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Methods

High-throughput functional screen of mouse gastrula cDNA libraries reveals new components of endoderm and mesoderm specification

Eric Chiao, Jeff Leonard, Kari Dickinson, and Julie C. Baker¹*Department of Genetics, Stanford University Medical School, Stanford, California 94062, USA*

This study describes a cross-species functional screen of mouse gastrula cDNA libraries for components of endoderm and mesoderm specification. Pools of 96 cDNAs from arrayed mouse gastrula cDNA libraries were transcribed into mRNA and injected into either the presumptive mesoderm or the ectoderm of one-cell *Xenopus laevis* embryos. Injected embryos were examined at gastrula stage by in situ hybridization with endoderm or mesoderm markers. Using this approach, we screened over 700 pools or ~60,000 cDNAs. We identified 17 unique cDNAs that function during mesoderm and/or endoderm specification and 16 that cause general morphology changes. Identified molecules fall into eight general functional groups as follows: cell cycle components (seven), transcription factors (four), extracellular secreted molecules (seven), transmembrane receptors (one), intracellular signaling components (five), microtubule components (two), metabolism molecules (three), and unknown (four). Several of the genes we identified would not have been predicted to be involved in endoderm or mesoderm specification, highlighting the usefulness of nonbiased screening approaches. This includes *Otx2*, which we show is a downstream target of *Xsox17β*. The speed, low cost, and high efficiency of this cross-species screen makes it an ideal method for examining cDNAs from difficult-to-obtain sources. Therefore, this approach complements the current mouse molecular genetics systems and provides a powerful means for the genome-wide examination of mammalian gene function.

[The sequence data from this study have been submitted to dbEST/GenBank under accession nos. CV736163–CV736199.]

The genomics revolution has provided biologists with the complete genome sequences of numerous organisms and a host of new technologies for the study of normal development and disease states. Tapping into genomic methodologies to understand the functional significance of genes has advanced several fields, including that of developmental biology. One window of time during mammalian development that is an ideal source for functional genomic studies is gastrulation. At this stage, which lasts only 24 h, the embryo undergoes complex differentiation events, beginning with only a few cell types and emerging with hundreds (Hogan et al. 1986; Beddington and Robertson 1998, 1999). Simultaneously, and unique to mammals, the embryo is also undergoing massive cellular proliferation, having a cell cycle rate of ~5 h and generating >8000 new cells (Snow 1977). These pivotal events make the mammalian gastrula a wealthy source to mine for key regulators of developmentally or pathologically important signaling pathways.

Due to the small size and inaccessibility of the mammalian gastrula, applying genomic approaches to elucidate gene function remains a challenge. Nevertheless, several approaches are currently being utilized to meet these challenges. The mouse is currently the most robust model for detailed molecular analysis of gene function in mammals. ENU mutagenesis, enhancer trap screens, and homologous recombination approaches have all been used to identify developmentally important genes, some as early as gastrulation (Justice et al. 1999; Anderson 2000; Mitchell et al. 2001). However, these approaches are resource intensive, time consuming, and primarily uncover loss-of-function muta-

tions. Important genes in functionally redundant pathways are difficult to identify, as are groups of molecules acting in concert. Recently, a large-scale in situ hybridization analysis of cDNA library clones identified potentially important developmental regulators and synexpression groups during mouse gastrulation (Sousa-Nunes et al. 2003). This approach, although clearly less resource intensive than the mutant screens, required a priori assumptions in order to increase efficiency. In any genomics approach, a reliance on such assumptions makes it unlikely that important genes with unexpected function would be found. For example, it is unlikely that a gene with an “uninteresting” expression pattern would be chosen for homologous recombination, or that a “housekeeping” gene would be selected for in situ analysis. Thus, although the approaches described above are all very effective tools for exploring gene function, they have limitations when applied to large-scale systematic genome-wide endeavors.

Signaling pathways, including those active during the formation of endoderm and mesoderm, are conserved between vertebrates, opening up the possibility of cross-species investigations of mammalian germ-layer formation (Shivdasani 2002). One very fruitful approach used to identify key vertebrate developmental regulators has been expression cloning in *Xenopus* (Lustig et al. 1997; Grammer et al. 2000). In this approach, mRNAs are synthesized from cDNA pools and then injected into *Xenopus* embryos. These embryos are then analyzed for changes caused by the protein products of the injected mRNAs. Alteration of developmental processes indicates that one or more cDNAs in the injected pool has a specified function. Pools with functional activity are subsequently split, or sib selected, until the single active clone is identified. *Xenopus* expression-cloning approaches have identified many key developmental molecules, including

¹Corresponding author.**E-mail** baker@genome.stanford.edu; **fax** (650) 736-1534.Article and publication are at <http://www.genome.org/cgi/doi/10.1101/gr.2993405>.

Smad2, Noggin, Wnt8, and Mixer, to name only a few (Smith and Harland 1991, 1992; Baker and Harland 1996; Henry and Melton 1998). The rapid macroscopic external development of *Xenopus* embryos coupled with the large clutch sizes makes expression cloning an ideal system for performing cost-effective high-throughput screens.

Only one study to date has used expression cloning as a high-throughput methodology (Grammer et al. 2000). That study used an arrayed neurula stage *Xenopus* cDNA library to screen for genes involved in apoptosis, neural development, somite formation, and blood development. By creating defined cDNA pools from their arrayed cDNA library, Grammer et al. (2000) greatly improved the sib-selection process. Previously sib selection entailed serially diluting the cDNA pools and reinjecting the diluted pools until the single activity was found, a process that was time consuming and frequently failed. Using arrayed libraries, subpools can be created in a controlled, reproducible fashion, thereby increasing the efficiency of sib selection. Here, we adapted the high-throughput method for use in a cross-species screen and further refined the sib-selection process so that single, active clones could be directly identified in one split. This is the first report of a high-throughput expression-cloning screen used to identify functional mammalian cDNAs, and the first such screen to target endoderm and mesoderm regulators in any species.

We have completed a high-throughput cross-species functional screen of >60,000 mouse gastrula cDNA clones and identified many endoderm and mesoderm specification molecules. We can categorize these molecules into eight general functional groups as follows: cell cycle components (seven), transcription factors (four), extracellular secreted molecules (seven), transmembrane receptors (one), intracellular signaling components (five), microtubule components (two), metabolism molecules (three), and unknown (four). Several of the results are unexpected and would only arise from a blind screening approach. This is apparent from our identification of two different isoforms of Fgf8, both of which induce mesoderm, and our cloning of the transcription factors *Otx2* and *Foxa2*, both extremely well-studied developmental molecules that have never been shown to be sufficient to induce endoderm. Here we show that *Otx2* is downstream of *Xsox17 β* in an endodermal specification pathway. Furthermore, this screen identified several cell cycle components that are sufficient for endodermal specification. These molecules would not have been predicted a priori to have this role, and their identification in this screen may shed light on the relationship between the rapid cellular proliferation and differentiation events occurring during mouse gastrulation. These data highlight the usefulness of nonbiased functional screening approaches for identifying new and unexpected molecules involved in the regulation of important developmental processes.

Results

To identify molecules that alter the fate of endoderm or mesoderm during mouse gastrulation, we performed a functional genomic screen using an expression-cloning approach. Two separate mouse cDNA libraries were generated, one from early stage (e6.5) and one from late stage (e7.5) gastrula (Baker et al. 1999). These cDNA libraries were arrayed on 384-well plates, and pools of 96 were generated from this resource. mRNA synthesized from these pools was injected into the presumptive mesoderm or ec-

toderm of the one-cell *Xenopus laevis* embryo (see Fig. 1 and Methods). By overexpressing pools in the mesoderm, our goal was to find molecules that could repress or expand mesodermal or endodermal cells. By overexpressing pools in the ectoderm, our goal was to identify molecules that ectopically induce either endoderm or mesoderm. Injected embryos were allowed to develop to gastrula stage (10.5). Then, the embryos were fixed and stained by in situ hybridization for the mesodermal marker, *Xbra*, or a combination of the endodermal markers, *Xsox17 β* and *endodermin* (*edd*) (see Fig. 3, below). During gastrulation, the transcription factor *Xbra* is expressed throughout the mesoderm in a ring around the equator of the embryo (Smith et al. 1991). *Xsox17 β* , also a transcription factor, is specifically and uniformly expressed throughout the endoderm (Hudson et al. 1997). *Edd* is expressed throughout the endoderm, but is expressed more strongly than *Xsox17 β* in the dorsal bottle cells and in the rim of the blastopore (Sasai et al. 1996). We chose these markers because they are the best studied of the endoderm and mesoderm markers available and make the most reliable probes, due to their high expression within the different domains. Throughout the screen, we observed morphological phenotypes in several pools, consisting mainly of cell cycle or patterning defects. Since these effects are frequently the result of high doses of potent cell fate-inducing molecules, these pools were also examined and split.

Using this approach, we screened through 368 pools from the e6.5 library and 344 pools from the e7.5 library for a total of ~60,000 cDNAs. Eighty-two pools (11.5%) had reproducible phenotypic effects on either endoderm and mesoderm (55) or general morphology (27). These pools were subsequently split using a gridding approach, whereby the rows and columns from the original pool are subpooled and assayed for activity. The overlap of activity reveals the single positive clone (see detailed explanation in Methods and in Fig. 2). Of the 82 positive pools, 23 single active clones were isolated by mesoderm and/or endoderm activities and 19 were isolated by general morphology changes. These clones were sequenced and their identity revealed by BLAST searches (Altschul et al. 1990). Forty-one pools either failed to split or the clone identified did not repeat the phenotypic effect reliably. Several clones were isolated more than once. A complete list of the clones identified, grouped by activity, is shown in Table 1A,B. The marker analysis of a selection of these clones is shown in Figures 3 and 4. It is interesting to point out that most of the clones were identified from the earlier staged library (e6.5). There is no indication from sequencing or clone

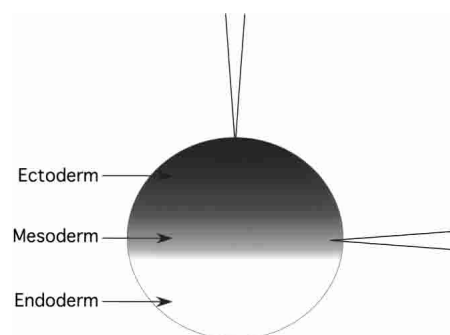


Figure 1. The *Xenopus* embryo is laid with a rudimentary polarity. The prospective ectoderm, (shaded) resides at the animal hemisphere, while the prospective endoderm resides at the vegetal hemisphere. The prospective mesoderm develops at the equator between these two cell types. Sites of injection are illustrated.

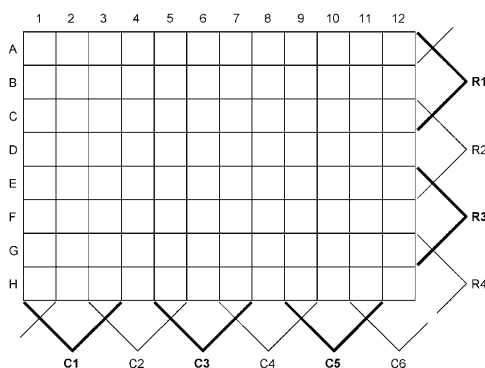


Figure 2. Positive pools were split as shown in this diagram. Ten subpools were generated of overlapping rows and columns. These subpools were injected and analyzed by in situ hybridization. Individual cDNA clones(s) within the intersection of phenotypically positive subpools were isolated and sequenced. This overlapping subpooling approach allows for the identification of positive clones in a single split.

analysis that might indicate that this is a superior library. Both appear to have similar numbers of full-length transcripts and have equivalent clone sizes.

Molecules identified that induce ectopic endoderm in whole embryos

Using this screen, we identified eight molecules that, when expressed ectopically in whole embryos, led to an increase in markers for endoderm. These include *Mix11*, *Hspa8*, *Kif22*, *Fzr1/Cdh1*, *Tcfcb*, *Habp2*, *Otx2*, and *Foxa2* (previously *HNF3β*). Since this analysis consisted of a combination *Xsox17β* and *edd* probes, we characterized which of these markers the eight molecules could induce. Four molecules were identified that led to the ectopic formation of both endodermal markers—*Kif22*, *Mix11*, *Hspa8*, and *Fzr1/Cdh1* (Fig. 3D–O)—while four were specific for induc-

tion of *edd*—*Tcfcb*, *Habp2*, *Otx2*, and *Foxa2* (Fig. 3P–ZZ). None were specific for induction of *Xsox17β* alone.

Molecules identified that induce ectopic mesoderm in whole embryos

We found two molecules that function as mesoderm inducers, *Actr11b*, and *Fgf8f*. *Actr11b* is a known and characterized mesoderm inducer. It is also a transmembrane receptor, which shows that the screen can recover even membrane-associated molecules (Hemmati-Brivanlou and Melton 1992; Nagaso et al. 1999). In addition, we identified another isoform of *Fgf8*, *Fgf8a*, in the gastrulation defective screen. At low concentrations, this clone can also induce mesoderm (see below and Fig. 5D).

Molecules identified that disrupt mesoderm and endoderm

We identified six molecules that disrupt mesoderm and/or endoderm without causing observable changes in cellular morphology or noticeable cellular death. The distinction between inhibition of marker expression and cellular changes is a critical one, since we wanted to be certain that inhibition was caused by a specific disruption of tissue and not a general death phenotype. Embryos injected with *Rsk4*, *Cks2*, *Lefty2*, *cyclin F*, *D7Ssb2*, and *geminin* looked phenotypically normal and healthy prior to in situ hybridization, with the exception that *Lefty2* and *geminin*-injected embryos displayed gastrulation delays and failure of bottle cell formation. To ensure that the disruption of marker was not caused by cell death, we performed a TUNEL assay on embryos injected with several concentrations of *Cks2*, *cyclin F*, and *D7Ssb2* (data not shown). Only *cyclin F* at concentrations >400 pg led to programmed cell death by gastrulation. Titration of *cyclin F* concentrations below 400 pg inhibited *Xbra* in the absence of TUNEL-positive cells. These results indicate that we observe inhibition of mesoderm and endoderm in the absence of cell death. *Lefty2*, *geminin*, and *D7Ssb2* inhibited both *Xbra* and *edd* expres-

Table 1A. Clones identified by endoderm or mesoderm phenotypic effect on *Xenopus* embryos

Gene	Group	Accession #	MGI #	Molecular marker			# cloned	Library
				<i>Xsox17β</i>	<i>edd</i>	<i>Xbra</i>		
<i>Hspa8</i>	un	NM_031165	MGI:105384	+	+	–	1	6.5
<i>Kif22</i>	mt	NM_145588	MGI:109233	+	+	–	1	7.5
<i>Fzr1; Cdh1</i>	cc	NM_019757	MGI:1926790	+	+	–	1	6.5
<i>Mix11</i>	tf	NM_013729	MGI:1351322	+	+	–	2	6.5
<i>Foxa2</i>	tf	NM_010446	MGI:1347476	–	+	–	1	7.5
<i>Tcfcb</i>	tf	NM_011549	MGI:103270	–	+	–	1	6.5
<i>Otx2</i>	tf	NM_144841	MGI:97451	–	+	–	1	6.5
<i>Habp2</i>	ex	NM_146101	MGI:1196378	–	+	–	1	7.5
<i>Ccnf; cyclin F</i>	cc	NM_007634	MGI:102551	–	–	–	1	6.5
<i>Cks2</i>	cc	NM_025415	MGI:1913447	–	–	–	1	7.5
<i>Rsk4</i>	sg	NM_025949	MGI:1914321	–	–	–	1	6.5
<i>D7Ssb2</i>	un	AK002578	MGI:1914859	–	–	–	1	6.5
<i>Gmnn; geminin</i>	cc	NM_020567	MGI:1927344	–	–	–	2	7.5
<i>Lefty2</i>	ex	NM_177099	MGI:2443573	–	–	–	5	6.5(4), 7.5(1)
<i>Fgf8a</i>	ex	NM_010205	MGI:99604	–	–	+	1	6.5
<i>Fgf8f</i>	ex	BC048734	na	–	–	+	1	6.5
<i>Actr11b</i>	tm	NM_007397	MGI:87912	–	–	+	1	6.5

Pluses indicate ectopic expression of the listed marker in whole embryos by in situ hybridization analysis. Minuses indicate loss of expression of listed marker in whole embryos by in situ hybridization. Table includes the gene name, functional group, accession number, mouse genomic resource number (MGI; see <http://www.informatics.jax.org>), number of times the clone was identified and the cDNA library that produced the clone. Functional group abbreviations are as follows: (cc) cell cycle component; (tf) transcription factor; (ex) extracellular secreted molecule; (sg) intracellular signaling molecule; (tm) transmembrane protein; (mt) microtubule assembly protein; (mb) metabolic protein; (un) unknown.

Table 1B. Clones identified in functional screen

Gene	Group	Accession #	MGI #	# cloned	Library
<i>Cdt1; Ris2</i>	cc	NM_026014	MGI:1914427	2	6.5
<i>Cox5b</i>	mb	NM_009942	MGI:88475	1	6.5
<i>Pcnt2</i>	mt	NM_008787	MGI:102722	1	6.5
<i>Apex1</i>	mb	NM_009687	MGI:88042	1	7.5
<i>Rnf26</i>	un	NM_153762	MGI:2388131	1	6.5
<i>Pkm2</i>	mb	NM_011099	MGI:97591	2	6.5
<i>Bop1</i>	cc	NM_013481	MGI:1334460	1	6.5
<i>Cdk8</i>	cc	NM_153599	MGI:1196224	1	6.5
2210403N09Rik	un	BC024804	MGI:1922915	1	6.5
<i>Rhoa</i>	sg	NM_016802	MGI:1096342	1	6.5
β catenin	sg	NM_007614	MGI:88276	1	6.5
<i>Wnt3a</i>	ex	NM_009521	MGI:98955	2	6.5
<i>Bmp2</i>	ex	NM_007553	MGI:88177	1	7.5
<i>Bmp4</i>	ex	NM_007554	MGI:88180	1	6.5
<i>Chn1</i>	sg	NM_175752	MGI:1915674	1	7.5
<i>Racgap1</i>	sg	NM_012025	MGI:1349423	1	6.5

Clones identified by morphological effect on *Xenopus* embryos. Table includes the gene name, functional group, accession number, mouse genomic resource number (MGI; see <http://www.informatics.jax.org>), number of times the clone was identified, and the cDNA library that produced the clone. Functional group abbreviations are as follows: (cc) cell cycle component; (tf) transcription factor; (ex) extracellular secreted molecule; (sg) intracellular signaling molecule; (tm) transmembrane protein; (mt) microtubule assembly protein; (mb) metabolic protein; (un) unknown.

sion, while *cyclin F*, *Cks2*, and *Rsk4* inhibited only *Xbra* (Fig. 4; see Myers et al. 2004).

Molecules identified that caused morphological changes

Cell-fate determination molecules frequently lead to morphology changes or cellular death when expressed at high levels in embryos. We identified 18 molecules that cause gastrulation defects, abnormal morphologies, or overt death (Table 1B). By diluting the concentration of these molecules, we determined which molecules led to specific cell-fate changes in germ-layer formation. Indeed, seven of these molecules, including β -catenin, *RhoA*, *D7Ssb2*, *Fgf8a*, *Wnt3a*, *Bmp4*, and *cyclin F* gave specific developmental phenotypes when diluted to a concentration that did not affect the general viability of the embryo (see Fig. 4 for *D7Ssb2* and *cyclin F*). Interestingly, *Wnt3a* and *Bmp4* were identified from the same pool, which led to gross embryonic death shortly after gastrulation. Splitting revealed previously described effects of both molecules; *Wnt3a* alone caused a duplication of embryonic axis and *Bmp4* alone ventralized the embryos (Hemmati-Brivanlou and Thomsen 1995; Hatta and Takahashi 1996). β -Catenin was able to dorsalize embryos and induce a secondary axis, and *RhoA* inhibited the formation of optic vesicles. Both of these phenotypic effects have previously been reported (Funayama et al. 1995; Guger and Gumbiner 1995; Wunnenberg-Stapleton et al. 1999). *Fgf8a* induced mesoderm at lower concentrations, while *D7Ssb2* and *cyclin F*, when diluted to appropriate concentrations, inhibited the formation of mesoderm.

Seven of the identified clones can directly transform naive ectoderm into endoderm and/or mesoderm

Our screening approach used intact *Xenopus* embryos as a readout for molecules that could activate the expression of mesodermal or endoderm markers. Although a useful approach, it does not discriminate between molecules that can directly induce (create de novo) these tissues and molecules that will simply expand or modify the tissue already present. Therefore, we used an explant approach to test which of our identified molecules could

directly induce endoderm or mesoderm from naive ectoderm (Sive et al. 2000). *Mixl1*, *Tcfcb*, *Kif22*, *Habp2*, *Hspa8*, *Fzr1*, *Otx2*, *Foxa2*, *Fgf8f*, and *Fgf8a* were all injected at different concentrations into the prospective ectoderm of the one-cell *Xenopus* embryo. At blastula stages (8–9), before any cell mixing, the ectoderm was excised and grown in isolation until intact siblings reached gastrula stage (10.5). The explants were harvested and analyzed with RT-PCR for marker expression. *Mixl1* was capable of directly inducing both *Xsox17 β* and *edd*, while *Fzr1* could induce *Xsox17 β* (Fig. 5B). This is the first report of *Fzr1*, a component of the anaphase-promoting complex, being involved in endodermal specification (Visintin et al. 1997). Conversely, *Mixl1* is well characterized as having this function (Lemaire et al. 1998). *Tcfcb*, *Otx2*, and *Foxa2* were all able to induce *edd*, but not *Xsox17 β* , consistent with the whole-embryo data (Fig. 5A,C). This indicates that they are downstream of *Xsox17 β* and the farthest downstream components of the early endoderm pathway known to date. Although we consistently saw very weak *Xsox17 β* bands in the *Kif22*-injected samples (data not shown), we believe that *Habp2*, *Hspa8*, and *Kif22* are not able to induce endoderm from naive ectoderm. *Fgf8a* and *Fgf8f* were both potent inducers of mesoderm in naive ectoderm (Fig. 5D).

Otx2 is induced by *XSox17 β*

Since *Otx2* could induce *edd*, but not *Xsox17 β* , we wanted to determine whether it was a target of *Xsox17 β* . Indeed, *Xenopus* ectodermal explants injected with *Xsox17 β* express *Otx2* at gastrula stages, suggesting that *Otx2* is downstream of *Xsox17 β* (Fig. 5E).

Discussion

The clones identified in our screen can be divided into three broad categories, known regulators of endoderm or mesoderm, uncharacterized genes, and genes that have been suggested to be involved, but have not been solidly implicated. Here, we further categorize the clones we identified based upon which markers they can induce or inhibit, discuss their potential roles in either mesoderm or endoderm formation, and define where they might function during germ-layer specification.

New downstream components of endoderm pathway emerge from screen

The largest group found in this screen was composed of molecules that could induce or expand endoderm. These molecules were identified by their ability to create ectopic *Xsox17 β* and/or *edd* expression in whole gastrula. The eight clones we identified, *Mixl1*, *Tcfcb*, *Kif22*, *Hspa8*, *Fzr1*, *Habp2*, *Otx2*, and *Foxa2*, all created ectopic *edd* expression when injected into embryos. Only four of these, *Kif22*, *Mixl1*, *Hspa8*, and *Fzr1* could also expand the marker *Xsox17 β* , and some of these induced *Xsox17 β* very weakly (see Fig. 3). None were capable of inducing *Xsox17 β* without *edd* in the whole embryo. Since *edd* is downstream of *Xsox17 β* , this result is not surprising (see Fig. 6).

Mixl1 was identified in this screen and is a well-studied member of the endodermal specification pathway (Lemaire et al. 1998; Shivdasani 2002; Stainier 2002). When overexpressed in

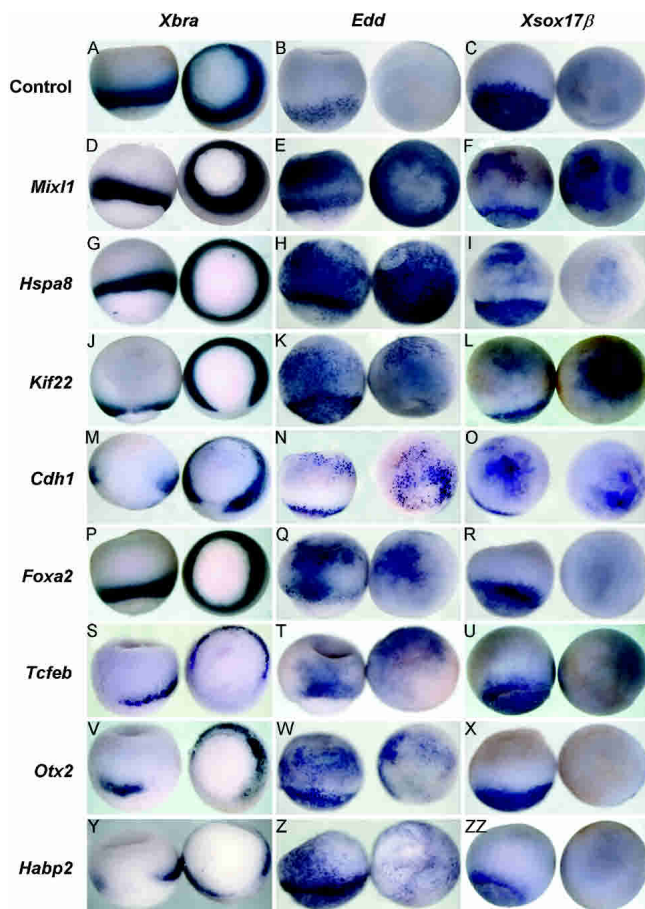


Figure 3. Positive clones that cause ectopic formation of endoderm or mesoderm in whole embryos. Gastrulating *Xenopus* embryos expressing positive clones were analyzed for the expression of *Xbra* (A,D,G,J,M,P,S,V,Y), *Edd* (B,E,H,K,N,Q,T,W,Z), or *Xsox17β* (C,F,I,L,O,R,U,X,ZZ). Identities of clones are listed at left and include *Mix11* (D–F), *Hspa8* (G–I), *Kif22* (J–L), *Fzr1/Cdh1* (M–O), *Foxa2* (P–R), *Tcfcb* (S–U), *Otx2* (V–X), and *Habp2* (Y–ZZ). (A–C) Uninjected controls. Concentrations of mRNAs injected into embryos are as follows: *Mix11* (D,F: 1 ng; E, 333 pg), *Hspa8* (G–I: 1 ng), *Kif22* (J,L: 200 pg; K, 100 pg), *Fzr1* (M–O: 200 pg), *Foxa2* (P–R: 200 pg), *Tcfcb* (S: 600 pg; T,U: 1 ng), *Otx2* (V: 500 pg; W: 1 ng; X: 200 pg), *Habp2* (Y: 200 pg; Z: 1 ng; ZZ: 400 pg). Embryos are oriented in *Xbra* column with left embryo vegetal hemisphere (endoderm) facing the bottom of the page, and right embryo vegetal hemisphere (endoderm) facing out. Embryos are oriented in both *Edd* and *Xsox17β* columns with left embryo vegetal hemisphere (endoderm) facing the bottom of the page and with the right embryo animal hemisphere (ectoderm) facing out.

Xenopus, it is a strong inducer of both *Xsox17β* and *edd*. Moreover, mutations in Zebrafish, *bon*, a close relative, result in severe disruption of endodermal domains during early development, providing genetic evidence that the Mix family plays an important role in endoderm specification (Kikuchi et al. 2000). Therefore, the identification of *Mix11* supports the notion that we can identify important endoderm regulators using this approach.

To date, little is known about how early endoderm is specified downstream of *Xsox17β* (Shivdasani 2002; Stainier 2002; Sinner et al. 2004). The identification of molecules that can directly induce *edd*, but not *Xsox17β*, is a discovery that fills a void in the downstream players of early endoderm specification (see Fig. 6). All known general endoderm specification molecules activate the expression of *Xsox17β*. Here, we describe three mol-

ecules, *Tcfcb*, *Otx2*, and *Foxa2*, that induce *edd*, but not *Xsox17β*, strongly suggesting that they act downstream of *Xsox17β*. Furthermore, we show that *Otx2* is induced by *Xsox17β*, suggesting a role directly downstream in a linear pathway. Recently, similar results were obtained for *Foxa2* (Sinner et al. 2004). Although *Otx2* and *Foxa2* have been shown to be important for endoderm formation in the mouse, they have not been described as sufficient to induce endoderm in any vertebrate, and have not been placed in the conventional endodermal pathway (Ang et al. 1993; Dufort et al. 1998; Jin et al. 2001; Kinder et al. 2001; Perea-Gomez et al. 2001a,b). *Tcfcb* has never been described as an endoderm regulator. Together, these three molecules lay the groundwork for studying the downstream regulation of endoderm specification.

We found several molecules that have never been implicated in endoderm formation in any animal model. These include *Tcfcb*, *Kif22*, *Hspa8*, *Fzr1*, and *Habp2*. It is yet unclear how these molecules might be involved in the pathways that specify endoderm (see Fig. 6). *Tcfcb* is perhaps the most “approachable” of the five, in that it is a helix-loop-helix transcription factor that has been shown to be critical for mouse embryonic survival. Homozygous mutations in mouse *Tcfcb* have defects in placental vascularization and die prior to e10.5 (Steingrimsson et al. 1998). No endodermal phenotype has been reported in these mutants. However, *Tcfcb* is a member of a family of closely related proteins, including *Mitf*, *Tfe3*, and *Tfec*, which have been shown to form heterodimers and are expressed throughout embryonic development (Steingrimsson et al. 2002). Therefore, it is plausible

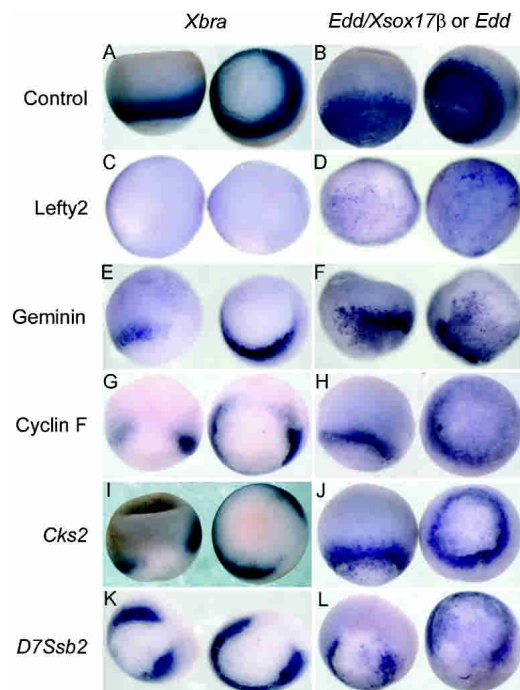


Figure 4. Positive clones that cause inhibition of mesoderm or endoderm in whole embryos. Gastrulating *Xenopus* embryos expressing positive clones were analyzed for the expression of *Xbra* (A,C,E,G,I,K), a combination of *edd* and *Xsox17β* (B,D,F) or *edd* alone (H,J,L). Identities of clones are listed at left and include *Lefty2* (C,D), *geminin* (E,F), *cyclin F* (G,H), *Cks2* (I,J), and *D7Ssb2* (K,L). (A,B) Uninjected controls. Concentrations of injected mRNAs are as follows: *Lefty2* (A,B: 1 ng), *geminin* (C,D: 400 pg), *cyclin F* (G,H: 200 pg), *Cks2* (I,J: 200 pg), and *D7Ssb2* (K: 10 ng; L: 500 pg).

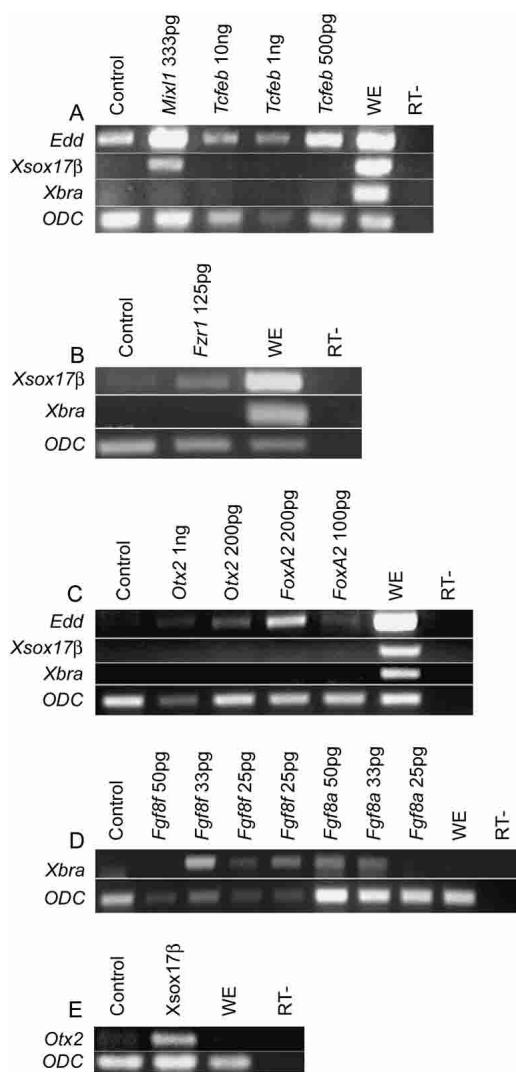


Figure 5. Identified molecules can directly induce endoderm or mesoderm from naive ectoderm. Capped synthetic mRNAs were injected at the indicated concentrations into the prospective ectoderm of the one-cell *Xenopus* embryo. The ectoderm was explanted and cultured in isolation until stage 10.5. Analysis of mesodermal and endodermal markers was performed by RT-PCR on the expressing tissues and uninjected controls. *Mix1* was capable of directly inducing both *Xsox17β* and *edd*, while *Fzr1* could induce *Xsox17β* (A and B). *Tcf1*, *Otx2*, and *FoxA2* were all able to induce *edd*, but not *Xsox17β* (A and C). *FGF8a* and *FGF8f* both induced *Xbra* in naive ectoderm (D). *Xsox17β* induces *Otx2* (E). Abbreviations are as follows: WE, whole embryo; RT-, reverse transcriptase minus. ODC is a loading control.

that an endoderm phenotype in the knock-out mouse might be masked by the redundant function of other members of this family. Double-mutant analysis is necessary to investigate this possibility. Here, we show that *Tcf1* can induce naive ectoderm to form endoderm, and that its activity lies downstream of *Xsox17β*. Therefore, like *FoxA2* and *Otx2*, *Tcf1* is a new candidate for the furthest known downstream specifier of early endodermal fate.

Fzr1, in addition to its endoderm-inducing properties, generated large cells when expressed in embryos. This is consistent with its extensively characterized role as a cell cycle regulator and as an adapter protein for the anaphase-promoting complex (APC)

(Visintin et al. 1997). *Fzr1/APC* was shown to ubiquitinate *SnoN*, which is a transcriptional repressor of TGFβ downstream targets. The proteolysis of *SnoN* then leads to the up-regulation of TGFβ signals (Stroschein et al. 2001; Wan et al. 2001). In light of this evidence, it is not surprising that this cell cycle regulator is a direct inducer of endoderm, given that TGFβ signaling plays a critical role in endoderm formation. We show that *Fzr1* induces *Xsox17β* in ectodermal explants, which suggests that it functions downstream of TGFβ and upstream of *Xsox17β*, most likely functioning at the level of direct TGFβ target genes (Fig. 6).

Kif22, *Habp2*, and *Hspa8* were the most intriguing molecules identified in the endodermal screen. All three can generate ectopic endoderm when overexpressed in whole embryos, but none of these can induce endoderm from naive ectoderm (with the possible exception of *Kif22*, which consistently generated very weak *Xsox17β* expression—data not shown). *Kif22* is a kinesin-like protein that belongs to the very large kinesin superfamily (Miki et al. 2001). This family also includes *Kif3*, which has been shown to be critical for left/right symmetry in the developing vertebrate embryo (Nonaka et al. 1998). *Kif22* contains an N-terminal kinesin motor and a forkhead homology-associated domain that is thought to mediate binding to phosphoproteins. Recently, it was suggested that a kinesin motor protein plays a role in the localization of *VegT* and *Vg1*, both vegetally localized endoderm specification molecules, during oogenesis. Mislocalization of these molecules would cause the formation of ectopic endoderm, which is the effect we observe with *Kif22* (Yoon and Mowry 2004). *Habp2*, Hyaluronan-binding-protein-2, contains a signal peptide, three EGF domains, a kringle domain, and a serine protease domain (Hashimoto et al.

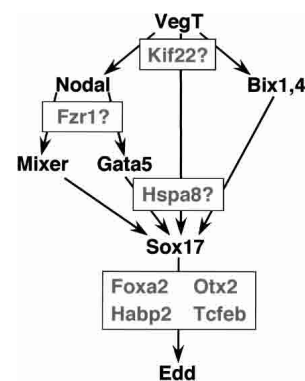


Figure 6. The endoderm specification pathway containing possible locations of identified clones. In *Xenopus*, the transcription factor, *VegT*, becomes vegetally localized during oogenesis and regulates downstream signals, including *Nodal*, to specify the endodermal lineage (Zhang et al. 1998; Xanthos et al. 2001). Cascades of transcription factors, forming downstream targets of *Nodal*, then regulate the commitment to endoderm. The Mixer-like homeodomain proteins, including *Milk/Bix2*, *Mix.1*, *Bix1/Mix.4*, and *Bix4*, and the GATA-factors, most notably *Gata4* and *Gata5*, all activate the expression of the downstream target *Xsox17β* (Hudson et al. 1997; Ecochard et al. 1998; Henry and Melton 1998; Tada et al. 1998; Casey et al. 1999; Reiter et al. 1999; Weber et al. 2000; Dickmeis et al. 2001). Molecules identified in this screen have been placed in possible pathway locations. A recent report has suggested that a kinesin motor protein localizes *VegT* and *Vg1* to the vegetal hemisphere during oogenesis; therefore, we place *Kif22* near *VegT* (Yoon and Mowry 2004). *Fzr1* acts directly downstream of *Nodal* (Stroschein et al. 2001; Wan et al. 2001). *FoxA2*, *Otx2*, *Habp2*, and *Tcf1* can induce *edd*, but not *Xsox17β*, indicating that they function between these two molecules in the endoderm-specification pathway. For reviews of endoderm specification, see Stainier (2002) and Shivdasani (2002).

1997). In the adult, *Habp2* is expressed in endodermally derived organs, including the pancreas, kidney, and liver. Virtually nothing is known about the function of the vertebrate proteins, although there is a *Drosophila* ortholog called *Masquerade* (*mas*). Total loss of *mas* function is lethal, resulting in defects in the central nervous system and in muscle development (Murugasu-Oei et al. 1995, 1996). *Hspa8* is a heat-shock protein that is highly expressed in the mouse pancreas. Little is known about this protein, other than it is not induced by heat and is thought to facilitate the correct folding of nascent polypeptides, thus acting as a chaperonin. How *Kif22*, *Habp2*, and *Hspa8* can induce endoderm has yet to be determined.

While we identified *Mixl1*, we did not identify several of the other known endoderm specification members—including *Nodal*, *Sox17*, and *Gata5*. From the onset, we did not expect to find either *Nodal* or *Sox 17*, since *Nodal* is a rare transcript at these stages of gastrulation, and therefore not well represented in the library, and *Sox17* requires large doses to induce endoderm in *Xenopus*. Nevertheless, it is clear that our efforts here have not yet reached screening saturation. Most of the genes we identified were found only once, and we have not yet arrayed enough independent clones from our libraries necessary for the redundancy needed to ensure full library coverage. Therefore, there remain future opportunities to use this approach to identify more novel genes involved in endoderm specification.

Fgf8f and Fgf8a are mesoderm-inducing molecules

Fibroblast growth factor (FGF) and TGF β are the two major signaling pathways involved in the formation of mesoderm (Heasman 1997). In this screen, we have identified three molecules, *Fgf8f*, *Fgf8a*, and *Actr11b*, that are components of these pathways. *Actr11b* is a well-described player in mesodermal specification and patterning in mice, fish, and frogs (Hemmati-Brivanlou and Melton 1992; Nagaso et al. 1999; Song et al. 1999). *Fgf8*, however, is more controversial. *Fgf8f* and *Fgf8a* are members of the FGF family, which is known to be involved in mesoderm formation. However, *Fgf8* has previously been reported to have distinctively different effects from the other FGFs, in that it induces neural tissue in the absence of mesoderm (Hardcastle et al. 2000; Pera et al. 2003). Therefore, we have found a different, albeit controversial, role for *Fgf8* in germ-layer formation. Certainly, a role in mesoderm formation and/or maintenance is consistent with the mouse *Fgf8* mutant phenotype, which has mesodermal migration defects during gastrulation (Sun et al. 1999). We have yet to determine why the *Fgf8f* and the *Fgf8a* clones behave differently from the published reports, although multiple isoforms of *Fgf8* exist and may have distinct functions.

Mesoderm inhibition screen reveals new molecules

Six molecules are described that can inhibit mesoderm in the absence of death, *Rsk4*, *Cks2*, *Lefty2*, *geminin*, *D7Ssb2*, and *cyclin F*. We were careful to distinguish during the screen between inhibition of marker expression and gross cellular abnormalities to ensure that we were looking at direct effects on the marker *Xbra*. Two of these molecules, *Lefty2* and *Rsk4*, have been characterized as inhibitors of TGF β and FGF signaling, respectively. *Lefty2* is a well-described inhibitor of TGF β signaling and therefore inhibits the formation of endoderm and mesodermal markers (Meno et al. 1999; Cheng et al. 2000). We have recently shown that *Rsk4* is a negative regulator of FGF signaling, and thus down-regulation of this pathway inhibits mesoderm locally

(Myers et al. 2004). The identification of both *Lefty2* and *Rsk4* provide compelling evidence that physiologically relevant molecules can be found using this approach.

D7Ssb2, *cyclin F*, *geminin*, and *Cks2* were identified by their mesoderm-repressing activities. *D7Ssb2* and *cyclin F* were originally found due to abnormal morphology changes in expressing embryos. After reducing their active concentration, we found that they could cause inhibition of mesoderm. *D7Ssb2* is contained in the 75Kd *Mesd* locus in the mouse genome. This region is vital for primitive streak and mesoderm formation (Wines et al. 2001). *D7Ssb2* has not been studied with respect to this effect, since it has been thought to be a pseudogene. Our sequencing results clearly show a 278aa ORF and suggest that *D7Ssb2* encodes a protein. To date, the gene residing in the *Mesd* locus responsible for the defect in mesoderm formation has not been identified, leaving the possibility that *D7Ssb2* may contribute to this phenotypic effect.

Cyclin F, *Cks2*, and *geminin* are all regulators of the cell cycle. Very little is known about *cyclin F*, except that—like other cyclins—its expression is “cycled” once per cell cycle (Bai et al. 1994). Mouse mutations in *cyclin F* have defects in placental development and in early embryonic development, particularly in the allantois, which becomes bulbous at e8.5 (Tetzlaff et al. 2004). This large allantois may be a result of an increase in posterior mesoderm, which would be consistent with our finding that *cyclin F* overexpression leads to a decrease in mesoderm. *Cks2* is a Cdc Kinase subunit and is involved in the progression of cells from interphase to M phase during mitosis. We have yet to determine how it interferes with mesoderm formation.

Geminin is one of the few molecules known to act as a link between the control of cell proliferation and the control of differentiation. By interacting with *Cdt1* and preventing its assembly onto chromatin, *geminin* controls cell proliferation by acting as a negative regulator of replication licensing, ensuring that DNA only replicates once (McGarry and Kirschner 1998). *Geminin*, however, also interacts with *Hox* gene products, which are essential for embryonic patterning. Recently, it has been shown that the interaction between *Hox* proteins and *geminin* can displace *geminin* from *Cdt1*, therefore promoting replication and cellular proliferation, while inhibiting gene transcription (Del Bene et al. 2004; Luo et al. 2004). These studies provide a tantalizing link between cell cycle control and differentiation. Furthermore—in support of this link—when overexpressed in *Xenopus*, *geminin* has been shown to inhibit DNA replication, as well as to act as a neurogenic molecule (Kroll et al. 1998; McGarry and Kirschner 1998; McGarry 2002). Both of these functions are distinct from the phenotypic affect we observe, which is a repression of both mesoderm and endoderm. This observation could reflect differences in activities between the frog and mouse clones, or simply be the result of concentration differences between experiments. It does not appear to be due to proliferation problems within the embryo, since the cells divide at the same rate as uninjected control embryos (data not shown). Interestingly, we also identified *Cdt1* in our screen; although it does have a distinct affect on cell cycle, it does not appear to have any differentiation properties.

Although not identified by their abilities to repress mesoderm, several of the endoderm-inducing clones—including *Kif22*, *Fzr1*, *Tcfcb*, *Otx2*, and *Habp2*—when further analyzed, could inhibit mesoderm. Further analysis is required to analyze whether the mesoderm is transformed into endoderm or whether these are separate phenomenon.

Conclusion

We have completed a high-throughput cross-species functional screen of >60,000 mouse gastrula cDNA clones and identified endoderm and mesoderm specification molecules. The ease and low cost of collecting large numbers of gastrula stage *Xenopus* embryos makes this screening approach particularly useful in identifying molecules that specify early cell fates. We calculate that two people, beginning with optimized in situ probes and pools of mRNA from an arrayed library, could screen through a maximum of 60,000 cDNAs in less than 2 mo by injecting 60 pools a day. This would include a single reinjection of all pools of 96 mRNAs that showed a phenotype during the first round of injections, and takes into account the time required to split the positive pools. When optimized, this method is truly high-throughput, limited only by the availability of interesting cDNA libraries and the choice of interesting cell-fate markers. The speed, low cost, and high efficiency make this cross-species screening an ideal method for examining cDNAs from difficult to obtain sources such as the early mouse gastrula. Therefore, this approach complements the current mouse molecular genetics systems and provides a powerful means for the genome-wide examination of mammalian gene function.

Methods

Construction of e6.5 and e7.5 cDNA libraries

E6.5 mouse embryos (291) and e7.5 (178) mouse embryos were dissected in M16 medium containing 10% FBS. Embryonic and extraembryonic tissues were maintained in e6.5 embryos, whereas extraembryonic tissues were removed in e7.5 embryos. Embryos were homogenized using 4 M guanadinium isothiocyanate, and total RNA isolated using acid phenol extraction (Chomczynski and Sacchi 1987). Total RNA from e6.5 (30 µg) and e7.5 (80 µg) embryos was used to generate cDNA using the Superscript plasmid system (Invitrogen). cDNA was cloned unidirectionally into pCS105. XL1Blue electrocompetent cells were transformed with ligated cDNAs. Each of the resulting libraries has >500,000 clones and an average cDNA insert size of 1.7 kb (Baker et al. 1999).

Generation of complex mRNA pools

The e6.5 and e7.5 cDNA libraries were arrayed on 92 and 132 384-well plates, respectively (Borchers et al. 2002). From these plates, we generated 368 pools of 96 colonies from the e6.5 library and 344 pools of 96 colonies from the e7.5 library. A 96-pin replicator was used to transfer clones from each plate onto LB plate with 100 µg/mL ampicillin, and plates were grown overnight at 37°C. Colonies from each pool of 96 were scraped from the LB plate and DNA prepared with Qiagen mini-prep columns. Pooled plasmid DNA was linearized with *AscI* and transcribed into capped mRNA with SP6 RNA polymerase using Ambion mMessage mMachine (Ambion). The total number of individual cDNAs screened was ~68,000.

Injection of *Xenopus* embryos with mRNA pools

Female adult frogs were ovulated by injection of human chorionic gonadotropin (Sigma-Aldrich) and eggs were fertilized in vitro. After treatment with 2.5% cysteine (pH 8.0) for 5 min to remove jelly, embryos were placed in 1/3 × MR (Sive et al. 2000). One-cell embryos were injected with 10 ng of each mRNA pool into either the animal hemisphere or the marginal zone (Fig. 1). These embryos were then cultured until gastrula stages (10.25–

11) (Nieuwkoop and Faber 1994), fixed using MEMFA (0.1 M Mops, 2 mM EGTA, 1 mM MgSO₄, 3.7% formaldehyde), and processed for in situ hybridization. Regardless of injection site, embryos were stained for both mesoderm and endodermal markers. The mesodermal marker, *Xbra*, was used at a concentration of 1 µg/mL (Smith et al. 1991). The endodermal markers, *Xsox17β* and *edd*, were used as a mixture at concentrations of 1 µg/mL and 1.5 µg/mL, respectively (Sasai et al. 1996; Hudson et al. 1997). In situ hybridizations were performed as previously described (Harland 1991; Sive et al. 2000). High-throughput processing was performed in 80-well Eppendorf tube racks that had been modified by replacing the bottom plastic with fine mesh and sawing off three rows, resulting in a final 65-well rack. All rinses, washes, and incubations were performed in these racks in glass Wheaton staining dishes (catalog #900403; Wheaton). Endodermal and mesodermal marker in situ hybridization staining patterns were compared between injected and uninjected control embryos from the same clutches. Pools and identified clones were considered phenotypically positive if >50% of the injected embryos were affected. For every phenotypically positive clone identified, in situ hybridization analysis was repeated more than three times using independent clutches of embryos.

Sib selection

Positive pools were split into 10 subpools of overlapping rows and columns, as shown in Figure 2. Subpools were injected and analyzed by in situ hybridization. If the phenotype of the original pool of 96 is due to a single cDNA within that pool, the intersection of positive subpools, in combination with the exclusion of negative subpools, defines that single cDNA. Individual cDNA clones within the intersection of phenotypically positive subpools were isolated and sequenced. The sequence was subsequently analyzed by BLAST (Altschul et al. 1990).

Ectodermal explants, RT-PCR assays, and TUNEL assay

Embryos were injected into the presumptive ectoderm at the one-cell stage, developed to stage 8–9, and transferred to 0.75 × NAM solution for tissue excision (Sive et al. 2000). Explants were cultured in 0.75 × NAM until stage 10.5. RNA was isolated, and reverse transcription RT-PCR was performed as previously described (Wilson and Melton 1994). Primer sequences were as follows: *Xbra* (F) GGATCGTTATCACCTCTG, (R) GTGTAGTCTGTAGCAGCA (Wilson and Melton 1994); *ODC* (F) CAGCTAGCTGTGGTGTGG (R) CAACATGGAACTCACACC (Agius et al. 2000); *Otx2* (F) CGGGATGGATTGTGCA, (R) TTGAACCAGACCTGGACT; *Xsox17β* (F) AACTCCCACAGCAGGCTACTTTG (R) TGTCAATGCTACTCTCCAGATGTCC (Myers et al. 2004); *edd* (F) TATTCTGACTCCTGAAGGTG (R) GAGAAGTCCCATGTGCTC (Sasai et al. 1996).

Whole-mount TUNEL analysis of *Xenopus* embryos was performed as described on http://www.xenbase.org/xmml/Marker_pages/pcd/apoptosisTUN.htm.

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