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The role of maternal CREB in early embryogenesis of *Xenopus laevis*

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Abstract

In *Xenopus* embryos, body patterning and cell specification are initiated by transcription factors, which are themselves transcribed during oogenesis, and their mRNAs are stored for use after fertilization. We have previously shown that the T-box transcription factor VegT is both necessary and sufficient to initiate transcription of all endoderm, and most mesoderm genes. In the absence of maternal VegT, no mesodermal organs (including the heart) or endodermal organs form. A second maternal transcription factor XTcf3 acts as a global repressor of transcription of dorsal genes, whose repression is inactivated on the dorsal side by a maternally encoded *Wnt* signaling pathway. In the absence of β-catenin, no mesodermal or endodermal organs form. We show here that the maternally encoded transcription factor CREB is also essential for development. It is required for the initiation of expression of several mesodermal genes, including *Xbra, Xcad2,* and *-3* and also regulates the cardiogenic gene *Nkx 2-5.* We show that maternal *CREB*-depleted embryos develop gastrulation defects that are rescued by the reintroduction of activated *CREB* mRNA. We conclude that maternal CREB must be added to the list of essential maternal transcription factors regulating cell specification in the early embryo.
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Keywords: VegT; β-Catenin; Wnt; CREB; *Nkx2-5*; Antisense oligo; Morpholino

Introduction

During vertebrate development, the heart is one of the first functional organs to form. In *Xenopus,* the heart is derived from the anterior lateral plate mesoderm. Although the heart tissue is fully derived from the mesoderm, it has been shown that endoderm signaling is required for its formation (Sater and Jacobson, 1989). The genes *Nkx2-5* and *Nkx2-3* are the first known markers of the cardiac primordia. These genes are homologues of the *tinman* gene in *Drosophila* that is essential for heart formation (Bodmer, 1993). Overexpression of either *Nkx2-5* or *Nkx2-3* in *Xenopus* causes an enlarged heart (Cleaver et al., 1996). Although *BMPs* are required for the maintenance of *Nkx* expression at later stages of cardiac development, the regulation of initiation of *Nkx* expression is not understood.

The candidate signaling pathways that may regulate *Nkx 2-5* expression in *Xenopus* include the maternal *VegT*-initiated nodal signaling pathway, the maternal *Wnt/β-catenin* pathway, and the cAMP/CREB-activated pathway. *Xenopus* embryos lacking either maternal VegT or β-catenin have no heart development. *VegT*-depleted embryos also lack all endodermal and most mesodermal differentiation markers (Zhang et al., 1998), while *β-catenin*-depleted embryos lack only dorsal derivatives of these germ layers (Heasman et al., 1994). A recent study showed that depletion of β-catenin caused the formation of multiple hearts in mice (Lickert et al. 2002).

Another candidate for the role of initiating *Nkx 2-5* expression is the cAMP response element binding protein, CREB, which has been suggested to initiate heart tube formation in *Drosophila* (Venkatesh et al., 2000). CREB was first identified as a factor that binds to DNA and activates transcription in response to the hormone somatostatin (Montminy and Bilezikjian, 1987). Subsequent studies have implicated CREB as a downstream effector in many signaling pathways that are activated in response to growth factors (Finkbeiner et al., 1997), neurotransmitters (Pende et al., 1997), and peptide hormones (Klemm et al., 1998). Transcriptional activation by CREB requires the phosphorylation of CREB on a consensus Ser133, which is

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essential for its dimerization as well as binding to its partner CBP. This complex then activates transcription by binding to CRE elements (TGACGTCA) that are present upstream of CREB target genes (Montminy, 1997; Shaywitz and Greenberg, 1999).

CREB homologues have been identified in vertebrates as well as many invertebrates. Two CREB homologues, *dCREB-A* and *dCREB-B,* have been identified in *Drosophila.* Absence of *dCREBA* leads to deletion of dorsal and ventral cuticular structures (Andrew et al., 1997). dCREB-B was shown to bind to multiple CRE elements present upstream of the *ubx* gene and mediates dpp signaling in the endoderm (Eresh et al., 1997). More recently, CREB was implicated in the regulation of *tinman,* the homologue of *Nkx2-5* gene in *Drosophila* (Venkatesh et al., 2000). The function of *CREB* in mammalian development has been analyzed in mice by generation of mice lacking one, two, or all three isoforms. Disruption of either the α or both α and Δ isoforms did not cause lethality, although these mice showed learning and memory disorders (Bourtchuladze et al., 1994). However, when all three isoforms were disrupted, the embryos survive to birth, but died soon after birth due to deficiency of surfactant proteins in the lung (Rudolph et al., 1998). These mice also show elevated levels of CREM and ATF1, proposed to compensate for CREB function during embryonic development. Mice lacking both CREB and ATF1 die at embryonic day 9.5, indicating that these proteins have important embryonic functions (Bleckmann et al., 2002). Recently, CREB has been shown to be required for the regulation of cell and mouse embryo size through its activation by Rho GTPase (Sordella et al., 2002). In *Xenopus,* the injection of mouse *dnCREB* mRNA caused severe posterior defects and strong spina bifida and inhibited activin-induced mesoderm formation in animal cap explants (Lutz et al., 1999). However, no direct study has been carried out on the function of endogenous CREB in *Xenopus.*

Here, we show firstly that, surprisingly, *Nkx 2-5* mRNA is robustly expressed in VegT$^-$ embryos, β-catenin$^-$ embryos, and VegT$^-$/β-catenin$^-$ embryos. Furthermore, inhibiting BMP signaling with a specific inhibitor, cmBMP7, does not block *Nkx2-5* expression. Next, we show that *CREB* is a maternal gene and that CREB phosphoprotein is abundant in the *Xenopus* oocyte and early embryo. To address the question of whether CREB regulates zygotic genes, we used antisense oligos to deplete the maternal protein. We show that maternal CREB regulates the expression of a specific subset of mesodermal genes, including the myocardial precursor marker, *Nkx.2-5,* the general mesodermal gene, *Xbra,* the ventrolateral mesodermal transcription factors, *Xcad2* and *-3* and the RNA binding protein, *Xpo.* These genes are not expressed or have reduced expression throughout the gastrula stage in maternal CREB-depleted embryos. The lack of expression of these early mesodermal genes correlates with gastrulation defects in maternal CREB$^-$ embryos, and these defects are partially rescued by the reintroduction of activated *CREB* mRNA. High-dose embryos arrest at gastrulation, while low-dose embryos have shortened axes, head defects, and fail to develop normal hearts.

Materials and methods

Oocytes and embryos

Full-grown oocytes were manually defolliculated and cultured in oocyte culture medium (OCM) as described in Wylie et al. (1996). Oocytes were injected with oligo using a Medical Systems picoinjector in OCM. Injection of oligo into the vegetal poles of oocytes was more effective at depleting *CREB* mRNA than injection into the equatorial region, so vegetal injections were used throughout these experiments. Oocytes were cultured a total of 48 h at 18°C before fertilization. In preparation for fertilization, they were stimulated to mature by the addition of 2 μM progesterone to the culture medium and cultured for 10–12 h. Oocytes were then labeled with vital dyes and introduced into a stimulated female host by using the host–transfer technique described previously (Heasman et al., 1991). Three hours after being placed in the frog's body cavity, the eggs were stripped and fertilized along with host eggs using a sperm suspension. Embryos were maintained in 0.1 $\times$ MMR, and all the colored, experimental embryos were sorted from host embryos. Unfertilized eggs and abnormally cleaving embryos were removed from all batches. For embryo injections, fertilized eggs were treated with cysteine for removal of the outer jelly coat and transferred to 2% Ficoll (Pharmacia) in 0.5% MMR. Oligo or RNAs were injected into embryos into the two- to four-cell-stage embryos vegetally. The embryos were later transferred to 0.1$\times$ MMR.

Oligos and mRNAs

The antisense oligo sequences (Sigma Genosys) used were as follows: VegT antisense oligo, 5′-C*A*G*CAGCATGTACTT*G*G*C-3′ (Zhang et al., 1998); β-catenin antisense oligo, 5′-T*G*C*C*TTTCGGTCTG*G*C*T*C-3′ (Wylie et al., 1996); CREB antisense oligos T1, 5′-C*A*T*C*TGTTGGGCTT*C*T*G*C-3′ and T2, 5′-A*G*T*T*GTATGGCTCC*T*C*C*T-3′, where * in all the sequences indicates a phosphorothioate bond. The other linkages were phosphodiester bonds. The morpholino oligo, MO targeted against CREB, had the sequence CACTGCCTCCTCTCCGTCACATTGG (Gene Tools). Oligos were resuspended in sterile water and were injected in doses of 4–6 ng per oocyte. The oocytes were cultured immediately at 18°C. RNAs were synthesized by linearizing the plasmid vectors containing subcloned cDNAs and transcribing the linear template with SP6 polymerase in the presence of cap analog and GTP using the Megascript kit (Ambion). RNAs were ethanol precipitated and resuspended in sterile, distilled water for injection.

Table 1
List of primers used in quantitative RT-PCR

PCR Primer Pair	Origin	Sequence	Denat Temp (°C)	Annealing Temp (°C)/Time (s)	Extension Temp (°C)/Time (s)	Acquisition Temp (°C)/Time (s)
BMP-4	Houston et al., 2002	U:5′-ACC CAT AGC TGC AAA TGG AC-3′ D:5′-CAT GCT TCC CCT GAT GAG TT-3′	95	55/5	72/12	81/3
CREB	New	U:5′-GCA GAAGCC CAA CAG ATGACA ATA C-3′ D:5′-TGT GTG GAG ACT GAA TGA CAG AAG G-3′	95	55/5	72/12	83/3
Chordin	XMMR	U:5′-AAC TGC CAG GAC TGG ATG GT-3′ D:5′-GGC AGG ATT TAG AGT TGC TTC-3′	95	55/5	72/12	81/3
GATA2	New	U:5′-CTA AAC AGA GGA GCA AGA GC-3′ D:5′-CCT AAG TTC CTC AAA AC-3′	95	55/5	72/15	83/3
GATA4	Xanthos et al., 2001	U:5′-AGT GCT ACT GCT GCT ACC TC-3′ D:5′-ACT GTA GGA GAC CTC TCT GC-3′	95	54/5	72/15	87/3
GATA5	Xanthos et al., 2001	U:5′ ACCTTCAGAGCTGCGACACT-3′ D:5′ CAGTGTATTGCCATACTGGTC-3′	95	60/5	72/20	86/3
GATA6	Xanthos et al., 2001	U:5′-CCA ACC GGG AGC CCC GAT A-3′ D:5′-GCT GCT GTA GCC TGT ATC C-3′	95	60/5	72/26	88/3
NCAM	Kofran et al., 1999	U:5′-CAC AGT TCC ACC AAA TGC-3′ D:5′-GGA ATC AAG CGG TAC AGA-3′	95	60/5	72/13	84/3
Nkx 2-3	Houston et al., 2002	U:5′-TCGTGTATGTTGGCAGCAGGAG-3′ D:5′-CCTCTTCATCTTCTTCTTTGGGGTC-3′	95	56/5	72/11	85/3
Nkx 2-5	New	U:5′-ACA CAG AGA GAG AGA TTG GGT GGC-3′ D:5′-TCA GTG AGT TCA GAG AGG CAA GGG-3′	95	55/5	72/13	83/3
ODC	Heasman et al., 2000	U:5′-GCC ATT GTG AAG ACT CTC TCC ATT C-3′ D:5′-TTC GGG TGA TTC CTT GCC AC-3′	95	55/5	72/12	83/3
Siamois	Heasman et al., 2000	U:5′-CTG TCC TAC AAG AGA CTC TG-3′ D:5′-TGT TGA CTG CAG ACT GTT GA-3′	95	55/5	72/16	81/3
Xbra	Sun et al., 1999	U:5′-TTC TGA AGG TGA GCA TGT CG-3′ D:5′-GTT TGA CTT TGC TAA AAG AGA CAG G-3′	95	55/5	72/8	75/3
Xcad2	New	U:5′-ACCACCACCAACGGTAAGAC-3′ D:5′-GAGTGGTTGTTGAGGCCTGT-3′	95	55/5	72/12	82/3
Xcad3	New	U:5′-TCT CCT CAT CCA TCT GGG ACT G-3′ D:5′-AGT TCT GTC TTC CGC CTG ATA GTG-3′	95	55/5	72/15	83/3
Xnr-3	Kofron et al., 1999	U:5′-CTT CTG CAC TAG ATT CTG-3′ D:5′-CAG CTT CTG GCC AAG ACT-3′	95	57/5	72/10	79/3
Xpo	New	U:5′-ACCTGGAGCATTGGGTTATG-3′ D:5′-GGGATAGGGAAGGATGCAAT-3′	95	55/5	72/11	80/3
Xsox17α	Xanthos et al., 2001	U:5′-GCA AGA TGC TTG GCA AGT CG-3′ D:5′-GCT GAA GTT CTC TAG ACA CA-3′	95	58/5	72/8	85/3
Wnt8	Ding et al., 1998	U:5′-CTG ATG CCT TCA GTT CTG TGG-3′ D:5′-CTA CCT GTT TGC ATT GCT CGC-3′	95	58/6	72/14	85/3

Analysis of gene expression using real-time RT-PCR

Total RNA was prepared from oocytes, embryos, and explants using proteinase K and then treated with RNase-free DNase as described (Zhang et al., 1998). Approximately one-sixth embryo equivalent of RNA was used for cDNA synthesis with oligo (dT) primers followed by real-time RT-PCR and quantitation using the “Light Cycler” System (Roche) as described in Kofron et al. (2001). The primers and cycling conditions used are listed in Table 1. Relative expression values were calculated by comparison to a standard curve generated by serial dilution of uninjected control cDNA. Samples were normalized to levels of ornithine decarboxylase (ODC), which was used as a loading control. Samples of water alone or controls lacking reverse transcriptase in the cDNA synthesis reaction failed to give specific products in all cases.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described (Harland, 1991) by using BM Purple as substrate (Roche). After satisfactory color development, embryos were fixed in MEMFA for 1 h at room temperature, washed, and stored in 100% ethanol. Some embryos were also treated in 10% H_2O_2 to bleach out the pigment.

Western blotting

Oocytes or embryos (2 each per sample) were suspended in PBS containing protease inhibitors and extracted with freon to remove yolk protein. The extracts were then precipitated with acetone and denatured by using 1× Laemmli buffer and electrophoresed on a denaturing SDS-PAGE.

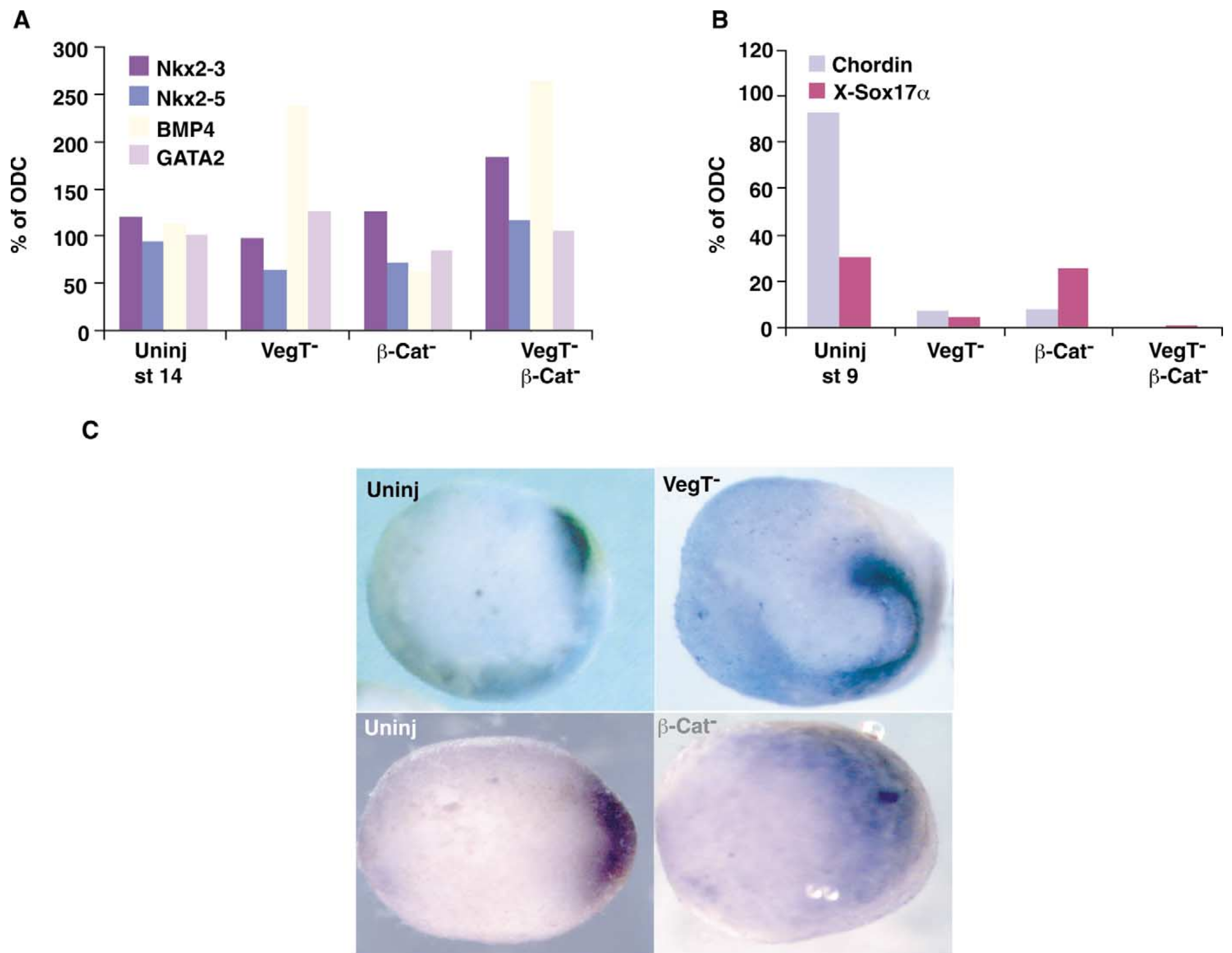

Fig. 1. Expression of early heart markers in VegT- and β-catenin-depleted embryos. (A, B) Oocytes were depleted with antisense oligonucleotides for VegT and/or β-catenin and a host transfer experiment was carried out as described in Materials and methods. The resulting embryos were collected at stage 14 (A) and analyzed for the expression of *Nkx2-3, Nkx2-5, BMP4,* and *GATA2* by real time RT-PCR. (B) The expression of *Chordin* and *Xsox17α* in the same samples. Expression levels were normalized to ODC. (C) In situ hybridization for *Nkx2-5* in VegT- and β-catenin-embryos: Embryos were injected at the two- to four-cell stage with antisense morpholino oligonucleotides for VegT or β-catenin as described in Materials and methods. The resulting embryos were fixed in MEMFA at neurula stage (stage 16) and probed for *Nkx2-5* expression pattern as described in Materials and methods.

The proteins were blotted on to PVDF and probed with anti-CREB antibody (1:500) (Calbiochem) or anti-phospho-CREB antibody (1:250) (Upstate) followed by appropriate HRP-conjugated secondary antibody. The protein bands were visualized by using ECL (Amersham) reagents. The blots were also probed with anti γ-tubulin antibody (Sigma) as a loading control.

Results

Nkx2-5 continues to be expressed in embryos lacking either Wnt- or VegT- initiated signaling pathways

Previous experiments showed that both the maternal VegT-initiated and Wnt signaling pathways are essential for the establishment of endodermal and mesodermal tissues (Kofron et al., 1999; Xanthos et al., 2001). VegT-depleted embryos lack all activin-type signaling as evidenced by the lack of Smad2 phosphorylation at the blastula stage (Lee et al., 2002). Embryos injected with morpholino targeting β-catenin lack all Wnt signaling activity (Heasman et al., 2000). However, *Nkx2-5* and *-2-3* continued to be expressed in these embryos. Fig. 1A shows that VegT^{-}, β-catenin^{-}, and β-catenin^{-}/VegT^{-} embryos express similar amounts of *Nkx2-5* and *Nkx2-3* mRNA as wild-type sibling embryos. They also express normal levels of ventral mesodermal gene *GATA2*. *BMP4* is overexpressed in VegT^{-} embryos. In comparison, other mesodermal genes and all endodermal genes are not transcribed in VegT^{-} embryos (Fig. 1B; Xanthos et al., 2001), while β catenin^{-} embryos fail to

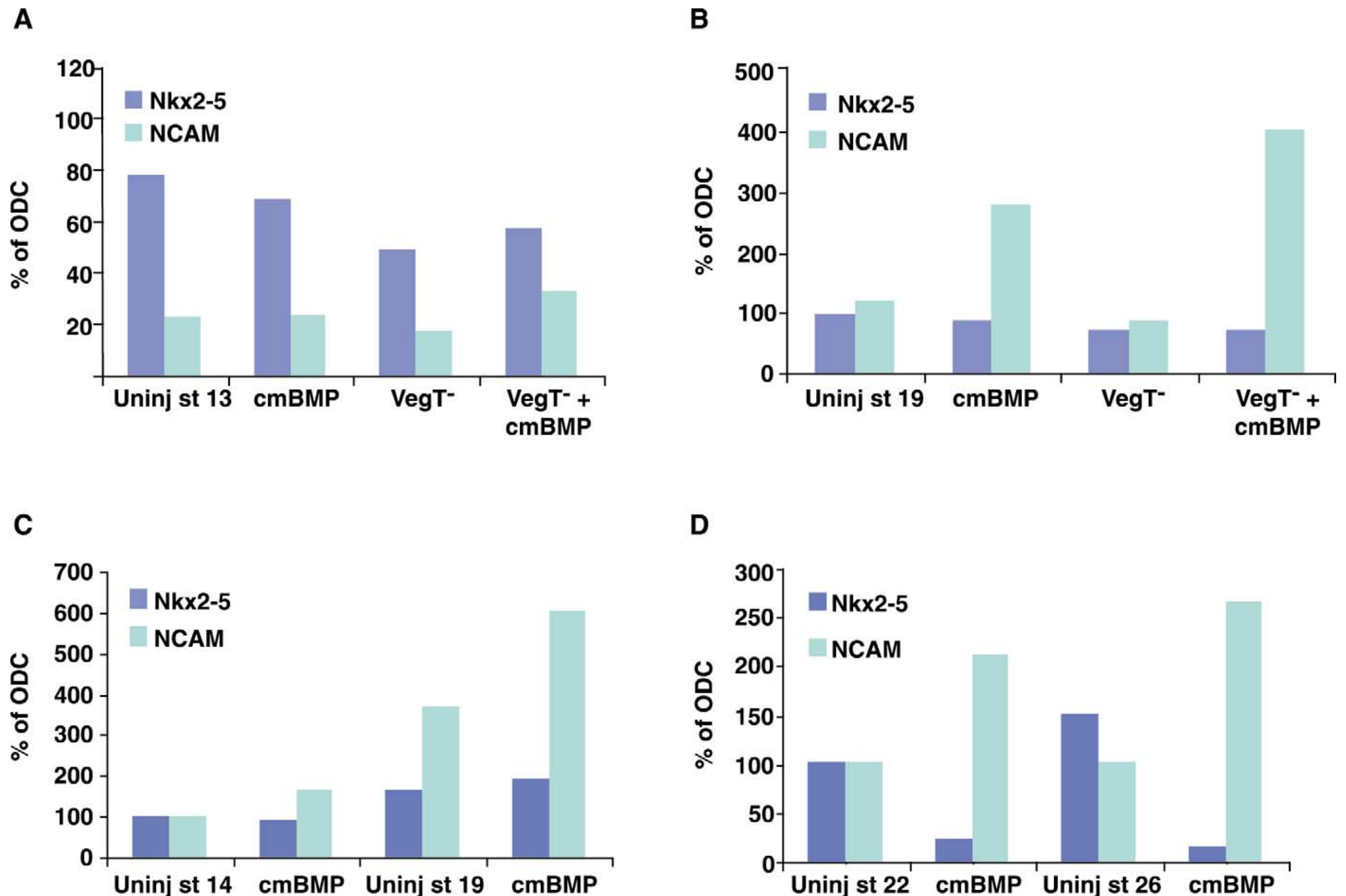

Fig. 2. Blocking BMP signals does not affect *Nkx2-5* expression. (A, B) Oocytes were injected with VegT antisense oligo (VegT$^-$) or cmBMP7 mRNA (cmBMP) or both (VegT$^-$+ cmBMP). After fertilization, the embryos were collected at stage 13 (A) and 19 (B) and analyzed for the expression of *Nkx2-5* and *NCAM* by real time RT-PCR and normalized for ODC expression. (C, D) 150 pg of cmBMP7 was injected into oocytes and a host transfer experiment was carried out as described. Embryos were collected at stages 14, 19 (C), 22, and 26 (D) to analyze the expression of *Nkx2-5* and *NCAM.*

express dorsal and anterior markers such as *chordin* (Fig. 1B; Kofron et al., 1999; Xanthos et al., 2002).

To compare the expression patterns of *Nkx2-5* mRNA in wild type, VegT$^-$, and β-catenin$^-$ embryos further, we carried out whole-mount in situ hybridization at the neurula stage. Fig. 1C indicates that *Nkx2-5* mRNA expression is in the same anterior, ventral heart-forming area but is more diffuse, occurring over a broader region in VegT$^-$ and β-catenin$^-$ embryos compared with sibling control embryos at the early neurula stage. These data show that there is an early signaling event independent of both the *Wnt*- and the *VegT*-initiated pathway that initiates the expression of *Nkx2-5* in a broad area of the anterior ventral mesodermal territory. This expression is more narrowly focused into the heart-forming region by signals downstream of the *Wnt*- and *VegT*-initiated pathway.

The BMP signaling pathway does not initiate Nkx2-5 mRNA expression in VegT$^-$ embryos

Previous studies from several groups have shown that *Nkx2-5* expression is regulated by BMP4 in *Xenopus* embryos (Shi et al., 2000; Walters et al., 2001). Since *BMP4* and *-7* mRNAs are overexpressed in VegT$^-$ embryos (Fig. 1A; Kofron et al., 1999; Xanthos et al., 2002), we next tested whether BMP signaling was responsible for initiating *Nkx2-5* expression in these embryos. We have shown previously that the cleavage mutant form of BMP7, cmBMP7, inhibits BMP signaling in a specific fashion since injection of *cmBMP7* mRNA into one blastomere of the eight-cell-stage embryo significantly reduced Smad1 phosphorylation at the early gastrula stage, without inhibiting Smad2 phosphorylation (Xanthos et al., 2002). To ensure that inhibition occurred throughout the embryos, we injected wild type and VegT$^-$ oocytes with *cmBMP7* mRNA prior to maturation. The oocytes were fertilized and analyzed by RT-PCR for the expression of *Nkx2-5* at the early and late neurula stages. Fig. 2 indicates that *Nkx2-5* is expressed normally in wild type and VegT$^-$ embryos, in which BMP signaling is inhibited by cmBMP7. To show that cmBMP7 was active in this experiment, we also tested the expression of the neural gene *NCAM,* since cmBMP7 is known to induce neural markers (Hawley et al., 1995). *NCAM* expression was in-

A

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Mouse CREB      1 MTMESGADNQQSGDAAVTEAENQQMTVQAQPQIATLAQVSMPAAHATSSA  50
Xenopus CREB    1 MTMES--ETQQSGD-AVTEAEAQQMTIQTQPQIATIAQVSMAAAHATSSA  47
                  *****  ..***** ****** ****.*.******.***** ********

Mouse CREB     51 PTVTLVQLPNGQTVQVHGVIQAAQPSVIQSPQVQTVQSSCKDLKRLFSGT 100
Xenopus CREB   48 PTVTLVQLPNGQTVQVHGVIQAAQPSVIQSPHIQTVQ-------------  84
                  *******************************..****

Mouse CREB    101 QISTIAESEDSQESVDSVTDSQKRREILSRRPSYRKILNDLSSDAPGVPR 150
Xenopus CREB   85 -ISTIAESEDSQESVDSVTDSQKRREILSRRPSYRKILNDLSSDVPGVPR 133
                   ******************************************* *****

Mouse CREB    151 IEEEKSEEETSAPAITTVTVPTPIYQTSSGQYIAITQGGAIQLANNGTDG 200
Xenopus CREB  134 IEEEKSEEETSAPAITTVTVPTPIYQTSSGQYIAITQGGAIQLANNGTDG 183
                  **************************************************

Mouse CREB    201 VQGLQTLTMTNAAATQPGTTILQYAQTTDGQQILVPSNQVVVQAASGDVQ 250
Xenopus CREB  184 VQGLQTLTMANTTASQQGTTILQYAQTTDGQQILVPSNQVVVPSCLKEMY 233
                  *********.*..*.* ************************* ..  ..

Mouse CREB    251 TYQIRTAPTSTIAP-GVVMASSPALPTQPAEEAARKREVRLMKNREAARE 299
Xenopus CREB  234 RHTRFAQHLPALLPQEWLMASSPALPAQPAEEAVRKREVRLMKNREAARE 283
                       .    .. *   .********.****** ****************

Mouse CREB    300 CRRKKKEYVKCLENRVAVLENQNKTLIEELKALKDLYCHKSD 341
Xenopus CREB  284 CRRKKKEYVKCLENRVAVLENQNKTLIEELKALKDLYCHKS  324
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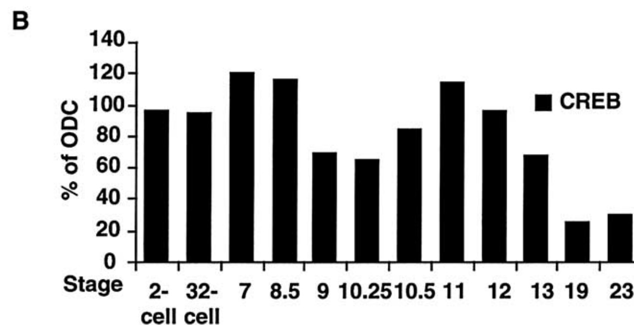


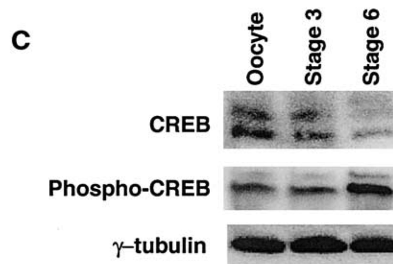


Fig. 3. Characterization of *Xenopus* CREB. (A) Comparison of the amino acid sequences of mouse and *Xenopus* CREB. The α-peptide missing in the *Xenopus* CREB sequence is highlighted. (B) Embryos were collected at indicated stages, and RT-PCR was carried out to analyze the expression of *CREB* RNA. (C) Oocytes and embryos were collected at indicated stages. Western blot analysis was carried out as described in Materials and methods, using a human α-CREB antibody (top) or a α-phospho-CREB monoclonal antibody (middle). γ-Tubulin was used as a loading control (lower).

duced in embryos injected with *cmBMP7* mRNA (Fig 2). Even when both Smad1 phosphorylation and Smad2 phosphorylation are inhibited in the same embryos (cmBMP7 + VegT$^-$ embryos; Fig. 2A), *Nkx2-5* is expressed at wild-type levels at the gastrula and neurula stages. While *Nkx2-5* mRNA expression is normal at the late neurula stage (stage 19, Fig. 2B and C), it is significantly reduced 3 h later at the early tailbud stage (stage 22) in BMP signaling-deficient embryos compared with control levels (Fig. 2D). These results confirm and extend those of Walters et al. (2001), showing that the BMP signaling pathway does not regulate the initiation of *Nkx 2-5* mRNA expression, but is required at the early tailbud stage to maintain its expression.

Cloning and characterization of Xenopus laevis maternal CREB

To address the possibility that the maternal transcription factor CREB is responsible for *Nkx2-5* expression, we next characterized the *Xenopus CREB* gene. Previous studies showed that a *CREB* transcript is present in oocytes (Lutz et al., 1999). Using a putative *CREB* EST present in a *X. laevis* maternal EST database (Blackshear et al., 2001), we designed primers to amplify the full-length *CREB* gene from a maternal cDNA library. The amplified sequences were isolated, cloned into appropriate vectors, and the coding sequence was identified. The sequence analysis revealed that

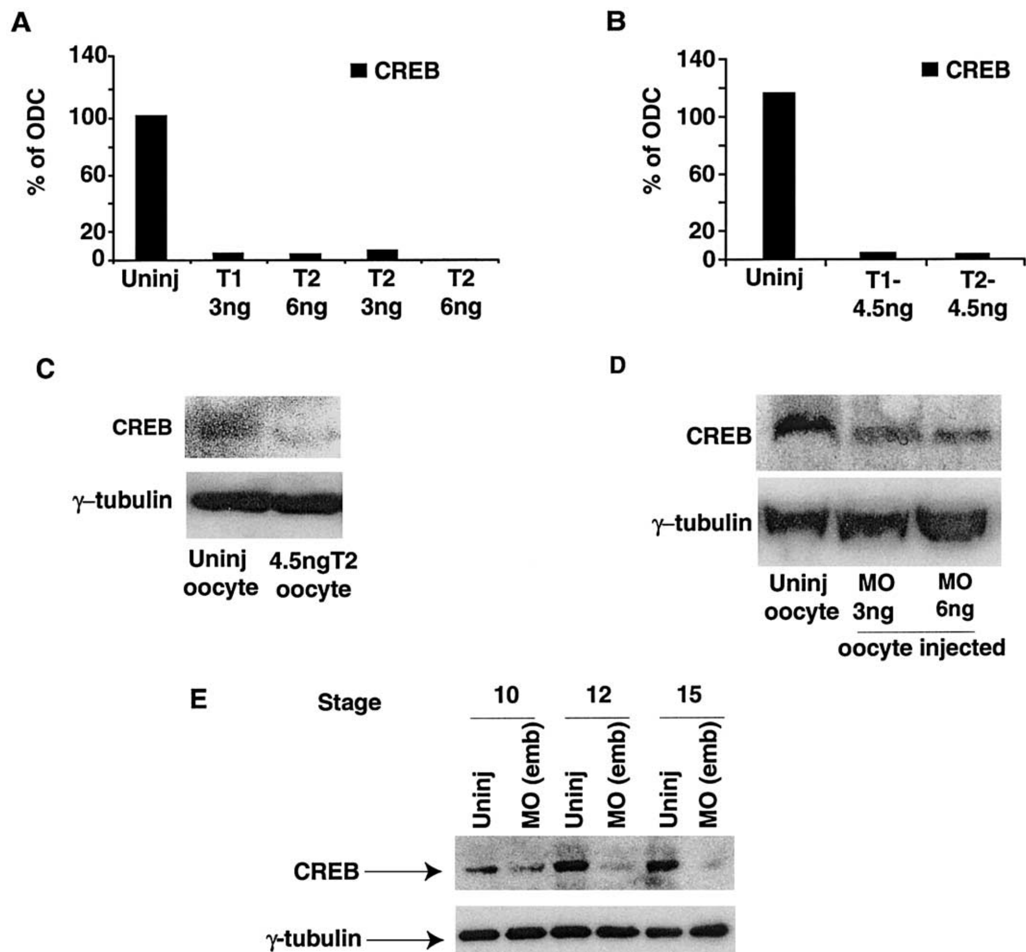


Fig. 4. Depletion analysis of CREB. (A, B) Oocytes were depleted with thiol modified antisense oligonucleotides, T1 or T2, for *CREB* mRNA. Oocytes were collected after 24–48 h and analyzed for level of depletion of *CREB* RNA by real time RT-PCR. The level of protein depletion was also analyzed by Western blotting using a α-CREB antibody for depletion with thioate oligoT2 (C) and depletion with the morpholino oligo, MO (D). (E) Embryos were injected with MO at the two-cell stage and collected at indicated stages and analyzed by Western blotting with α-CREB antibody. γ-Tubulin was used as a loading control in (C–E).

the *Xenopus CREB* gene has an ORF of 925 bp. The putative amino acid sequence shows high homology to the mouse CREB protein (Fig. 3A). The maternal *X. laevis* CREB differs from mouse CREB in that it lacks the α-peptide that connects the KID domain and the Q1 domain. Thus, the *Xenopus* CREB is more similar to the second isoform of CREB known in mammals (Montminy, 1997).

In a developmental series (Fig. 3B) we found that *CREB* mRNA is present at high levels at the cleavage and blastula stages. A second peak of expression occurs at the mid-gastrula stage. Analysis of early embryos indicated that *CREB* mRNA is expressed throughout the early embryo in animal, equatorial, and vegetal cells (data not shown). We confirmed that the *CREB* mRNA is translated, by Western blotting analysis (Fig. 3B). We identified two bands about ~43 kDa, one possibly a posttranslationally modified form of *Xenopus* CREB protein. Since CREB is functional only as a phosphorylated protein, we then looked for phosphorylated CREB using a phospho-CREB antibody. Fig. 3C shows that phosphorylated CREB is present in oocytes as well as in cleavage and early blastula-stage embryos. Although the amount of CREB protein decreased compared with the oocyte level by the early blastula stage, the amount of active phospho-CREB increased (Fig. 3C).

Antisense mediated depletion of maternal CREB mRNA

To target maternal CREB, we used two chimeric phosphorothioate/phosphodiester antisense oligos (see Materials and methods). Phosphorothioate/phosphodiester antisense oligos are unstable and are ineffective at degrading mRNA when injected into embryos (Woolf et al., 1990). However when phosphorothioate/phosphodiester antisense oligos are injected into oocytes, they degrade maternal mRNA and do not affect zygotic transcription of the gene of interest. We have shown previously that when oocytes injected with chimeric oligos are fertilized and develop to the gastrula stage, the maternal target mRNAs are degraded but zygotic transcription occurs normally (Zuck et al., 1998; Wylie et al., 1996; Zhang et al., 1998). Here, oligos were injected at

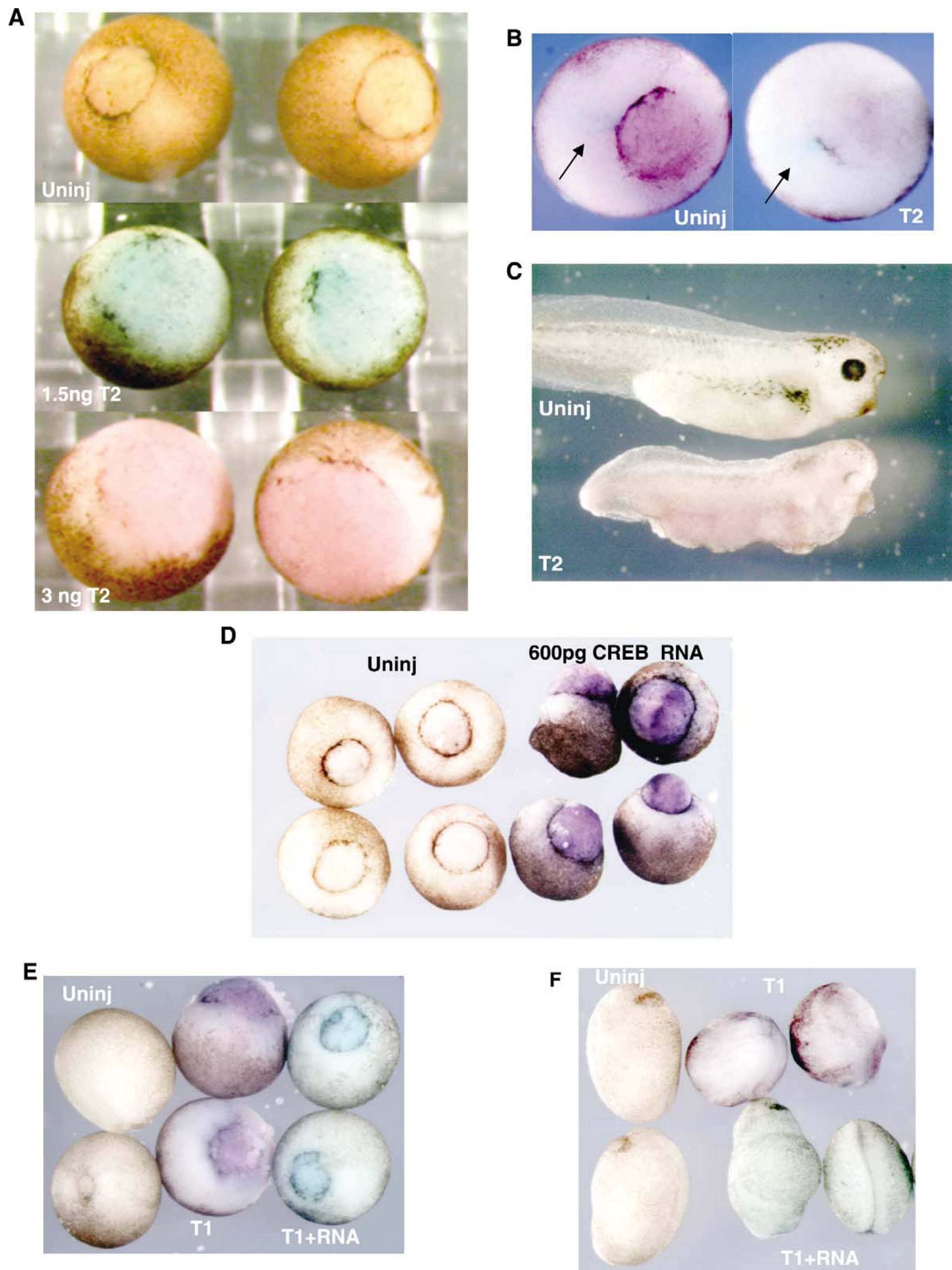


Fig. 5. Phenotypic analysis of CREB depletion and ectopic expression of CREB mRNA. (A) Oocytes were depleted with antisense thioate oligonucleotide T2 at the indicated doses, and a host transfer experiment was carried out as described. The resulting embryos showed a delayed gastrulation. In (B), embryos were marked on the dorsal side with Nile blue and then observed during gastrulation. Arrows indicate the Nile blue staining on the dorsal side (C) A comparison of late-stage embryos. (D) Synthetic *CREB* mRNA was injected at indicated doses into oocytes, and a host transfer experiment was carried out. The effect of ectopic *CREB* expression on gastrulation was observed. (E, F) 600 pg of *CACREB* RNA was injected 48 h after oligo injections, and a host transfer experiment was carried out as described. *CREB*-depleted embryos injected with *CACREB* were able to gastrulate (E) and were able to develop into more normal neurula stage embryos than those depleted of *CREB* (F).

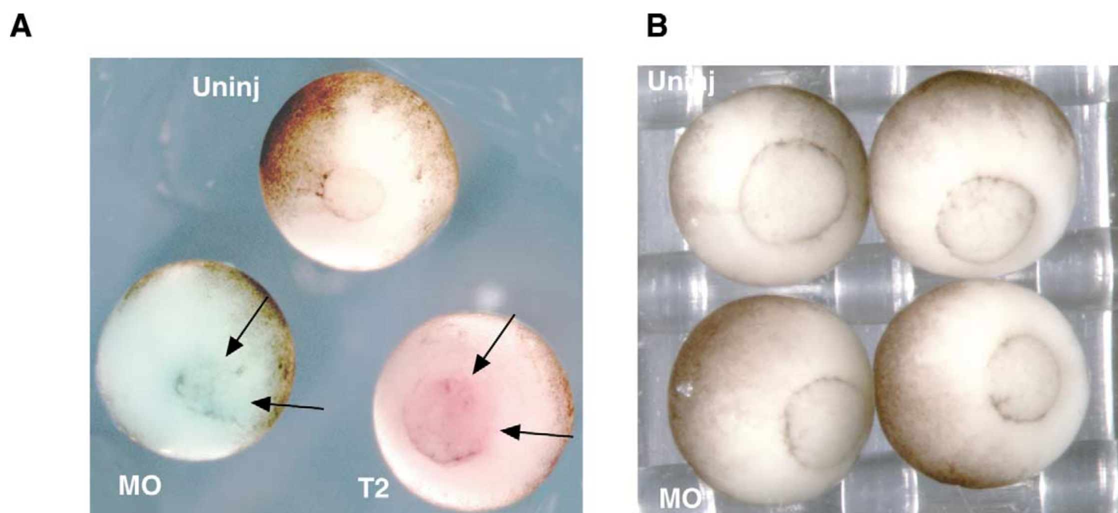


Fig. 6. Effect of morpholino oligo injection in oocytes and embryos. (A) Oocytes were injected with 6 ng MO or 4.5 ng of T2, and a host transfer experiment was carried out after 48 h. Both MO- and T2-injected embryos were delayed in gastrulation. The arrows indicate the delayed ventral balstopore region. (B) 6 ng MO was injected into embryos at the two- to four-cell stage. The resulting embryos show no delay in gastrulation.

various concentrations into oocytes and, after culturing the oocytes for 24–48 h, the depletion of maternal *CREB* mRNA was analyzed by RT-PCR. Two oligos were found to be very efficient in depleting maternal *CREB* mRNA to less than 5% control levels when injected at 3–6 ng/oocyte (Fig. 4A and B; oligos T1 and T2). Western blotting confirmed that the level of maternal CREB protein decreased by 60% in oocytes injected with these oligos (Fig. 4C; and data not shown).

We also used a morpholino oligo (MO) to target both maternal and zygotic *CREB* mRNA. In contrast to standard oligos, morpholino oligos work by forming a stable complex with the target mRNA to prevent its translation (Heasman et al., 2000). The efficacy of the oligo was analyzed by Western blotting for CREB protein. We confirmed that when this oligo is injected into oocytes, it also depleted maternal CREB protein to less than 30% control levels in the oocytes (Fig. 4D). We also used this oligo to target the zygotic CREB protein by injecting it at the two-cell stage. Fig. 4E shows that embryos injected with MO at the two-cell stage have significant depletion of CREB protein at the gastrula and neurula stages.

Maternal CREB is required for normal gastrulation

Next, we carried out oocyte transfer experiments to determine the phenotypic effect of maternal CREB depletion on embryonic development. Using various doses of the antisense thioate oligo T2 (1.5–6 ng), we depleted the maternal pool of CREB from oocytes and transferred them to host females and fertilized them. The maternal CREB$^-$ embryos developed normally until gastrulation. The first abnormality that we observed occurred at gastrulation. Embryos that were injected with higher doses of oligo (more than 6 ng) were completely inhibited at gastrulation, although the cells continued to divide and survived until sibling embryos reached the tailbud stage (data not shown). Embryos injected with lower doses of oligo formed the dorsal blastopore lip in a delayed fashion, but the ventral and lateral blastopore lips did not appear when control embryos had complete blastopores (Fig. 5A). To confirm that the ventral lip was affected, we marked the dorsal side of these embryos with Nile blue at the four-cell stage and observed that the absence of lip formation was always on the ventral side of the embryo (Fig. 5B). These embryos did neurulate and eventually developed to tailbud stages and formed abnormal tadpoles (Fig. 5C).

To confirm that the effect of the phosphorothioate oligos on development was specific, we tested whether injecting *CREB* mRNA into embryos depleted of maternal CREB reversed this effect. First, we analyzed the effect of overexpressing *CREB* mRNA alone by injecting 600 pg into oocytes before maturation. Embryos overexpressing *CREB* mRNA develop normally to the gastrula stage and have gastrulation abnormalities, including more rapid formation of the blastopore ring and protrusion of the blastopore (Fig. 5D). Next, oocytes were injected with T1 CREB oligo and, after 48 h, half of the oocytes were injected with 600 pg activated *CREB* mRNA. Embryos injected with CREB oligo alone showed the delayed gastrulation phenotype, while maternal CREB$^-$ embryos injected with CA*CREB* mRNA were not arrested in gastrulation and developed with more normal body axes (Fig. 5E and F). These data confirm that the effect of T2 oligo on gastrulation is specifically due to the depletion of maternal CREB.

To examine whether zygotic CREB protein played an essential role in early development, we investigated the effects of injecting the morpholino oligo (MO) against CREB. When MO is injected into oocytes and then cultured for 2 days before fertilization, there is a significant reduction

of maternal CREB protein (Fig. 4D). Embryos derived from these oocytes have the same phenotype as that caused by injecting T1 or T2 oligo (Fig. 6A). However, MO injected into the two-cell-stage embryo targets the zygotic CREB protein predominantly (Fig. 4E). Embryos treated in this fashion have no gastrulation defects and develop normally (Fig. 6B). Taken together, these results show that the gastrulation phenotype is the result of the depletion of maternal protein and that zygotic CREB protein is not essential for early development.

Maternal CREB regulates the expression of specific zygotic ventrolateral and general mesodermal genes

We next studied candidate genes that might be affected by CREB depletion. We performed RT-PCR on wild type and CREB$^-$ embryos at the early and mid-gastrula stages to compare the expression of zygotic regionally expressed genes, including *siamois, Xnr3* (dorsal mesodermal); *Xbra* (general mesodermal); *Xpo,* the *Cdx* genes, *Xcad2* and *Xcad3, BMP4, Xwnt 8* (ventral mesodermal); *Xsox17, GATA4* and *-6* (endodermal) and *chordin* (dorsal endodermal). As shown in Fig. 7A, a subset of zygotic genes, *Xbra, Xpo, Xcad2, and -3,* are not expressed, or expressed at 10% control levels in CREB$^-$ embryos. In comparison, *BMP4,* a ventral mesodermal gene, is not affected. The expression of zygotic *CREB* is slightly delayed but otherwise normal in maternal CREB$^-$ embryos. *Xwnt8* is delayed in its expression but is expressed at normal levels at later stages of gastrulation.

Since *Xbra* has been shown to be required for gastrulation movements (Conlon and Smith, 1999) and therefore may be important in the CREB$^-$ phenotype, we further studied its expression in CREB$^-$ embryos by in situ hybridization. Fig. 7B confirms that *Xbra* is not expressed in a halo around the blastoporal ring (embryo on the right in Fig. 7B) unlike control, uninjected embryos (embryo in the center).

To confirm that *Xcad2, Xcad3, Xpo,* and *Xbra* were regulated by maternal *CREB,* we analyzed these markers in embryos rescued by the reintroduction of activated *CREB* mRNA (Fig. 7C; siblings of the embryos shown in Fig. 5E). While there was no rescue of *Xbra, Xpo, Xcad2,* and *-3* at the early gastrula stage in CREB$^-$ embryos injected with *CACREB* mRNA, all four zygotic genes were significantly rescued by the late gastrula stage. This delay in expression coincided with the observed delay in rescue of gastrulation movements.

Nkx2-5 is downstream of maternal CREB

Nkx2-5 expression starts at the neurula stage in *Xenopus.* Fig. 8A shows that *Nkx2-5* expression is initiated at the correct time in CREB$^-$ embryos, at the early neurula stage, but is not maintained at the correct level. In six experiments, we observed that the expression of *Nkx2-5* was always about 40% of uninjected control embryo levels at the late neurula stage (Fig. 8A). This lower expression was maintained at later stages of development (Fig. 8B). This suggests that maternal CREB regulates, directly or indirectly, the expression of *Nkx2-5* mRNA during the neurula stage. Injection of *Xcad2* or *Xcad3* did not increase the level of *Nkx2-5* in CREB$^-$ embryos, suggesting that these transcription factors do not regulate *Nkx2-5* (data not shown). Since the morpholino oligo prevents zygotic as well as maternal CREB activity we next compared the level of *Nkx2-5* expression in embryos derived from oocytes injected with CREB MO and cultured for 2 days. We observed a similar downregulation of *Nkx2-5* expression as for embryos depleted only for maternal CREB (Fig. 8C). However, when the morpholino oligo MO was injected after fertilization and therefore targeted mainly the zygotic CREB, there was no phenotypic effect and no reduction in the expression of *Nkx2-5* (Fig. 8D). This shows that zygotic CREB does not play a part in the regulation of *Nkx2-5* expression.

As the expression of *Xcad* genes and *Xpo* are reduced throughout gastrulation, we asked whether they were expressed normally at the neurula stage. We used CREB$^-$ embryos derived from MO-injected oocytes to prevent both maternal and zygotic CREB synthesis through neurula stage. Fig. 8E and F shows that, at the neurula stage, the expression of *Xpo* and Xcad *genes* was at normal levels compared with uninjected control embryos. This suggests that a second CREB-independent wave of expression of these genes occurs at this time.

Discussion

Three novel observations are made in this study. First, we show that the expression of the early marker of myocardial precursor, *Nkx2.5,* is regulated by the maternally stored, leucine zipper transcription factor, CREB. Second, we show that maternal CREB is essential for gastrulation in *Xenopus.* Third, we demonstrate that CREB specifically controls the expression of several ventrolateral and general mesodermal transcription factors, including *Xbra, Xcad2,* and *Xcad3.* These results place maternal CREB as an essential determinant of cell specification in the gastrula.

CREB- a regulator of Nkx2.5 expression

This study was initiated because we observed that embryos in which Wnt-, BMP-, or VegT-initiated signaling was interrupted, continued to express the myocardial precursor marker, *Nkx2-5.* This was unexpected because all three pathways have been implicated in myocardial development. *Xenopus* embryos in which maternal and/or zygotic Wnt signaling is interrupted by depletion of β-catenin (Heasman et al., 2000) or Wnt11 (Pandur et al., 2001) lack heart formation. Loss of BMP signaling leads to heart defects and a reduction of *Nkx2.5* expression in zebrafish

(Reiter et al., 2001) and *Xenopus.* The maternal transcription factor VegT regulates the signaling molecule FGF8 (Kofron et al., 1999) and the transcription factor GATA5 (Xanthos et al., 2001), both of which have been shown to regulate *Nkx2-5* (Alsan et al., 2002; Sparrow et al., 1998; Reiter et al., 1999). Therefore, we tested whether maternal and/or zygotic CREB might be the missing *Nkx2-5* regulator. We found that *Nkx2-5* is initiated properly, but is not sustained correctly in both maternal and maternal/zygotic CREB embryos. We have not investigated here whether this is the result of a direct interaction of CREB with *Nkx2-5* promoter, although potential CRE-binding sites have been reported in the mouse gene (Searcy et al., 1998). Although no functional CRE binding sites have been reported in the 4.3-kb fragment of the *Xenopus Nkx2-5* promoter, the 4.3-kb region has been shown to be sufficient to drive transgene expression in the correct location (Sparrow et al., 2000). Furthermore, we report here that CREB is ubiquitously expressed in the early *Xenopus* embryo (data not shown). This suggests that CREB maintains the level of expression of *Nkx2.5* rather than regulating the location of Nkx2-5 activity. This work suggests that an unknown, maternal or zygotic initiator of *Nkx2-5* expression remains to be identified in *Xenopus.*

Maternal CREB is essential for gastrulation

We show here that CREB is required for successful gastrulation, and that ventrolateral lip formation is particularly sensitive to the levels of CREB expression. While high doses of oligo prevent gastrulation completely, lower doses only affect the lateral and ventral lip formation. Further work is required to determine whether the defect is one of adhesion, migration, or shape change. One likely downstream pathway is the convergent extension pathway that has been shown to require the expression of the T-box gene, *Xbra* (Conlon et al., 1996), which in turn regulates the myocardial signaling molecule, Xwnt11 (Tada et al., 2000). We show here that *Xbra* is downregulated in CREB-depleted embryos. The relative resilience of the dorsal lip compared to ventrolateral blastopore lip formation may be explained by the fact that the dorsal segment of *Xbra* expression is regulated independently of the rest, by the maternal *Wnt* pathway (Lerchner et al., 2000; Yokota et al., 2003). However, it is unlikely that *Xbra* is the only CREB target gene involved in gastrulation. In preliminary experiments, reintroducing *Xbra* mRNA into CREB$^-$ embryos did not rescue the gastrulation phenotype (data not shown). In mouse, CREB activity has been shown to be downstream of the Rho-inhibitory protein p190-B Rho GAP (Sordella et al., 2002). If Rho GAP modulates CREB phosphorylation in a similar fashion in *Xenopus,* then CREB$^-$ embryos may be expected to phenocopy the increased adhesion phenotype of embryos expressing constitutively active Rho.

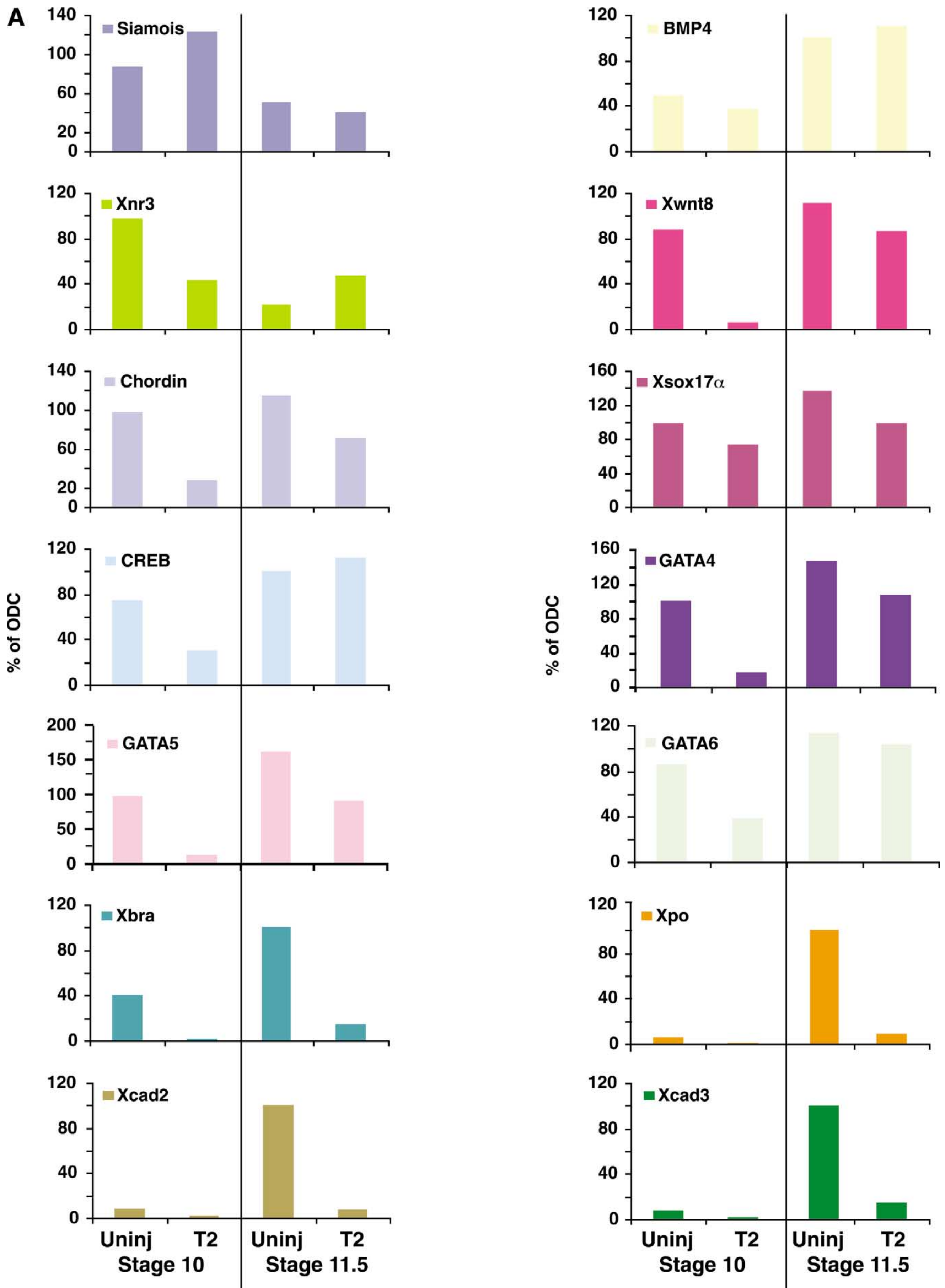
CREB-dependent ventral mesodermal specification

The most generally accepted mechanism by which ventral mesodermal cells are thought to be specified is by the activation of BMP target genes through the Smad1 signaling cascade. We demonstrate here that this picture is too simple, since maternal CREB regulates a subset of ventrolaterally expressed mesodermal genes, specifically the caudal-type homeobox genes *Xcad2* and *-3* and the gene encoding the RNA binding protein Xpo. This regulation is independent of the BMP pathway since *Xcad2* and *-3* continue to be expressed in embryos in which Smad1 phosphorylation is blocked (data not shown). Pillemer et al. (1998) also demonstrated that *Xcad2* positively regulated ventral genes, including *Xpo, Xvent1,* and *Xvent2,* and also inhibited secondary axis formation, in the absence of BMP signaling.

It has been suggested that *Xcad2* controls the competence of cells' ability to respond to organizer inducing signals (Levy et al., 2002). In this study, we saw no abnormally sustained or increased expression levels of dorsal organizer genes during gastrulation in CREB embryos, as might be expected if *Xcad2* acted as competence terminator. However, a detailed in situ analysis may need to be performed to see more subtle changes in the localization of organizer genes in CREB-depleted embryos.

We found here that the expression of *Xcad2* and *-3* was at normal levels in neurula-stage CREB$^-$ embryos, when both maternal and zygotic CREB were depleted. It is unlikely that this is a nonspecific effect. Antisense oligos can cause a nonspecific developmental delay, resulting in the late appearance of gene expression. However, we show here that reintroduction of *CREB* mRNA, in embryos depleted of CREB with a high dose of antisense oligo, rescues the expression of *Xcad2* and *-3* at the gastrula stage. Furthermore, although *Xcad2* and *Xcad3* are delayed 6 h in their expression in CREB$^-$ embryos, other zygotic genes, including *siamois* and *BMP4,* are expressed at the correct times. It seems likely, therefore, that the later expression of *Xcad2* and *-3* is regulated by a second pathway independent of CREB. A candidate for the regulator of this *Xenopus* neurula pathway is the fibroblast growth factor, FGF, which has been shown to be required for both *Xcad3* and *Hox* gene regulation in the development of the posterior trunk and tail in *Xenopus* neurulae (Pownall et al., 1996).

Despite the rapid emergence of pattern in both the dorsal/ventral and primary germ layer (animal/vegetal) axes of the embryo, only two maternally encoded transcription factors (XTcf3 and VegT) have been shown to be necessary and sufficient to initiate patterning of the embryo. We show here that maternally encoded CREB should be included in this list of pattern initiators in the early *Xenopus* embryo.


A
Siamois
Xnr3
Chordin
CREB
GATA5
Xbra
Xcad2
BMP4
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Xsox17α
GATA4
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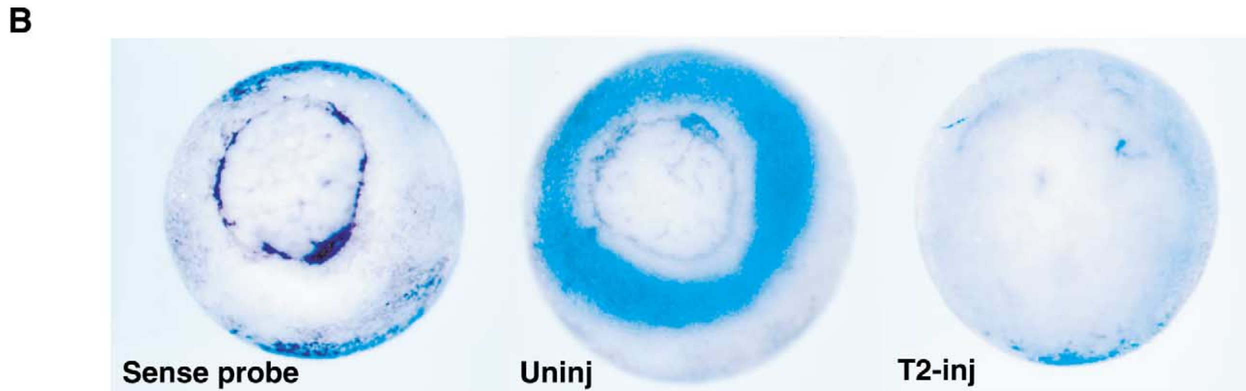
Xpo
Xcad3
% of ODC
Uninj
T2
Stage 10
Stage 11.5



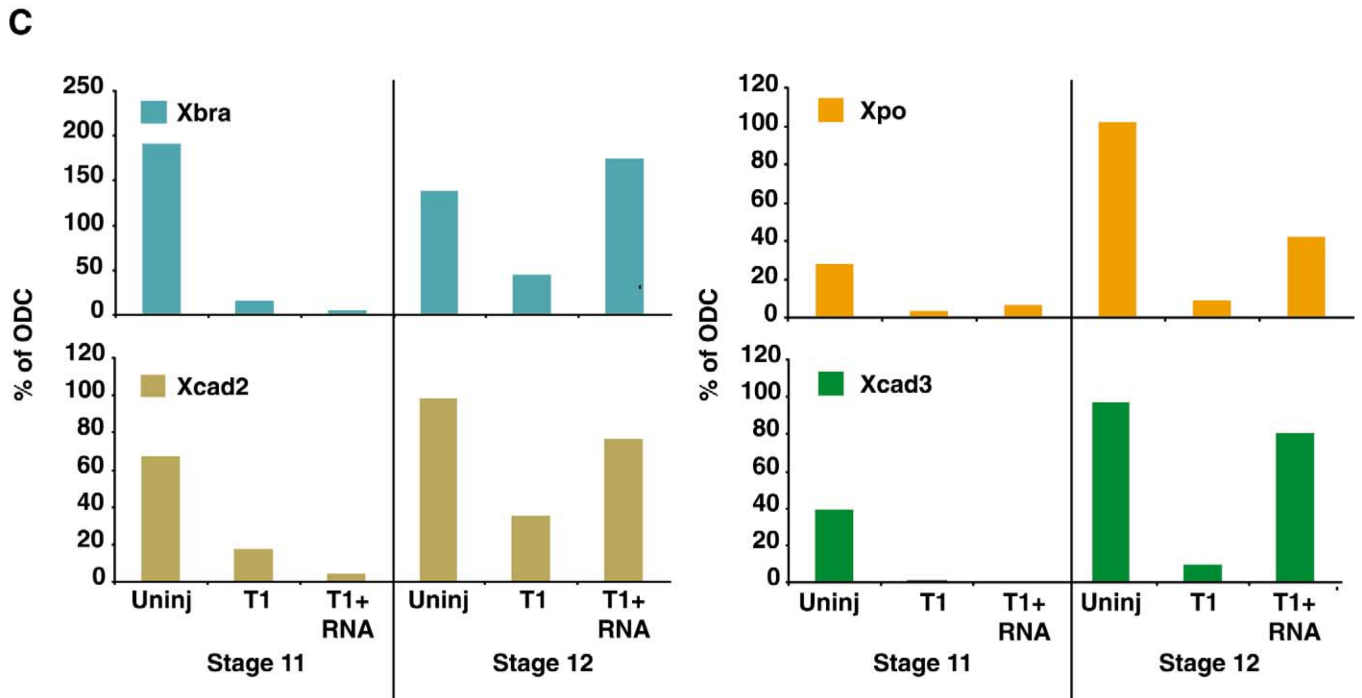


Fig. 7 (*continued*)

Fig. 7. Expression analysis of CREB depleted early gastrula embryos. Oocytes were injected with 4.5 ng of T2 and a host transfer experiment was carried out. (A) The resulting CREB-depleted embryos were collected at indicated stages and analyzed for the expression of various mesodermal markers, *Xpo, Xcad2, Xcad3, Xbra, Xwnt8, Siamois, Xnr3, BMP4, Xsox17α, GATA4, -5, -6,* and *CREB* by real time RT-PCR and normalized for expression of ODC. (B) CREB-depleted embryos were collected at early gastrula and fixed in MEMFA. In situ hybridization was carried out as described in Materials and methods, and expression of *Xbra* mRNA was analyzed. (C) Oocytes were injected with 4.5 ng antisense oligo T1. Forty-eight hours later, 600 pg of CACREB RNA was injected and a host transfer experiment was carried out as described. Embryos were collected at indicated stages, and RT-PCR analysis was carried out to analyze the expression of Xbra, *Xpo, Xcad*2, and -3. Expression levels were normalized to ODC.

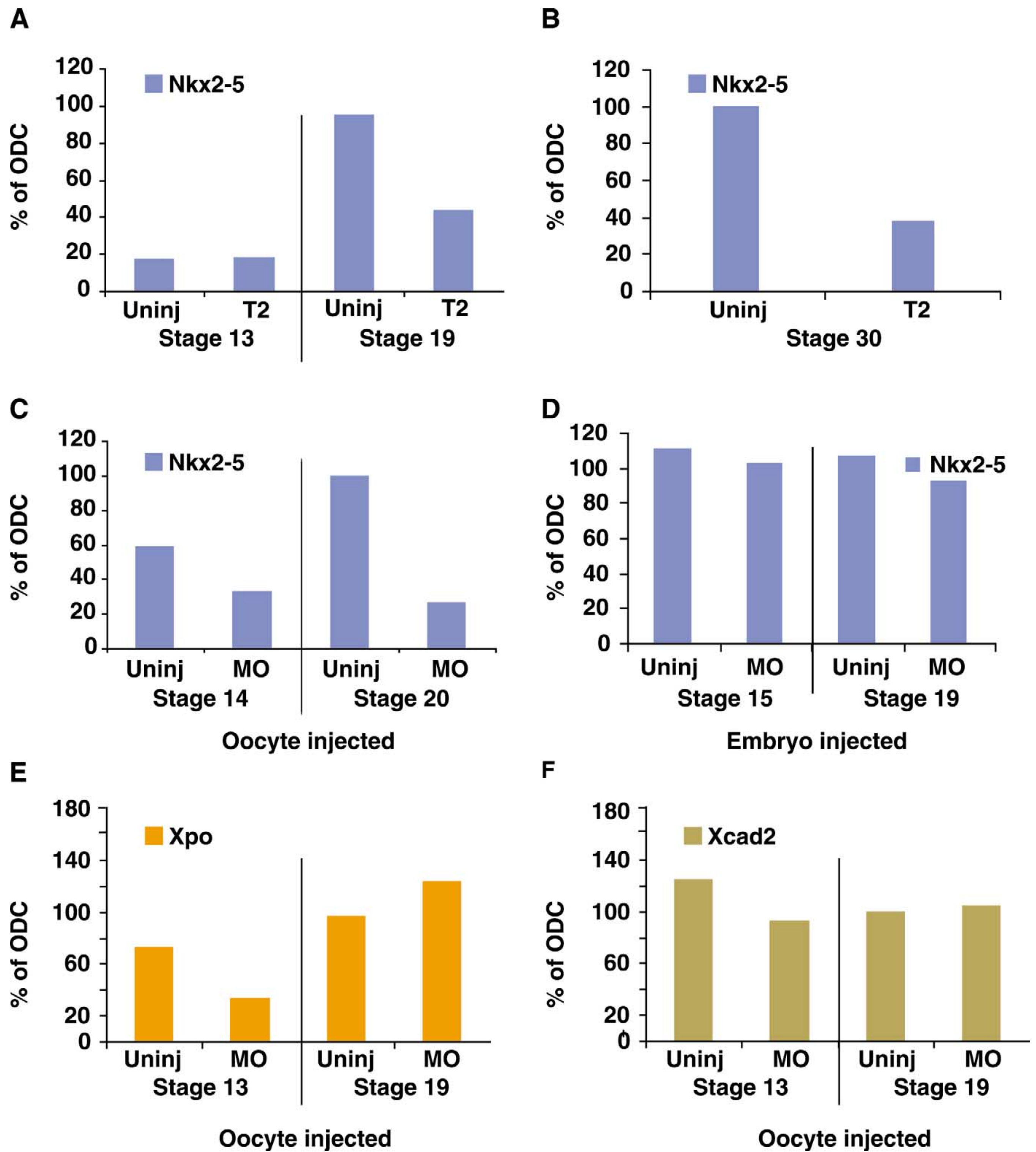


Fig. 8. Expression of *Nkx2-5* in CREB-depleted embryos. Oocytes were injected with 4.5 ng of T2 (A, B) or 6 ng of MO (C), and a host transfer experiment was carried out as described. Embryos were collected at indicated stages and analyzed for the expression of *Nkx2-5* by real time RT-PCR. (D) Two-to four-cell-stage embryos were injected with 6 ng of MO, and the embryos were analyzed at the indicated stages for the expression of *Nkx2-5*. (E, F) CREB-depleted embryos were collected at neurula stages for the expression of *Xcad2* and *Xpo* by real time RT-PCR. Expression levels were normalized for the expression of ODC.

Acknowledgments

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References

Alsan, B.H., Schultheiss, T.M., 2002. Regulation of avian cardiogenesis by Fgf8 signaling. Development 129, 1935–1943.

Andrew, D.J., Baig, A., Bhanot, P., Smolik, S.M., Henderson, K.D., 1997. The Drosophila dCREB-A gene is required for dorsal/ventral patterning of the larval cuticle. Development 124, 181–193.

Blackshear, P.J., Lai, W.S., Thorn, J.M., Kennington, E.A., Staffa, N.G., Moore, D.T., Bouffard, G.G., Beckstrom-Sternberg, S.M., Touchman, J.W., Bonaldo, M.F., Soares, M.B., 2001. The NIEHS Xenopus maternal EST project: interim analysis of the first 13,879 ESTs from unfertilized eggs. Gene 267, 71–87.

Bleckmann, S.C., Blendy, J.A., Rudolph, D., Monaghan, A.P., Schmid, W., Schutz, G., 2002. Activating transcription factor 1 and CREB are important for cell survival during early mouse development. Mol. Cell. Biol. 22, 1919–1925.

Bodmer, R., 1993. The gene tinman is required for specification of the heart and visceral muscles in Drosophila [published erratum appears in Development 1994 Nov; 119(3):969]. Development 118, 719–729.

Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., Silva, A.J., 1994. Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. Cell 79, 59–68.

Cleaver, O.B., Patterson, K.D., Krieg, P.A., 1996. Overexpression of the tinman-related genes XNkx-2.5 and XNkx-2.3 in Xenopus embryos results in myocardial hyperplasia. Development 122, 3549–3556.

Conlon, F.L., Sedgwick, S.G., Weston, K.M., Smith, J.C., 1996. Inhibition of Xbra transcription activation causes defects in mesodermal patterning and reveals autoregulation of Xbra in dorsal mesoderm. Development 122, 2427–2435.

Conlon, F.L., Smith, J.C., 1999. Interference with brachyury function inhibits convergent extension, causes apoptosis, and reveals separate requirements in the FGF and activin signalling pathways. Dev. Biol. 213, 85–100.

Ding, X., Hausen, P., Steinbeisser, H., 1998. Pre-MBT patterning of early gene regulation in Xenopus: the role of the cortical rotation and mesoderm induction. Mech. Dev. 70, 15–24.

Eresh, S., Riese, J., Jackson, D.B., Bohmann, D., Bienz, M., 1997. A CREB-binding site as a target for decapentaplegic signalling during Drosophila endoderm induction. EMBO J. 16, 2014–2022.

Finkbeiner, S., Tavazoie, S.F., Maloratsky, A., Jacobs, K.M., Harris, K.M., Greenberg, M.E., 1997. CREB: a major mediator of neuronal neurotrophin responses. Neuron 19, 1031–1047.

Harland, R.M., 1991. In situ hybridization: an improved whole-mount method for Xenopus embryos. Methods Cell Biol. 36, 685–695.

Hawley, S.H., Wunnenberg-Stapleton, K., Hashimoto, C., Laurent, M.N., Watabe, T., Blumberg, B.W., Cho, K.W., 1995. Disruption of BMP signals in embryonic Xenopus ectoderm leads to direct neural induction. Genes Dev. 9, 2923–2935.

Heasman, J., 2002. Morpholino oligos: making sense of antisense? Dev. Biol. 243, 209–214.

Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C.Y., Wylie, C., 1994. Overexpression of cadherins and underexpression of β-catenin inhibit dorsal mesoderm induction in early Xenopus embryos. Cell 79, 791–803.

Heasman, J., Holwill, S., Wylie, C.C., 1991. Fertilization of cultured Xenopus oocytes and use in studies of maternally inherited molecules. Methods Cell Biol. 36, 213–230.

Heasman, J., Kofron, M., Wylie, C., 2000. β-Catenin signaling activity dissected in the early Xenopus embryo: a novel antisense approach. Dev. Biol. 222, 124–134.

Houston, D.W., Kofron, M., Resnik, E., Langland, R., Destree, O., Wylie, C., Heasman, J., 2002. Repression of organizer genes in dorsal and ventral Xenopus cells mediated by maternal XTcf3. Development 129, 4015–4025.

Klemm, D.J., Roesler, W.J., Boras, T., Colton, L.A., Felder, K., Reusch, J.E., 1998. Insulin stimulates cAMP-response element binding protein activity in HepG2 and 3T3-L1 cell lines. J. Biol. Chem. 273, 917–923.

Kofron, M., Demel, T., Xanthos, J., Lohr, J., Sun, B., Sive, H., Osada, S., Wright, C., Wylie, C., Heasman, J., 1999. Mesoderm induction in Xenopus is a zygotic event regulated by maternal VegT via TGFbeta growth factors. Development 126, 5759–5770.

Kofron, M., Klein, P., Zhang, F., Houston, D.W., Schaible, K., Wylie, C., Heasman, J., 2001. The role of maternal axin in patterning the Xenopus embryo. Dev. Biol. 237, 183–201.

Lee, M.A., Heasman, J., Whitman, M., 2001. Timing of endogenous activin-like signals and regional specification of the Xenopus embryo. Development 128, 2939–2952.

Lerchner, W., Latinkic, B.V., Remacle, J.E., Huylebroeck, D., Smith, J.C., 2000. Region-specific activation of the Xenopus brachyury promoter involves active repression in ectoderm and endoderm: a study using transgenic frog embryos. Development 127, 2729–2739.

Levy, V., Marom, K., Zins, S., Koutsia, N., Yelin, R., Fainsod, A., 2002. The competence of marginal zone cells to become Spemann's organizer is controlled by Xcad2. Dev. Biol. 248, 40–51.

Lickert, H., Kutsch, S., Kanzler, B., Tamai, Y., Taketo, M.M., Kemler, R., 2002. Formation of multiple hearts in mice following deletion of β-catenin in the embryonic endoderm. Dev. Cell 3, 171–181.

Lutz, B., Schmid, W., Niehrs, C., Schutz, G., 1999. Essential role of CREB family proteins during Xenopus embryogenesis. Mech. Dev. 88, 55–66.

Montminy, M., 1997. Transcriptional regulation by cyclic AMP. Annu. Rev. Biochem. 66, 807–822.

Montminy, M.R., Bilezikjian, L.M., 1987. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature 328, 175–178.

Nantel, F., Sassone-Corsi, P., 1996. CREM: A transcriptional master switch during the spermatogenesis differentiation program. Front. Biosci. 1, d266–d269.

Pandur, P., Lasche, M., Eisenberg, L.M., Kuhl, M., 2002. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. Nature 418, 636–641.

Pende, M., Fisher, T.L., Simpson, P.B., Russell, J.T., Blenis, J., Gallo, V., 1997. Neurotransmitter- and growth factor-induced cAMP response element binding protein phosphorylation in glial cell progenitors: role of calcium ions, protein kinase C, and mitogen-activated protein kinase/ribosomal S6 kinase pathway. J. Neurosci. 17, 1291–1301.

Pillemer, G., Yelin, R., Epstein, M., Gont, L., Frumkin, Y., Yisraeli, J.K., Steinbeisser, H., Fainsod, A., 1998. The Xcad-2 gene can provide a ventral signal independent of BMP-4. Mech. Dev. 74, 133–143.

Pownall, M.E., Tucker, A.S., Slack, J.M., Isaacs, H.V., 1996. eFGF, Xcad3 and Hox genes form a molecular pathway that establishes the anteroposterior axis in Xenopus. Development 122, 3881–3892.

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

Reiter, J.F., Verkade, H., Stainier, D.Y., 2001. Bmp2b and Oep promote early myocardial differentiation through their regulation of gata5. Dev. Biol. 234, 330–338.

Rudolph, D., Tafuri, A., Gass, P., Hammerling, G.J., Arnold, B., Schutz, G., 1998. Impaired fetal T cell development and perinatal lethality in

mice lacking the cAMP response element binding protein. Proc. Natl. Acad. Sci. USA 95, 4481–4486.
Sater, A.K., Jacobson, A.G., 1989. The specification of heart mesoderm occurs during gastrulation in *Xenopus laevis*. Development 105, 821–830.
Searcy, R.D., Vincent, E.B., Liberatore, C.M., Yutzey, K.E., 1998. A GATA-dependent nkx-2.5 regulatory element activates early cardiac gene expression in transgenic mice. Development 125, 4461–4470.
Shaywitz, A.J., Greenberg, M.E., 1999. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu. Rev. Biochem. 68, 821–861.
Shi, Y., Katsev, S., Cai, C., Evans, S., 2000. BMP signaling is required for heart formation in vertebrates. Dev. Biol. 224, 226–237.
Sordella, R., Classon, M., Hu, K.Q., Matheson, S.F., Brouns, M.R., Fine, B., Zhang, L., Takami, H., Yamada, Y., Settleman, J., 2002. Modulation of CREB activity by the Rho GTPase regulates cell and organism size during mouse embryonic development. Dev. Cell 2, 553–565.
Sparrow, D.B., Cai, C., Kotecha, S., Latinkic, B., Cooper, B., Towers, N., Evans, S.M., Mohun, T.J., 2000. Regulation of the tinman homologues in Xenopus embryos. Dev. Biol. 227, 65–79.
Sparrow, D.B., Kotecha, S., Towers, N., Mohun, T.J., 1998. Xenopus eHAND: a marker for the developing cardiovascular system of the embryo that is regulated by bone morphogenetic proteins. Mech. Dev. 71, 151–163.
Tada, M., Smith, J.C., 2000. Xwnt11 is a target of Xenopus Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. Development 127, 2227–2238.
Venkatesh, T.V., Park, M., Ocorr, K., Nemaceck, J., Golden, K., Wemple, M., Bodmer, R., 2000. Cardiac enhancer activity of the homeobox gene tinman depends on CREB consensus binding sites in Drosophila. Genesis 26, 55–66.
Walters, M.J., Wayman, G.A., Christian, J.L., 2001. Bone morphogenetic protein function is required for terminal differentiation of the heart but not for early expression of cardiac marker genes. Mech. Dev. 100, 263–273.
Woolf, T.M., Jennings, C.G., Rebagliati, M., Melton, D.A., 1990. The stability, toxicity and effectiveness of unmodified and phosphorothioate antisense oligodeoxynucleotides in Xenopus oocytes and embryos. Nucleic Acids Res. 18, 1763–1769.
Wylie, C., Kofron, M., Payne, C., Anderson, R., Hosobuchi, M., Joseph, E., Heasman, J., 1996. Maternal β-catenin establishes a "dorsal signal" in early Xenopus embryos. Development 122, 2987–2996.
Xanthos, J.B., Kofron, M., Tao, Q., Schaible, K., Wylie, C., Heasman, J., 2002. The roles of three signaling pathways in the formation and function of the Spemann Organizer. Development 129, 4027–4043.
Xanthos, J.B., Kofron, M., Wylie, C., Heasman, J., 2001. Maternal VegT is the initiator of a molecular network specifying endoderm in Xenopus laevis. Development 128, 167–180.
Yokota, C., Kofron, M., Zuck, M., Houston, D.W., Isaacs, H., Asashima, M., Wylie, C.C., Heasman, J., 2003. A novel role for a nodal-related protein; Xnr3 regulates convergent extension movements via the FGF receptor. Development 130, 2199–2212.
Zhang, J., Houston, D.W., King, M.L., Payne, C., Wylie, C., Heasman, J., 1998. The role of maternal VegT in establishing the primary germ layers in Xenopus embryos. Cell 94, 515–524.
Zuck, M.V., Wylie, C.C., Heasman, J., 1998. Maternal mRNAs in Xenopus embryos: an antisense approach, in: Richter, J.D. (Ed.), A Comparative Methods Approach to the Study of Oocytes and Embryos. Oxford Univ. Press, Oxford, UK, pp. 341–354.