

Analysis of Spemann organizer formation in *Xenopus* embryos by cDNA macroarrays

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Abstract

The understanding of vertebrate development has greatly benefited from the study of gastrulation in the *Xenopus* embryo. Over the years, the molecular dissection of the Spemann organizer has proven to be a very fruitful source for gene discovery. Here, we report a comprehensive screen of gene expression in the *Xenopus* gastrula using cDNA macroarrays. Nylon filters containing more than 72,000 cDNAs from a gastrula stage library were hybridized with differential probes from embryos in which organizer induction had been inhibited by reducing Nodal-related or maternal β -Catenin signaling. Combining the changes in gene expression levels caused by these two major signaling pathways in a single graph identified both known and novel dorsoventral regulated genes. The most highly enriched organizer-specific genes were the secreted molecules *chordin* and *Xnr-3*, followed by the transmembrane protein *paraxial protocadherin (PAPC)*. Ventral-specific abundant cDNAs included *S10-40-H5*, members of the Hyaluronan synthase family, *Xvent-2* and *XFD2/FoxI1*. A differential probe of dorsal and ventral lips identified many more organizer-specific cDNAs than the screens inhibiting Nodal-related and β -Catenin signaling, suggesting that additional, as yet uncharacterized signaling pathways, contribute to organizer formation. Finally, extension of this approach to the blastula preorganizer signaling center identified the transcription factor *pintallavis/FoxA2* as a new preorganizer component. © 2004 Elsevier Inc. All rights reserved.

Keywords: β -Catenin; Cerberus; Chordin; FoxA4; FoxI1; Goosecoid; Hyaluronan synthase; Macroarray; Mix; Nodal-related; PAPC; Preorganizer; Spemann organizer; *Xenopus*; *Xvent-2*

Introduction

In their classic transplantation experiment, [Spemann and Mangold \(1924\)](#) showed that a small region in the gastrula stage embryo determines the dorsoventral body axis of the amphibian embryo. The dorsal lip of the blastopore—upon transplantation to the ventral side—can induce a secondary body axis in neighboring cells, including a central nervous system (CNS), dorsal mesoderm, and a secondary gut. Subsequently, a homologous gastrula organizing center was found in all model vertebrates, such as Hensen's node in chick and mouse and the embryonic shield in zebrafish (for a review, see [De Robertis et al., 2000](#)). As shown in [Fig.](#)

[1A](#), the formation of the Spemann organizer in *Xenopus* results from a sequential process that is regulated by molecules deposited in the unfertilized egg. Sperm entry induces a cortical rotation in the egg, which subsequently results in the stabilization and nuclear localization of maternal β -Catenin on the future dorsal side of the embryo ([Larabell et al., 1997](#); [Schneider et al., 1996](#)). Inhibition of cortical rotation by UV irradiation of fertilized eggs, or inhibition of the maternal β -Catenin pathway by, for example, microinjection of a dominant-repressive version of the transcription factor XTcf-3 (Δ N-XTcf-3), abolishes all dorsal development. Conversely, LiCl treatment, which inhibits Glycogen Synthase Kinase-3 β (GSK3 β), stabilizes β -Catenin throughout the embryo, leading to a radialized organizer and hence to dorsalized embryos ([Kao and Eliason, 1988](#)). At blastula stage, maternal β -Catenin leads to the expression of the BMP antagonists *chordin*, *noggin*, *follistatin*, and *Xnr-3* on the dorsal side of the animal cap, in a region that has been designated the preorganizer ([Wessely et al., 2001](#)). At gastrula, Nodal-related signals emanating from the endoderm or the Nieuwkoop center maintain the

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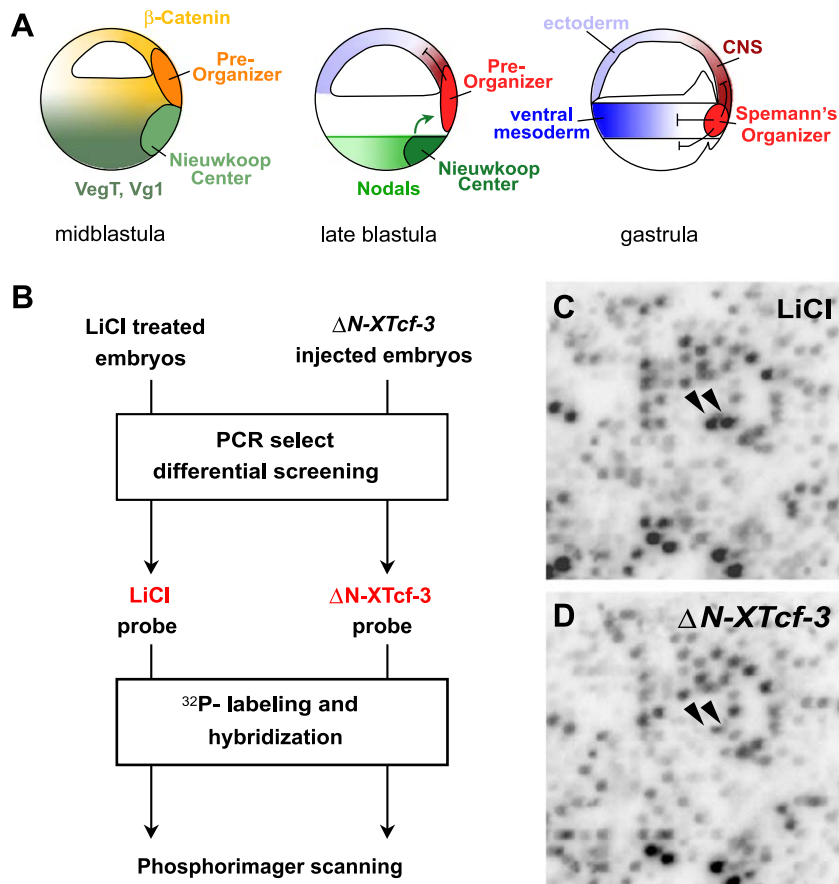


Fig. 1. Screening of cDNA macroarrays. (A) Diagram of organizer formation in *X. laevis*. At blastula stage, two signaling centers the preorganizer in the animal region and the Nieuwkoop center in the endoderm pattern the embryo. Both are established after the mid-blastula transition and are dependent on the nuclear localization of β -Catenin on the dorsal side of the embryo. The Nieuwkoop Center also depends on maternal vegetal mRNAs (*VegT*, *Vg1*). Although the preorganizer is involved in the formation of neural tissue, the Nieuwkoop center establishes a gradient of Nodal-related signals in the endoderm and is required for mesoderm induction and maintenance of the Spemann organizer. Both blastula-organizing centers cooperate in the formation of the Spemann organizer at gastrula stage, which then patterns all three germ layers. (B) Flow diagram of generation of subtractive hybridization. *Xenopus* embryos were either injected radially with 800 pg ΔN -XTcf-3 mRNA to inhibit the maternal β -Catenin pathway or treated with LiCl to induce a radialized Spemann organizer. At gastrula, polyA⁺ RNA was isolated and processed to generate subtractive probes, which were then used as probes for the hybridization of the macroarray filters. (C, D) Partial view of a macroarray filter hybridized with the “LiCl” and the “ ΔN -XTcf-3” probe, respectively. Arrowheads indicate the two twin spots of a *chordin* clone present in C, but absent in D.

expression of these preorganizer genes and induce others such as *gooseoid* (*gsc*), *cerberus* (*cer*), *Frzb-1*, and *Dickkopf-1* (*Dkk-1*), thereby establishing the Spemann organizer in dorsal mesendoderm (Agius et al., 2000).

The distinct roles of the preorganizer center and the Spemann organizer in patterning the *Xenopus* embryo can be revealed by selectively inhibiting the formation of the Spemann organizer. The C-terminal fragment of the secreted head inducer Cerberus (Cer-S) specifically inhibits Nodal-related signaling but does not affect signaling by other TGF- β mesoderm inducers such as Activin, Derriere, or Vg-1 (Agius et al., 2000; Piccolo et al., 1999). Microinjection of *cer-S* mRNA in the *Xenopus* embryo results in embryos completely lacking mesoderm, including the Spemann organizer, but still containing a CNS (Agius et al., 2000). These brain structures derive from cells of the blastula preorganizer center (Kuroda et al., 2004). Removing gene expression in the dorsal

animal cap either by preorganizer extirpation or by antisense morpholino oligos against *chordin* or *noggin* showed that the preorganizer gene expression domain predisposes the animal cap to neural induction and is required for the planar neural induction phenomenon (Kuroda et al., 2004). At gastrula, the Spemann organizer is the main signaling center in the *Xenopus* embryo and patterns all three germ layers, reinforcing the predisposition towards neural development in the animal cap and dorsalizing both the mesoderm and the endoderm (De Robertis et al., 2000; Harland and Gerhart, 1997; Fig. 1A).

Several differential screens have identified many novel transcription factors and secreted molecules required to maintain the identity and inducing activity of the organizer (for review, see De Robertis et al., 2000; Harland and Gerhart, 1997). Over the past few years, new technologies for isolating differentially expressed genes have emerged.

cDNA arrays in the form of micro- or macroarrays allow the systematic characterization of thousands of clones. In the case of microarrays, DNAs are printed onto glass slides and hybridized using fluorescence probes. In the case of macroarrays, cDNA libraries are gridded as bacterial colonies on nylon filters and hybridized using radioactively labeled probes (Clark et al., 1999; Rast et al., 2000). One key advantage of the macroarray is that the analyzed gene pool is determined by the choice of the arrayed cDNA library and not by the previous identification of a gene, its EST, or its prediction from genomic sequence. Furthermore, abundant cDNAs are represented multiple times on the filters and provide a form of quality control since a successful screen should identify all of its copies. The macroarray methodology has been used successfully to identify novel genes and gene regulation networks in sea urchin, zebrafish, and *Xenopus* (Bellmeyer et al., 2003; Dickmeis et al., 2001; Rast et al., 2000, 2002).

In the present study, we systematically analyze Spemann organizer formation in *Xenopus laevis* using macroarrays of cDNAs present in a gastrula stage library. We show that *chordin* and *Xnr-3* are among the most abundantly regulated genes of the maternal β -Catenin pathway, whereas genes such as *Xvent-2* and *XFD2/FoxI1a* are negatively regulated. A separate analysis of the Nodal-related signaling pathway identified several additional regulated genes. By integrating quantitative data from both these analyses into a single plot, we could identify genes co-regulated by β -Catenin and Nodal-related signaling. This combined plot provides a much-improved resolution of the data. *Paraxial protocadherin* (*PAPC*; Kim et al., 1998) emerged as a highly regulated target gene of these two principal signaling pathways. In comparing these data with data from a differential screen using dorsal lips, it became evident that the β -Catenin and Nodal-related pathways cannot account for regulating all gene expression in the Spemann organizer. This suggests that other yet unidentified signaling pathways are also involved in Spemann organizer formation.

Material and methods

Embryo manipulations and whole-mount in situ hybridization

Xenopus embryos obtained by in vitro fertilization were maintained in $0.1 \times$ modified Barth saline (Sive et al., 2000) and staged according to Nieuwkoop and Faber (1994). Dorsal and ventral marginal zones were dissected in $0.5 \times$ MMR solution (Sive et al., 2000) and directly lysed for RNA preparation. Synthetic mRNA was injected marginally or vegetally into each blastomere at the four-cell stage. For synthetic mRNA synthesis, pCS2-*cer-S* (Agius et al., 2000) was linearized with *NotI*, and pCS2-*siamois-EN^{rk}* and pCS2-*siamois* (Kessler, 1997) were linearized with *SacII* before transcription with Sp6 RNA polymerase as described (Pic-

colo et al., 1999). pCDNA- ΔN -*XTcf-3* (Molenaar et al., 1996) was linearized with *XbaI* and transcribed with T7 RNA polymerase. Whole-mount in situ hybridization was performed as described (Sive et al., 2000; <http://www.hhmi.ucla.edu/derobertis>).

Arraying of cDNA libraries

Two directional plasmid libraries from early cleavage stage (stage 6) and gastrula stage (stage 11) were used for macroarrays. The libraries contained inserts with an average length of 1500 bp and were cloned into the pCS2⁺ vector (Pera et al., 2001). Filter spotting was performed as previously described (Rast et al., 2000; Sea Urchin Genome Project Web site, <http://www.sugp.caltech.edu/resources/methods/array.psp>). Briefly, approximately 72,000 clones of the stage 11 library and 28,000 clones of the stage 6 library were spotted in duplicate onto six 22×22 cm Hybond N⁺ nylon filters (Amersham Pharmacia Biotech, Piscataway, NJ) with a “Q-Bot” robot (Genetix Ltd., New Milton, Hampshire, UK). Each cDNA was spotted in duplicate in a defined pattern to distinguish specific hybridization signals from unspecific background (Rast et al., 2000). Filters were grown on 3% agar plates and colony size monitored. Colonies were grown for approximately 12 h at 37°C and processed for screening according to standard protocols (Clark et al., 1999; Nizetic et al., 1991). The macroarrays were generated at CalTech; we could not have carried out this work without the generous help of Prof. Eric Davidson and his expert team.

RNA isolation and probe preparation

Embryos and explants were lysed in RNA STAT-60 and total mRNA isolated following the manufacturer’s protocol (Tel-Test, Inc., Friendswood, TX). PolyA⁺ mRNA was isolated from total mRNA using Oligotex (Qiagen, Hilden, Germany). cDNA synthesis and subtracted probes were prepared according to published procedure (PCR-Select Subtraction Kit, BD Biosciences Clontech, Palo Alto, CA; Wessely and De Robertis, 2000).

Hybridization of macroarray filters

Probe labeling

Complex probes (100–200 ng) were labeled with α -³²P dCTP according to manufacturer’s procedure (PCR-Select Differential Screening Kit, BD Biosciences Clontech). For gene-specific probes, 50–100 ng of restriction fragments were labeled with the Prime-It II kit (Stratagene, La Jolla, CA).

Filter hybridization

Filters were hybridized with radioactive probe for 48 h and then washed two times at medium stringency (65°C; $0.3 \times$ SSPE, 0.1% SDS, 0.05% sodium pyrophosphate, 15

min each) and two times at high stringency (65°C; 0.1× SSPE, 0.1% SDS, 0.05% sodium pyrophosphate, 15 min each). Filters were exposed to a phosphor screen for 1–2 days to allow near-saturation for the most intense spots and scanned using a phosphorimager (Storm Model 860, Molecular Dynamics, Sunnyvale, CA). Following quantitative data acquisition for each spot, filters were stripped by alkaline treatment (Clark et al., 1999) and successful stripping verified by phosphorimager analysis. After stripping, the same filters were hybridized with the second differential probe and the procedure repeated. Comparison of data from the same filter is essential to avoid variability arising during the preparation of the individual nylon filters.

Filter analysis

Filters were analyzed using the VisualGrid program, <http://www.gpc-biotech.com>; Genome Pharmaceuticals, Munich, Germany). Since every spotted 384-well plate did not contain DNA in its A1 position, a background value was calculated by averaging all the A1 values in the filter and this value was subtracted from all spot intensities. All the calculations were performed using Microsoft Excel. The presented data are derived, unless indicated otherwise, from the one filter containing 18,000 duplicated gastrula cDNA clones. Although not shown, examination of the other three filters containing gastrula stage cDNAs produced similar results. For sequencing, the positive clones were streaked out on LB-Ampicillin plates, miniprep DNA isolated, and sequenced from the 5′ using the Sp6 promoter primer. Although we aimed to cover the full repertoire of genes expressed at gastrula, some transcripts must have been missed because they were not represented in the 72,000 clones spotted. Upon hybridization of the filters with specific probes to known genes (*chordin*, *noggin*, *Xnr-3*, *siamois*, *Frzb-1*, *Dkk-1*), only *siamois* was not present (data not shown).

Results

Throughout the years, the Spemann organizer has been a fertile hunting ground for new gene discovery (De Robertis et al., 2000; Harland and Gerhart, 1997). To explore whether most of the genes specifically expressed in this signaling center have already been identified, we undertook a screening for organizer-specific genes in a genome-wide approach using macroarrays of a gastrula stage 11 library. This method (Rast et al., 2000) was found to be a very efficient way of identifying differentially expressed clones. This is because the picking of bacterial colonies of cDNA clones into 384-well plates and their subsequent arraying avoids the error-prone secondary screening of putative positive colonies required in traditional cDNA library platings (e.g. Bouwmeester et al., 1996). This allows rapid identification of the positive cDNAs by sequencing (Rast et al., 2000). As a modification to the standard technique generally used for

cDNA arrays, we did not label total mRNA, but instead generated differential probes using a PCR-based subtraction method. This greatly increased the sensitivity. Because the protocol included an equalization step, it also reduced the representation of highly expressed transcripts, such as those of the housekeeping machinery, in the final probe.

Macroarray analysis of the maternal β -Catenin pathway

To examine target genes of the two main signaling pathways known to regulate organizer formation, we first analyzed maternal β -Catenin signaling (Fig. 1A). Treatment of embryos at the 32-cell stage with LiCl results in radially dorsalized embryos due to the induction of organizer-specific genes in the entire marginal zone (Kao and Elinson, 1988). The opposite result, ventralization, is caused by injection of synthetic mRNA encoding a dominant-repressive version of XTcf-3 (ΔN -XTcf-3), which does not bind β -Catenin and constitutively represses β -Catenin target genes and blocks organizer formation (Molenaar et al., 1996; Wessely et al., 2001). Similar ventralization can be obtained by UV irradiation of the egg, but microinjection of ΔN -XTcf-3 mRNA was found to be more reproducible. PolyA⁺ mRNAs from gastrula stage embryos treated with LiCl or injected with ΔN -XTcf-3 mRNA at the four-cell stage were used as the starting material for the PCR-based subtraction procedure (Fig. 1B). The cDNAs from LiCl-treated embryos were used as tester and the cDNA from ΔN -XTcf-3 injected embryos as the driver to generate the “LiCl” probe, and vice versa for the “ ΔN -XTcf-3” differential probe. Figs. 1C and D show a portion of the filters analyzed, indicating with arrowheads a duplicated spot that is present when probed with the “LiCl” probe, but absent with the “ ΔN -XTcf-3” probe. Subsequent sequencing analysis revealed that this particular clone encoded *chordin*, a gene expressed in the Spemann organizer and regulated by the maternal β -Catenin pathway (Wessely et al., 2001), thereby validating the experimental approach.

To analyze all 18,000 duplicated cDNAs present on each filter, the intensity of each spot with the “LiCl probe” was quantified and used as the x -value (ordinate) and the intensity with the “ ΔN -XTcf-3 probe” as the y -value (abscissa) for an xy -scatter plot (Fig. 2A). Each dot on the graph represents one spot on the filter. The resulting graph showed a clear separation of five clone populations (Fig. 2A, regions I–V). The majority of the cDNAs were found in the median (region III). Those clones had the same signal intensity with both probes and represented cDNAs whose abundance was not affected by modulation of the β -Catenin signaling pathway. Clones drawn on the lower side of this median in regions IV and V were positively regulated by β -Catenin signaling. While those in region V were highly dependent on the activation of the β -Catenin pathway, clones present in region IV were regulated by β -Catenin to a lesser extent. Similarly, regions I and II in Fig. 2A corresponded to

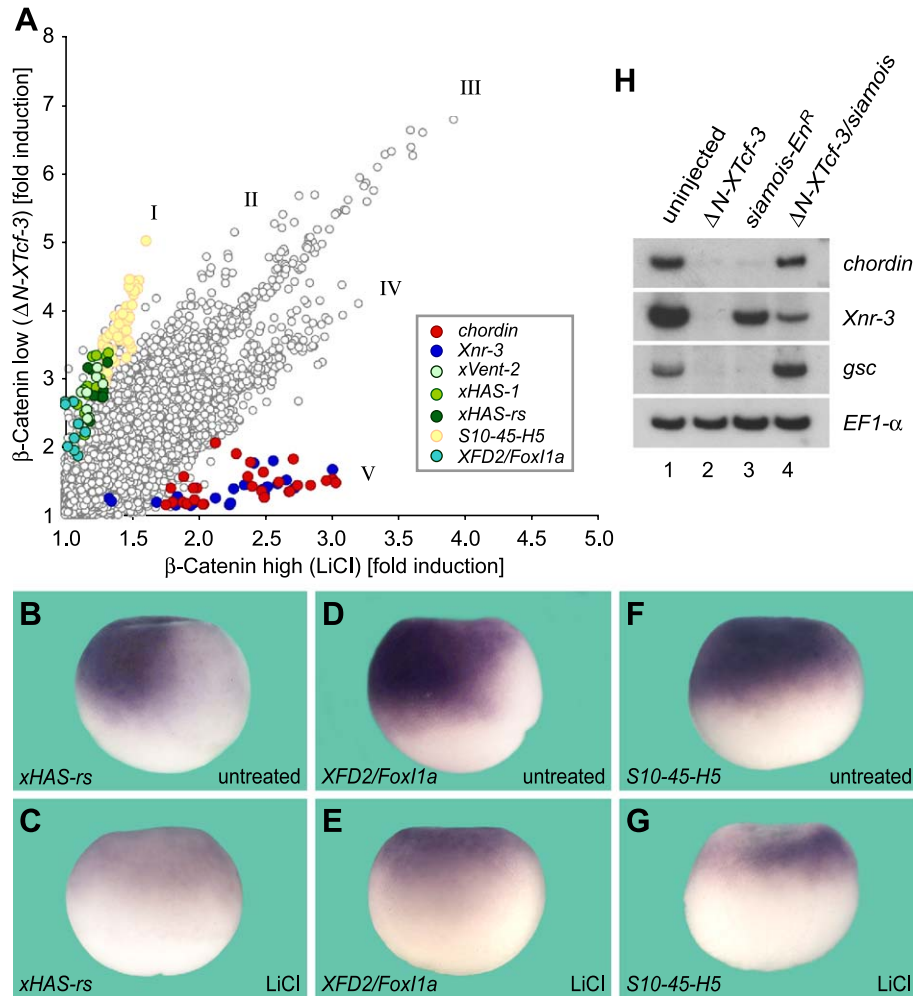


Fig. 2. Macroarray screen of the maternal β -Catenin pathway. (A) xy -scatter plot of the intensities of the individual spots after hybridization with “LiCl” and “ ΔN -XTcf-3” probes. Clones are categorized into five regions (I–V) depending on the extent of regulation by the maternal β -Catenin pathway. Note that *chordin* and *Xnr-3* comprise most of the clones strongly regulated by β -Catenin (region V). (B–G) Whole-mount in situ hybridization of gastrula stage embryos left untreated (B, D, F) or treated with LiCl (C, E, G) and hybridized with *xHAS-rs* (B, C), *XFD2/Fox11a* (D, E), and *S10-45-H5* (F, G) probes. All embryos are shown from the side with dorsal to the right. (H) RT-PCR analysis of whole gastrula stage embryos injected radially with ΔN -XTcf-3 (800 pg, lane 2), *siamois-EN^R* (120 pg, lane 3), and a combination of ΔN -XTcf-3 and *siamois* (200 pg, lane 4). EF1- α serves as RNA loading control.

clones that were, to different degrees, inhibited by the β -Catenin pathway. Using a subtractive probe of stages 5 and 10 *Xenopus* embryos to distinguish between maternal and zygotic genes, we observed that all the clones in regions I and V were purely zygotic, whereas many of the clones present in regions II and IV were of maternal origin (data not shown). This distribution may reflect the fact that the expression of zygotic genes is a de novo induction by the maternal β -Catenin pathway, although the maternal transcripts are still present at gastrula and their expression levels can therefore only be partially up- or down-regulated.

cDNAs regulated by β -Catenin

To reveal the identity of the individual spots, we sequenced the most extreme clones in regions I and V on all filters. Among the clones inhibited by β -Catenin (region I)

were *Xvent2*, *Hyaluronan Synthetase-1* (*xHAS-1*), *Hyaluronan Synthetase-related sequence* (*xHAS-rs*), *XFD2/Fox11a*, *XFD2/Fox11b*, and *S10-45-H5*.

Xvent2/Vox2/Xom

This homeobox gene is a known transcriptional repressor acting downstream of BMP signaling that prevents dorsal gene expression on the ventral side of the embryo (Ladher et al., 1996; Melby et al., 1999; Onichtchouk et al., 1996; Schmidt et al., 1996). *Xvent2* is expressed in ventral mesoderm and ectoderm and is down-regulated by LiCl (Onichtchouk et al., 1996).

xHAS-1 and xHas-rs

Hyaluronan synthetase-1/DG42 was first cloned in a pioneering differential screen aimed at identifying differential gastrula stage mRNAs (Sargent and Dawid, 1983). *xHAS-1* mRNA is detected just after midblastula, peaks at

late gastrula, and decays by the end of neurulation in a dorsal to ventral direction (Rosa et al., 1988; Sargent and Dawid, 1983). Hyaluronan is an extremely hydrophilic glycosaminoglycan of the extracellular matrix of vertebrates. It forms spaces that contain large amounts of water facilitating the migration of cells. It also binds growth factors and cell surface proteins during embryogenesis and controls cell proliferation, migration, and differentiation (Toole, 2000). xHAS-rs is unique to *Xenopus*, and its amino acid sequence is closely related to xHAS-1 (Spicer and McDonald, 1998). Whole-mount in situ hybridization of xHAS-rs showed that at the gastrula stage, it is expressed in the animal region, sparing the dorsal side (Fig. 2B). Since LiCl treatment greatly reduced xHAS-rs mRNA levels throughout the animal cap (Fig. 2C), it is likely that in untreated embryos, the dorsal expression of xHAS-rs is down-regulated by the activity of the maternal β -Catenin pathway.

XFD2/FoxI1a and XFD2/FoxI1b

These winged helix/forkhead transcription factors are expressed in similar fashion to xHAS-rs. At gastrula, transcripts are present in the animal hemisphere with lower expression in the dorsal side, and expression levels are down-regulated by LiCl treatment (Figs. 2D, E and Lef et al., 1994).

S10-45-H5

The most abundant of the ventral clones was a novel gene, which has recently also been identified in a microarray analysis of genes differentially expressed during neural induction in *Xenopus* (accession number: , AF549891, Munoz-Sanjuan et al., 2002). In contrast to xHAS-rs and XFD2/FoxI1a, this clone was expressed throughout the animal cap without apparent dorsal–ventral asymmetry (Fig. 2F). However, S10-45-H5 was strongly down-regulated by LiCl (Fig. 2G), indicating that it is negatively regulated by β -Catenin signaling.

Transcripts increased by maternal β -Catenin

Analysis of the cDNAs induced by β -Catenin revealed that the 19 most highly regulated spots encoded only two genes, *chordin* and *Xnr-3* (Fig. 2A, region V). Furthermore, hybridization of the filters with specific probes for *chordin* and *Xnr-3* showed that all *chordin* and *Xnr-3* clones present on the filters were actually identified by the differential screen. Examination of the other filters (not shown, but see Fig. 5B below) also identified multiple isolates of *goosecoid* (*gsc*) among the highly activated targets of β -Catenin signaling in region V.

The appearance of *Xnr-3* in region V is consistent with the identification of two TCF/Lef-1 binding sites in its promoter (McKendry et al., 1997). Similarly, the *gsc* promoter contains binding sites for the homeobox transcription factor Siamois/Twin (Laurent et al., 1997), which is in turn activated by the early β -Catenin signaling pathway. The

chordin gene was initially isolated as a gene up-regulated by LiCl treatment and was known to be inhibited at the gastrula stage upon microinjection of ΔN -XTcf-3 mRNA (Sasai et al., 1994; Wessely et al., 2001).

To examine further whether *chordin* (like *Xnr-3*) is directly regulated by β -Catenin action, or indirectly (like *goosecoid*) via Siamois/Twin, we injected *Xenopus* embryos with synthetic mRNAs for ΔN -XTcf-3, *siamois-engrailed-repressor* (*siamois-EN^R*) (Kessler, 1997), or ΔN -XTcf-3 together with *siamois* wild-type mRNA. RT-PCR analysis (Fig. 2H) showed that *chordin* expression was inhibited by ΔN -XTcf-3 and *siamois-EN^R* and could be induced by *siamois* mRNA in embryos in which the maternal β -Catenin pathway was blocked by ΔN -XTcf-3 (Fig. 2H). This regulation was identical to that of *gsc*. These results indicate that *chordin* is not directly induced by β -Catenin, but rather is activated indirectly through the Siamois/Twin transcription factor. *Xnr-3* expression was also slightly down-regulated by *siamois-EN^R* (Fig. 2H, compare lanes 1 and 3) and up-regulated by *siamois* even in the presence of ΔN -XTcf-3 (Fig. 2H, lanes 2 and 4). The *Xnr-3* promoter contains not only two Lef1/TCF binding sites but also a homeobox binding site (McKendry et al., 1997). It is conceivable that the expression of *Xnr-3* may be initiated by the maternal β -Catenin pathway, which is then reinforced by the homeobox genes Siamois/Twin.

We conclude from these data that the maternal β -Catenin pathway regulates gene expression at the gastrula stage embryo both positively and negatively. The two previously identified genes, *chordin* and *Xnr-3*, constitute the main differential output of the maternal β -Catenin signal on the dorsal side of the gastrula.

Macroarray analysis of the Nodal-related signaling pathway

Examination of the plotted β -Catenin data indicated that up to 6000 clones could be differentially regulated in a positive way by β -Catenin in region IV (Fig. 2A). However, sequencing of these candidates proved impractical, not only because of the large numbers, but also due to the interspersed false positives that were not regulated by the maternal β -Catenin pathway in whole-mount in situ hybridizations. This led us to examine Nodal-related signaling, the second main pathway implicated in the expression of organizer-specific genes.

Nodal-related signaling is required for the formation of mesendoderm, including the Spemann organizer, and can be inhibited by the Nodal-specific antagonist Cer-S (Fig. 1A; Agius et al., 2000). We generated two differential probes from gastrula stage embryos either injected with *cer-S* mRNA or left uninjected using the same PCR-based subtraction method as for the LiCl and ΔN -XTcf-3 probes. Macroarray filters were hybridized, and individual clones quantified and plotted as described above. The most highly

differentially regulated clones were sequenced. All the spots strongly up-regulated upon inhibition of Nodal-related signaling encoded *cerberus* (Fig. 3A). This was an expected false positive result because *cer-S* mRNA had been injected to inhibit Nodal-related signaling; the signals reflected merely the persistence of the injected *cer-S* mRNA in the low Nodal sample.

The presence of *chordin* clones among the genes dependent on Nodal-related signaling (Fig. 3A) validated the effectiveness of the chosen approach. Endogenous *chordin* transcripts accumulate when animal cap cells are treated with Activin/Nodal-related growth factors and are down-regulated at gastrula upon microinjection of *cer-S* (Agius et al., 2000; Sasai et al., 1994). In addition, we identified a series of bona fide endomesodermal transcription factors such as the winged helix/forkhead transcription factor *pintallavis/FoxA4a*, a close homologue of *HNF3 β /FoxA2*

(Kaestner et al., 2000; Ruiz i Altaba and Jessell, 1992), and multiple isolates of homeobox genes of the Mix class (*Mix.1*, *Mixer*, and *Mix.4*) (Henry and Melton, 1998; Mead et al., 1998; Rosa, 1989), *Xbra* (Smith et al., 1991), *VegT/Antipodean* (Stennard et al., 1999), and *Sox17 β* (Hudson et al., 1997) (Fig. 3A and data not shown). All of these had been identified by earlier screens as Activin-responsive genes, with *Mix.1* being the first direct Activin target gene to be identified (Rosa, 1989).

Paraxial protocadherin

In addition to the above expected clones, a strong positive clone dependent on Nodal signaling was a transmembrane protein of the protocadherin family, *PAPC*, which is expressed in the mesodermal mantle, first in the organizer and then in the paraxial mesoderm. *PAPC* is a cell adhesion molecule that also has the remarkable property of driving

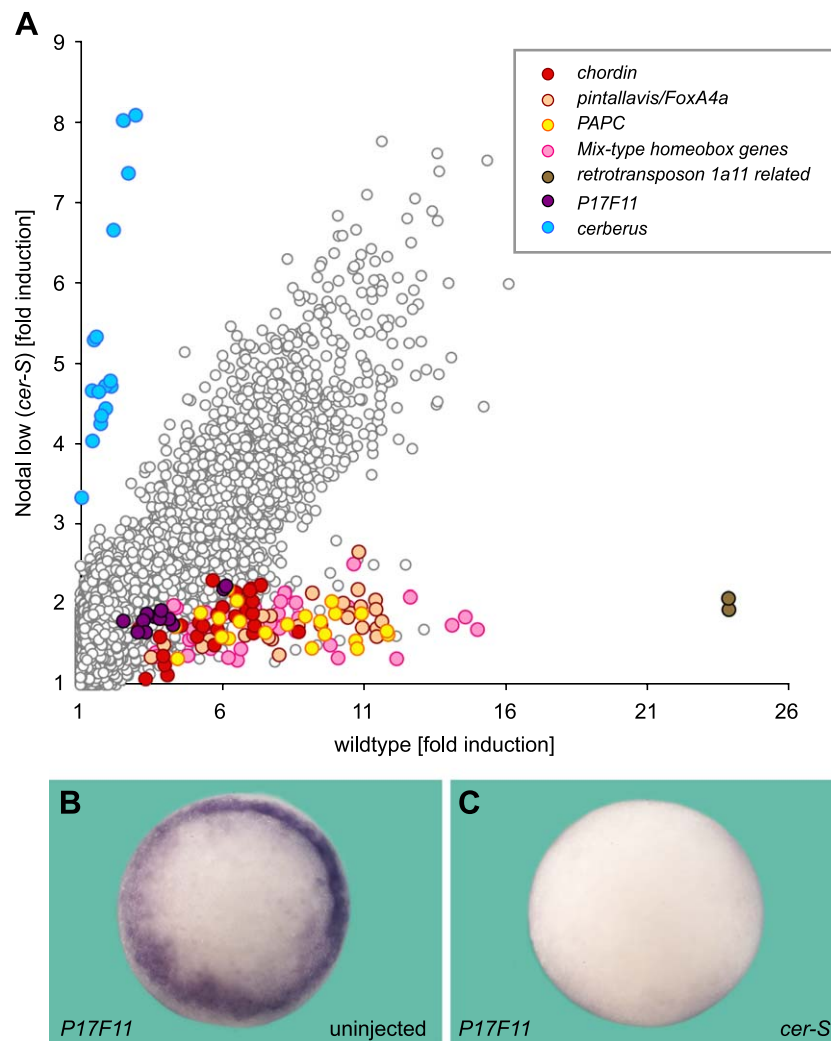


Fig. 3. Macroarray analysis of the Nodal-related signaling pathway. (A) *xy*-scatter plot of the spot intensities after hybridization of the macroarray filters with probes comparing wild-type embryos to those in which Nodal-related signaling was inhibited by injection of 150 pg *cer-S* mRNA into each blastomere at the four-cell stage. (B, C) Whole-mount in situ hybridization of gastrula stage embryos left uninjected (B) or injected with *cer-S* mRNA (C) with the novel gene *P17F11* in vegetal view.

morphogenetic gastrulation movements in overexpression assays (Hukriede et al., 2003; Kim et al., 1998).

IAl1

Interestingly, the gene most strongly regulated (over 20-fold) by Nodal-related signaling was *IAl1*, a retrotransposon-like cDNA originally identified by the Dawid group during a screen for genes induced by Activin and FGF in *Xenopus* animal caps (Greene et al., 1993).

P17F11

Another gene expressed in endomesoderm and activated by the Nodal-related signaling pathway was the *P17F11* (accession number: BAB79593). This cDNA had been previously identified in a screen for genes involved in head induction (Shibata et al., 2001) and encodes a novel protein with no detectable protein motifs or signal peptide, which is related in sequence to the human hypothetical protein *MGC13045* (accession number: NP_115720). Whole-mount in situ hybridization showed that *P17F11* is strongly expressed in the mesendoderm (Fig. 3B, Shibata et al., 2001). This expression could be blocked by inhibition of Nodal-related signaling via microinjection of *cer-S* mRNA (Fig. 3C).

Taken together, these results suggest that the *cer-S* screen was able to accurately identify genes activated by Nodal-related signaling in the embryo.

The combined organizer graph

The Spemann organizer is formed at gastrula at the intersection of Nodal-related and β -Catenin signaling (Fig.

1A). We therefore asked whether the combined representation of both signaling pathways would improve the identification of Spemann organizer-specific genes. To this end, the behavior of each spot in the β -Catenin/ Δ N-XTcf-3 differential screen was converted into a single quantitative value. This was achieved by calculating the natural logarithm of the ratio of the intensity of each spot after hybridization with the “LiCl” probe and the “ Δ N-XTcf-3” probe. In this calculation, genes positively regulated by β -Catenin had positive values, whereas genes strongly repressed by Δ N-XTcf-3 had negative values. The same calculation was carried out for each spot from the data set from the wild type/*cer-S* screen. The critical step came when the Nodal screen values were plotted as the ordinate and those of the β -Catenin screen in the abscissa of the same graph. Fig. 4 shows the resulting *xy*-scatter plot. The combination of the two individual screens into one graph allowed a much better overall picture of the gene regulation in the *Xenopus* gastrula.

All the *Xnr-3* spots were located on the right side of the *x*-axis because of their dependence on β -Catenin signaling, but did not shift upwards on the *y*-axis (Fig. 4). Thus, *Xnr-3* expression is regulated by β -Catenin, but not by Nodal-related signaling (McKendry et al., 1997; Wessely et al., 2001). Similarly, the genes expressed in the endomesoderm such as *pintallavis/FoxA4a* and the Mix-type homeobox genes were strongly regulated by Nodal-related, and mildly or not at all by β -Catenin signaling (Fig. 4).

The organizer quadrant

All the spots encoding *chordin* were in the upper-right quadrant of the graph (Fig. 4). This region of the plot

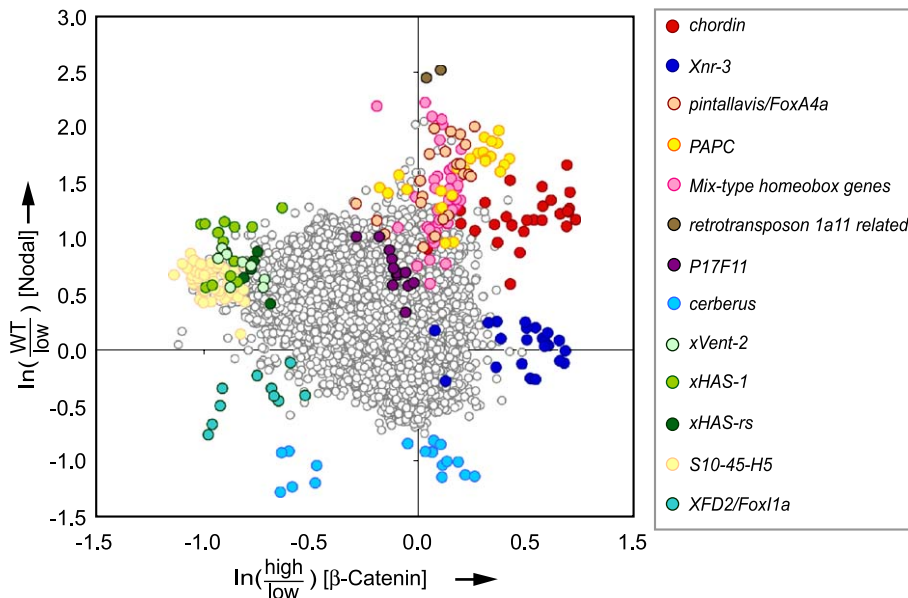


Fig. 4. The combined organizer graph. The values of the β -Catenin differential screening were converted into a single value by taking the natural logarithm of the ratio of the intensities of each spot after hybridization with “LiCl” and “ Δ N-XTcf-3” differential probes. This was plotted on the *x*-axis. The same transformation was performed for the results of the Nodal-related screen and plotted on the *y*-axis. Color coding indicates some of the genes identified by sequencing. This combined graph provides a much improved resolution of the macroarray results.

corresponds to genes regulated by both Nodal-related and β -Catenin signaling. Given that these two pathways regulate gene expression in the Spemann organizer, this indicates that the clones found in the upper-right quadrant represent organizer-specific genes. This notion was confirmed by the fact that all cDNAs encoding the organizer gene *gsc* (present on other filters, but not in the one shown in Fig. 4) were found in this quadrant.

One surprise was the strong representation of *PAPC* in the organizer quadrant. *PAPC* was known to be expressed in the dorsal marginal zone at gastrula (Kim et al., 1998), but it was unexpected that *PAPC* is regulated so strongly by both the maternal β -Catenin and Nodal-related signaling pathway. Only one copy of *PAPC* had been isolated in the original differential screen (Bouwmeester et al., 1996), whereas most of the spots in the organizer quadrant located between *chordin* and the Nodal-inducible genes (such as Mix family and *pintallavis/FoxA4a*) were encoded for *PAPC*. In addition, we obtained several isolates of *xBtg-x*, a member of the Btg/Tob gene family of antiproliferative genes, which is expressed in this quadrant and will be the

subject of its own study (Wessely et al., manuscript in preparation).

The ventral genes

The clones negatively regulated by β -Catenin also showed a more refined resolution in the combination plot (Fig. 4). *xHAS-1*, *xHAS-rs*, *XVent-2*, and *S10-45-H5* were co-localized in an area corresponding to genes inhibited by β -Catenin and moderately induced by Nodal-related. All of these genes are expressed in the ectoderm and also in the mesoderm (Ladher et al., 1996; Onichtchouk et al., 1996; Rosa et al., 1988; Schmidt et al., 1996; and data not shown). On the other hand, *XFD2/FoxI1a* was found in the lower left quadrant of the combined graph (Fig. 4). This indicates that *FoxI1a* is repressed by β -Catenin but was either unaffected or negatively regulated by Nodal-related signaling. In situ hybridization showed that *XFD2/FoxI1a* was expressed in the ectoderm but not in the mesoderm (data not shown).

We conclude from these data that the combined plot, which integrates the targets of Nodal-related and β -Catenin signaling, allows a much finer resolution of the genes

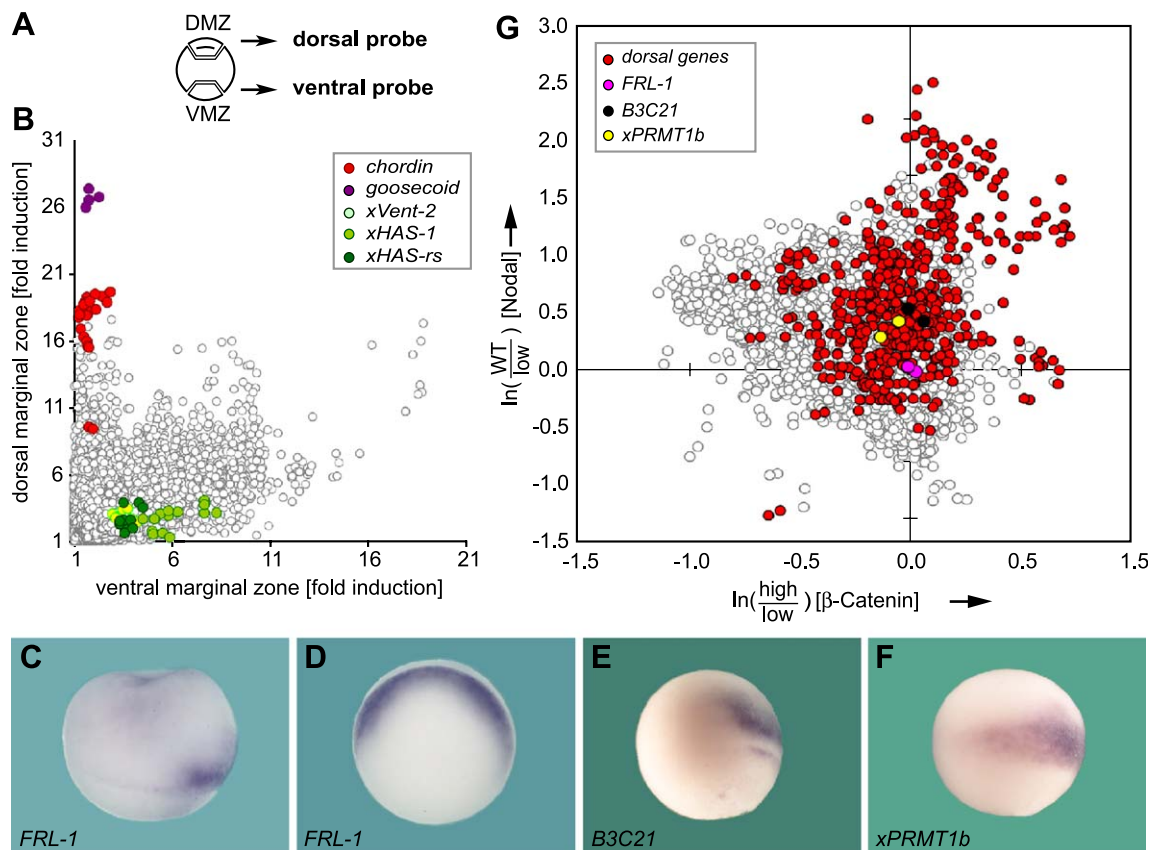


Fig. 5. Macroarray analysis of dissected dorsal and ventral tissue. (A) Dorsal marginal zones (DMZ) and ventral marginal zones (VMZ) of stage 10.5 embryos were excised and dorsal- and ventral-specific probes prepared. (B) Graph of screen using DMZ and VMZ probes. *Chordin* and *goosecoid* were the two genes with greatest differential dorsal expression. The ventral genes *XVent2*, *xHAS-1*, and *xHAS-rs* are also indicated. The graph shows the analysis of a different filter from the one shown in the previous figures. (C–F) Whole-mount in situ hybridization of gastrula stage embryos with *FRL-1* (C, D), *B3C21* (E), and *xPRMT1b* (F) in lateral view (C, E, F) or vegetal view (D). (G) The 600 dorsal-most enriched spots were projected onto the combined organizer graph of Fig. 4. The results of three different differential screens of 18,000 gastrula cDNAs plated in duplicate onto a single nylon filter are integrated in this one graph. The wide range of expression of the 600 spots suggests that there are additional pathways besides β -Catenin and Nodal-related that affect dorsal-specific gene expression.

differentially expressed in the *Xenopus* gastrula. This method has the additional attraction of providing a semi-quantitative measure of the representation of each cDNA in the differential probe (Fig. 4). It should be noted that sequence analysis of the clones present in the upper-right quadrant failed to identify all known organizer-specific genes. For example, clones encoding the BMP antagonist *noggin* were

present on the macroarray filters, but were only found in the area of mildly regulated genes (data not shown).

Direct analysis of dorsal and ventral gene expression

To determine whether the β -Catenin and Nodal-related signaling pathways were sufficient to explain organizer

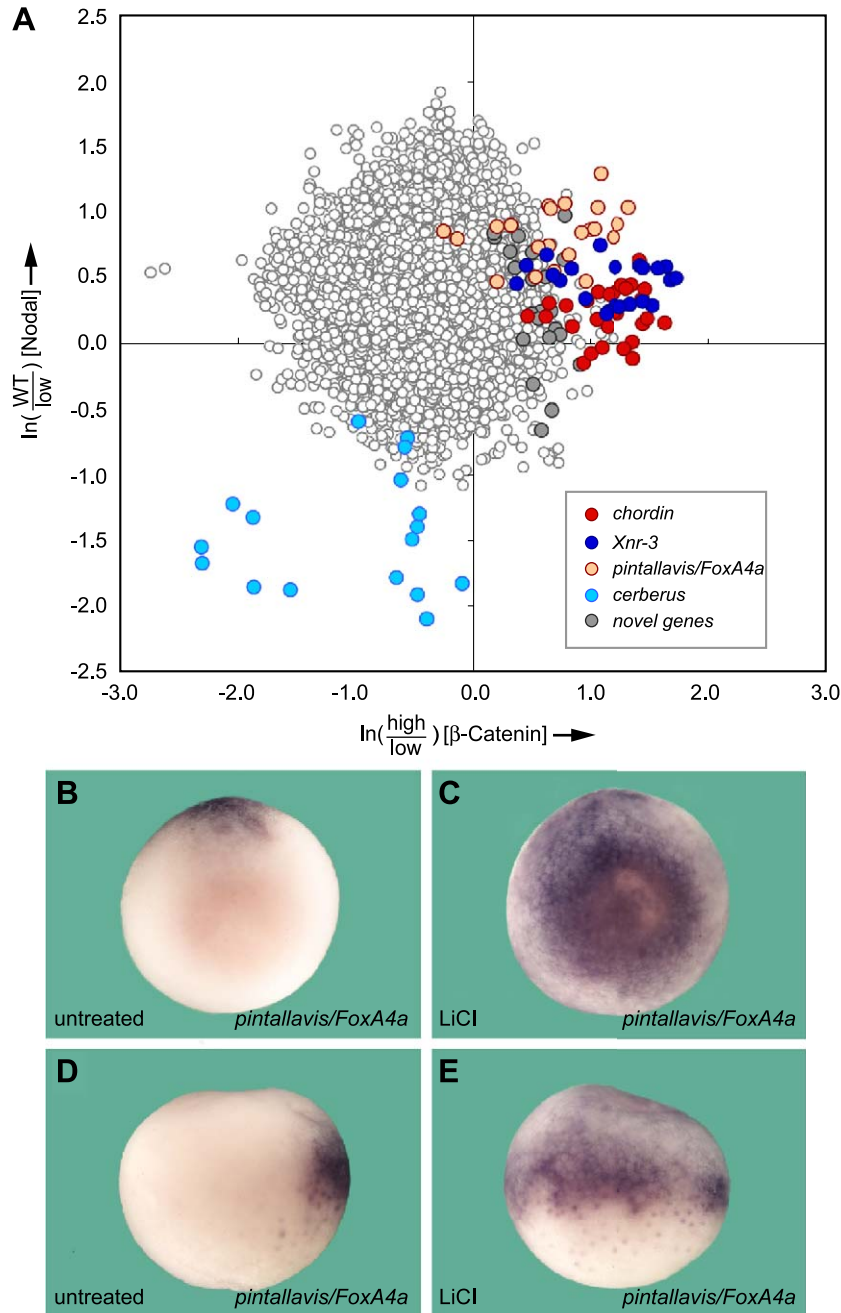


Fig. 6. The preorganizer graph. (A) The data for the maternal β -Catenin and the Nodal-related signaling pathways at blastula stage (8.5 h post fertilization) were plotted as in Fig. 4. Color coding indicates the spots encoding *chordin*, *Xnr-3*, *pintallavis/FoxA4a*, and several novel genes. Since the spot intensities using blastula stage RNAs were weaker than those at gastrula stage, background correction distorted the clone distribution and therefore this step was omitted. (B–E) Whole-mount in situ hybridizations with *pintallavis/FoxA4a* on untreated (B, D) and LiCl-treated (C, E) blastula stage embryos. (B, C) animal (D, E) and lateral views. Like other preorganizer genes, *pintallavis/FoxA4a* expression is expanded over the entire animal cap upon activation of the maternal β -Catenin pathway.

formation as represented in our working model (Fig. 1A), we manually dissected 1400 dorsal and ventral lips of gastrula stage embryos, prepared polyA⁺ mRNA, and generated by PCR-based subtraction an organizer-specific probe (DMZ) as well as the complementary ventral marginal zone (VMZ) probe (Fig. 5A). The macroarray filters were hybridized, spots quantified, and plotted as DMZ vs. VMZ signal intensity. As expected, *chordin* and *gsc* were among the most dorsal-enriched clones, and *Xvent2*, *xHAS-1*, and *xHAS-rs* were among the ventral-enriched clones (Fig. 5B). Sequencing of additional dorsal-enriched cDNAs identified known organizer genes such as *cerberus* (Bouwmeester et al., 1996), *pintallavis/FoxA4a*, *PAPC*, and *XRnd1* (Wunnenberg-Stapleton et al., 1999).

FRL-1

Another gene identified by the DMZ probe was the EGF-CFC molecule *FRL-1*. Previous studies had described *FRL-1* as a gene expressed in the mesoderm and a dorsal preference was not mentioned in these studies (Kinoshita et al., 1995; Yabe et al., 2003). However, our in situ hybridization showed a clear preferential expression in the dorsal mesendoderm at gastrula stage (Figs. 5C, D).

B3C21 and xPRMT1b

Similarly, *B3C21* (UniGene Cluster X1.24723), a novel gene that as of yet has not been identified in other species and has no recognizable protein domains, as well as the arginine methyltransferase *xPRMT1b* (accession number: AY330768), were found to be expressed in the dorsal side (Figs. 5E, F).

Evidence for additional signaling pathways

These data indicated that the dorsal lip probe identified more organizer-specific clones than the Nodal-related or β -Catenin probes. To further investigate this, the 600 dorsal-most spots identified by the organizer-specific probe were superimposed onto the combined organizer graph used in Fig. 4. As shown in Fig. 5G, almost all of the spots of the upper-right quadrant were identified in the screen for dorsal lip genes. However, many other dorsal-specific spots were also found in other regions of the graph. These results suggest that, in addition to the β -Catenin and Nodal-related pathways, additional signaling pathways must participate in the formation of the Spemann organizer at gastrula.

The preorganizer genes

The Spemann organizer patterns all three germ layers of the *Xenopus* embryo at the gastrula stage. However, the formation of the CNS requires the expression of molecules such as *chordin* and *noggin* at an earlier stage, in the blastula preorganizer (Kuroda et al., 2004; Wessely et al., 2001). To investigate preorganizer formation, we generated

the same set of probes (LiCl/*ΔN-XTcf-3* and control/*cer-S*) from stage 9.5 blastula embryos (8.5 h post fertilization). The data were plotted in the same way as described for the combined organizer graph of Fig. 4. As shown in Fig. 6A, at the blastula stage, the *chordin* clones were not found in the upper-right quadrant as in the case of the gastrula organizer graph (Fig. 4), but instead lay along the β -Catenin axis interspersed with *Xnr-3* clones. This confirmed that the β -Catenin pathway, but not Nodal-related signaling, regulates *chordin* expression at the blastula stage (Wessely et al., 2001).

pintallavis/FoxA4a

This analysis identified *pintallavis/FoxA4a* as another gene strongly regulated by the β -Catenin signal (Fig. 6A). In contrast to *chordin* and *Xnr-3*, the clones encoding *pintallavis/FoxA4a* were shifted upwards on the Nodal axis suggesting that at the blastula stage there is already some regulation by Nodal-related signaling. Later on at gastrula, *pintallavis/FoxA4a* is very strongly regulated by Nodal-related, and only mildly by β -Catenin signaling (Fig. 4). In situ hybridization of blastula stage embryos showed that *pintallavis* is expressed dorsally in the preorganizer and is strongly up-regulated by LiCl treatment (Figs. 6B–E). We conclude from these data that *pintallavis* is a previously unidentified component of the preorganizer.

Sequence analysis of the other spots activated by β -Catenin signaling identified several other cDNAs (Fig. 6A). Those include *strabismus* (GenBank accession number AY069979), a clone similar to cathepsin B (UniGene Cluster X1.5191), a clone similar to RNA-binding motif protein 14 (UniGene Cluster X1.16766), the hypothetical protein MGC53321 (GenBank accession number BC043949), and three other unknown proteins (UniGene Cluster X1.11673, X1.14640, and GenBank accession number CA790223). In the future, it will be interesting to determine whether those genes are expressed dorsally, and whether they, as well as *pintallavis*, contribute to the function of the preorganizer in CNS formation.

Discussion

In this manuscript, we describe a systematic exploration of Spemann organizer formation in *X. laevis* using cDNA macroarrays. By generating differential cDNA probes, we analyzed three aspects of organizer formation: first, differential gene expression by modulating the maternal β -Catenin and the zygotic Nodal-related signaling pathways; second, the regional specification of the gastrula by comparing dorsal and ventral marginal zone tissues; third, the temporal regulation of gene expression at blastula and gastrula stage. The integration of the semiquantitative analysis of the response of 72,000 individual cDNAs to Nodal-related and β -Catenin signaling

into a single graph (Fig. 4) provided fresh insights into the regulation of gene networks in each of the aspects analyzed.

Organizer formation is regulated by multiple pathways

The Spemann organizer is in the dorsal mesendoderm of the gastrula embryo. This region is where high Nodal-related and β -Catenin signaling meet, and it is thought that these two signal transduction pathways are the main regulators of Spemann organizer formation (Fig. 1A and De Robertis et al., 2000). The combined organizer graph integrated quantitatively gene expression changes mediated by these two pathways at the gastrula stage. Genes whose transcription was up-regulated by both pathways were in the upper-right quadrant and included *chordin*, *pintallavis*/*FoxA4a*, *gooseoid*, and *PAPC* (Fig. 4). All of these genes are expressed in dorsal mesendoderm and are regarded as bona fide organizer genes (De Robertis et al., 2000; Harland and Gerhart, 1997).

However, the genes identified make up only a small subset of the genes known to be expressed in the Spemann organizer. For example, we did not identify *Dkk-1*, *Frzb-1*, and *ADMP* (De Robertis et al., 2000). There are three possible explanations for this observation. First, some of the organizer genes may not be present among the 72,000 clones spotted on the four nylon filters analyzed. Second, identification of the individual cDNAs relies on the intensity of the signals. Thus, if the overall expression level of a given gene in the probe is too low or if the different treatments do not result in large enough expression differences, a clone will escape detection. Third, one might miss organizer genes because the main thrust of our approach was based on the assumption that Nodal-related and β -Catenin signaling are the predominant regulators of gene expression in the Spemann Organizer. By directly comparing the transcripts enriched in the dorsal lip of a gastrula stage embryo to those regulated by Nodal-related and β -Catenin signaling (Figs. 4 and 5), we could show that some genes, such as *FRL-1*, are expressed dorsally but are not strongly regulated by the β -Catenin pathway.

The overlay of dorsal-enriched clones onto the combined organizer graph provides a way of integrating the results of three differential screens into a single plot (Fig. 5G). The results suggest that other signaling pathways required for dorsal development must exist. These pathways will have to be investigated in the future. Some of them have been suggested by the study of other model organisms. In the chick, fibroblast growth factor 8 (Fgf-8) signaling regulates gene expression in Hensen's node (Streit et al., 2000) and before gastrulation cross-talk among Fgf-8, Fgf-3, and Wnt signals is required for neural induction (Streit et al., 2000; Wilson et al., 2000, 2001). In *Xenopus*, Fgf-3 is downstream of the maternal β -Catenin pathway (Schohl and Fagotto, 2003)

and, like *Fgf-8*, upon microinjection into *Xenopus* embryos induces neural differentiation (Hardcastle et al., 2000 and our unpublished observations). Another signaling pathway involved in dorsoventral patterning in *Drosophila* is the EGF signaling pathway (for review, see Van Buskirk and Schupbach, 1999). The EGF and FGF pathways signal in part via activation of mitogen-activated protein kinase (MAPK). Therefore, an interesting approach in the future will be to determine the effect of MAPK signaling on gene expression in the Spemann organizer. This could best be done, for example, by inhibiting MAPK using low-molecular-weight inhibitors such as U0126 (Favata et al., 1998) and performing macroarray analysis.

Chordin and Xnr-3 are the most abundant β -Catenin target genes

The activation of the maternal β -Catenin signaling pathway is required for dorsal development in the *Xenopus* embryo. Inhibition of the pathway by UV irradiation, depletion of the β -catenin mRNA pool by antisense oligonucleotides, or microinjection of a dominant-repressive ΔN -XTcf-3 mRNA results in completely ventralized embryos lacking CNS and other dorsal structures (Moleenaar et al., 1996; Scharf and Gerhart, 1980; Wylie et al., 1996). Therefore, the identification of the transcriptional targets of the maternal β -Catenin pathway is important to understand dorsal development. Interestingly, in our macroarray analysis of the β -Catenin signaling pathway, only three genes, *chordin*, *Xnr-3*, and *gooseoid*, were strongly up-regulated at the gastrula stage. *Xnr-3* is a direct target of β -Catenin and binding sites for Tcf/Lef have been found in its promoter (McKendry et al., 1997). In the case of *gooseoid*, promoter analysis has identified a binding site for the homeobox gene *Siamois/Twin* (Laurent et al., 1997). *Siamois/Twin* is itself a known direct target of the maternal β -Catenin pathway (Laurent et al., 1997), but was not identified in our screen. Hybridization with a specific probe for *siamois* showed that it was not present among all the arrayed gastrula cDNAs, presumably because *siamois* is mainly expressed at earlier stages (data not shown).

Expression of *chordin* is regulated by the maternal β -Catenin pathway (Sasai et al., 1994; Wessely et al., 2001). We now show that *chordin* expression, like *gsc*, is not regulated directly by β -Catenin, but instead by *Siamois/Twin* (Fig. 2H). Knock-down experiments have shown that *Chordin* has an important role in the early β -Catenin output (Oelgeschläger et al., 2003). Treatment of *Xenopus* embryos with LiCl at the 32-cell stage leads to radially dorsalized embryos as a result of the nuclear localization of β -Catenin throughout the embryo. Microinjection of antisense morpholino oligos against *chordin* blocked the effects of LiCl, showing that the dorsalizing activity of LiCl requires *chordin* expression (Oelgeschläger et al., 2003). The fact

that, as reported here, accumulation of *chordin* transcripts is one of the main outputs of LiCl treatment is consistent with this requirement of *chordin*. It will be interesting to examine whether any of the three other β -Catenin target genes, *Xnr-3*, *siamois/twin*, and *goosecoid*, are also required for the dorsalizing effect of LiCl.

Pintallavis/FoxA4a and the preorganizer

The preorganizer is characterized by the expression of *chordin*, *noggin*, *Xnr-3*, *siamois* and, as shown in this study, *pintallavis/FoxA4a* in the dorsal animal region of the blastula stage embryo (Fig. 6 and Wessely et al., 2001). Each gene presumably has a specialized function in the preorganizer. The transcription factor Siamois/Twin regulates gene expression in the preorganizer (see above). The BMP antagonists Chordin and Noggin are required for planar neural induction and brain formation in embryos lacking mesoderm (Kuroda et al., 2004). *Xnr-3* has anti-BMP activity and may contribute to neural induction since *Xenopus* embryos lacking *Xnr-3* show a reduction in neural markers such as *NCAM* and *En2* (Hansen et al., 1997; Haramoto et al., 2004; Yokota et al., 2003).

The function of *pintallavis/FoxA4a* has not yet been elucidated. *Pintallavis/FoxA4a* is closely related to *HNF3 β /FoxA2*. In *Xenopus*, these two genes together recapitulate the expression of a single gene, *HNF3 β /FoxA2*, in mouse (Ruiz i Altaba and Jessell, 1992; Ruiz i Altaba et al., 1993b). Mouse embryos lacking *HNF3 β /FoxA2* do not develop a morphological node or node derivatives such as notochord, but still undergo neural induction (Klingensmith et al., 1999). Therefore, a requirement of *pintallavis/FoxA4a* in neural induction seems unlikely. Fate map studies of the blastula preorganizer have shown that the dorsal animal cap gives rise, in addition to CNS, to the notochord and the floorplate (Kuroda et al., 2004). It is possible that the expression of *pintallavis/FoxA4a* subdivides the preorganizer into different cell populations, with those expressing *pintallavis/FoxA4a* predominantly giving rise to floorplate and notochord. This view is supported by the observation that overexpression of *pintallavis/FoxA4a* in *Xenopus* embryos can induce both floorplate and notochord (Ruiz i Altaba et al., 1993a; Saka et al., 2000). This regionalization of the preorganizer might be under the control of Nodal-related signaling since, in contrast to *chordin* and *Xnr-3*, *pintallavis/FoxA4a* is already under partial control of Nodal-related signaling at the blastula stage (Fig. 6A).

Concluding remarks

The exploration of differential gene expression by microarray analysis presented here provides information that advances our understanding of the overall regulation of gene expression in the *Xenopus* model embryo. It appears that most of the abundant genes specifically

expressed in the Spemann organizer have been cloned in previous screens. Perhaps this type of analysis is close to saturation for new gene discovery. Our results are of course influenced by the choice of the cDNA library and its clone diversity, as well as the chosen screening methods, and these may be improved. However, it appears that future studies on Spemann organizer function will concentrate on understanding how the physiological activities of a known set of genes are integrated. In addition, other regions of the embryo, such as the ventral side of the gastrula stage embryo, in which Hyaluronan synthetase and *XFD2/FoxI1a* were found, and the blastula preorganizer, have been investigated less thoroughly and might still harbor undiscovered genes.

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