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Genomic Profiling of Mixer and Sox17β Targets During *Xenopus* Endoderm Development

Kari Dickinson, **Jeff Leonard**, and **Julie C. Baker***

*Department of Genetics, Stanford University Medical School, Stanford, CA 94062

Abstract

The transcription factors Mixer and Sox17β have well characterized roles in endoderm specification during *Xenopus* embryogenesis. In order to more thoroughly understand the mechanisms by which these endodermal regulators act, we expressed Mixer and Sox17β in naïve ectodermal tissue and, using oligonucleotide-based microarrays, compared their genomic transcriptional profile to that of unaffected tissue. Using this novel approach, we identified 71 transcripts that are upregulated by Mixer or Sox17β, 63 of which have previously uncharacterized roles in endoderm development. Furthermore, an in situ hybridization screen using antisense probes for several of these clones identified six targets of Mixer and/or Sox17β that are expressed in the endoderm during gastrula stages, providing new and regional markers of the endoderm. Our results contribute further insight into the functions of Mixer and Sox17β and bring us closer to understanding at the molecular level the pathways that regulate endoderm development.

Keywords

Mixer; Sox17; endoderm; *Xenopus*; microarray; Xtwik2; Borg4; March8; Gpr4; Cxcr4

Introduction

The endoderm is one of the three primary germ layers established during early vertebrate embryogenesis. The integrity of this germ layer is crucial to an organism's survival as the cells of the endoderm will go on to form the gut epithelium and associated organs such as the liver and pancreas. The endoderm is also a source of instructive cues, providing developmental signals to structures in the embryo such as the head and heart (Nascone and Mercola, 1995; Bouwmeester et al., 1996; Beddington and Robertson, 1998; Couly et al., 2002).

Recent interest in vertebrate endoderm development has launched several studies over the past few years in which a handful of molecules involved in endoderm formation were identified (reviewed in Shivdasani, 2002; Stainier, 2002). VegT, a T-box transcription factor, is the primary maternal regulator of endoderm specification in *Xenopus* (Horb and Thomsen, 1997; Zhang et al., 1998; Xanthos et al., 2001). The maternal localization of

Corresponding Author, Julie C. Baker, Genetics Department, Stanford University, 300 Pasteur Drive, MC 5120, Stanford, CA 94062, 650-723-1082, 650-725-1534 (fax), jbaker@stanford.edu.

VegT mRNA to the vegetal pole and the subsequent initiation of zygotic factors including Nodal-like molecules and transcription factors such as Mixer and Sox17 are the forces governing early endoderm development. Mixer is a paired-like homeodomain transcription factor identified in a functional screen for endodermal determinants in *Xenopus* (Henry and Melton, 1998). Sox17α and Sox17β are HMG domain-containing transcription factors identified in a subtractive PCR screen for endodermally enriched genes in *Xenopus* (Hudson et al., 1997). Both *Mixer* and *Sox17*β are expressed exclusively in the *Xenopus* endoderm, can induce endodermal cell fate in naïve ectodermal tissue and are required for proper endogenous endoderm development (Hudson et al., 1997; Henry and Melton, 1998). Mixer and Sox17 β are functionally conserved, as they are involved in Zebrafish and mouse endoderm specification (Kikuchi et al., 2000; Alexander and Stanier, 1999; Pearce and Evans, 1999; Kanai-Azuma et al., 2002). Both *Mixer* and *Sox17*β are induced by Nodal-like signals in the *Xenopus* embryo and have been placed in a hierarchical pathway leading to endoderm specification, with Mixer upstream of Sox17β based on its ability to induce *Sox17*β in naïve ectodermal explants (Henry and Melton, 1998; Shivdasani, 2002; Stainier, 2002). Genetic evidence in Zebrafish supports this epistatic relationship (Alexander et al., 1999; Alexander and Stainier, 1999; Aoki et al., 2002; Shivdasani, 2002).

Despite the extensive studies on the roles of molecules like Mixer and Sox17 β in endoderm specification, little is known about the genes that are regulated by these transcription factors. It is likely that each induces a set of molecules that define endodermal cell fate. In this report, we use oligonucleotide-based microarray technology developed by Affymetrix to identify molecules that are upregulated by Mixer, Sox17β or both. Interestingly, despite the somewhat linear progression of events thought to establish endodermal cell fate, most of the transcripts we identified were downstream of Mixer or Sox17β with few downstream of both.

Results

Experimental set-up and data interpretation

We used a genomic microarray approach to identify molecules involved in early endoderm formation. Our experimental strategy was to transform naïve ectodermal tissue into endoderm and assay the change in genome-wide gene expression (see Fig. 1). Although VegT is the farthest known upstream endoderm inducer, we did not select this molecule for our experiments because VegT can also induce mesoderm (Stennard et al., 1996). Mixer and Sox17β, on the other hand, induce endoderm in the absence of mesoderm induction and were thus selected for our experiments based on their specificity.

To transform ectoderm into endoderm, we injected 500 pg of *Mixer* or *Sox17*β into the animal hemisphere of one-cell *Xenopus* embryos. At stage 8.0 (just prior to the onset of zygotic gene expression), we performed ectodermal explants and cultured the isolated tissue to stage 10.5. This is the point at which the endodermal germ layer is well established and is involuting in intact sibling embryos. We then isolated total RNA and prepared it for array hybridization (see Experimental Procedures). This entire process was repeated to generate a second set of data, which we will refer to as experiment 2.

Using the Gene Chip Operating Software provided by Affymetrix, the arrays were scanned and individual intensities for each oligo spot were assigned numerical values and averaged for each probe set. The data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE3334. We interpreted these values using DNA-Chip Analyzer (Li and Wong, 2001; see Experimental Procedures) and created three lists based on changes in intensities for individual probe sets within an experimental sample when compared to the uninjected control. The first list contains genes and ESTs that are induced by Mixer and Sox17β (Table 1). The second list contains genes and ESTs that are induced by Mixer, but not reliably by Sox17β (Table 2). The third list contains genes that are induced by Sox17β, but not reliably by Mixer (Table 3).

10 transcripts were induced by Mixer and Sox17β

To identify molecules that are induced by Mixer and Sox17β, we generated four initial lists of genes and ESTs from experiments 1 and 2 that had 1.8 fold or higher intensities in the Mixer and Sox17β injected samples versus control with a false detection rate (FDR) ≤ 0.10. Transcripts that appeared on all four lists were incorporated into a final list shown in Table 1. Using this screening method, six known genes and four ESTs were identified as downstream targets of both Mixer and Sox17β. Of the known genes, *FoxA1* (*Xfkh2*, *Hnf3*α) has previously been shown to be expressed in the early endoderm and activated by Sox17β in ectodermal explants (Bolce et al., 1993; Sinner et al., 2004), but this is the first report of FoxA1 as a target for Mixer. The remaining five genes and four ESTs have not been characterized in terms of endoderm development.

28 transcripts were induced by Mixer

A wealth of evidence indicates that Mixer is upstream of Sox17β in the pathway of gene expression leading to endoderm specification (Henry and Melton, 1998; Alexander et al., 1999; Alexander and Stainier, 1999; Aoki et al., 2002; Shivdasani, 2002). Using data from our microarrays, we found additional molecules downstream of Mixer. We initially generated two list of genes and ESTs from experiments 1 and 2 that contained probe sets with a ≥ 2.0 increase in intensity in the Mixer samples versus controls. Transcripts that appeared on both lists with a FDR ≤ 0.10 were selected for Table 2. One exception is *EST-21*, which appeared twice in our data for experiment 2, with a fold change of 11.5 and 6.4 above control, but in experiment 1 had a fold change of 1.6 and 1.5. Despite the lower fold changes in experiment 1, *EST-21* had a FDR of 0.01 and 0.03 and was therefore included in the list. Other transcripts that appeared more than once on our list are *Msx-2a*, *EST-9* and *EST-17* and are indicated with an asterisk in Table 2. It is also worth noting the few transcripts that had fold changes of greater than 5.0 in both experiments (indicated with a †). These include *Msx-2a* (6.9, 8.2), *Xeel* (10.1, 21.9), *EST-17* (7.2, 6.7; 7.3, 6.9) and *EST-19* (7.1, 23.9).

We expected to see several of the genes in Table 2, including *Sox17*α, *Sox17α2*, *Sox17*β, *Gata-5a* and *Mig30*. All of these are known targets of Mixer or have been previously characterized for their roles in endoderm formation. All three *Sox17-like* transcripts have

large fold change values ranging from 6.3 to 17.4. The remaining genes and ESTs in Table 2 have not been investigated for their role in endoderm formation.

33 transcripts were induced by Sox17β

For several years after Sox17β was discovered, the only known transcriptional targets for Sox17β during gastrula stage were *Endodermin* and *Hnf1*β (*Tcf2*; Hudson et al., 1997; Clements et al., 2003). Recently investigators used a candidate gene approach to screen for Sox17β targets and identified nine additional downstream genes (Sinner et al., 2004). In our array experiment, we identified 34 potential targets for Sox17β using the same selection criteria we described for identifying Mixer targets (Table 3). Of the 16 known genes identified, *Otx2* and *Goosecoid* have been shown to be targets for Sox17β (Chiao et al., 2005; Sinner et al., 2004). In fact, genetic experiments in the mouse suggest a role for Otx2 in early endoderm development (Perea-Gomez et al., 2001). *Otx2* and *Goosecoid* appear twice in each data set and have fold changes greater than 5.0. Other transcripts that have fold changes ≥ 5.0 include *Irx4* (14.5, 18.6), *Crgb* (10.1, 21.2), *Ash1* (5.4, 14.3), *Xtwi* (9.3, 17.7), *EST-22* (84.2, 106.2), *EST-35* (8.5, 11.4) and *EST-34* (6.3, 6.3). *EST27* appears twice in this data set. All of the genes and ESTs in Table 3, except for *Goosecoid* and *Otx2*, are novel targets for Sox17β and do not have a known role in endoderm development.

6 transcripts exhibit novel expression in the early endoderm

We have identified 71 targets for Mixer and/ or Sox17β, 63 of which may have a completely novel role in endoderm development. To determine whether these are targets for Mixer and Sox17β within the endoderm, we analyzed the expression pattern at gastrulation and later stages for 34 of the most promising transcripts identified. During gastrulation, six of the transcripts were expressed in the endoderm, two in the mesoderm, and eleven were ubiquitous. The remaining fifteen did not have detectable expression (see Table 4). Several of these clones had unique and detectable patterns of expression at later developmental times (Fig. 2). Since the molecules expressed within the endoderm during gastrulation were more likely to be endogenous targets of Mixer and Sox17β, we investigated these further and describe them in detail below (Fig. 3). They include the following molecules: *Cxcr4*, *EST1* (*March8*), *EST15* (*Borg4*), *EST-21*, *Gpr-4* and *EST35* (*Xtwik2*). The sequence conservation between several of these ESTs and proteins in the mouse and human database strongly suggest that they are the correct homologs. Therefore, from this point forward we will refer to EST1 as March8, EST15 as Borg4 and EST35 as Xtwik2.

Cxcr4—In our array experiments, we observed an increase in the expression of the C-X-C motif chemokine receptor 4 (*Cxcr4*) in ectoderm expressing Mixer or Sox17β. Our analysis of *Cxcr4* expression in *Xenopus* at stage 10.5 identified novel endodermal expression. In Fig. 3A, we observe *Cxcr4* expression scattered throughout the yolky vegetal cells and a high level of expression in the endodermal cells most proximal to the blastopore ring. In the lateral view of a hemisected stage 10.5 embryo stained with *Cxcr4* probe (Fig. 3C), we observe *Cxcr4* expression throughout the deep endodermal cells and a high level of expression in the cells lining the endoderm/ mesoderm boundary extending down to the involuting cells of the blastopore ring. We also observed a low level of scattered expression throughout the ectoderm (data not shown). Cxcr4 is a member of the superfamily of

heterotrimeric-G-protein-coupled receptors originally identified in leukocytes and also known to act as a co-receptor for the entry of HIV into CD4+ lymphocytes (Murphy, 1996). A role for Cxcr4 in the development of the hematopoietic system and vascularization of the gastrointestinal tract has been characterized in mice (Tachibana et al., 1998; Zou et al., 1998). In *Xenopus*, analysis of *Cxcr4* expression from stage 13 and beyond revealed a potential role for Cxcr4 in embryonic neural development and adult B cell differentiation (Moepps et al., 2000).

March8—We identified another transcript that was increased in the presence of either Mixer or Sox17β, which we refer to as *March8*. Our analysis of *March8* revealed expression in the superficial cells of the endoderm (Fig. 3E). We also detected transcript in the deeper cells adjacent to the blastopore ring (Fig. 3G). Some expression of *March8* was observed in the ectoderm (data not shown). The predicted amino acid sequence for this transcript is 86% identical to human cellular modulator of immune recognition (c-MIR or March8) and is likely the *Xenopus* ortholog of this gene. Human March8 was identified based on its secondary structure similarity to two related proteins, MIR1 and 2, encoded by Kaposi's sarcoma associated-herpes virus (Goto et al., 2003). This protein functions as a membrane-bound E3 ubiquitin ligase and contains a BKS-PHD catalytic domain responsible the E3 mediated ubiquitination and degradation of immune recognition-related molecules.

Borg4—Our analysis of *Borg4*, which is induced by Mixer but not by Sox17β, revealed expression in the outer endodermal cells of the blastopore ring (Fig. 3I). Significant expression in the deep endoderm cells beyond the blastopore ring was not observed (Fig. 3K). The predicted amino acid sequence of Borg4 shares 55% identity with the human Cdc42 effector protein, binder of Rho GTPase 4 (Borg4). Borg4 was identified in a two-hybrid screen of a mouse embryo library for molecules that bound to the TC10 GTPase (Joberty et al., 1999). The Rho family of GTPases regulates multiple biological processes including cell motility, morphogenesis, protein kinase cascades, gene expression and cell cycle progression. GTPase specificity is thought to be regulated by downstream effector proteins such as those of the Borg family, which are proposed negative regulators of Rho GTPase signaling (Joberty et al., 1999). The mouse homolog of *Borg4* is ubiquitously expressed in adult tissue (Osada et al., 2000).

EST-21—A second transcript we identified as a target for Mixer is *EST-21*. Our analysis of *EST-21* revealed only selective staining of cells in the deep endoderm domain (Fig. 3M, O). This transcript does not share sequence homology with any clone in the database and may be a frog-specific gene.

Gpr-4—In addition to targets for Mixer, we also identified several transcripts induced only by Sox17β. One of these is the *Xenopus* ortholog of the human G protein-coupled receptor 4 (Gpr-4). Our analysis of *Gpr-4* in *Xenopus* revealed expression in the inner cells of the superficial endoderm layer with little or no expression around the blastopore ring region (Fig. 3Q). In the hemisected embryos, we observed scattered expression throughout the deep endodermal tissue (Fig. 3S). G protein-coupled receptors are known to transduce numerous extracellular signals into cells and regulate various aspects of cell proliferation (Marinissen

and Gutkind, 2001). Recently, Gpr-4 was identified in a separate microarray experiment as a target gene for FGF signaling in *Xenopus* (Chung, et al., 2004). In this study, *Gpr-4* transcript was also observed in the endoderm and functional analysis of this gene revealed a potential role for Gpr-4 in regulating gastrulation movements (Chung et al., 2004). Gpr-4 also has oncogenic properties and has been shown to regulate pH and ERK activity (Sin et al., 2004; Ludwig et al., 2003; Bektas et al., 2003). Expression of human *Gpr-4* has been observed in the kidney, heart and lung (Mahadevan et al., 1995). Interestingly, Gpr-4 shares some sequence homology with the angiotensin receptor, a gene we found to be induced by Mixer and Sox17β (see Table 1).

Xtwik-2—A second transcript we identified as a potential target for Sox17β is *Xtwik-2*. Our analysis of *Xtwik-2* revealed expression in the endodermal cells surrounding the blastopore ring (Fig. 3U). In the hemisected embryos, we observed expression in the cells proximal to the adjacent mesoderm, extending from the blastopore ring up to the blastocoel floor with much fewer cells expressing *Xtwik-2* in the innermost endoderm tissue (Fig. 3W). The predicted amino acid sequence of this clone is nearly identical Xtwik-2. However, the nucleotide sequence is more divergent and thus may be a close paralog of Xtwik-2. Human Twik-2 is known to regulate cell electrogenesis and is expressed in the pancreas, stomach, spleen and uterus (Chavez et al., 1999; Medhurst et al., 2001).

EST-21 and Gpr-4 have novel expression patterns beyond stage 10.5

We analyzed the expression patterns of the six clones expressed in the early endoderm at later stages. *Cxcr4* displayed expression patterns identical to those published (Moepps et al., 2000). *EST-1*, *EST-15* and *EST-35* did not have specific expression patterns at stages beyond 10.5. At tailbud stage, *EST-21* is expressed throughout the epidermis in a scattered punctate pattern with less staining in the head region (Fig. 4A). *Gpr-4* is expressed along the neural tube in neurula stage embryos and in the eye and brachial arches in tailbud stage embryos (Fig. 4C, E).

New endoderm markers respond as predicted by array to endoderm inducers

We identified six genes that have novel endoderm expression. We next examined the transcriptional response of these genes to endoderm inducers, Mixer, Sox17β, Smad2 and VegT in ectoderm via RT-PCR in order to confirm the array data and test whether these genes also respond to other known inducers of endodermal cell fate. Based on our array data, we expected to see increased expression of *Cxcr4*, *March8*, *Borg4* and *EST-21* in ectoderm expressing Mixer and increased expression of *Cxcr4*, *March8*, *Gpr-4* and *Xtwik-2* in ectoderm expressing Sox17β. As shown in Fig. 5, all six genes responded to Mixer and Sox17β as predicted. We also observed induction of *Gpr-4* by Mixer, which is reflected in the microarray data for experiment 2 (2.2 fold increase), but below our threshold in experiment 1 (1.3 fold increase). Because this fold change did not meet our standards for induction by Mixer, we placed *Gpr-4* in Table 3 instead of Table 1.

We also examined the transcriptional response of the six new endoderm genes to other endoderm inducers such as Smad2 and VegT. Fig. 5 shows induction or increased expression of *Cxcr4*, *March8*, *Gpr-4* and *Xtwik-2* by Smad2, a subtle increase in *March8*

expression by Smad2 and no induction of *EST-21* by Smad2. VegT increased the expression of *Cxcr4*, *March8* and *Gpr-4* above background and clearly induced expression of *Borg4* and *Xtwik-2*. Like Smad2, VegT did not induce *EST-21*, indicating that it is a Mixer specific target.

Discussion

Aside from adopting a cellular fate capable of becoming the epithelium and organ system of the gastrointestinal tract, the *Xenopus* endoderm cell has several functions in the early embryo. These include nourishing the embryo through its yolk protein stores, acting as a signaling source to instruct the overlying mesoderm to adopt specific cell fates and migrating to the interior of the embryo while establishing an anterior posterior axis during gastrulation. All of these functions require a unique set of molecules that define endodermal cell fate. Using microarrays, we identified 71 transcripts that are upregulated during the transformation of ectoderm to endoderm. Eight of these had previously described roles during endoderm development providing evidence that we were identifying known targets.

This is the first report that utilizes microarray technology to identify molecules specifically involved in endoderm formation. Many of the known genes that comprise the endoderm pathway have been identified through functional and differential expression screens, candidate gene approaches or genetics (Jones et al., 1995; Baker and Harland, 1996; Lustig et al., 1996; Hudson et al., 1997; Joseph and Melton, 1997; Henry and Melton, 1998; Lemaire et al., 1998; Sun et al., 1999; Yasuo and Lemaire, 1999; Weber et al., 2000; Xanthos et al., 2001; Afouda et al., 2005). Through these various studies, many of the important players in endoderm specification were identified including VegT, Mixer, Sox17α/β, Gata4, 5 and 6, Mix.1, and various components of the Nodal-like TGFβ signaling pathway (Jones et al., 1995; Baker and Harland, 1996; Lustig et al., 1996; Hudson et al., 1997; Joseph and Melton, 1997; Henry and Melton, 1998; Lemaire et al., 1998; Sun et al., 1999; Yasuo and Lemaire, 1999; Weber et al., 2000; Xanthos et al., 2001; Shivdasani, 2002; Stainier, 2002; Afouda et al., 2005). Many of these genes affect the transcription of one another and, perhaps more importantly, an undefined number of downstream target genes (Hudson et al., 1997; Henry and Melton, 1998; Clements et al., 1999; Yasuo and Lemaire, 1999; Engleka et al., 2001; Shivdasani, 2002; Stainier, 2002; Loose and Patient, 2004). It is this collection of unknown target genes that ultimately defines the early endoderm cell. Using microarrays, we were able to take a genome-wide snapshot of the changes in gene expression for a group of cells instructed to become endoderm. We identified 71 potential targets for Mixer and/ or Sox17β, 63 of which may have a completely novel role in defining endodermal cell fate.

The collection of signaling molecules and transcription factors, including Mixer and Sox17, that lead to endoderm specification is often depicted as a linear progression of inductive events (Yasuo and Lemaire, 1999; Shivdasani, 2002; Stainier, 2002). Therefore, we expected to see most of the genes induced by Sox17β also induced by Mixer, given that Mixer is known to induce Sox17β. However, we observed most genes in our array experiments to be downstream of Mixer *or* Sox17β, with only ten transcripts upregulated by both. Our results may simply reflect the limitations within the assay, with induction of

transcripts by Sox17β directly being more robust than the induction of these same transcripts by Mixer via Sox17β. However, we believe our results reflect a non-linear branching of the pathway and highlight the distinct functions of Mixer and Sox17β as they lead to endoderm specification.

We identified six genes that are expressed in the early endoderm through an in situ hybridization screen of 34 transcripts found by our microarray analysis: an 18% success rate. Criticisms will be raised that this is considered a low hit rate due to experimental error from Microarray data. A closer look at the data suggests otherwise. First, of the transcripts that produced expression patterns, 32% were expressed within the endoderm. This is because of the 34 transcripts analyzed, 15 had no detectable expression at gastrula stages. Five of these did demonstrate expression in later staged embryos, suggesting that the probe could detect transcript. The other ten transcripts were not detected at any embryonic stage suggesting that the lack of signal was due to probe failure. Due to penetration problems, insitu hybridizations are less effective in the endoderm than elsewhere in the embryo and extensive troubleshooting is sometimes required to identify the best sequence from each probe for hybridizations. Therefore, we are left questioning whether there are more endoderm specific genes to be uncovered within this grouping. Second, we found 11 transcripts to be ubiquitously expressed. This does not rule out any of these genes as downstream targets of Sox17β or Mixer. Many important regulators of specific tissues are expressed ubiquitously and activated specifically in particular cell types. Whether these eleven are true targets, we have yet to know, but certainly, although they are not exclusively expressed within the endoderm, they cannot be ruled out.

Caveats exist within these interpretations and there are several reasons why not all of the targets identified in this screen will be endogenous players. First, certainly overexpression of molecules as potent as Sox17β and Mixer may lead to non-specific effects by promiscuously activating promoters containing HMG box or homeodomain motifs. Second, indirect inductions may occur between the start of zygotic transcription (stage 8) and the time of analysis (st10.5). In order to obtain direct targets a temporally activated system would need to be employed. Third, our FDR cut-off was < 0.10, indicating that 10% of the transcripts analyzed are indeed background. Be that as it may, we used Microarray technology as a first pass screen to obtain candidate endoderm specific genes and used the secondary in situ screen to validate. This approach effectively identified six uncharacterized endoderm genes, which display regional specific patterns. One of the great difficulties in studying endoderm has historically been a dearth of markers. Here we present the community with six new ones, more than doubling the current collection.

The endoderm specific genes identified in this screen are expressed in discrete locations within the endoderm, representing either subblastoporal or marginal regions (Fig. 3 and Fig. 6). Interestingly, both Mixer and Sox17β induce genes that are expressed in each region. For example, Mixer induces specifically Borg4, which is expressed strongly in the boundary with the mesoderm, and EST21, which is restricted to the subblastoporal cells. Although both genes are induced selectively by Mixer, they display non-overlapping patterns of expression within the endoderm. This indicates that Mixer can induce may complementary

cell types within the endoderm and further reflects that endoderm is a heterogeneous population of cells, comprising at least several distinguishable cell types.

The proposed locations of these six genes within the endoderm pathway is summarized in Fig. 6. Borg4 and EST-21 are induced by Mixer, but not by Sox17β. It is possible that these genes may also have a function within the pathway and serve as intermediates leading to the induction of Sox17β or other downstream endoderm molecules (Fig. 6, gray arrow). *Gpr-4* and *Xtwik2* are induced by Sox17β, but not by Mixer, and are therefore placed farthest downstream. *Cxcr4* and *March8* are induced by both Mixer and Sox17β. The upregulation of *Cxcr4* and *March8* may be due independent inductive events by Mixer and Sox17β, or may simply reflect a by-product of Sox17β initiation by Mixer.

We can begin to make hypotheses about the roles of some of these genes in endoderm development based on their structure and specific expression pattern. Four of the six endodermally expressed genes are either known receptors (Cxcr4 and Gpr-4) or membrane spanning proteins (March8 and Xtwik2) (Chavez et al., 1999; Moepps et al., 2000; Goto et al, 2003; Chung et al, 2004). Borg4 may be a member of a signal transduction cascade (Joberty et al., 1999). These proteins may play a role in transducing extracellular signals secreted from within the endoderm itself or from the neighboring mesoderm. We observed a heavy concentration of expression around the blastopore ring for *Cxcr4*, *March8*, *Borg4* and *Xtwik-2*. These may be involved in integrating the instructional cues for endoderm cells to begin involution during gastrulation. Cxcr4, for example, has been characterized for its role in neural crest migration (Moepps et al., 2000) and may operate through a similar mechanism to direct endodermal cell movements during gastrulation. Borg4 may regulate cell motility within the endoderm through its association with Rho GTPases, integrins and the extracellular matrix.

The putative downstream targets of Mixer and Sox17β described in this paper may also be transcriptionally upregulated by other inducers of endoderm. We found that four of the six endodermally expressed clones were induced by VegT, and all six were induced by Smad2. Recently, *Cxcr4 and Borg4*, in addition to *c-myc*, *Xmsr*, *FoxA1* and *FoxC1*, were identified in a cDNA-based microarray experiment as possible targets of VegT (Taverner et al., 2005). *Xmsr* was found to be a direct target of VegT (Taverner et al., 2005), and although its transcription is induced by Mixer and Sox17β, it is unknown whether *Xmsr* is a direct target of these transcription factors. It will be interesting to sort out the hierarchy of induction for these molecules during endoderm formation.

We have laid the foundation for many future studies with our list of 63 potentially novel regulators of endoderm formation, especially the six we found to be expressed in the endoderm. At this point we can only speculate what these molecules are doing in the context of endoderm development. Overexpression analysis of these genes will reveal possible inductive roles in endoderm specification. These studies would be complemented by morpholino or dominant-negative loss of function analyses to identify molecules within our list that are required for the formation of endoderm. The ultimate goal will be to explore epistatic and biochemical relationships between these genes and other known components of the endoderm pathway.

Experimental Procedures

mRNA synthesis and injection for array samples

DNA plasmid constructs for *Mixer* (pCS300) (Henry and Melton, 1998) and *Sox17*β (pSPJC2L) (Hudson et al., 1997) were linearized with AscI and XhoI, respectively. SP6 transcription of mRNA was performed using mMESSAGE machine (Ambion, Austin, TX). Female frogs were primed for ovulation with human chorionic gonadotropin (Condie and Harland, 1987). Embryos were collected into 0.1× MR solution, fertilized in vitro, and de-jellied with 2.5% cysteine, pH 8.0. Embryos were transferred onto mesh grid plates containing 1/3× MR with 2.5% ficoll for injection. Embryos were injected with 500 pg of *Mixer* or *Sox17*β mRNA at the one-cell stage in the presumptive ectoderm.

Ectodermal explants

Injected and control uninjected embryos were cultured to stage 8 and transferred to agarose coated dishes containing 3/4× NAM solution (Peng, 1991) for tissue excision. 90 explants per sample were performed; 80 to be used for the array experiment and 10 for a control RT-PCR assay. Explants were harvested at stage 10.5.

Total RNA isolation

Stage 10.5 explants were homogenized in lysis buffer (0.5% SDS, 5 mM EDTA, 50 mM Tris pH 7.5, 50 mM NaCl) containing 0.2 mg/ml proteinase K and incubated at 42°C for 30 minutes. Samples were then extracted with equal volumes of phenol:chloroform:IAA, ethanol precipitated (with 0.1 volume 3 M NaOAc, 2.5 volume ethanol and 1 μl glycogen), washed and resuspended in 15 μl DEPC water. DNAse treatment (added to samples): 2.5 μl 10× DNase buffer, 0.4 μl DNase1 (Ambion, Austin, TX), 1.25 μl 20 mM DTT, 0.5 μl RNase inhibitor, 5.4 μl water. Samples were incubated at 37°C for 30 minutes, then brought up to a volume of 100 μl with water. Phenol extraction, ethanol precipitation (minus glycogen) and wash were performed as above. Samples were resuspended in 100 μl water. A second ethanol precipitation was performed with 10 μl 3 M NaOAc, 1 μl glycogen and 250 μl ethanol and incubated overnight at −20°C. Samples were centrifuged for 20 minutes at 4°C. Pellets were washed twice with 80% ethanol followed by a 5 minute centrifugation, then dried and resuspended in 12 μl DEPC water.

cDNA synthesis

1 μl T7(dT) 24 primer (100 pmol/μl) (Affymetrix, Santa Clara, CA) was added to 10 μg total RNA and incubated at 70°C for 10 minutes. First and second strand cDNA synthesis was performed using SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen, Carlsbad, CA). cDNA was then phenol chloroform extracted using Phase Lock Gel tubes (Eppendorf, Hamburg, Germany); equal volume of phenol chloroform was added to cDNA and centrifuged for 2 minutes. Upper phase was added to 0.75 volume 5 M NH4OAc and 2.5 volume cold ethanol, vortexed and centrifuged for 20 minutes. Pellets were washed twice in 80% cold ethanol followed by a 5 minute centrifugation, dried and resuspended in 12 μl DEPC water.

In vitro transcription (IVT) and fragmentation of cRNA

Biotin-labeled cRNA was synthesized using BioProbe T7 RNA Transcript Labeling kit (ENZO Biochem Inc., Farmingdale, NY) and purified using RNeasy Mini Protocol for RNA Cleanup (Qiagen, Valencia, CA). An adjusted cRNA yield was calculated using the following formula: adjusted cRNA yield = μg cRNA after IVT- (μg total RNA used initially) (fraction of cDNA reaction used in IVT). 20 μg (adjusted value) cRNA was incubated in fragmentation buffer (0.2 M Tris-acetate 8.1, 0.5 M KOAc, 0.15 M MgOAc) at 94°C for 35 minutes.

Array hybridization

Hybridization of biotin-labeled cRNA to *Xenopus laevis* Genome Arrays (Affymetrix, Santa Clara, CA) was performed using procedures previously described (Wodicka et al., 1997).

Array analysis

Arrays were scanned and data was imported using Gene Chip Operating Software version 1.0 (Affymetrix, Santa Clara, CA). Data was analyzed using DNA-Chip Analyzer (dChip) (Li and Wong, 2001; www.dchip.org). Data from six arrays (duplicated experiments from control explants and explants injected with *Mixer* or *Sox17*β) was normalized to the baseline array containing the median probe intensity (*Sox17*β, experiment 2). Data was analyzed based on the Perfect Match-only model based expression index (Li and Wong, 2001). Low values were truncated to an intensity of 33.22 (10^{th} percentile of expressions called “A”). Samples were compared based on fold increases in intensity for individual probe sets in experimental samples (*Mixer* and *Sox17*β) versus baseline (control). False detection rates for each list were generated using the rank products (RP) method (Breitling et al., 2004).

In situ hybridization screen

Dioxygenin-labeled anti-sense probes were generated from clones obtained from Open Biosystems (www.openbiosystems.com) and NIBB (xenopus.nibb.ac.jp). *Xenopus* embryos were developed to various stages between mid-gastrulation (10.5) and early tadpole (30) and fixed in MEMFA. For bi-sected embryos: Embryos were harvested at stage 10.5, fixed in MEMFA, and dehydrated in MeOH. Embryos were then bisected with a scalpel in MeOH and subsequently processed by In situ hybridization. In situ analysis was performed as described (Harland, 1991).

mRNA synthesis and injection for RT-PCR analysis

*Sox17*β and *Mixer* were cut and transcribed as above. *VegT* (cs105) (Zhang et al., 1998) and *Smad2* (cs105) (Baker and Harland, 1996) were linearized with AscI and transcribed with SP6 as above. 500 pg *Sox17*β, *Mixer*, *VegT* or *Smad2* was injected into the presumptive ectoderm of one-cell embryos. Explants were performed as above (20 per sample).

RT-PCR

Explants were cultured in 3/4× NAM to stage 10.5. RNA was isolated and cDNA was synthesized as previously described (Wilson and Melton, 1994). 48 μl PCR reactions were assembled with the following ingredients: 34.5 μl dH2O, 5 μl 10X PCR buffer, 4 μl 25 mM

MgCl2, 1 μl 10mM dNTP mix, 1 μl cDNA (from 20 μl reaction), 1 μl each 0.1 μg /μl primer, 0.5 μl Taq Polymerase (Applied Biosystems, Foster City, CA). PCR was performed using the following parameters: denature 94°C 2 minutes, 24–28 cycles of (denature 94°C 1 minute, anneal 62–64°C 1.5 minutes, elongate 72°C 1.5 minutes), final elongation 72°C 5 minutes. The following primers were used:

Gene	Primer Sequence	Reference
ODC	F-CAGCTAGCTGTGGTGTGG	(Agius et al., 2000)
	R-CAACATGGAAACTCACACC	
Sox17β	F-AACTCCCACCAGCAGGCTACTTTG	(Myers et al., 2004)
	R-TGTCAATGTCACTCTCCAGATGTCC	
Xbra	F-AACTGGTCTACCCTTCAAATGCC	(New)
	R-CGTGACATCATACTGGTTTTCTGC	
March8	F-TCCTCGGACATCAGTGACTCCATC	(New)
	R-AAGAACATACAGGGACCAAACGAC	
Cxcr4	F-GGCTATCAAAAGAAATCCAGGACC	(New)
	R-GCAGGAATCTAAACCCAAACAGTC	
Borg4	F-CGGGTGATGCCTTTGGAGATAC	(New)
	R-GGAACAGTTGCTGGACTTGAGC	
Gpr-4	F-AGGGAAACATCTTGGGCATCTAC	(New)
	R-TCCTTGAACGGAGTGGGAAAAC	
EST-21	F-ACACTTCACCACAATACCAGGGAG	(New)
	R-CTTTTCCATCGGGGCTCAAG	
Xtwik-2	F-GGAAGCAGAACACAGTAACAATCCG	(New)
	R-CACAAGTAGCGTGAGTAACAGCCAG	

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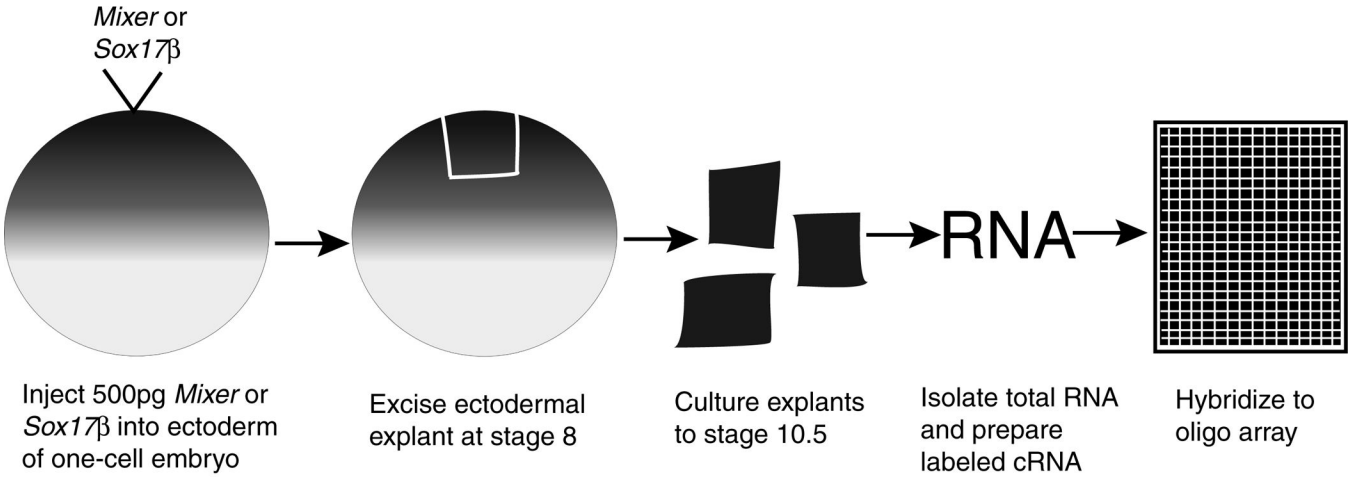

Fig. 1. Schematic of experimental strategy

This is a simplified visualization of the experimental procedure, which begins with injection of *Mixer* or *Sox17*β into the embryo and ends with an output of data from microarrays.

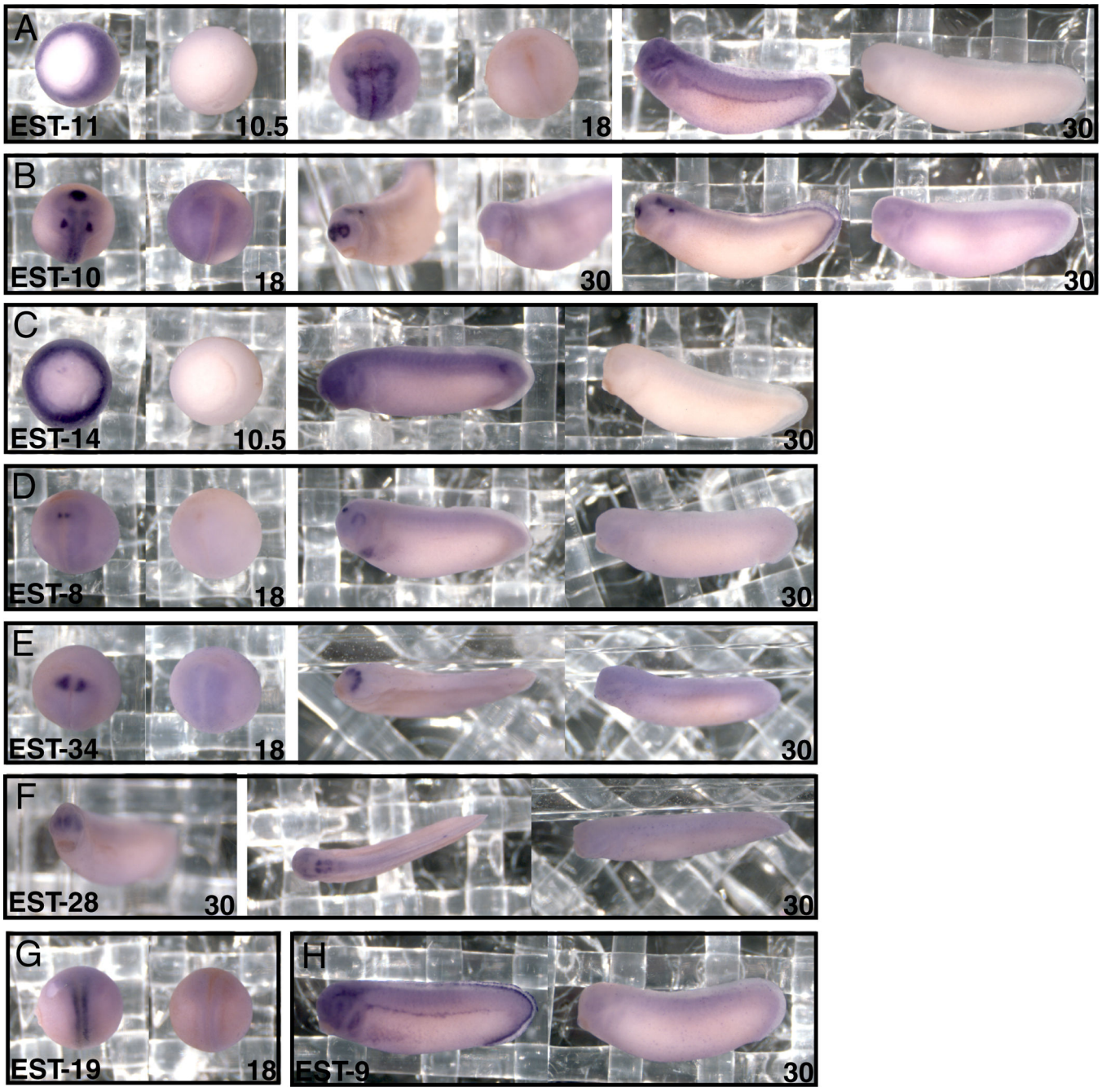

Fig. 2. In situ hybridization screen reveals additional expression patterns

A: Embryos at gastrula (10.5), neurula (15) and tailbud (30) stage are stained with antisense (left panel) and sense (right panel) probes for *EST-11* showing expression in the mesoderm, neural tube, neural crest and pronephros. B: Embryos at neurula and tailbud stage are stained with antisense and sense probes for *EST-10* demonstrating expression in cement gland, neural tube nasal placode, otic placod and forebrain. C: Embryos at gastrula and tailbud stage are stained for antisense and sense probes for *EST-14* demonstrating expression in mesoderm and in a single posterior somite. D: Embryos at neurula and tailbud stage are stained with antisense and sense probes for *EST-8*, indicating expression within forebrain

and heart. E: Embryos at neurula and tailbud stage are stained with antisense and sense probes for *EST-34*, showing expression in the placodes. F: Embryos at tailbud stage are stained with antisense and sense probes for *EST-28* showing expression in nasal placodes and hindbrain. G: Embryos at neurula stage are stained with antisense and sense probes for *EST-19*, indicating expression within the neural tube. H: Embryos at tailbud stage are stained with antisense and sense probes for *EST-9*, demonstrating expression within pronephros and neural crest.

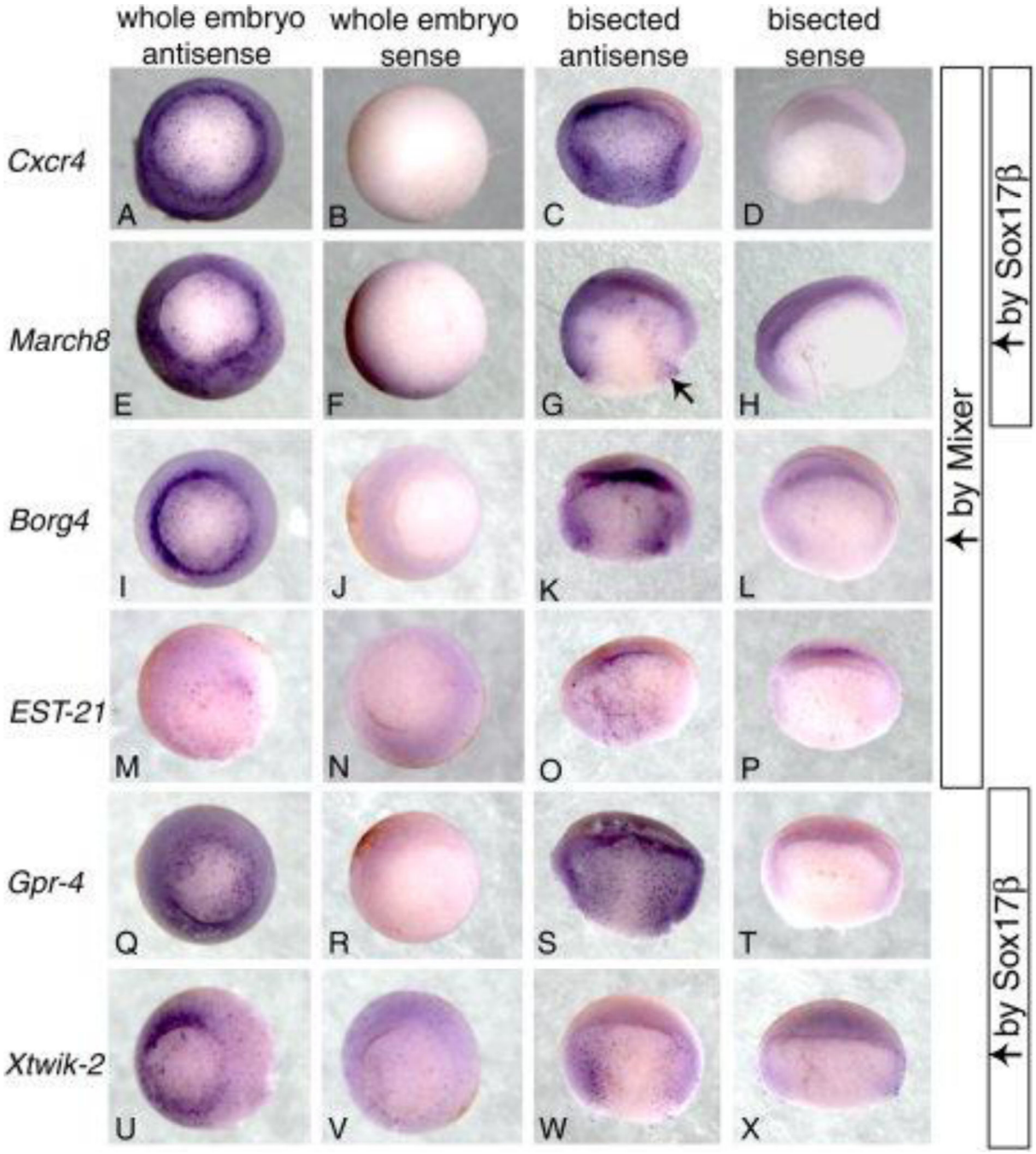

Fig. 3. Six transcripts are expressed in the early endoderm

The first two columns display stage 10.5 embryos, vegetal view, stained with antisense and sense probes for *Cxcr4 March8 Borg4 EST-21 Gpr-4* and *Xtwik-2*. The last two columns display hemisected stage 10.5 embryos, lateral view, stained with antisense and sense probes for the same transcripts above. The descriptions along right side of figure indicate which transcripts were upregulated with either Mixer, Sox17β or both. Arrow points to the deeper cells adjacent to blastopore ring expressing March8.

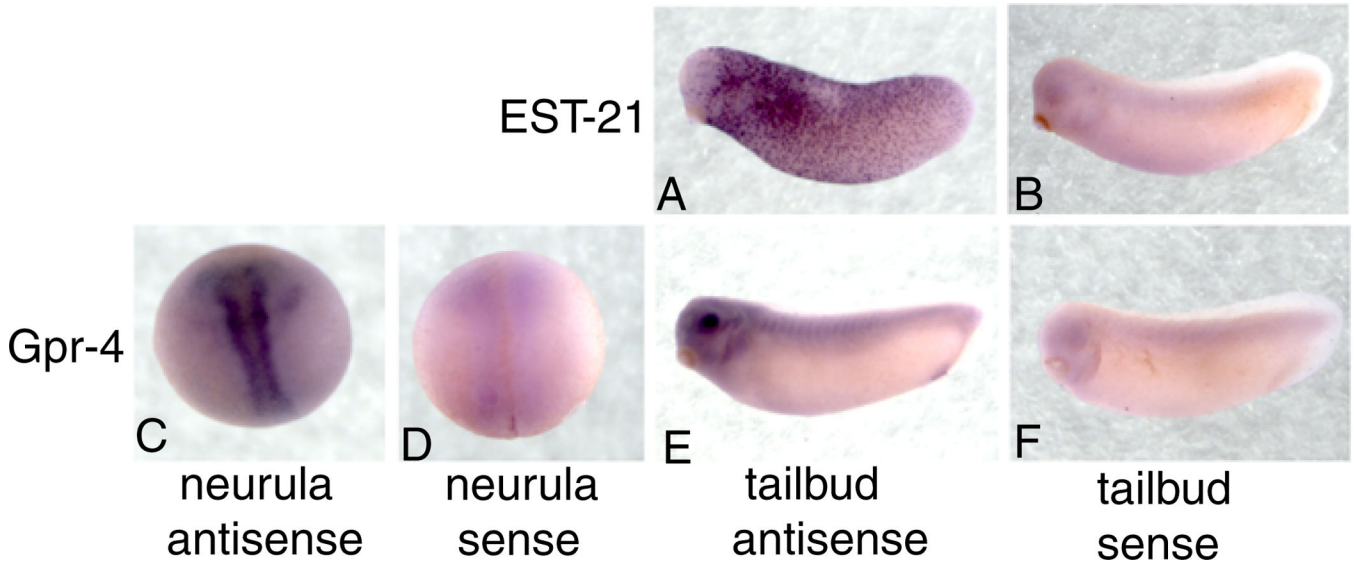

Fig. 4. EST-21 and Gpr-4 have additional patterns during later embryonic stages

A and B: Embryos at tailbud stage are stained with antisense and sense probes for *EST-21*. C and D: Neurula stage embryos are stained with antisense and sense probes for *Gpr-4*. E and F: Tailbud stage embryos are stained with antisense and sense probes for *Gpr-4*.

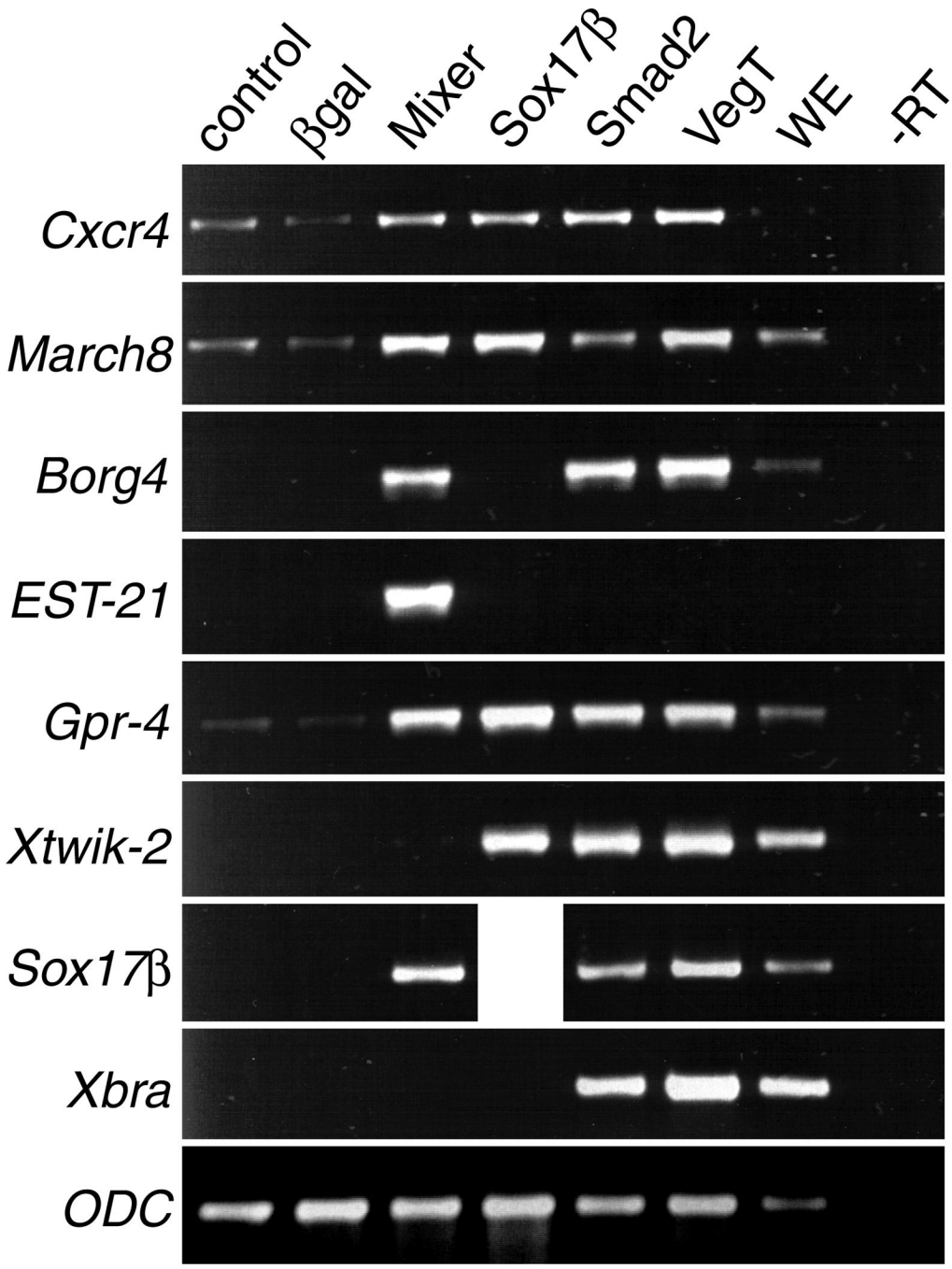

Fig. 5. Semiquantitative RT-PCR analysis confirms array data and supports an endogenous role for six new molecules in the endoderm pathway

RT-PCR was performed on cDNA synthesized from ectoderm explants expressing Mixer, Sox17β, Smad2 or VegT with primers for *Cxcr4 March8 Borg4 EST-21 Gpr-4* and *Xtwik-2* β-*gal* was injected as a control. Primers for *Sox17*β and *Xbra* were used as positive controls. *ODC* was used as a loading control. WE, whole embryo; -RT, minus reverse transcriptase.

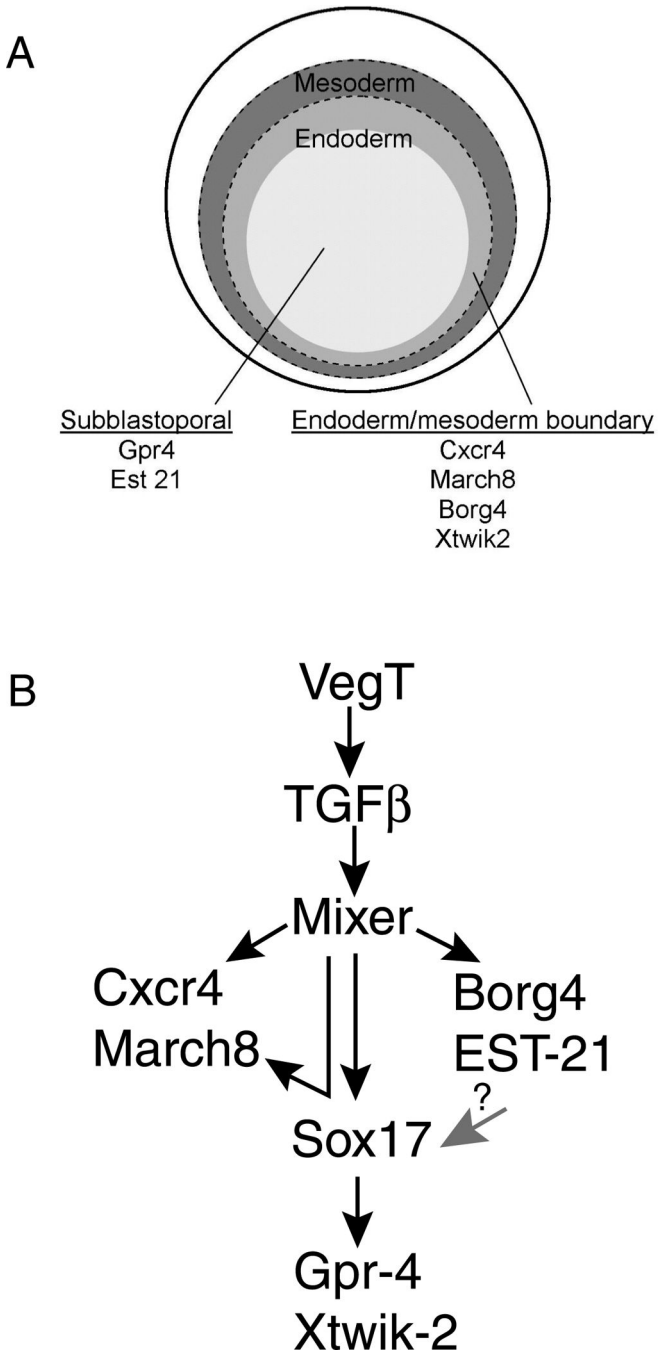

Fig. 6. Six new genes are expressed in discrete regions of the endoderm and may play roles in the endoderm pathway

A. This cartoon illustrates the vegetal hemisphere of a Xenopus embryo during gastulation. The most vegetal cells (off white) are referred to as subblastoporal endoderm and express *Gpr4* and *Est21*. A population of endoderm (light grey) lies adjacent to the mesoderm and expresses *Cxcr4 March8, Borg4* and *Xtwik2*. Mesoderm is depicted in dark grey. B. A simplified version of the endoderm pathway is diagramed hypothesizing how the endoderm specific targets may be involved. *Cxcr4* and *March8* are placed downstream of Mixer and

Sox17β. *Borg4* and *EST-21* are placed downstream of Mixer. It is unknown whether *Borg4* and *EST-21* activate Sox17β (gray arrow with ?). *Gpr-4* and *Xtwik-2* are placed downstream of Sox17β.

TABLE 1

Genes induced by Mixer and Sox17β

Name	Accession number	Unigene cluster	Domains/ homology/ description	Fold Change by Mixer	Fold Change by Sox17β
FoxA1	BC047130	Xl.888	Forkhead box A1, (*Xfkh2, Hnf3* β)	2.0, 2.6	5.0, 4.0
Cxcr4	BC044963	Xl.11336	C-X-C motif chemokine receptor 4	2.4, 2.6	2.2, 3.3
Frzb-1	U68059	Xl.212	Frizzled-related protein precursor	5.0, 7.1	3.9, 3.0
Cpeb	U14169	Xl.984	Cytoplasmic polyadenylation element binding protein	2.7, 8.0	5.0, 26.5
X-msr	U72029	Xl.23649	Angiotensin receptor related protein (AKA *Xangio1*)	3.6, 10.6	2.6, 5.5
p30 B9.10	X73317	Xl.1244	Maternal B9.10 protein	2.3, 2.0	3.5, 3.1
EST-1	CA986927	Xl.8458	similar to human March8	1.8, 4.0	2.0, 3.7
EST-2	BJ048011	Xl.16876	similar to human March8	2.5, 4.0	2.2, 3.3
EST-3	AW199159	Xl.25556	similar to human c-Myc target JPO1	2.9, 5.7	4.6, 15.2
EST-4	CA790591	Xl.21726	frog gene	6.3, 11.1	3.2, 4.7

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TABLE 2

Genes induced by Mixer only

Name	Accession number	Unigene cluster	Domains/ homology/ description	Fold Change by Mixer	Fold Change by Sox17β
Msx2a[*†]	AW766492	Xl.31078	Msh homeobox homolog 2 (*Hhox-7.1'*)	6.9, 8.2	−1.5, −1.4
				3.9, 2.6	1.1, −1.2
P7E4	AB072006	Xl.34957	BTB (POZ) domain containing 3	4.7, 2.8	−1.2, −2.0
Wnt-11	L23542	Xl.44504	Maternally expressed wnt gene	3.8, 5.9	1.3, 1.0
*Sox17*α	AJ001730	Xl.3831	SRY-related HMG-box transcription factor	6.3, 9.7	1.3, 1.2
Sox17-α2	AB052691	Xl.11957	SRY-related HMG-box transcription factor	7.1, 11.8	1.3, 1.1
Sox17-β	BC070615	Xl.44	SRY-related HMG-box transcription factor	13.0, 17.4	163.4, 280.4[‡]
Mab-21	AF040992	Xl.279	Xenopus Mab21l1	3.9, 7.9	1.4, 3.2
Gata-5a	L13701	Xl.578	Zinc finger transcription factor	2.0, 3.0	−1.2, −1.7
Xeel[†]	AB105372	Xl.6266	Embryonic epidermal lectin	10.1, 21.9	1.3, −1.1
Mig30	AB035379	Xl.34912	Mixer inducible gene 30	2.5, 4.6	−1.1, −1.0
c-myc	M14455	Xl.826	Myelocytomatosis oncogene	2.6, 3.5	1.1, −1.3
EST-5	BJ055630	Xl.9623	likely ortholog of mouse Epsin 2	2.2, 2.5	−1.3, −1.2
EST-6	BG555687	Xl.1321	similar to Centromere/kinetochore protein Zw10	2.4, 7.6	−1.1, −1.9
EST-7	CB561069	Xl.7842	similar to Serum/glucocorticoid regulated kinase	3.0, 6.4	−2.3, −1.4
EST-8	BC060483	Xl.14214	similar to human transmembrane protein Claudin 5	3.5, 2.1	1.0, 1.0
EST-9[*]	BC045272	Xl.8630	similar to human Serum inducible kinase	3.0, 5.6	1.3, 1.4
				2.6, 4.7	1.2, 1.0
EST-10	BJ046058	Xl.9284	similar to human GT box-binding protein Sp3	4.2, 2.0	1.4, 1.7
EST-11	BG020193	Xl.2466	similar to human I38026 MLN 62 protein	2.6, 3.6	−1.1, 1.1
EST-12	BJ087388	Xl.16135	similar to human K-sam precursor	2.0, 2.0	−1.0, −1.5
EST-13	BG486882	Xl.4337	similar to human Sec4 GTP binding protein	2.1, 4.4	1.1, 1.0
EST-14	BC041294	Xl.13019	similar to human RAS-like protein	2.3, 3.8	−1.9, −1.3
EST-15	BJ044041	Xl.16547	similar to human Borg4	2.0, 2.8	−1.2, −2.7
EST-16	BJ100112	Xl.13033	similar to human Chemokine-like super family 8	3.0, 9.1	1.1, 1.8
EST-17[*†]	BJ085828	Xl.3435	frog gene	7.2, 6.7	−2.3, −2.1

Name	Accession number	Unigene cluster	Domains/ homology/ description	Fold Change by Mixer	Fold Change by Sox17β
				7.3, 5.9	−2.1, −2.0
EST-18	BJ051730	Xl.15365	frog gene	2.0, 4.3	−1.7, −1.2
EST-19 †	BJ056057	Xl.10150	frog gene	7.1, 23.9	1.4, 1.0
EST-20	BJ086610	Xl.13363	frog gene	3.0, 2.1	−1.1, −1.1
EST-21 *	BJ075680	Xl.15089	frog gene	1.6, 11.5	1.2, 1.1
				1.5, 6.4	1.2, 1.2

*Indicates transcripts represented more than once on the array and meet the required standards twice in our data set for experiments 1 and 2.

†Represents transcripts whose induction by Mixer was greater than 5.0 for experiments 1 and 2.

‡Number reflects measure of mRNA present due to injection.

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TABLE 3

Genes induced by Sox17β only

Name	Accession number	Unigene cluster	Domains/ homology/ description	Fold Change by Mixer	Fold Change by Sox17β
ElrD	u17599	Xl.1036	RNA-binding protein HuD	1.0, 1.0	2.6, 10.1
Clast3	CB561662	Xl.10510	CD40 ligand-activated specific transcript 3	1.2, −1.0	2.6, 3.0
*Otx2**†	AW199379	Xl.3004	Orthodenticle-A like homeobox protein	1.7, 1.2	6.2, 8.1
				1.2, 1.1	2.9, 5.2
Irx4 †	AF338157	Xl.12086	Iroquois-4 homeobox transcription factor	1.3, 1.1	14.5, 18.6
Nr2f2	BC044975	Xl.14532	Nuclear receptor subfamily 2, group F, member 2	−1.0, 1.0	2.0, 2.8
FoxC1	AF116844	Xl.180	Winged helix transcription factor (*Xfd-11*)	1.8, 1.2	2.5, 4.1
Pdgf	BC043948	Xl.20029	Platelet derived growth factor receptor alpha	1.2, 1.4	2.4, 2.3
Crgb †	AF071563	Xl.21441	Crystallin, gamma B	1.1, 1.1	10.1, 21.2
Eomes	U75996	Xl.373	T-domain gene Eomesodermin	1.5, 1.1	2.8, 3.0
Ash1 †	M98272	Xl.450	Achaete-scute protein homologue	1.3, 1.6	5.4, 14.3
Xgam	M63446	Xl.5871	Gamma-tubulin	1.3, 1.6	2.1, 2.9
Foxd1-A	AJ011652	Xl.66	Forkhead box D1 (brain factor 2, *Xbf-2*)	1.3, 1.2	2.6, 4.1
*Gsc**†	BJ056432	Xl.801	Goosecoid homeobox protein	1.1, −1.1	5.0, 4.1
				2.5, 1.3	16.3, 17.5
Gpr-4	AY553187	Xl.45565	G-protein coupled receptor 4	1.3, 2.2	2.6, 4.3
Xlim-1	CB562197	Xl.32655	LIM domain-containing homeobox protein	1.2, 1.1	2.4, 2.5
Xtwi	M27730	Xl.879	bHLH Twist homolog 1	2.2, −1.1	9.3, 17.7
EST-22 †	BG810694	Xl.2565	similar to alpha-Tubulin at 84B	−1.1, 1.0	84.2, 106.2
EST-23	BI478249	Xl.19057	similar to human amino acid transporter	−1.1, −1.1	2.8, 5.7
EST-24	BM191866	Xl.7085	similar to human hypothetical protein FLJ20511	1.1, 1.2	2.0, 2.3
EST-25	BJ044317	Xl.1419	similar to human Interferon regulatory factor 1	−1.0, 1.1	8.0, 2.3
EST-26	BC041234	Xl.16040	similar to human Phosphatidylserine decarboxylase	1.2, 1.5	2.0, 3.0
*EST-27**	BG021407	Xl.34405	similar to human new Ets-related factor	1.4, 1.1	2.3, 3.8
				1.1, 1.1	2.9, 4.9
EST-28	BJ043709	Xl.8559	similar to human Protocadherin 10, isoform 1 precursor	1.1, 1.4	4.6, 9.2

Name	Accession number	Unigene cluster	Domains/ homology/ description	Fold Change by Mixer	Fold Change by Sox17β
EST-29	CA792418	Xl.1295	similar to human HRAS1-related cluster protein 1	−1.1, −1.0	3.7, 5.1
EST-30	BJ054524	Xl.16561	similar to human Chloride channel protein CLC-KA	−1.1, −1.0	2.9, 16.0
EST-31	BM172631	Xl.16695	similar to human Elongation of very long chain fatty acids	1.3, −1.0	3.5, 4.4
EST-32	BI348356	Xl.18627	similar to human hypothetical protein DKFZp586N041.1	1.4, 1.5	2.1, 2.1
EST-33	BQ385449	Xl.19414	similar to human Rsu-1 homolog	1.1, 1.1	2.8, 3.7
EST-34 †	BJ044473	Xl.15931	similar to mouse Doublesex- and mab-3-related txn factor	1.3, 1.1	6.3, 6.3
EST-35 †	BJ048106	Xl.23586	likely paralog of *Xtwik2*	1.3, 1.7	8.5, 11.4
EST-36	BJ085642	Xl.13426	similar to human C2orf17 unnamed protein	1.3, 1.1	2.4, 2.2
EST-37	BJ079356	Xl.14106	frog gene	1.4, 2.3	2.7, 2.6
EST-38	BG023545	Xl.2439	frog gene	1.3, 2.0	2.5, 5.2

*Indicates transcripts represented more than once on the array and meet the required standards twice in our data set for experiments 1 and 2.

†Represents transcripts whose induction by Sox17β was greater than 5.0 for experiments 1 and 2.

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TABLE 4

In situ hybridization results

Name	Accession number	Expression			Figure Reference
		St 10.5	St 15	St 30	
Cxcr4	BC044963	endo	X*	X*	2 A,C
Cpeb	U14169	ubiq			
p30 B9.10	X73317	none			
March8	CA986927	endo			2 E,G
EST-2	BJ048011	ubiq			
EST-3	AW199159	none			
EST-4	CA790591	ubiq			
Xeel	AB105372	ubiq			
EST-8	BC060483	none	X	X	4 D
EST-9	BC045272	none		X	4 H
EST-10	BJ046058	none	X	X	4 B
EST-11	BG020193	meso	X	X	4 A
EST-14	BC041294	meso		X	4 C
Borg4	BJ044041	endo			2 I,K
EST-16	BJ100112	ubiq			
EST-17	BJ085828	ubiq			
EST-19	BJ056057	none	X		4 G
EST-20	BJ086610	ubiq			
EST-21	BJ075680	endo		X	2 M,O; 3 A
Gpr-4	AY553187	endo	X	X	2 Q,S; 3 C,E
EST-22	BG810694	ubiq			
EST-23	BI478249	ubiq			
EST-24	BM191866	ubiq			
EST-25	BJ044317	none			
EST-26	BC041234	none			
EST-27	BG021407	none			
EST-28	BJ043709	none		X	4 F

Name	Accession number	Expression			Figure Reference
		St 10.5	St 15	St 30	
EST-30	BJ054524	none			
EST-33	BQ385449	none			
EST-34	BJ044473	none	X	X	4 E
Xtwik-2	BJ048106	endo			2 U, W
EST-36	BJ085642	none			
EST-37	BJ079356	none			
EST-38	BG023545	ubiq			

* Expression at these stages previously determined (Moepps et al., 2000).