig. 3. *Xombi* is expressed maternally and zygotically. Embryos were harvested at the indicated stage and assayed by RT-PCR for expression of *Xombi*. Elongation factor 1- α (EF-1 α) expression was also assayed as a loading control (after blastula stages) and to illustrate the timing of the onset of general zygotic transcription. Each PCR reaction (23 cycles) contained the cDNA equivalent of one-tenth of an embryo.

Fig. 4. Spatial expression of *Xombi* during normal development. Oocytes or embryos were hybridized with a digoxigenin-labeled *Xombi* antisense RNA probe. (A) Animal-vegetal section of a stage III oocyte. (B) Higher magnification view of a different section from the same oocyte. (C) Vegetal view of a stage V oocyte. (D) Animal-vegetal (side) view of a stage VI oocyte. (E) Vegetal view of a stage 9.6 embryo. (F) Vegetal view of a stage 10+ embryo. The arrow denotes the blastopore groove. (G) Vegetal view of a stage 10.5 embryo. (H) Vegetal view of a stage 11 to 11.5 embryo. (I) Parasagittal section through a mid-gastrula embryo. (J) Parasagittal view of a mid-gastrula embryo that has been split open. In I and J, the black arrows denote the forming archenteron and the white arrows denote the boundary of *Xombi* expression. (K) Dorsal view of a mid-gastrula stage stained for *Xombi* (light blue stain) and *brachyury* (magenta stain). Where both co-localize in the marginal zone, a dark blue stain is detected. In dorsal notochord-forming cells, where only *brachyury* is expressed, only a magenta stain is detected (arrow at anterior end). (L) Dorsal view of a neurula stage embryo. Note that *Xombi* is still specifically excluded from the notochord. (M) Lateral view of an early tailbud stage embryo. (N) Differential interference contrast (DIC) image of a posterior transverse section of an early tailbud stage embryo showing *Xombi* staining only within cells in the neural tube. In A, B and D, the animal pole is at the top. In E, F, G, H, I and J, dorsal is to the right. In K, L and M, anterior is to the right. In M and N, dorsal is at the top. All oocytes and embryos except for those in G, H, J and K were cleared in a 2:1 mixture of benzyl benzoate and benzyl alcohol.



that generates the bulk of the posterior mesoderm. During neurula and tailbud stages, faint diffuse staining is also detected in the posterior and anterior ends of the embryo (Fig. 4L,M).

By the early tailbud stages, *Xombi* transcripts become clustered in patches of cells found along the dorsal midline of the trunk (Fig. 4M). Between ten and twenty of these patches are typically observed, about half of which are paired symmetrically across the dorsal midline. These patches of *Xombi* expression are detected at least until the tadpole stage of development, although it is not clear whether expression in any one persists over time. Sections of tailbud stage embryos localize *Xombi* expression to the region of the neural tube where interneurons are found, at its lateral edge above the midline (Fig. 4N).

Correlation between *Xombi* expression and blastopore formation in dorsalized and ventralized embryos

UV treatment of fertilized eggs or immersion of 64-cell embryos in lithium chloride causes *Xenopus* embryos to take on uniformly ventralized or dorsalized fates, respectively (Kao and Danilchik, 1991). Both aberrant types of embryos still undergo bottle cell and blastopore formation and at least limited tissue involution. *Xombi* is expressed at high levels in both dorsalized and ventralized embryos (Fig. 5). In dorsalized embryos, *Xombi* transcripts are expressed around the entire marginal zone at least 90 minutes prior to the onset of gastrulation (Stage 9.6; Fig. 5D), the same time they are detected in the dorsal marginal zone in normal embryos (Fig. 5A). In sibling ventralized embryos, *Xombi* is expressed in a punctate, nuclear pattern (Fig. 5G), the same pattern of expression

detected concomitantly in the ventral marginal zone of normal embryos (Fig. 5A). Bottle cells form by stage 10.5 in dorsalized embryos but, as in normal embryos, *Xombi* transcripts are

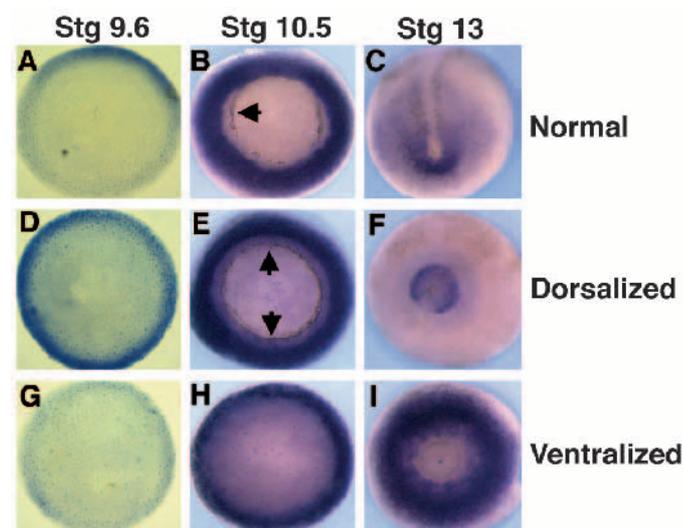


Fig. 5. *Xombi* expression in ventralized and dorsalized embryos. Embryos were hybridized with a digoxigenin-labeled *Xombi* antisense RNA probe. (A-C) Uninjected embryos. (D-F) Dorsalized embryos. (G-I) Ventralized embryos. (A,D,G) Vegetal view of stage 9.6 embryos (about 90 minutes prior to the onset of gastrulation). (B,E,H) Vegetal view of stage 10.5 embryos. (C, F,I) Posterior view of stage 13 embryos. Except for A, D and G, which show cleared sibling albino embryos, sibling pigmented embryos were used. The arrows in B and E indicate the location of the blastopore pigment line.

never detected in the anterior aspect of the forming lip or in the bottle cells themselves (Fig. 5E). By stage 10.5, *Xombi* transcripts are expressed at high levels around the entire marginal zone of ventralized embryos, although bottle cells have not yet formed (Fig. 5H). During all gastrula stages of development, *Xombi* staining is slightly less intense in ventralized embryos than in dorsalized embryos and is restricted to the marginal zone. By the beginning of neurulation (stage 13), *Xombi* expression drops considerably in normal (Fig. 5C) and dorsalized (Fig. 5F) embryos and is confined to a small region of cells surrounding the constricted blastopore. In ventralized embryos, *Xombi* expression is maintained at high levels even after the end of gastrulation (Fig. 5I).

Xombi is a general mesoderm inducer

To assess its effect on cell differentiation, *Xombi* was expressed in presumptive ectodermal explants otherwise fated to form epidermis. The explants were cultured until sibling embryos reached the mid to late gastrula stages, and then gene expression was analyzed by RT-PCR. *Xombi* induces the dose-dependent expression of three *Xenopus* genes that are zygotically expressed within the marginal zone (Fig. 6A): *brachyury*, a pan-mesodermal gene (Smith et al., 1991); *Xwnt8*, a ventrolateral mesodermal gene encoding a secreted signaling protein (Christian et al., 1991; Smith and Harland, 1991); and *goosecooid*, a dorsal-specific mesodermal gene encoding a homeodomain protein (Blumberg et al., 1991). The lowest

active dose of *Xombi* RNA (30 pg) induces *brachyury* expression and low level *Xwnt8* expression, but not *goosecooid* expression. Higher RNA doses induce all three genes: *brachyury* expression is maximal at 100 pg; *Xwnt8* expression is maximal at 300 pg and *goosecooid* expression is maximal at 1000 pg. None of the three mesodermal genes are expressed in control uninjected explants. We also left intact embryos that had been injected with *Xombi* RNA in both sides of the animal pole. Whole embryos ectopically expressing *Xombi*, express higher levels of *brachyury*, *Xwnt8* and *goosecooid* than do control sibling embryos (not shown).

Xombi is thus capable of ectopically inducing mesoderm in explanted tissue and intact embryos. To determine where mesoderm forms relative to the ectopic invagination, normal or ventralized embryos were injected with *Xombi* RNA at the four-cell stage, harvested at early gastrula stages and stained by double in situ hybridization for *Xwnt8*, *brachyury*, or *goosecooid*, to identify mesodermal cells, and then for *Xombi*, to identify the site of RNA injection. In over 50 embryos analyzed, the region of ectopic expression of *Xombi* closely overlapped the region where ectopic mesoderm is induced (not shown). In the example shown in Fig. 6B, the expression of *Xwnt8* occurs on the animal pole side of both the ectopic invagination and the endogenous blastopore lip. Similar to the endogenous blastopore lip, which defines the vegetal limit of the involuting mesoderm, ectopic invaginations induced by low doses of *Xombi* generally form at the vegetal border of the ectopic mesoderm.

Xombi expression is induced by mesoderm-inducing proteins

RT-PCR analysis was used to determine whether a number of known mesoderm-inducing proteins are capable of inducing *Xombi* expression in ectodermal explants. We found that *Xombi* expression is induced by members of both TGF β and FGF signaling families and by *brachyury*, an inducer of ventral mesoderm. Treatment of ectodermal explants with as low as 0.3 ng/ml activin A protein or microinjection as low as 0.25 pg *activin β B* RNA or 50 pg *Xnr1* RNA strongly induces *Xombi* expression (Fig. 7). Microinjection of 10 pg *eFGF* RNA induces *Xombi* expression in ectodermal explants, although even five-fold lower doses were generally ineffective and higher doses were toxic to the injected cells. *Brachyury* also induces the dose-dependent expression of *Xombi* (Fig. 7). A low level of *Xombi* transcript is reproducibly detected in control uninjected explants.

Not all of the signaling proteins that induce *Xombi* expression in ectodermal explants are also able to induce ectopic invaginations in intact embryos. Whereas the dorsal mesoderm inducers *activin β B* and *Xnr1* induce both *Xombi* expression and the formation of ectopic invaginations, the ventral mesoderm inducers *eFGF* and *brachyury* induce *Xombi* expression but not invagination. Ectopic invaginations are induced by the same doses of *activin β B* and *Xnr1* that induce *Xombi* expression, both in explants and in injected embryos assayed for *Xombi* expression by in situ hybridization (not shown). At the lowest effective doses of *activin β B* and *Xnr1* RNA, the ectopic invaginations are similar in appearance to those induced by *Xombi*. At higher doses, the invagination sites are larger and more darkly pigmented than those induced by *Xombi* and the entire region of cells with constricted apical

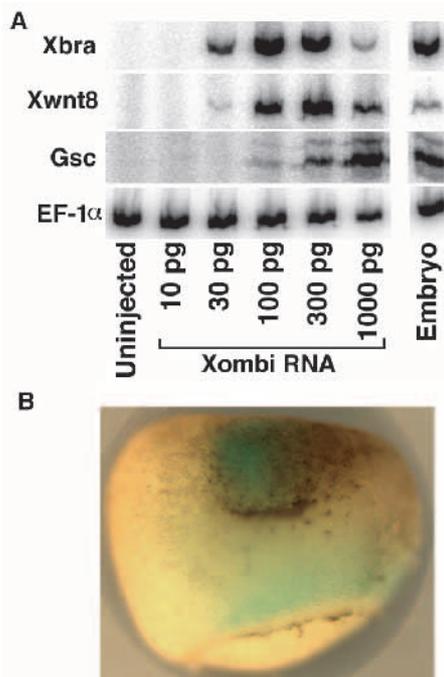


Fig. 6. *Xombi* is a general mesoderm inducer. (A) Presumptive ectoderm was explanted from embryos injected with the indicated dose of *Xombi* RNA and assayed by RT-PCR for the expression of *brachyury* (*Xbra*), *Xwnt8* or *goosecooid* (*Gsc*). *EF-1 α* expression was assayed as a loading control. (B) Lateral view of a *Xombi* RNA-injected gastrula embryo that has been stained by in situ hybridization for *Xwnt8*. *Xwnt8* expression (light blue stain) is detected in the endogenous marginal zone above the forming blastopore lip as well as in a region of cells above the forming ectopic lip. Dorsal is to the left.

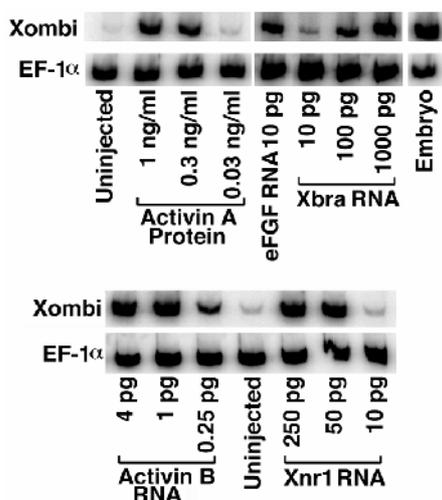


Fig. 7. Induction of *Xombi* expression by mesoderm-inducing proteins. Ectodermal explants were harvested from uninjected blastula-stage embryos and then treated with the indicated concentration of activin A protein. In parallel, presumptive ectoderm was also explanted from embryos that had been injected in the animal pole at the two-cell stage with the indicated amount of *activinβB*, *Xnr1*, *eFGF* or *brachyury* (*Xbra*) RNA. Explants were harvested when sibling embryos reached the mid-gastrula stage, and then assayed by RT-PCR for *Xombi* expression. *EF-1α* expression was assayed as a loading control.

pigment invaginates to form a darkly pigmented pit within the animal pole (not shown).

In ectodermal explants, the induction of some (but not all) mesodermal markers by activin requires an intact FGF signaling pathway (Labonne and Whitman, 1994; Cornell and Kimelman, 1994). Thus, even though FGF itself does not induce ectopic invaginations, it seemed possible that FGF might play a role in mediating the changes in cell behavior that underly their formation. To address this possibility, transcripts encoding *activinβB* and *Xombi* were co-injected with transcripts encoding a dominant negative inhibitor of the FGF receptor (XFD), and embryos were scored at mid-gastrula stages for formation of ectopic invaginations. Expression of XFD, but not UHAV ϕ , an inactive XFD construct missing just three amino acids in the ligand-binding domain (Amaya et al., 1991), strongly inhibited ectopic lip formation by *activinβB* and *Xombi* (Table 2), and partially inhibited ectopic lip formation by higher doses of *activinβB* (data not shown). *ActivinβB* RNA-injected embryos frequently formed a clearly defined partial secondary body axis, as previously shown (Thomsen et al., 1990), whereas *Xombi* RNA-injected embryos almost never did (Table 2).

Dependence of *Xombi* expression and blastopore lip formation on FGF signaling in vivo

In the intact *Xenopus* embryo, the FGF signalling pathway is required for normal gastrulation movements laterally and ventrally, though, surprisingly, not for gastrulation and subsequent head morphogenesis dorsally (Amaya et al., 1991, 1993; Isaacs et al., 1994). To determine whether *Xombi* expression in the presumptive mesoderm requires FGF signaling in vivo, we injected transcripts encoding XFD into the dorsal or ventral

Table 2. XFD inhibits ectopic invagination caused by *Xombi* and *ActivinβB*

RNA	% with invagination	% with secondary axis	(n)
<i>Xombi</i> + UHAV ϕ	100	0	56
<i>Xombi</i> + XFD	11	0	55
<i>Activin</i> + UHAV ϕ	84	60	45
<i>Activin</i> + XFD	0	2	54

Transcripts encoding *Xombi* (500 pg) or *activinβB* (1 pg) were co-injected with 500 pg transcripts encoding XFD, a dominant negative inhibitor of the FGF receptor, or UHAV ϕ , an inactive form of XFD. RNAs were injected into the animal pole region of a ventral blastomere at the eight-cell stage of development and embryos were visually scored at mid-gastrula stages for formation of ectopic invaginations, and then again during tailbud stages for the presence of partial secondary axes. Embryos injected with XFD or UHAV ϕ alone never formed ectopic invagination sites or secondary axes.

marginal zone and then examined *Xombi* and *brachyury* expression by double in situ hybridization analysis just prior to and during gastrulation. Since *brachyury* expression is inhibited anywhere in the marginal zone that XFD is expressed (Amaya et al., 1993), *brachyury* expression is used here as a measure of XFD function.

As shown in Fig. 8, a functional FGF signaling pathway is not required for initial expression of *Xombi* in the dorsal marginal zone, but is required for its continued expression during mid to late gastrula stages. Just prior to the onset of gastrulation (stage 10-), *Xombi* is expressed at high levels dorsally and throughout much of the marginal zone in uninjected embryos or embryos injected dorsally or ventrally with XFD RNA (magenta stain in Fig. 8A,D,G). At this time embryos have not yet begun to express detectable levels of *brachyury* (light blue stain). Later, when involution begins dorsally (stage 10.25) and ventrally (stage 11.5), *brachyury* expression is completely inhibited in the region of the mesoderm where XFD is expressed (dark blue stain is due to the overlap of *Xombi* and *brachyury* expression). *Xombi* expression is partially inhibited by XFD at stage 10.25 (Fig. 8B,E,H), and is almost completely inhibited at stage 11.5 (Fig. 8C,F,I).

Blastopore lip formation exhibits the same dependence on FGF signaling as does *Xombi* expression. *Xombi* expression and lip formation occur early in gastrulation at the dorsal side regardless of where XFD is expressed. During mid to late gastrula stages, however, XFD interferes with the maintenance of *Xombi* expression and also appears to inhibit the process by which the dorsal blastopore lip extends laterally and ventrally. XFD-expressing embryos exhibit defects in lateral and ventral blastopore lip formation of varying severity; embryos injected dorsally generally have more extreme defects than embryos injected ventrally. The role of FGF in the extension of the blastopore lip is illustrated in Fig. 8F,I. In Fig. 8F, XFD has been injected near the dorsal lip (as denoted by the absence of *brachyury* expression); there is no FGF signaling in the tissue flanking either side of the lip and lateral extension of the lip is not observed. In Fig. 8I, FGF signaling occurs on one side of the dorsal blastopore lip, but not on the other. In this case, the lip extends laterally on the left side of the marginal zone, where there is intact FGF signaling, but not on the other side, where XFD is expressed.

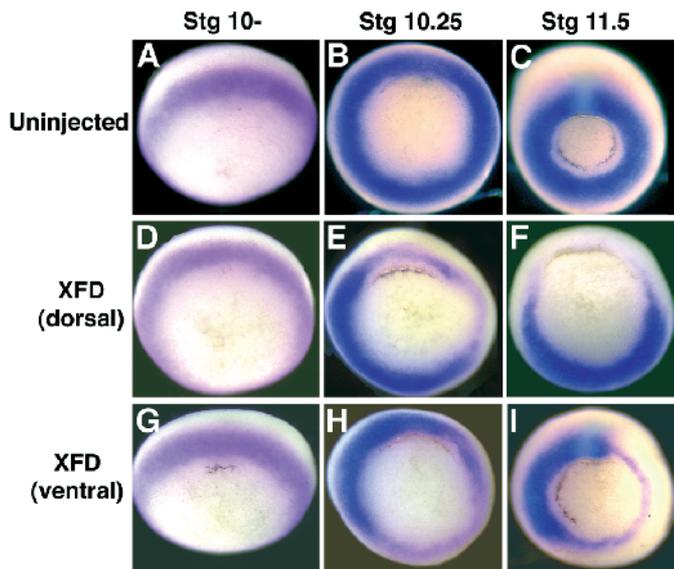


Fig. 8 *Xombi* expression and blastopore lip formation are FGF-independent early in gastrulation but FGF-dependent late in gastrulation. Embryos were co-stained by double in situ hybridization for *Xombi* (magenta stain) and *brachyury* (light blue stain). A dark blue stain is detected wherever the genes are co-expressed. (A-C) Uninjected embryos. (D-F) Embryos injected dorsally with *XFD* RNA (1 or 2 ng). (G-I) Embryos injected ventrally with *XFD* RNA (1 or 2 ng). (A,D,G) Stage 10 embryos in which apically pigmented superficial cells are just becoming apparent. (B,E,H) Stage 10.25 embryos in which the dorsal lip has formed but not extended very far laterally. (C,F,I) Stage 11.5 embryos. All embryos are shown in vegetal view with dorsal at the top.

DISCUSSION

The role of *Xombi* in blastopore lip formation

We describe here the isolation and characterization of *Xombi*, a novel T-box gene, which has an expression pattern and biological properties that reflect a possible role in blastopore lip formation. *Xombi* is expressed within the marginal zone soon after the mid-blastula transition in a dorsal to ventral wave of expression, preceding a similar wave of bottle cell and blastopore lip formation at the vegetal border of the marginal zone. *Xombi* expression also precedes blastopore lip formation in both dorsalized and ventralized embryos, where bottle cell formation and (subsequently) tissue involution occur simultaneously around the ring of marginal zone tissue, a strikingly different pattern than in normal embryos. When expressed ectopically in intact embryos, *Xombi* mimics the activity of the endogenous blastopore inducer, causing formation of an ectopic invagination that resembles the endogenous blastopore lip. This ectopic invagination frequently forms at the border of the *Xombi* injection site; the endogenous blastopore lip likewise forms at the border of *Xombi* expression in the marginal zone. *Xombi* is thus expressed at the correct time and place and appears sufficient to induce lip formation.

Activin β B and Xnr1, two secreted TGF β proteins expressed in the *Xenopus* embryo during gastrulation (Thomsen et al., 1990; Jones et al., 1995; Lustig et al., 1996), induce both *Xombi* expression and ectopic invagination, and thus may act upstream of *Xombi* in the blastopore lip induction pathway.

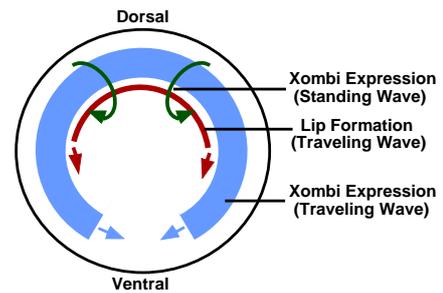


Fig. 9 Kinetic model of *Xombi* expression and blastopore lip formation. A vegetal view of a schematized late blastula to early gastrula *Xenopus* embryo is shown. *Xombi* expression (blue) begins dorsally during late blastula stages and has moved circumferentially towards the ventral side of the embryo by early gastrula stages. Blastopore lip formation (red) follows this traveling wave although the site of lip formation is always several cell diameters from the border of *Xombi* expression in the mesoderm. As mesoderm involution proceeds through the nascent lip (green), *Xombi* is expressed in a standing wave that forms orthogonal to the traveling wave of lip formation.

Ventral mesoderm inducers such as eFGF are also capable of inducing *Xombi* expression but, surprisingly, do not induce invagination. This may reflect the timing of *Xombi* induction, lower levels of *Xombi* induced or perhaps an inhibitory effect of ventral mesoderm that is concomitantly induced. Although FGFs do not induce ectopic invagination, FGF signaling is required for *Xombi* expression and ventrolateral blastopore lip formation in vivo. The induction of ectopic invagination by both *Xombi* and activin β B is also dependent upon FGF signaling. It is not yet clear when FGF signaling is required in vivo or whether this requirement reflects a direct role for FGF or an indirect role for FGF in the TGF β signaling pathway.

Xombi injection recreates many of the characteristics of normal invagination. Ectopic expression of *Xombi* induces the formation of cells with similar constricted pigmentation as bottle cells, and induces changes in cell behavior that also precede bottle cell formation and involution at the endogenous lip (Hardin and Keller, 1988). These include the accumulation of pigment granules in the constricted apices of superficial epithelial cells, the fusion of the apical ends of these cells to form a pigment line and the invagination of superficial cells within this line to form a groove. Since it is not expressed in bottle cells, but only adjacent to them, *Xombi* may induce expression of genes encoding secreted proteins that act in a paracrine manner to induce these cell behaviors. *Xombi* expression in the marginal zone may first lead to bottle cell formation and the initial invagination of the blastopore lip, but subsequent expression of other signaling proteins in the epithelial layer of the organizer, such as the Wnt-inducible secreted protein Xnr3 (Smith et al., 1995), are probably required for the convergence and extension movements that provide the driving force for much of the later tissue involution.

Xombi is expressed in superficial and deep cells of the involuting marginal zone that are fated to form the lining of the archenteron and the mesodermal mantle, respectively. The absence of *Xombi* staining in involuted tissue and noninvolved cells close to the lip, however, suggests that *Xombi* is expressed transiently as the cells move toward the blastopore lip, and is shut off as they near the inflection point. Thus, in addition to

the dorsal to ventral traveling waves of *Xombi* expression and blastopore lip formation, there is a standing wave of *Xombi* expression that forms at right angles to the traveling waves (Fig. 9). A number of other mesodermal genes expressed during gastrulation, including *Xwnt8* (Fig. 6B), also exhibit a similar standing wave of expression. A standing wave of *Xombi* expression is observed in dorsalized and ventralized embryos, suggesting that the mechanism of down regulation in cells near the lip is independent of normal dorsal-ventral patterning.

Although injection of a bolus of *Xombi* will induce bottle cell formation and ectopic invagination, in the context of the normal process of gastrulation there is a precise temporal progression from the dorsal to the ventral side of both *Xombi* expression and lip formation. This progression suggests either inhibitory or activating signals propagating from the expanding site of involution. The strong effect of disrupting FGF signaling on the dorsal side, not on dorsal invagination, which proceeds normally, but on the lateral propagation of involution, which is inhibited, provides evidence for a role for FGF in the propagation of this traveling wave. Propagating waves of morphogenesis are important in developing pattern in such systems as the insect eye (Heberlein et al., 1993; Ma et al., 1993). That *Xombi* expression is only dependent on FGF signaling during mid to late gastrula stages provides a possible explanation for the differential sensitivity of dorsal invagination and ventrolateral lip propagation to XFD inhibition. Thus, regardless of whether XFD is expressed dorsally or ventrally, *Xombi* is expressed dorsally and the dorsal blastopore lip forms normally. When FGF signaling is inhibited dorsally, however, *Xombi* is not expressed laterally during mid to late gastrula stages and gastrulation movements are inhibited.

Distinct and overlapping roles of *Xombi* and *brachyury* in mesoderm induction and maintenance

The *Xenopus* genes that have been previously cloned using functional assays (*Xwnt8*, *siamois*, *noggin* and *Xnr3*) all rescue axial structures by ectopically inducing formation of a Nieuwkoop center (Christian et al., 1991; Lemaire et al., 1995; Smith and Harland, 1991, 1992; Smith et al., 1995). *Xombi* does not induce formation of a similar dorsalizing center but instead appears to act only as a general mesoderm inducer. *Xombi* transcripts are expressed maternally in the vegetal cortex and cytoplasm. Though the maternal function of these transcripts remains obscure, *Xombi* could play a role in early steps in mesoderm formation following the cortical rotation. When mesodermal genes begin to be expressed in the marginal zone, zygotic *Xombi* transcripts colocalize with transcripts of three mesodermal genes it ectopically induces: *gooseoid*, *Xwnt8* and *brachyury*. Thus the time course and site of *Xombi* expression, its general mesoderm-inducing properties, its induction by activin A, activin β B, *Xnr1* and eFGF in vitro, and its partial dependence on FGF signaling, are all consistent with a model in which *Xombi* expression is induced during late blastula stages by mesoderm-inducing signals from the Nieuwkoop center and then is maintained during gastrulation by vegetal signals or signals within the marginal zone itself. According to this view, the role of *Xombi* is not to mediate the induction of *gooseoid*, *Xwnt8* or *brachyury* by primary mesoderm-inducing signals but rather to maintain and amplify the response to these primary signals.

The extent to which overlapping mesodermal genes within the marginal zone regulate each other's expression remains unclear. The finding that *brachyury* and eFGF each induce the expression of the other has led to the proposal that the two genes form a regulatory circuit within the marginal zone and play a role in the maintenance of mesodermal gene expression (Isaacs et al., 1994). In the present study, we show that *Xombi* and *brachyury* induce each other's expression, suggesting this putative positive feedback loop could involve *Xombi* as well. *Xombi* is expressed prior to *brachyury* on the dorsal side, and thus may initiate the loop.

Although *Xombi* and *brachyury* are both T-box-containing proteins and induce the expression of each other, they appear to carry out distinct functions during most of early *Xenopus* development. Unlike *Xombi*, *brachyury* is not expressed at high levels maternally, and outside of gastrula stages there is no significant overlap between *Xombi* and *brachyury* expression. At the neurula stage of development, *Xombi* is specifically excluded from notochord-forming cells along the dorsal midline, whereas *brachyury* is not only expressed in these cells but in fact is believed to be required for notochord formation in most vertebrates (Yasuo et al., 1995). Conversely, *brachyury* is not detected along the dorsal midline during tailbud stages of development, when *Xombi* is expressed in cells in a region of the trunk neural tube fated to form interneurons. Finally, ectopic expression of *brachyury*, unlike ectopic *Xombi* expression, does not lead to dorsal mesoderm induction or to changes in cell behavior that lead to the formation of ectopic invaginations.

The role of *Xombi* in endoderm development

The family of T genes may be derived from an original T gene involved in gut formation in the worm-like animals from which all living animals have evolved (Reuter, 1995; Yasuo et al., 1995). T genes are still involved in gut endoderm development in various phyla today; for example, a T-related gene (*Trg*) is expressed in the hindgut primordium in arthropods and is necessary for hindgut formation (Kispert et al., 1994). In more highly evolved organisms, T genes are also expressed in other embryonic structures, although many of these, such as the stomochord of hemichordates and the notochord of chordates, are believed to be phylogenetically derived from the gut endoderm (Reuter, 1995; Yasuo et al., 1995). A role for *Xombi* in gut development would not be surprising since recent work has shown that the gut endoderm in *Xenopus* is induced and patterned by many of the same genes that induce and pattern the mesoderm (Gamer and Wright, 1995; Henry et al., 1996). Although *Xombi* is not detected at high levels in the gut endoderm after gastrulation in *Xenopus*, several findings are consistent with the idea that it may be an early marker in the endodermal differentiation pathway. In normal development, *Xombi* transcripts are expressed in the vegetal pole region of the oocyte, which is fated to form the bulk of the endoderm, and are expressed throughout the superficial involuting marginal zone, which is fated to form the endodermal lining of the gut. *Xombi* also appears to be part of the pathway that leads to induction of bottle cells, which are fated to form pharyngeal endoderm at the anterior end of the gut. Finally, we have found that *Xombi* expression occasionally leads to the ectopic formation of hollow tubes several hundred microns in length (unpublished results) that are reminiscent of the endodermally

lined tube that forms early in gut development. The nature of these tubes and their relationship to *Xombi* function in vivo, if any, remain to be determined. The ancient role for T genes in endoderm differentiation and tubulation in gut morphogenesis suggests that *Xombi* may stand at an interesting intersection point between the evolution of morphogenesis and cell fate determination in complex metazoa.

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