Chapter II

Expression and function of *blimp1/krox,* an alternatively transcribed regulatory gene of the sea urchin endomesoderm network

Carolina B. Livi and Eric H. Davidson

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Abstract

The *blimp1/krox* gene of Strongylocentrotus purpuratus, formerly *krox1*, encodes zinc finger transcription factors that play a central role in both early and late endomesoderm specification. Here, we show that there are two alternative splice forms transcribed under the control of different regulatory regions. The *blimp1/krox1b* form was previously unknown, and is the form expressed during cleavage, beginning 6–9 h postfertilization. This form is required for the early events of endomesoderm specification. A different splice variant, *blimp1/krox1a*, is expressed only from gastrula stage onward. During cleavage stages, the *blimp1/krox* gene is expressed in the large micromeres and veg2 descendents. Soon after, it is expressed in the ring of specified mesoderm cells at the vegetal pole of the blastula. Its expression is later restricted to the blastopore region and the posterior of the invaginating archenteron, and finally to the midgut and hindgut of the pluteus larva. The expression of *blimp1/krox* is dynamic, and involves several distinct spatial territories. A GFP-recombinant BAC was created by substituting the GFP coding sequence for that of the second exon (1b) in order to distinguish the expression pattern of the early form from that of the late form. This construct closely mimics *blimp1/krox1b* expression during early stages of sea urchin development. To expand our knowledge of the downstream linkages of this gene, additional experiments were carried out using antisense morpholino oligos (MASO). We confirmed previously-published data that Blimp1/krox autoregulates its own expression, but discovered, surprisingly, that this gene represses rather than activates itself. This negative autoregulation is restricted to the mesodermal and probably skeletogenic territories during the blastula stage, as shown by

in situ hybridization analysis of MASO-injected embryos. The MASO perturbation analysis also revealed Blimp1/krox inputs into other genes of the endomesoderm regulatory network.

Keywords: Strongylocentrotus purpuratus; SET domain; PR domain; Cys2His2 zinc finger transcription factor; Endomesodermal specification; Gene regulatory network; Alternative splice forms

JEL classification: *blimp1/krox*; *krox1*; *blimp1*; *blimp-1*; *blmp-1*; *prdm1*; *prdi-bf1*; *znfpr1a1*; *ubo*; *odd-3*

Introduction

In sea urchins, territorial specification is initiated early in cleavage. The endomesoderm lineages originate from the vegetal half of the embryo. This process involves maternal determinants segregated to this area as well as intercellular signaling interactions (Davidson, 1986 and Davidson, 1989; for recent reviews Ransick and Davidson, 1998, Davidson, 2001 and Angerer and Angerer, 2003). General understanding of the transcriptional control of endomesoderm specification in the sea urchin embryo is summarized in a gene regulatory network (EM-GRN) connecting more than 40 regulatory genes that are expressed between egg and late blastula stage embryos (Davidson et al., 2002a, Davidson et al., 2002b, Oliveri and Davidson, 2004 and Levine and Davidson, 2005). Individual genes encoding regulatory factors have been linked into the GRN by means of experimental perturbations in which expression of the gene is either knocked down or its product is replaced with a dominant negative form. The linkages between genes indicated in the GRN provide predictions of the *cis*-regulatory interactions that drive the process. These predictions are tested through *cis*-regulatory analysis of functional elements capable of generating expression in the correct spatial and temporal domains (for current review, Levine and Davidson, 2005; a continually updated version of the network, and supporting data, are at http://sugp.caltech.edu/endomes/).

Among the early zygotic regulatory genes required for specification of the sea urchin embryo endomesoderm is the *blimp1/krox* gene. This gene (initially called *krox1*) was cloned from a gastrula stage cDNA library in a screen for zinc finger transcription

factors, and was reported to be transiently expressed in the vegetal plate territory (Wang et al., 1996). Its encoded protein is most similar to B-lymphocyte-induced maturation protein 1 (Blimp-1) in vertebrates, a transcriptional regulator of B-cells (Chang and Calame, 2002, Chang et al., 2000 and Siammas and Davis, 2004). Here, we rename the gene *blimp1/krox*, as it is in fact not a member of the krüppel gene family, as originally claimed. The *blimp1/krox* factor is a member of the SET domain family of proteins that recruits methyltransferases, which may directly modify histones (Jenuwein, 2001). "SET" stands for Su(var)3–9, Enhancer-of-zeste, and Trithorax, all proteins which contain this functional domain. The structure of the SET domain has been established in yeast to have an overall fold rich in beta-strands, a potential active site consisting of a SAM binding pocket, and a connected groove that could accommodate the binding of the N-terminal tail of histone H3 (Min et al., 2002). The "PR" subset of SET domains, the one present in Blimp1 family members, is somewhat divergent. It mediates protein–protein interactions, and its orthologue in mice (synonyms: Prdm1, and PrdI-bf) has been shown to have a modular structure, such that particular domains are required for the regulation of subsets of its downstream target genes (Siammas and Davis, 2004). In humans, the PrdI–Bf1 (Blimp1) protein products act as transcriptional repressors in myeloid cells and recruit methyltransferases to promoter sites where they induce histone H3 methylation (Györy et al., 2003 and Györy et al., 2004). *blimp1* null (-/-) mice die during early embryogenesis, as this gene plays an important role in gastrulation. Conditional knockouts in B-cell lines show that it is essential for their differentiation into plasma cells (Shaffer et al., 2004 and Siammas and Davis, 2004). In mice, it is the balance of expression between the two alternatively spliced isoforms, one of which is missing the PR domain, that regulates

cellular proliferation and differentiation (Gyory et al., 2003). Mouse blimp-1 is also required for germ cell development (Ohinata et al., 2005). In chicks, this gene is expressed in the apical ectodermal ridge and posterior dorsal ectoderm of developing limb buds (Ha and Riddle, 2003). Xenopus *blimp1* has an important role in endomesoderm specification, acting to promote anterior endoderm development and spatially restricting mesoderm formation (de Souza et al., 1999). Recently, the zebrafish orthologue of *blimp-1*, also called *ubo*, has been shown to be important for gastrulation, muscle specification, and neural crest development (Baxendale et al., 2004 and Roy and Ng, 2004). The *blimp-1* gene is essential for slow-twitch muscle fiber specification, and besides repressing fast MyHC, it also acts as a positive activator of the slow MyHC isoform and Prox1 proteins (Baxendale et al., 2004). Thus, this gene has many different functions, a feature that, as we shall see, it displays in sea urchin embryos as well.

As previously reported, injection of mRNA into sea urchin eggs encoding the DNA binding domain of Blimp1/krox fused to the repressor domain of the Drosophila Engrailed factor revealed some of its downstream target genes in the EM-GRN (Davidson et al., 2002a and Davidson et al., 2002b). We showed that *blimp1/krox* is necessary for initiation and maintenance of the expression of *otx* in the endomesoderm, and this was subsequently confirmed to be a direct *cis*-regulatory function (Yuh et al., 2004). It is also important for the specification of endodermal cells from the veg1 tier, where it regulates *eve* and *hox11/13b*. In the present work, we demonstrate that the *blimp1/krox* gene produces splice isoforms that are alternatively transcribed, and alternative splicing is a conserved feature of this gene in deuterostomes. The two

isoforms are expressed in a different spatial–temporal pattern. The early form is *blimp1/krox1b*, and this is the form present during endomesoderm specification in the period modeled by the EM-GRN. Expression of the late form, *blimp1/krox1a*, begins only in the early gastrula. Therefore, the *cis*-regulatory control system operating the 1b transcription unit is the one relevant to the EM-GRN.

Materials and methods

5′ Race and sequencing

A 10-h postfertilization (hpf) race library made using the GeneRacer Kit (Invitrogen, Carlsbad, CA) was used to establish the sequence of the message further 5′ of the known *blimp1/krox* mRNA. A primer in exon 2 (blimp1/krox race R: 5′-TGTCAGACGGCACGGCGTTGTCGTTGCA-3′) was used. The resulting fragments were subcloned into a TA cloning vector (pGEMTeasy, Invitrogen, Carlsbad, CA) and sequenced in an ABI 377 sequencer using ABI Prism BigDye Terminator Cycle Sequencing (Applied Biosystems, Foster City, CA). The resulting sequences were blasted against the blimp1/krox cDNA and Spblimp1/krox BAC sequences using the BLAST feature in Family Relations (Brown et al., 2002 and Brown et al., 2005).

Embryo handling

Adult Strongylocentrotus purpuratus (S. purpuratus) animals were maintained at the Caltech Marine Biological Laboratory, and gametes were shed according to standard procedures (Leahy, 1986). Embryos were obtained, cultured, collected and microinjected as previously described (Foltz et al., 2004 and Cheers and Ettensohn, 2004) with minor modifications. All injection solutions contained 15% glycerol and 0.12M KCl, and were injected into fertilized eggs using a Picospritzer (Parker Instrumentation, Fairfield, NJ). When injecting, we attempted to cause a clearing in the egg cytoplasm of 1/5 to 1/4 of the egg diameter corresponding to a 2- to 4-pl injection volume.

Morpholino oligonucleotide sequences

The sequence of the anti-blimp1/krox1b MASO which is targeted against the translation initiation site of the early form is 5′-CTCCCTTTCGCTTGAAAAACACCGC-3′ (complementary to nucleotide positions -27 to -3 with respect to the translational start site of blimp1/krox1b mRNA). We injected 2 to 4 pl at a concentration of 100 μM of this morpholino when indicated, in conjunction with a MASO targeting the late form, anti-blimp1/krox1a M1: 5′-AGACGGCACGGCGTTGTCGTTGCAC-3′ (nt position +6 to +31 of *blimp1/krox1a* mRNA) or Anti-blimp1/krox1a M2: 5′-CGGCGTTGTCGTTGCACCCCATCGC-3′ (nt position -3 to +22 of *blimp1/krox1a* mRNA) at 200 μM concentrations. In all experiments, as a negative control, embryos injected with 300 μM of the standard control morpholino (SCM) were included. All morpholinos were acquired from Gene Tools (Corvallis, OR).

blimp1/krox knockdown perturbation

Morpholino oligos were resuspended in water to a concentration of 500 μM or 1 mM. A working solution of 100 to 300 μM of morpholino oligos in 15% glycerol and 0.12 M KCl was injected into fertilized eggs. The standard control morpholino (SCM) from GeneTools (Corvallis, OR) was used at equal or greater concentration as a control in every experiment and compared side-by-side with uninjected and MASO embryos (Angerer and Angerer, 2004). The efficacy of the anti-blimp1/krox1a and anti-blimp1/krox1b morpholinos was assessed in initial experiments through co-injection with GFP mRNA containing sequence complementary to the respective morpholino (data not shown).

BAC recovery and sequencing

Spblimp1/krox (clone 163O19) and Lvblimp1/krox (clone 60B16) BACs were obtained by hybridization of a Spblimp1/krox cDNA fragment to arrayed genomic BAC libraries for S. purpuratus and Lytechinus variegatus (L. variegatus) respectively (Cameron et al., 2000 and Cameron et al., 2004). Spblimp1/krox and Lvblimp1/krox BACs were sequenced by the DOE's Joint Genome Institute (Genebank accession nos. AC131508; AC131502).

BAC sequence annotation

The BAC sequences were annotated using the Sea Urchin Genome Annotation Resource (SUGAR) as well as Family Relations (Brown et al., 2002 and Brown et al., 2005).

BAC homologous recombination

Using the homologous recombination machinery from bacterial cells, the sequence coding for exon1b from Spblimp1/krox BAC 163o19 was substituted for that of green fluorescent protein (Yu et al., 2000). To create the cassette containing GFP and kanamycin with flanking regions homologous to the BAC, a PCR approach was taken. Briefly, 45 bp of sequence homologous to the BAC on the 5′ end of the region to be recombined is attached to the 5′ end of the GFP primer (Sp_blimp1/krox_(1b)right: 5′CTGCCCCATTCATCACATTTTCAACAATCTGAGTCGACAGATGACTCGAAGA GCTATTCCAGAAGTAGTGA-3′) such that it is added to the product when the primer is used to amplify the construct containing the GFP/Km cassette. In the same manner, 45 bp of sequence homologous to the 3′ end of the region to be flipped out is attached to the 5′ end of the kanamycin primer (Sp_blimp1/krox_(1b)left: 5′TTGTTGTGATTTTGTACCGCGGTGTTTTTCAAGCGAAAGGGAGAAATGAGC AAGGGCGAGGAACT-3′). After PCR amplification using the two primers containing the homologous sequence tails and the construct containing the cassette as the template, the product was purified using MiniElute PCR Purification Kit (Qiagen, Valencia, CA), and subsequently digested with DpnI (New England Biolabs, Ipswich, MA) to remove traces of the original cassette. This fragment was used to transform competent EL250

cells containing the BAC construct, and the kanamicyn gene was removed as previously described (Yu et al., 2000). Recombinant BACs were screened by sizing the inserts using PCR and subsequently sequenced using outside flanking primers (Out-Sp1b-F: 5′-CTCATCTACTTTCGCTGCCAGTACT-3′, and Out-Sp1b-R: 5′-CTCATTATAGTTGATGGACATACTCATATC-3′).

Recombinant GFP-BAC transgenesis

Spblimp1/krox1b-GFP BAC was purified using Maxi NucleoBond® Plasmid Kit according to instructions from the manufacturer (Clontech, Mountain View, CA). After linearizing using AscI (New England Biolabs, Ipswich, MA), the digested BAC was loaded onto a CL4b Sepharose column (Pharmacia, Uppsala, Sweden) and fractionated into small aliquots (Hammes and Schedl, 2001). Optical densities were taken from each fraction at 260 and 280, and the first to contain a significant amount of DNA was used for microinjection. The size and quality of the fractions were accessed by pulse-field gel electrophoresis. The injection solution contained 500 molecules per 2 pl of Spblimp1/krox1b-GFP BAC. Embryos were injected as described by Rast (2000), with minor modifications. No carrier DNA was added, as BACs are long enough not to require it for linear incorporation. A final concentration of 15% glycerol with 0.12 M KCl was used.

WMISH probes

Digoxigenin-labeled RNA probes were made as previously described (Yuh et al., 2002). Briefly, gene fragments were amplified by PCR and subcloned into and TA cloning vector (Invitrogen, Carlsbad, CA). Constructs were linearized 3′ of the probe sequence in relation to the transcriptase promoter used. All probes corresponded to the antisense as well as the sense direction. No staining was observed using the sense probes (Fig. 12, see Supplemental Materials in online version of this article). blimp1/krox Probe Primer F: 5′-TTCTTCCGATCACCTTGCTG-3′, and blimp1/krox Probe Primer R: 5′-GAAAGATAGCCATTGGAATCTGC-3′.

WMISH

Whole mount in situ hybridizations were performed as previously described (Minokawa et al., 2004), with minor modifications. Embryos were collected at different developmental stages and fixed in 4% paraformaldehyde, 32.5% filtered seawater, 32.5 mM MOPS (pH 0.7) and 162.5 mM NaCl. When looking at endogenous message distribution, a hybridization buffer containing 70% formamide was used. When looking at message generate from a transgene, such as GFP, the hybridization buffer contained 50% formamide. In both cases, the embryos were hybridized for 5 to 8 days at 48°C with occasional mixing. An additional high-temperature wash in MOPS buffer was added after the high-temperature wash in hybridization buffer. Embryos were mounted in 50% glycerol, visualized using Nomarsky optics, and imaged with a color digital camera. Images were collected and processed using Adobe Photoshop.

QPCR

Temporal accumulation of messages was monitored using real-time quantitative polymerase chain reaction (QPCR). Approximately 500 embryos from different stages were collected. RNA was isolated using an RNAeasy micro kit (Qiagen, Valencia, CA), and DNase treated using a DNA-free kit (Ambion, Austin, TX) according to instructions from the manufacturer. Reverse transcription into cDNA was performed using a Taqman Gold RT kit following instructions from the manufacturer (Applied Biosystems, Foster City, CA). Quantitation of the mRNA was performed as described by Oliveri et al. (2002).

The expression of putative downstream targets of *blimp1/krox* was monitored by QPCR. Either 100 or 200 injected embryos were collected for RNA isolation into RNA-Bee (Leedo Medical Laboratories, Houston TX). Reverse transcription into cDNA was performed using a Taqman Gold RT kit following instructions from the manufacturer (Applied Biosystems, Foster City, CA). Fold changes in expression in control and morpholino-injected embryos were calculated as previously described (Davidson et al., 2002a and Davidson et al., 2002b).

Briefly, the equivalent of two embryos was used as a template in each reaction in the presence of 5 pmol of each primer (forward and reverse). Primer sequences can be found at http://sugp.caltech.edu/resources/methods/q-pcr.psp or are listed below. SYBR Green was used to monitor product accumulation in real time and ROX was used as a measure

of background fluorescence in a 7900 (Applied Biosystems, Foster City, CA) as previously described (Rast et al., 2000 and Ransick et al., 2002).

The very short length of the 1a and 1b exons (116 bp, and 226 bp, respectively) made finding appropriate primer pairs that would amplify only one or the other splice form difficult. For this reason, we used more than one set of primers. Three independent oligo primer pairs were synthesized to amplify the *blimp1/krox1a* message, and two independent oligo primer pairs were designed for *blimp1/krox1b*. They were utilized in QPCR experiments to measure the transcripts present in sea urchin embryos over time.

Spblimp1/krox exon1a only F: 5′-AAGCACTTGCTTGCTGTTACC-3′

Spblimp1/krox exon1a only R: 5′-AAAATAGCTTGCGGTTTCAATC-3′

Spblimp1/krox exon1a + 2 F1: 5′-GGAAAGCACTTGCTTGCTGT-3′

Spblimp1/krox exon1a + 2 R1: 5′-CGAAGACCTGATCGAAGACC-3′

Spblimp1/krox exon1a + 2 F2: 5′-CGATTGAAACCGCAAGCTAT-3′

Spblimp1/krox exon1a + 2 R2: 5′-ATCGACCTCGGTCATGTCA-3′

Spblimp1/krox exon1b only F: 5′-GCGAGGGTGTTCAACGATA-3′

Spblimp1/krox exon1b only R: 5′-TCAAGGATAGCGGACACTCA-3′

Spblimp1/krox exon1b + 2 F: 5′-CTAGCAATGCGGGATCTCTACT-3′

Spblimp1/krox exon1b + 2 R: 5′-CGAAGACCTGATCGAAGACC-3′

Protein sequence alignment and phylogenetic tree

Nucleotide sequences were translated using the Expasy-translate tool (http://us.expasy.org/tools/dna.html). Available protein sequences (as indicated below) were aligned using ClustalX version 1.81 (Thompson et al., 1997). Alignment output file was formatted using Boxshade (http://www.ch.embnet.org/software/BOX_form.html). A neighbor-joining tree was constructed using MEGA version 2.1 or 3 (Kumar et al., 2001 and Kumar et al., 2003) and tested by bootstrapping using default parameters. Protein domains were mapped using InterProScan (http://www.ebi.ac.uk/InterProScan/).

Species abbreviations: Sp = *Strongylocentrotus purpuratus*; Lv = *Lytechinus variegatus*; Am1 = *Asterina miniata*; Tn = *Tetraodon nigroviridis*; Fr = *Fugu rubripes* (*Takifugu rubripes*); Dr = Danio rerio; Xl = *Xenopus laevis*; Gg = *Gallus gallus*; Mm = *Mus musculus*; Rn = *Rattus norvegicus*; Pt = *Pan troglodytes*; Hs = *Homo sapiens*; Ce = *Caenorhabditis elegans*; Cb = *Caenorhabditis briggsae*; Dm = *Drosophila melanogaster*; Ag = *Anopheles gambiae*; Am2 = *Apis mellifera*.

Accession Numbers for sequences used in alignment: GeneBank accession nos. DQ225099, DQ177152, AY196329, AY196905, AY497217, CAG11080, AB126229, AF182280, AC147720, AF305534S6, XM_228320, XM_518658, AF084199, Z78418, CAE58934, AY071225, XM_391847, XP_316619.

Spblimp1/krox1a: DQ225099; Spblimp1/krox1b: DQ177152; Amblimp/krox-alpha: AY196329; Amblimp/krox-beta: AY196905; Drblimp1: AY497217; Tnblimp1: CAG11080; Frblimp1: AB126229; Xlblimp1: AF182280; Ggblimp1: AC147720;

Mmblimp1: AF305534S6; Rnblimp1: XM_228320; Ptblimp1: XM_518658; Hsblimp1: AF084199; Ceblmp-1: Z78418; Cbblmp-1, CAE58934; Dmblimp-1: AY071225, Amblimp-1: XM_391847, Agblimp-1: XP_316619.

Diagrams, graphs and line drawings

Figures were made using Adobe Illustrator CS or Adobe Photoshop CS. Gene network diagrams were made using BioTapestry version 2.1 (Longabaugh et al., 2005). Temporal expression graph was drawn using GraphPad Prism 4.

Results

Gene structure and isolation of early splice form

The blimp1/krox locus is 42 kbp and the gene is split into seven exons (Figs. 1A, B). A nucleotide alignment between the BAC sequence and cDNA sequences can be found in Supplemental Materials. Exons 1a and 6 contain only nontranslated sequence, and the 3′ UTR is quite long. The genomic organization of the locus is conserved between S. purpuratus and L. variegatus (data not shown). There are no other genes predicted within this region.

Fragments obtained from a 5′ race library contained a novel sequence that aligned to the blimp1/krox BAC genomic sequence between the previously known blimp1/krox

exon 1 and exon 2 (see Fig. 1 of Supplemental Materials). We named the 5′ most exon of blimp1/krox "1a" and the following one "1b" as they are alternatively used. The blimp1/krox1b form was found to be an alternative splice form by QPCR amplification of cDNAs from different embryonic stages, using primers that would recognize this new sequence. This exon contains a 5′ UTR as well as coding sequence for 50 extra amino acids when spliced to exon 2 (Fig. 2B and Supplemental Materials). Therefore, this isoform utilizes a different translation initiation sequence from that of the other splice form, in which the first exon contains only 5′ UTR (Fig. 1 and Fig. 2). There are three ATGs in frame within exon 1b that could act as translation initiation sites (Fig. 1B), but only the most upstream one has a consensus Kozac sequence. As the predicted amino acid sequence from L. variegatus also corresponds to this longer form, we assume that the most-upstream ATG, yielding the longer peptide, is the one utilized. Exons 1a and 1b utilize different transcription initiation sites, and, as will be described elsewhere, distinct regulatory apparatus. All other exons are included in both of the splice forms (sequence from exon 2 was part of the isolated fragment from the race library). The newly-described exon 1b is highly conserved between *S. purpuratus* and *L. variegatus* (full alignment is shown in Supplemental Materials; *L. variegatus* diverged from *S. purpuratus* approximately 50 mya; Smith, 1988 and Lee, 2003). *Asterina miniata* (*A. miniata*), a starfish, likewise has two similar alternative splice forms (Hinman and Davidson, 2003). However, the additional N-terminal amino acids encoded by sea urchin exon 1b are not well-conserved in starfish (Supplemental Materials).

The proteins encoded by the blimp1/krox are 703 and 753 amino acids long, and correspond to the late and early forms respectively (Figs. 2A, B). The translation is different from that previously published on the basis of a cDNA clone (Wang et al., 1996) due to a stop codon in the BAC sequence in the absence of which the peptide would be 837 amino acids in length. It is unclear if this difference is the result of a polymorphism in the population, a sequencing error, or a mutation in the clone isolated. All recognized protein domains are present using either translation. The protein includes classic Cys2His2 (C2H2) zinc fingers that are characterized by the sequence C(X)2–4,C(X)8, H(X)3–5, H (Evans and Hollenberg, 1988).

Phylogenetic analysis

The multiple alignment of Fig. 2C and the phylogenetic analysis of Fig. 3 indicate that sea urchin blimp1/krox is indeed the orthologue of the vertebrate blimp1/prdm1/prdI-bf1 genes. The two most prominent domains present in the blimp1/krox protein are the SET domain (more specifically a PR domain) and the four DNA-binding C2H2 zinc fingers followed by a fifth divergent zinc finger. As shown in Figs. 2C, D, both of these domains are highly conserved (Fig. 2 and Fig. 3, and Supplemental Materials).

The taxonomic distribution that can be seen in the protein sequence tree matches what would be expected from a clade-built tree. The ecdysozoan proteins all group together, as do the deuterostome Blimp1 proteins. If the zinc fingers are used alone to build the tree, the relationship between the groups does not hold and many groupings appear

polyphyletic. All three echinoderm sequences (i.e., Strongylocentrotus, Lytechinus, and Asterina) are more closely related to the vertebrate sequences than to the ecdysozoan sequences, thereby forming a monophyletic deuterostome group.

Temporal expression of the alternative splice forms

We looked at the temporal expression pattern of the two splice forms by QPCR, utilizing primers that distinguish them (see Material and Methods for details). The time-course of accumulation of their respective transcripts is shown in Fig. 4. The blue line represents the expression of the early form, *blimp1/krox1b*. This message can first be detected between 6 and 9 hpf (cleavage stage), and its expression peaks at 42 hpf (late gastrula stage) at around 7500 molecules per embryo. There are about 60 cells of the endoderm in the late blastula and early gastrula, so there are about 125 molecules per cell of transcript from the early transcription unit. Thereafter, it rapidly declines. Thus, blimp1/krox1b is the isoform expressed during the time period when the endomesoderm territory is being specified, from soon after the birth of the large micromeres to gastrulation.

The green line represents the expression of *blimp1/krox1a*, the late form. It is not expressed until sometime between 30 and 36 hpf, and its transcription persists past 84 hpf into the late pluteus stage. It is expressed at the highest levels between 54 and 72 hpf, accumulating around 1500 molecules per embryo. At this time, there are approximately 60 midgut and 60 hindgut cells, bringing the expression of Spblimp1/krox1a to 12

molecules per average cell. Thus, *blimp1/krox1b* is expressed at about a 5-fold higher level per embryo, comparing respective peak expression levels, than is *blimp1/krox1a*.

Spatial expression of the blimp1/krox gene

The *blimp1/krox* gene is expressed in multiple tiers of cells in the vegetal plate, including the large micromeres and the veg2 and veg1 lineages, at different stages of development. A whole mount in situ hybridization series is displayed in Fig. 5A. Between 6 and 9 h postfertilization *blimp1/krox* mRNA accumulates in the large micromeres, but this gene is not expressed in the small micromeres, nor in any of their descendants during embryogenesis (note unstained vegetal-most cells in the 10 h vegetal view (VV) of Fig. 5A). By 10 hpf, it is also expressed in the veg2 tier of endomesodermal precursors, but its expression disappears from the micromeres soon after this time, as can be seen in the 18-h VV of Fig. 5A. It has been cleared from the mesodermal lineages of these territories by a few hours later, when it also begins to be expressed in a new territory, a subset of veg1 tier descendants that will become part of the gut (21 and 25 h embryos in VV, Fig. 5A). Expression is strongest in the blastopore region of the early and mid gastrula (36 and 48 h embryos, Fig. 5A), and encompassing the midgut as well as the hindgut of the later gastrula and larva (72 h embryo, Fig. 5A). Midgut expression is very likely activated by Brn1/2/4, as anti-brn1/2/4 morpholino antisense oligo (MASO) down-regulates blimp1/krox expression at 36 hpf (Yuh et al., 2005), and brn1/2/4 is expressed at the right time and place.

A diagrammatic summary of *blimp1/krox* expression throughout the whole course of development is shown in Fig. 5B, as viewed from the side of the embryo, and in Fig. 5C, the pattern of expression is portrayed as seen from the vegetal pole for the cleavage–blastula period. From here, the dynamic distribution pattern of *blimp1/krox* message is most obvious. This gene is not only activated in different lineages, but in the skeletogenic and mesodermal territories, it is also repressed in successive concentric domains some hours after its activation.

Expression of a blimp1/krox1b-GFP knock-in BAC

According to the measurements in Fig. 4, transcripts accumulated before 30 hpf are solely the product of the early 1b transcription unit. Nonetheless, to provide an independent indication of the spatial expression of the 1b regulatory system, we created a GFP knock-in (Yu et al., 2000) that would specifically report the activity of the early transcription unit. Thus, the GFP coding sequence was inserted in place of the exon1b coding sequence immediately following the ATG start codon (Fig. 6A). The expression of the transgene was monitored by GFP WMISH so that the location of the stain would indicate the contemporary expression domain rather than the accumulation of the long-lived GFP protein. Fig. 6B shows examples of WMISH embryos injected with Spblimp1/krox1b-GFP BAC in side view. Stages are indicated in the top right-hand corner. It can be seen that in these examples, the reporter construct has been incorporated in one-half or one-fourth of the embryo, which is not infrequently seen with injected BACs (S. Damle and E. Davidson, unpublished results). At 17 hpf, GFP message is

found throughout the vegetal plate, i.e., in micromere as well as veg2 lineages (top row in Fig. 6B), while at 24 hpf, the signal is present in both veg2 and veg1 endoderm but has cleared from the now ingressed micromere descendants (bottom row Fig. 6B). These results are exactly as expected for the early blimp1/krox transcription unit.

Functional characterization of the early form of blimp1/krox

The initial predictions for inputs of blimp1/krox within the endomesodermal gene regulatory network relied on perturbation data from experiments using a fusion construct in which the Drosophila Engrailed repressor domain was joined to the DNA binding domain of blimp1/krox (Bl1/K-En). In embryos injected with Bl1/K-En mRNA, all direct targets of blimp1/krox should be strongly down-regulated with respect to controls, since the Engrailed domain acts as a dominant repressor. Indeed, most genes affected displayed strong down-regulation in these experiments, though in rare cases, an up-regulation occurred, necessarily an indirect effect. In order to determine the real polarity of the endogenous blimp1/krox effects on these genes, we studied them again, using MASOs targeted against the blimp1/krox1b early gene product, or in combination with blimp1/krox1a MASO.

The two alternatively transcribed isoforms utilize different translation initiation sites. Different MASOs can therefore be used in order to block the translation of either message, so that the function of the early and late forms can be established separately. The antisense blimp1/krox1a MASO has no noticeable phenotype until the gastrula stage.

Thereafter, the treated embryos produce pencil-like guts that fail to form the normal tripartite structure (data not shown). This phenotype correlates well with the temporal expression of the gene, since *blimp1/krox1a* mRNA accumulates only after 36 hpf. A similar phenotype is displayed in embryos bearing brn1/2/4 MASO; as noted above, we believe that Brn1/2/4 could be a driver of the blimp1/krox1a transcription unit (Yuh et al., 2005).

The blimp1/krox1b MASO, by contrast, has a strong early phenotype, including a decrease in the thickening of the vegetal plate, and subsequently, a lack of a clear veg1 descendant tier of cells in the blastula stage. This is reminiscent of the phenotype of embryos expressing mRNA encoding blimp1/krox-En fusions (Davidson et al., 2002a and Davidson et al., 2002b), but it is not as strong. At 40 hpf, it is easy to distinguish controls from anti-blimp1/krox1b MASO-injected embryos. As can be seen in Figs. 7C, D, anti-blimp1/krox1b MASO-injected embryos may display less- and more-severe phenotypes (compare controls, Figs. 7A, B). In about 50% of cases, a small invagination does appear as in Fig. 7C, although it is much delayed when compared to controls, and it does not ever extend to form a gut. In the remaining 50% of MASO embryos, no invagination occurs at all, and in some cases, cells in the embryos begin to exogastrulate instead (Fig. 7D).

Treatment of embryos with anti-blimp1/krox1b MASO affects expression of this gene itself in a striking way. The controls in Figs. 8A–C show normal WMISH patterns of expression observed with blimp1/krox probes (cf. Fig. 5). The remaining five panels of

Fig. 8 display embryos injected with a MASO targeted to the *blimp1/krox1b* message. These embryos lack the torus of endoderm expression formed in normal embryos by the clearance after 18 hpf of transcripts from the central veg2 mesoderm domain. The inner boundaries of this taurus, i.e., the mesoderm/endoderm interface, are indicated in the controls of Figs. 8A–C, by arrows. In the MASO-treated embryos, *blimp1/krox* expression never clears from the mesoderm, and at 26 hpf, intense expression is continuing across the whole of the vegetal plate. The arrow in Fig. 8E, for example, points to the center of the mesodermal domain in a MASO-treated embryo, displaying heavy *blimp1/krox* expression in this region. Though we did not explicitly address the requirement of blimp1/krox expression for the initial clearance of transcripts from the skeletogenic micromeres, it may operate by a similar mechanism. Thus, for instance, Fig. 8D shows a MASO-treated embryo in which ingressed micromeres can be seen expressing *blimp1/krox* ectopically (arrow), which is never normally observed. The subsequent expression of *blimp1/krox* message in the endodermal portion of the veg1 tier also appears to be missing in MASO-treated embryos (not shown). In summary, blimp1/krox is certainly required to repress its own expression in mesodermal cells of the veg 2 tier. It may also be required for its earlier down-regulation in the large micromeres, and, directly or indirectly, for the activation of its own expression in the endodermal cells of the veg 1 tier. Note that blimp1/krox does not repress its own expression within cells of the veg1 or veg2 endodermal territories. The difference in the response of the gene to the blimp1/krox factor in the mesoderm, where it acts as a repressor, and in the endoderm, where it does not, must be due to the presence of different co-regulators in these domains.

Computational binding-site searches and gel shift analyses indicate that the negative autoregulation of the blimp1/krox gene is likely to be direct. There are several sites in the intergenic region surrounding the blimp1/krox exons that correspond to the consensus target site sequence for blimp1/krox factors, G(A/G)AA(G/C)(G/T)GAAA (Gupta et al., 2001). We found that these sites are bound by a factor the mobility of which is very similar to that of the factor that binds the blimp1/krox sites in a known otx cis-regulatorymodule (Yuh et al., 2004); and that the Blimp1/krox sites of the blimp1/krox gene and those from the otx gene regulatory module compete reciprocally (details are given in Figs. 4 and 5 of Supplemental Materials).

Discussion

Alternative regulation of the blimp1/krox splice isoforms

The early and late transcripts of the blimp1/krox gene have different lead exons that are positioned at widely different locations in the genome (Fig. 1) and transcribed at different stages in development (Fig. 4). The proteins derived from the two isoforms could be functionally distinct, since their N-terminal sequence differs by the exon 1b-specific peptide (Fig. 2). However, the highly conserved domains, the SET domain and the DNA binding zinc fingers are present in both blimp1/krox1a and blimp1/krox1b proteins. As will be described elsewhere, transcription of the 1a and 1b forms is controlled by entirely distinct regulatory modules, which respond to distinct inputs.

Whatever the significance of the N-terminal peptide, if any, the alternative regulatory systems enable the gene to be deployed in very distinct circumstances: early on, it functions as one of the β-catenin/Tcf1 cohort of regulatory genes, and as such, blimp1/krox is among the primary regulators of the zygotic gene regulatory network. The general role of these very early regulators is interpretation of cytoplasmic spatial cues at the beginning of development - here vegetal nuclearization of β-catenin - and installation of the zygotic transcriptional control system. In contrast, the late or 1a form is expressed only in the definitive endoderm of the hindgut and midgut. According to perturbation data from Yuh et al. (2005), the regulatory system controlling 1a expression responds to a Brn1/2/4 input, which is a midgut and hindgut, and later a midgut-specific regulator. Brn1/2/4 controls expression of endo16, a downstream differentiation gene, and blimp1/krox1a might likewise be used to operate differentiation genes in this phase of its function. The phenotype of the embryos treated with MASO targeted to the late form indicates that expression of this form is required for gut regionalization.

Downstream targets of blimp1/krox expression

As demonstrated in Fig. 5 and Fig. 6, *blimp1/krox* message is first expressed in the skeletogenic micromere lineage, then in the secondary mesenchyme mesodermal domain, the cells of which are of independent origin (fourth cleavage micromere vs. macromere), and subsequently in the veg2 endoderm, veg1 endoderm, and gut. The expression domains of the early and late transcripts together account for the overall pattern summarized in Fig. 5C. This gene plays a series of roles in endomesoderm

specification. Its initial function in the skeletogenic and then the veg2 endomesodermal cells is to drive expression of the *wnt8* gene, into which it has a direct *cis*-regulatory input (Minokawa et al., 2005). Thus, blimp1/krox contributes to the essential intercellular Wnt8-β catenin feedback loop required for expression of important regulatory genes in all of the domains of the endomesoderm (Davidson et al., 2002a, Davidson et al., 2002b and Oliveri and Davidson, 2004). Later, the blimp1/krox gene generates an essential input into the regulatory apparatus that governs endoderm specification. Here, it operates upstream of an important, highly conserved network subcircuit composed of otx, gatae, brachury and foxa genes that drives endoderm specification, as discovered earlier (Davidson et al., 2002a, Davidson et al., 2002b and Hinman et al., 2003). Of these genes, it provides a direct input into the *cis*-regulatory module controlling endodermal expression of the β 1/2otx transcript. In addition, in the veg2 and veg1 endoderm, blimp1/krox is apparently a transcriptional activator of *eve* and of *hox11/13b*. From the mesenchyme blastula stage on, expression of both of these genes ceases in the veg2 endoderm, and appears instead in the veg1 endoderm. These are all functions mediated by the early or 1b form of blimp1/krox protein. With respect to the gut-specific late form, only the Brn1/2/4 input into the cis-regulatory module controlling its expression is so far-established, and none of its downstream targets are yet known.

The network linkages of blimp1/krox can now be summarized as in Fig. 9, which includes genes known or suspected to be either immediately upstream or downstream of this gene. This diagram is in the form of a view from the genome (Bolouri and Davidson, 2002 and Longabaugh et al., 2005), such that all regulatory linkages between genes are

seen at once irrespective of the time or subdomain where they are expressed. For views that specify the interactions occurring in any particular spatial and temporal domain, and which include all genes in the network, the reader may consult the interactive model on the gene regulatory network website (http://sugp.caltech.edu/endomes/).

The blimp1/krox negative autoregulatory loop

We found earlier (Davidson et al., 2002a) that the blimp1/krox gene is strongly repressed by an Engrailed-blimp1/krox fusion, an indication that a cis-regulatory module of this gene might include autoregulatory target sites for its own product. The MASO experiments reported here (Fig. 8) decisively demonstrate that this autoregulation is negative: blimp1/krox represses itself, directly or indirectly. Very possibly, the interaction is a direct one, though the demonstration that this is the case awaits the *cis*-regulatory identification of the responsible target sites. Thus, there are nearby potential target sites that match the canonical sequence recognized by Blimp1 proteins, and competition gel shift experiments show that these indeed specifically interact with the sea urchin blimp1/krox factor (Supplemental Materials, Figs. 4 and 5). Direct or not, the negative autoregulation of *blimp1/krox* is evidently the cause of the dynamic clearance of its transcripts from the veg2 mesodermal domain in the late blastula stage, since elimination of expression at the protein level by MASO treatment blocks this clearance (Fig. 8). As discussed in text, it is likely that the same mechanism is responsible for the earlier clearance of *blimp1/krox* transcripts from the skeletogenic micromere lineage as well. In spatial terms, the expression of this gene describes a wave-like form, in that it is

activated in the micromere lineage between 6 and 9 hpf, but the transcripts have disappeared from this lineage sometime prior to 18 h; similarly, it is activated in the veg2 mesodermal lineage by about 10 hpf, and the transcripts have disappeared from this domain by 21 hpf, as discussed above. Were the mRNA to vanish the moment the gene is turned off in consequence of its own transcription, the periodicity of this wave would be expected to be about 2 to 3 h rather than at least 9 h (Bolouri and Davidson, 2003). The difference might indicate that the negative autoregulation is in fact indirect, but the most likely explanation is that the mRNA has a several-hour half-life. If this were true, then in each domain the gene might be expressed only for a short burst of a few hours. This is the phase of its activity when blimp1/krox drives the wnt8 self-reinforcing loop (Minokawa et al., 2005). The wnt8 gene similarly begins operation in the skeletogenic micromeres, expands to the veg2 mesoderm, turns off in the micromeres, and later turns off in the veg2 mesoderm while expanding into the veg2 endoderm and then veg1 endoderm. Another blimp1/krox target gene, *eve,* describes a very similar dynamic pattern of expression (Ransick et al., 2002). Thus, the negative autoregulation of blimp1/krox could provide part of the causal explanation of this progressive spatial expression pattern; that is, it could explain the progressive, concentric clearance of transcripts that all three genes display.

In summary, this work illustrates the multiplicity of functions that a single regulatory gene may execute over the course of a couple of days of embryonic development. The blimp1/krox gene is alternatively transcribed, under diverse regulatory controls, at different stages and in different places. After gastrulation, the late form

participates in gut regionalization. The early transcript form has diverse roles: initially, it provides a spatially and temporally dynamic input into the Wnt8–Tcf1 regulatory loop, which literally defines the endomesoderm; then, a few hours later, it operates to drive an endoderm specification network subcircuit.

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References

Angerer, L.M., Angerer, R.C., 2003. Patterning the sea urchin embryo: gene regulatory networks, signaling pathways, and cellular interactions. Curr. Top. Dev. Biol. 53, 159–198.

Angerer, L.M., Angerer, R.C., 2004. Disruption of gene function using antisense morpholinos. In: Ettensohn, C.A., Wessel, G., Wray, G. (Eds.), Developmental Biology of Sea Urchins, Ascidians, and Other Invertebrate Deuterostomes: Experimental Approaches. Methods in Cell Biology, vol. 74. Academic Press/Elsevier Science, pp. 699–711.

Baxendale, S., Davison, C., Muxworthy, C., Wolff, C., Ingham, P.W., Roy, S., 2004. The B-cell maturation factor Blimp-1 specifies vertebrate slow-twitch muscle fiber identity in response to Hedgehog signaling. Nat. Genet. 36, 88–93.

Bolouri, H., Davidson, E.H., 2002. Modeling DNA sequence-based cisregulatory gene networks. Dev. Biol. 246, 2–13.

Bolouri, H., Davidson, E.H., 2003. Transcriptional regulatory cascades in development: initial rates, not steady state, determine network kinetics. Proc. Natl. Acad. Sci. U. S. A. 100, 9371–9376.

Brown, C.T., Rust, A.G., Clarke, P.J., Pan, Z., Schilstra, M.J., De Buysscher, T., et al., 2002. New computational approaches for analysis of cis-regulatory networks. Dev. Biol. 246, 86–102.

Brown, C.T., Xie, Y., Davidson, E.H., Cameron, R.A., 2005. Paircomp, Family

Relations II and Cartwheel: tools for interspecific sequence comparison. BMC Bioinform. 24, 70–76.

Cameron, R.A., Mahairas, G., Rast, J.P., Martinez, P., Biondi, T.R., Swartzell, S., et al., 2000. A sea urchin genome project: sequence scan, virtual map, and additional resources. Proc. Natl. Acad. Sci. U. S. A. 97, 9514–9518.

Cameron, R.A., Oliveri, P., Wyllie, J., Davidson, E.H., 2004. cis-Regulatory activity of randomly chosen genomic fragments from the sea urchin. Gene Expression Patterns 4, 205–213.

Chang, D.H., Calame, K.L., 2002. The dynamic expression pattern of B lymphocyte induced maturation protein-1 (Blimp-1) during mouse embryonic development. Mech. Dev. 117, 305–309.

Chang, D.H., Angelin-Duclos, C., Calame, K., 2000. BLIMP-I: trigger for differentiation of myeloid lineage. Nat. Immunol. 1, 169–176.

Cheers, M.S., Ettensohn, C.A., 2004. Rapid microinjection of fertilized eggs. Development of sea urchins, ascidians, and other invertebrate deuterostomes: experimental approaches. In: Ettensohn, C.A., Wessel, G., Wray, G. (Eds.), Developmental Biology of Sea Urchins, Ascidians, and Other

Invertebrate Deuterostomes: Experimental Approaches. Methods in Cell Biology, vol. 74. Academic Press/Elsevier Science, pp. 287–310.

Davidson, E.H., 1986. Gene Activity in Early Development, 3rd edition. Academic Press, Inc., Orlando, FL.

Davidson, E.H., 1989. Lineage-specific gene expression and the regulative capacities of the sea urchin embryo: a proposed mechanism. Development 105, 421–445.

Davidson, E.H., 2001. Genomic Regulatory Systems: Development and Evolution. Academic Press, San Diego, CA.

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.H., et al., 2002a. A provisional regulatory gene network for specification of endomesoderm in the sea urchin embryo. Dev. Biol. 246, 162–190.

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.H., 2002b. A genomic regulatory network for development. Science 295, 1669–1678.

de Souza, F.S.J., Gawantka, V., Gómez, A.P., Delius, H., Ang, S., Niehrs, C., 1999. A zinc finger gene Xblimp1 controls anterior endomesodermal cell fate in Spemann's organizer. EMBO J. 18, 6062–6072.

Evans, R.M., Hollenberg, S.M., 1988. Zinc fingers: gilt by association. Cell 52, 1–3.

Foltz, K.R., Adams, N.L., Runft, L.L., 2004. Echinoderm eggs and embryos: procurement and culture. In: Ettensohn, C.A., Wessel, G., Wray, G. (Eds.), Developmental Biology of Sea Urchins, Ascidians, and Other Invertebrate Deuterostomes: Experimental Approaches. Methods in Cell Biology, vol. 74. Academic Press/Elsevier Science, pp. 41–53.

Gupta, S., Anthony, A., Pernis, A.B., 2001. Stage-specific modulation of IFNregulatory factor 4 function by Krüppel-type zinc finger proteins. J. Immunol. 166, 6104–6111.

Györy, I., Fejer, G., Ghosh, N., Seto, E., Wright, K.L., 2003. Identification of a functionally impaired positive regulatory domain I binding factor 1 transcription repressor in myeloma cell lines. J. Immunol. 170, 3125–3133.

Györy, I., Wu, J., Fejer, G., Seto, E., Wright, K.L., 2004. PRDI-BF1 recruits the histone H3 methyltransferase G9a in transcriptional silencing. Nat. Immunol. 5, 299–308.

Ha, A.S., Riddle, R.D., 2003. cBlimp-1 expression in chick limb bud

development. Gene Expression Patterns 3, 297–300.

Hammes, A., Schedl, A., 2001. Isolation of Linearized BAC DNA for Microinjection to Produce Transgenic Animals. Practical Approach Series. Oxford Univ. Press.

Hinman, V.F., Davidson, E.H., 2003. Expression of AmKrox, a starfish ortholog of a sea urchin transcription factor essential for endomesodermal specification. Gene Expression Patterns 3, 423–426.

Hinman, V.F., Nguyen, A.T., Cameron, R.A., Davidson, E.H., 2003. Developmental gene regulatory network architecture across 500 million years of echinoderm evolution. Proc. Natl. Acad. Sci. U. S. A. 100, 13356–13361.

Hough-Evans, B.R., Britten, R.J., Davidson, E.H., 1988. Mosaic incorporation and regulated expression of an endogenous gene in the sea urchin embryo. Dev. Biol. 129, 198–208.

Jenuwein, T., 2001. Re-SET-ing heterochromatin by histone methyltransferases. Trends Cell Biol. 11, 266–273.

Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA2: molecular

evolutionary genetics analysis software. Bioinformatics 17, 1244–1245.

Kumar, S., Tamura, K., Nei, M., 2003. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 5, 150–163.

Leahy, P.S., 1986. Laboratory culture of Strongylocentrotus purpuratus adults, embryos and larvae. Methods Cell Biol. 27, 1–13.

Lee, Y.H., 2003. Molecular phylogenies and divergence times of sea urchin species of Strongylocentrotidae, Echinoida. Mol. Biol. Evol. 20, 1211–1221.

Levine, M., Davidson, E.H., 2005. Gene regulatory networks for development. Proc. Natl. Acad. Sci. U. S. A. 102, 4936–4942.

Longabaugh, W.J., Davidson, E.H., Bolouri, H., 2005. Computational representation of developmental genetic regulatory networks. Dev. Biol. 283, 1–16.

Min, J., Zhang, X., Cheng, X., Grewal, S.I., Xu, R.M., 2002. Structure of the SET domain histone lysine methyltransferase Clr4. Nat. Struct. Biol. 9, 828–832.

Minokawa, T., Rast, J.P., Arenas-Mena, C., Franco, C.B., Davidson, E.H., 2004. Expression patterns of four different regulatory genes that function during sea urchin development. Gene Expression Patterns 4, 449–456.

Minokawa, T., Wikramanayake, A.H., Davidson, E.H., 2005. cis-Regulatory inputs of the wnt8 gene in the sea urchin endomesoderm network. Dev. Biol. 288, 545–558.

Ohinata, Y., Payer, B., O'Carroll, D., Ancelin, K., Ono, Y., Sano, M., et al., 2005. Blimp1 is a critical determinant of the germ cell lineage in mice. Nature 436, 207–213.

Oliveri, P., Davidson, E.H., 2004. Gene regulatory network controlling embryonic specification in the sea urchin. Curr. Opin. Genet. Dev. 14, 351–360.

Oliveri, P., Carrick, D.M., Davidson, E.H., 2002. A regulatory gene network that directs micromere specification in the sea urchin embryo. Dev. Biol. 246, 209–228.

Ransick, A., Davidson, E.H., 1998. Late specification of Veg1 lineages to endodermal fate in the sea urchin embryo. Dev. Biol. 195, 38–48.

Ransick, A., Rast, J.P., Minokawa, T., Calestani, C., Davidson, E.H., 2002. New early zygotic regulators expressed in endomesoderm of sea urchin embryos discovered by differential array hybridization. Dev. Biol. 246, 132–147.

Rast, J.P., 2000. Transgenic position of teh sea urchin embryo. Developmental Biology Protocols, Vol. II. Methods in Molecular Biology, vol. 136. Humana Press Inc., Totowa, NJ, pp. 365–373.

Rast, J.P., Amore, G., Calestani, C., Livi, C.B., Ransick, A., Davidson, E.H., 2000. Recovery of developmentally defined gene sets from high-density cDNA macroarrays. Dev. Biol. 228, 270–286.

Roy, S., Ng, T., 2004. Blimp-1 specifies neural crest and sensory neuron progenitors in the zebrafish embryo. Curr. Biol. 14, 1772–1777.

Shaffer, A.L., Shapiro-Shelef, M., Iwakoshi, N.N., Lee, A.H., Qian, S.B., Zhao, H., et al., 2004. XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. Immunity 21, 81–93.

Siammas, R., Davis, M.M., 2004. Modular nature of Blimp-1 in the regulation of gene expression during B cell maturation. J. Immunol. 172, 5427–5440.

Smith, A.B., 1988. Phylogenetic relationship, divergence times, and rates of

molecular evolution for camarodont sea urchins. Mol. Biol. Evol. 5, 345–365.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

Wang,W.,Wikramanayake, A.H., Gonzalez-Rimbau, M., Vlahou, A., Flytzanis, C.N., Klein,W.H., 1996. Very early and transient vegetal-plate expression of SpKrox1, a Krüppel/Krox gene from Stronglyocentrotus purpuratus. Mech. Dev. 60, 185–195.

Yu, D., Ellis, H.M., Lee, E.C., Jenkins, N.A., Copeland, N.G., Court, D.L., 2000. An efficient recombination system for chromosome engineering in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 97, 5978–5983.

Yuh, C.H., Brown, C.T., Livi, C.B., Rowen, L., Clarke, P.J., Davidson, E.H., 2002. Patchy interspecific sequence similarities efficiently identify positive cis-regulatory elements in the sea urchin. Dev. Biol. 246, 148–161.

Yuh, C.H., Dorman, E.R., Howard, M.L., Davidson, E.H., 2004. An otx cisregulatory module: a key node in the sea urchin endomesoderm gene regulatory network. Dev. Biol.

269, 536–551.

Yuh, C.H., Dorman, E.R., Davidson, E.H., 2005. Brn1/2/4, the predicted midgut regulator of the endo16 gene of the sea urchin embryo. Dev. Biol. 281, 286–298.

Figure Legends:

Fig. 1. Structure of blimp1/krox gene and splicing isoforms. (A) Annotation of BAC 169o19 showing the position of blimp1/krox exons. The antisense morpholino against the early form binds in the region surrounding the ATG in exon 1b, while the late form morpholino binds in the region surrounding the ATG in exon 2. Numbered boxes represent the location of exon sequences on BAC. (B) Structure of the alternatively transcribed and spliced cDNAs. In the blimp1/krox1a transcript, exon 1a is spliced to exon 2. Thus, the late form is (exon 1a + 2 + 3 + 4 + 5 + 6). In the blimp1/krox1b transcript, exon 1b is spliced to exon 2; thus, the early form is (exon 1b + 2 + 3 + 4 + 5 + 6). The blimp1/krox1a form is that described previously (as Spkrox1; Wang et al., 1996).

Figure 1

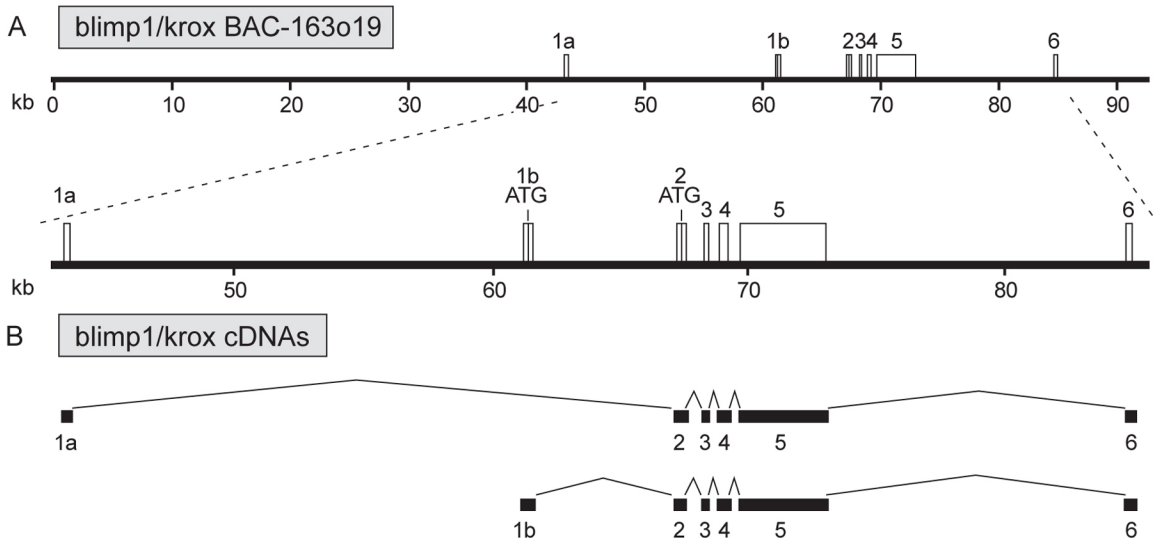
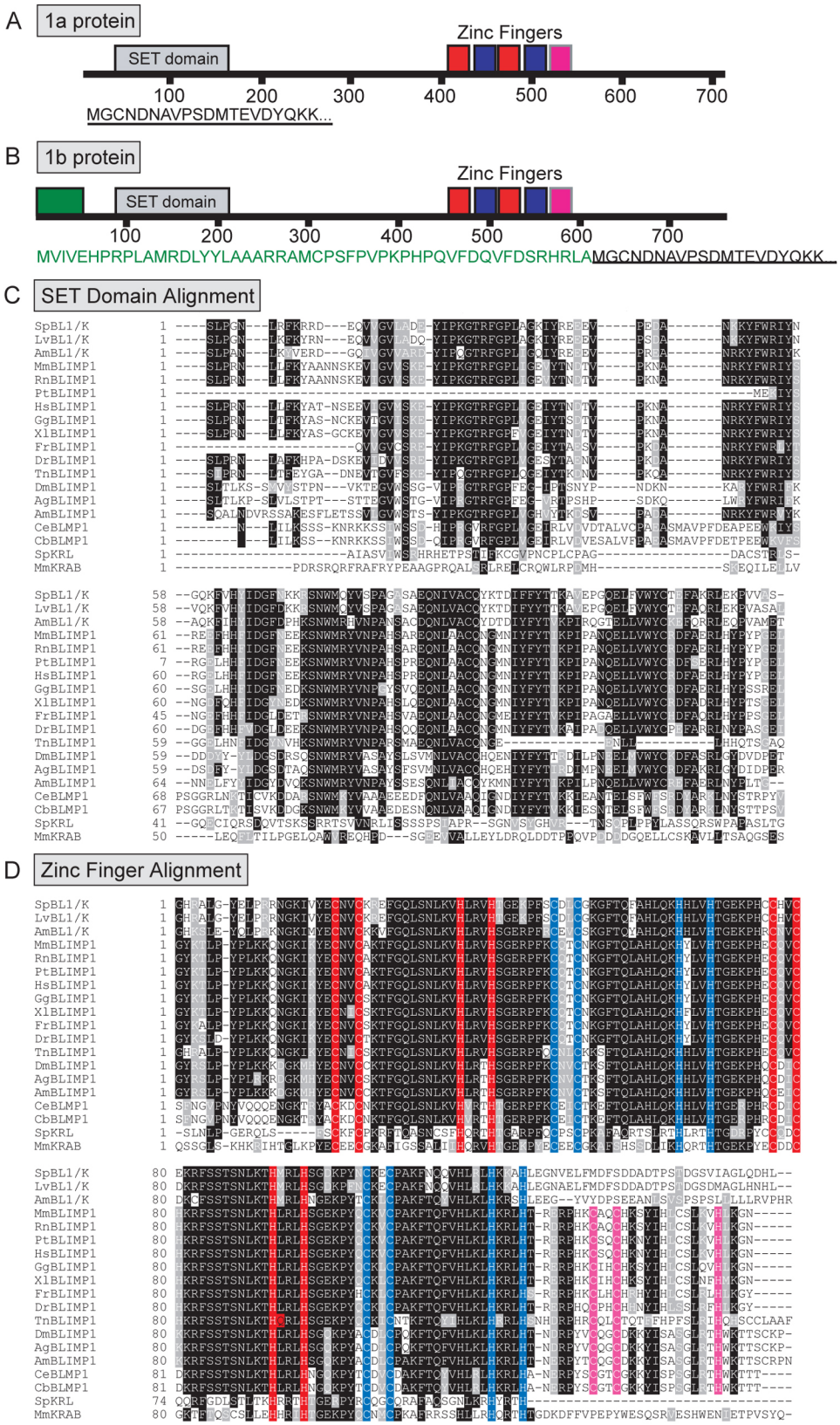

A
blimp1/krox BAC-163o19
1a
1b
2 3 4
5
6
kb
0
10
20
30
40
50
60
70
80
90
1a
1b
ATG
2
ATG
3 4
5
6
kb
50
60
70
80
B
blimp1/krox cDNAs
1a
2 3 4
5
6
1b
2 3 4
5
6

Fig. 2. blimp1/krox protein isoform domains. (A) 1a (late) form; (B) 1b (early) form. Four complete Cys2His2 Zn fingers and one degenerate Zn finger are indicated by colored boxes. The N-terminal amino acid sequences are shown beneath; the first 50 amino acids are unique to the 1b protein (green). (C) SET domain alignment (see Materials and methods for abbreviations and sources). All orthologues of blimp1/krox contain an N-terminal SET domain, mediating protein–protein interaction, and possibly conferring protein–methylating enzyme activity. (D) Zn finger alignment. The degenerate 5th zinc finger no longer follows the consensus sequence, but can easily be identified. Global alignment including isoform sequences can be seen in Supplemental Materials. Colored bars, coded as in (A) and (B) denote the individual Zn fingers. Cysteins and histidines forming the C2H2 zinc finger structure are highlighted.

Figure 2


A
1a protein
SET domain
Zinc Fingers
100
200
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400
500
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700
MGCNDNAVPSDMTEVDYQKK...
B
1b protein
SET domain
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100
200
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MVIVEHPRPLAMRDLYYLAAARRAMCPSFPVPKPHPQVFDQVFDSRHRLAMGCNDNAVPSDMTEVDYQKK...
C
SET Domain Alignment
SpBL1/K
LvBL1/K
AmBL1/K
MmBLIMP1
RnBLIMP1
PtBLIMP1
HsBLIMP1
GgBLIMP1
XlBLIMP1
FrBLIMP1
DrBLIMP1
TnBLIMP1
DmBLIMP1
AgBLIMP1
AmBLIMP1
CeBLMP1
CbBLMP1
SpKRL
MmKRAB
D
Zinc Finger Alignment
SpBL1/K
LvBL1/K
AmBL1/K
MmBLIMP1
RnBLIMP1
PtBLIMP1
HsBLIMP1
GgBLIMP1
XlBLIMP1
FrBLIMP1
DrBLIMP1
TnBLIMP1
DmBLIMP1
AgBLIMP1
AmBLIMP1
CeBLMP1
CbBLMP1
SpKRL
MmKRAB

Fig. 3. Phylogenetic tree of the Blimp1 gene family. A neighbor-joining tree was constructed using MEGA version 2 (Kumar et al., 2001) and tested by bootstrapping using 1000 replicates to infer the reliability of branching points. The scale bar represents the number of amino acid substitutions per site, and is based on the amino acid sequences with Poisson-corrected distances. The tree is based on a multiple alignment shown in Supplemental Materials Fig. 2.

Figure 3

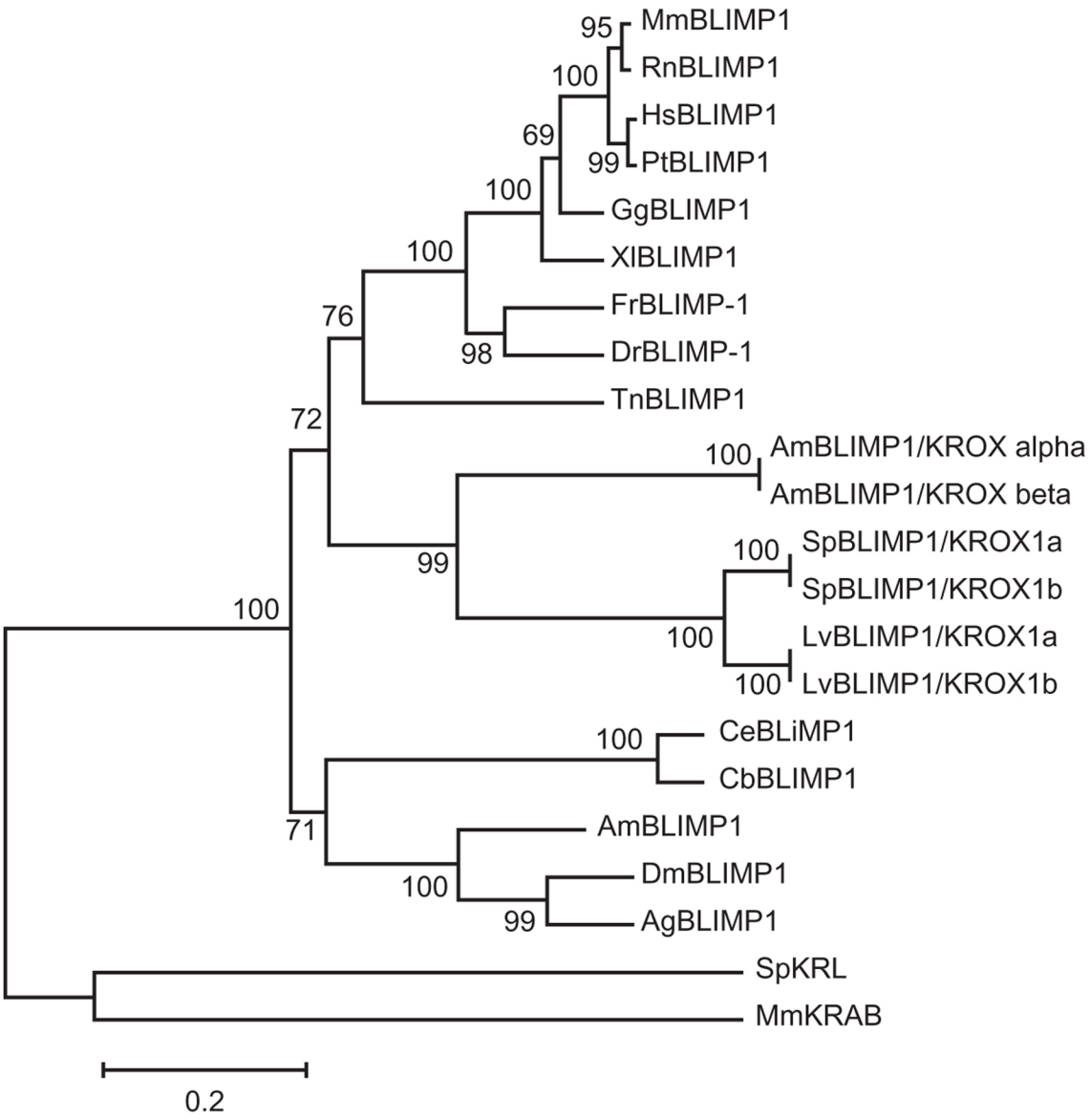

MmBLIMP1
RnBLIMP1
HsBLIMP1
PtBLIMP1
GgBLIMP1
XlBLIMP1
FrBLIMP-1
DrBLIMP-1
TnBLIMP1
AmBLIMP1/KROX alpha
AmBLIMP1/KROX beta
SpBLIMP1/KROX1a
SpBLIMP1/KROX1b
LvBLIMP1/KROX1a
LvBLIMP1/KROX1b
CeBLiMP1
CbBLIMP1
AmBLIMP1
DmBLIMP1
AgBLIMP1
SpKRL
MmKRAB
95
100
69
99
100
100
76
98
72
100
99
100
100
100
100
71
100
99
0.2

Fig. 4. Temporal expression of endogenous blimp1/krox gene. Quantitative real-time PCR measurements show levels of expression of *blimp1/krox1a* and *blimp1/krox1b* mRNAs at different developmental stages. Results were normalized to levels of the transcript of the Z12-1 gene, the abundance of which remains relatively constant throughout the stages monitored, in order to obtain the number of molecules per embryo (Oliveri et al., 2002). Two independent primer sets were used to measure expression of *blimp1/krox1b*, and three independent primer sets were used to measure *blimp1/krox1a*. Error bars represent one standard deviation. *blimp1/krox1b* begins to be being expressed after 6 hpf (some time points are not shown in graph). *blimp1/krox1a* starts being expressed after 30 hpf.

Figure 4

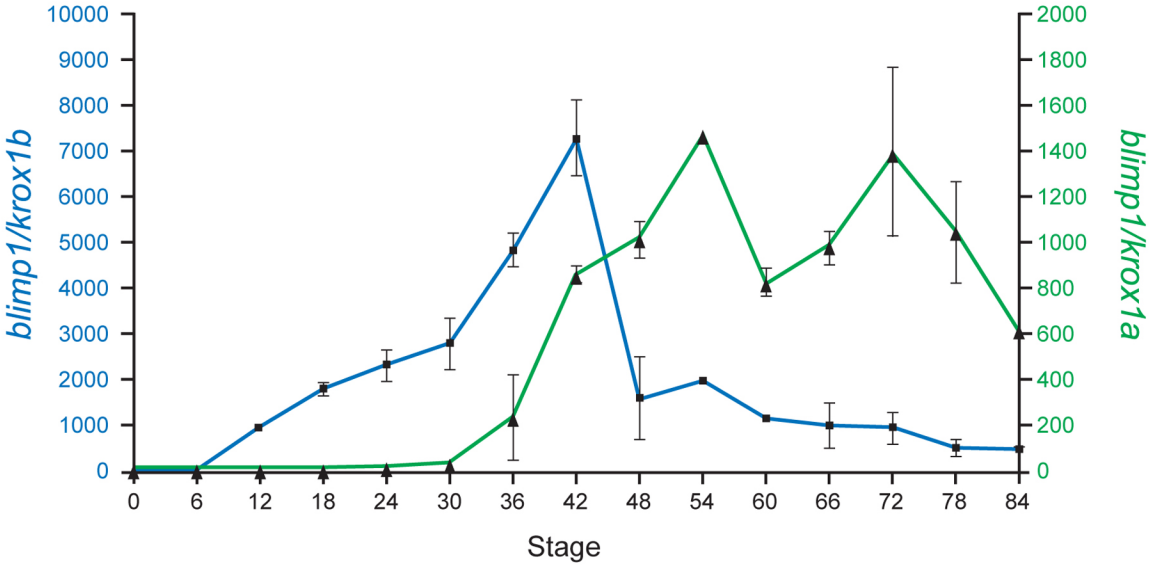

blimp1/krox1b
blimp1/krox1a
Stage
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
0
200
400
600
800
1000
1200
1400
1600
1800
2000
0
6
12
18
24
30
36
42
48
54
60
66
72
78
84

Fig. 5. Spatial expression of blimp1/krox. (A) Spatial distribution of *blimp1/krox* transcripts detected by whole-mount in situ hybridization (WMISH) using digoxigenin-labeled antisense probe; images of WMISH embryos mounted in 50% glycerol are shown. At 10 hpf, the gene is expressed in the large micromere descendents; at 18 hpf, it is expressed in the veg2 tier of cells, but is no longer observed in the large micromeres; at 21 hpf, it is expressed in the veg1 tier of cells, and expression has faded from the veg2 mesoderm. A similar pattern is seen at 25 hpf. At 36 hpf the gene is expressed in the cells surrounding the blastopore; at 48 hpf, the gene is expressed in the hind and mid gut; and at 72 hpf it is expressed in the mid and hind-gut of the pluteus larva. Bottom right hand corner indicates the view of the embryo displayed (SV, side view; VV, vegetal view). (B) Side view diagram of *blimp1/krox* spatial expression during embryogenesis, based on (A) and on many additional images not shown. (C) Vegetal view diagram of *blimp1/krox* spatial expression.

Figure 5

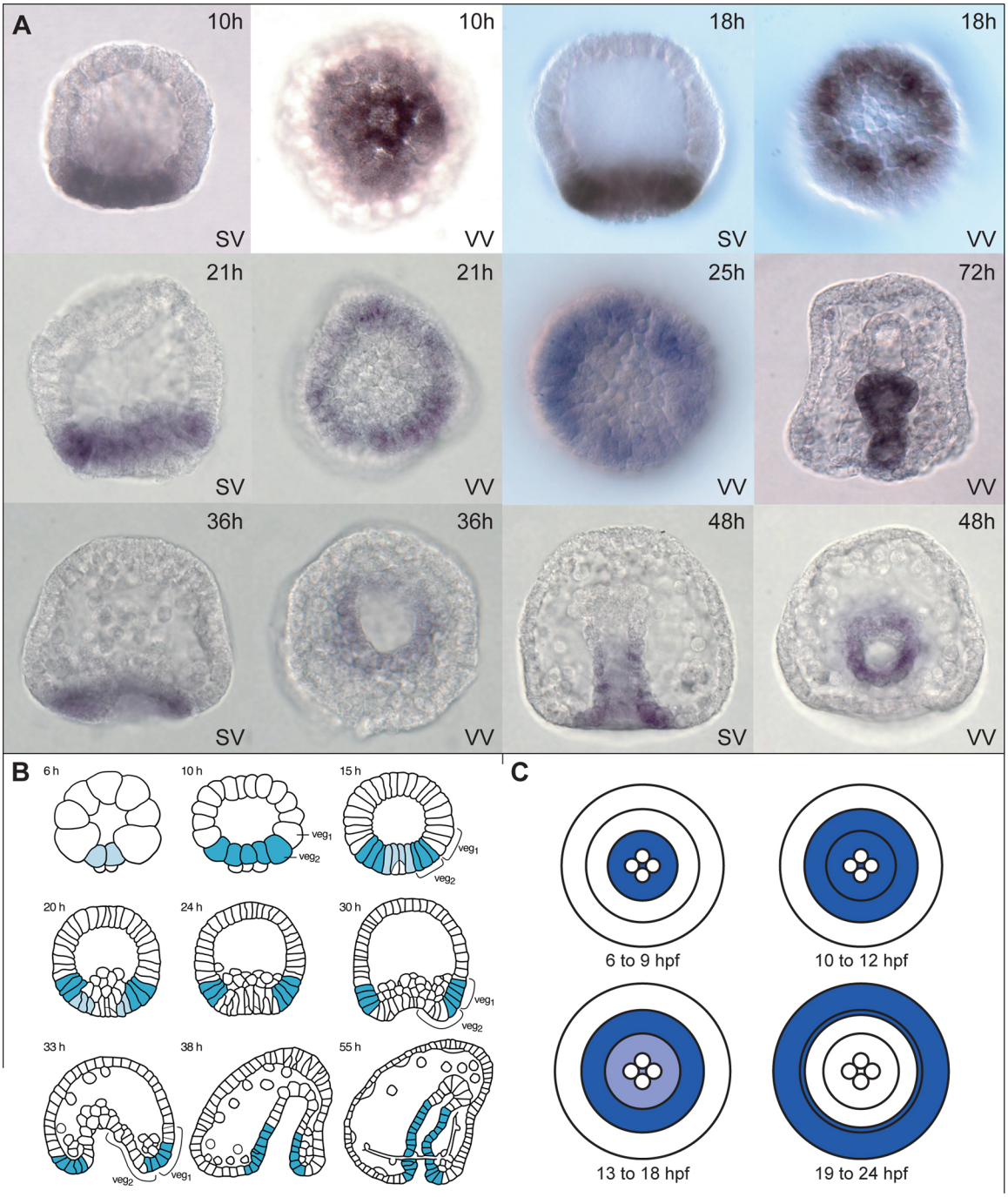

A
10h
SV
10h
VV
18h
SV
18h
VV
21h
SV
21h
VV
25h
VV
72h
VV
36h
SV
36h
VV
48h
SV
48h
VV
B
6 h
10 h
15 h
veg1
veg2
20 h
24 h
30 h
33 h
38 h
55 h
C
6 to 9 hpf
10 to 12 hpf
13 to 18 hpf
19 to 24 hpf

Fig. 6. Expression of blimp1/krox1b-GFP knockin BAC in transgenic embryos. (A) Diagram of blimp1/krox1b-GFP knockin BAC. The GFP coding sequence was inserted by homologous recombination in place of the coding region for exon 1b. The 5′ UTR was maintained, and only a few nonconserved nucleotides from the 3′ end of the exon were removed in the recombination process. (B) GFP expression patterns generated by the blimp1/krox1b-GFP knockin BAC are shown, visualized by WMISH using antisense GFP probe. Embryos were 17 and 24 hpf as indicated, and are all shown in side view. Bottom right-hand corner indicates the view displayed of the embryo. At 17 hpf, GFP mRNA is present in the large micromeres, but it is absent from their descendants in the 24 hpf embryos, as can clearly be seen once these cells have ingressed. DNA constructs injected into sea urchin zygotes are incorporated in a mosaic fashion (Hough-Evans et al., 1988), thus the transgene expression observed in an individual embryo is a fraction of the complete pattern assembled from observing many embryos.

Figure 6

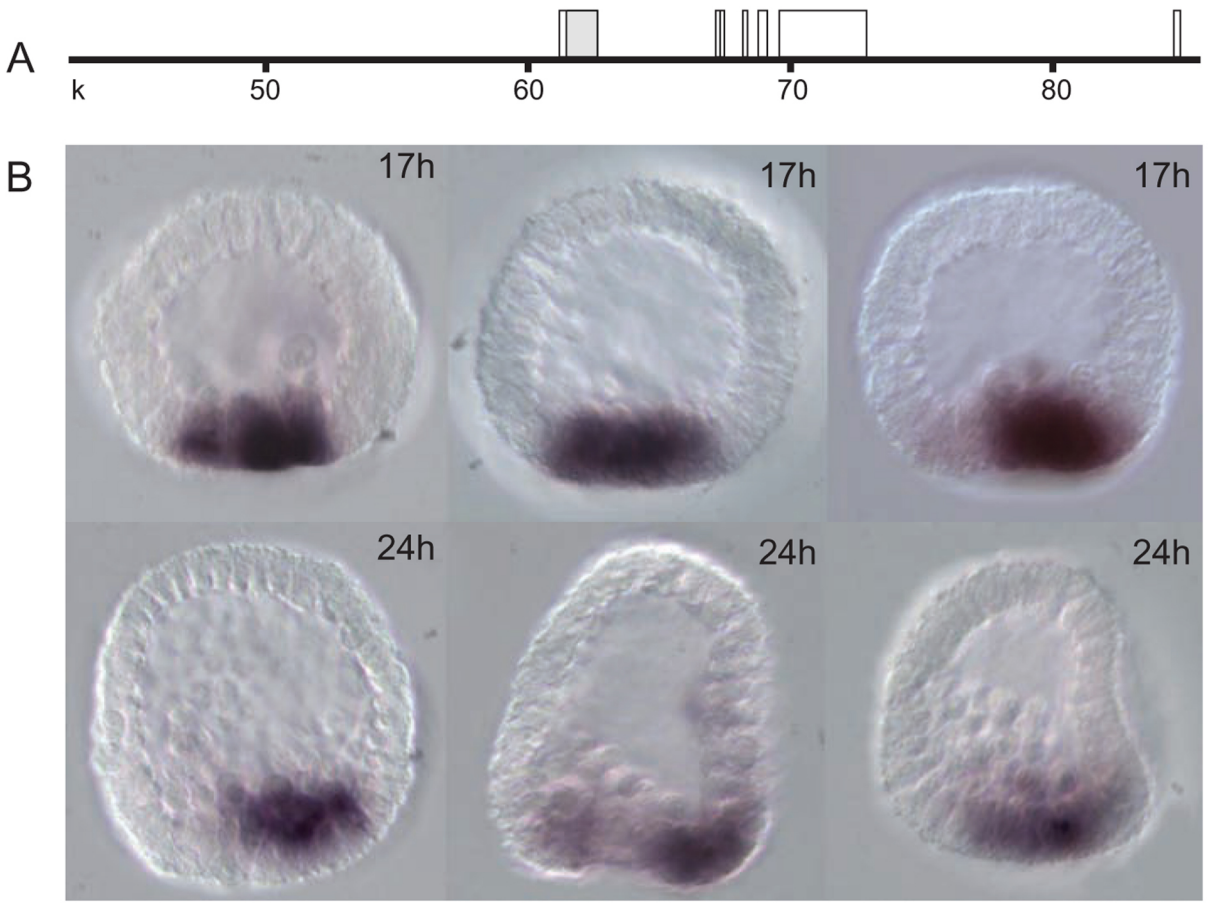


Fig. 7. Phenotypes of embryos bearing blimp1/krox1b MASO. Embryos were injected with 2 to 4 pl of a 100-μM stock of the MASO (see Materials and methods for details). (A) Uninjected (uninj), (B) standard control morpholino (scm), and (C, D) blimp1/krox MASO embryos. A well-developed gut can be observed in controls (panels A and B). The embryo shown in panel C has formed an invagination, but gut elongation did not proceed beyond formation of a short, truncated archenteron. The embryo shown in panel D formed no invagination, and instead the vegetal-most cells are exogastrulating.

Figure 7

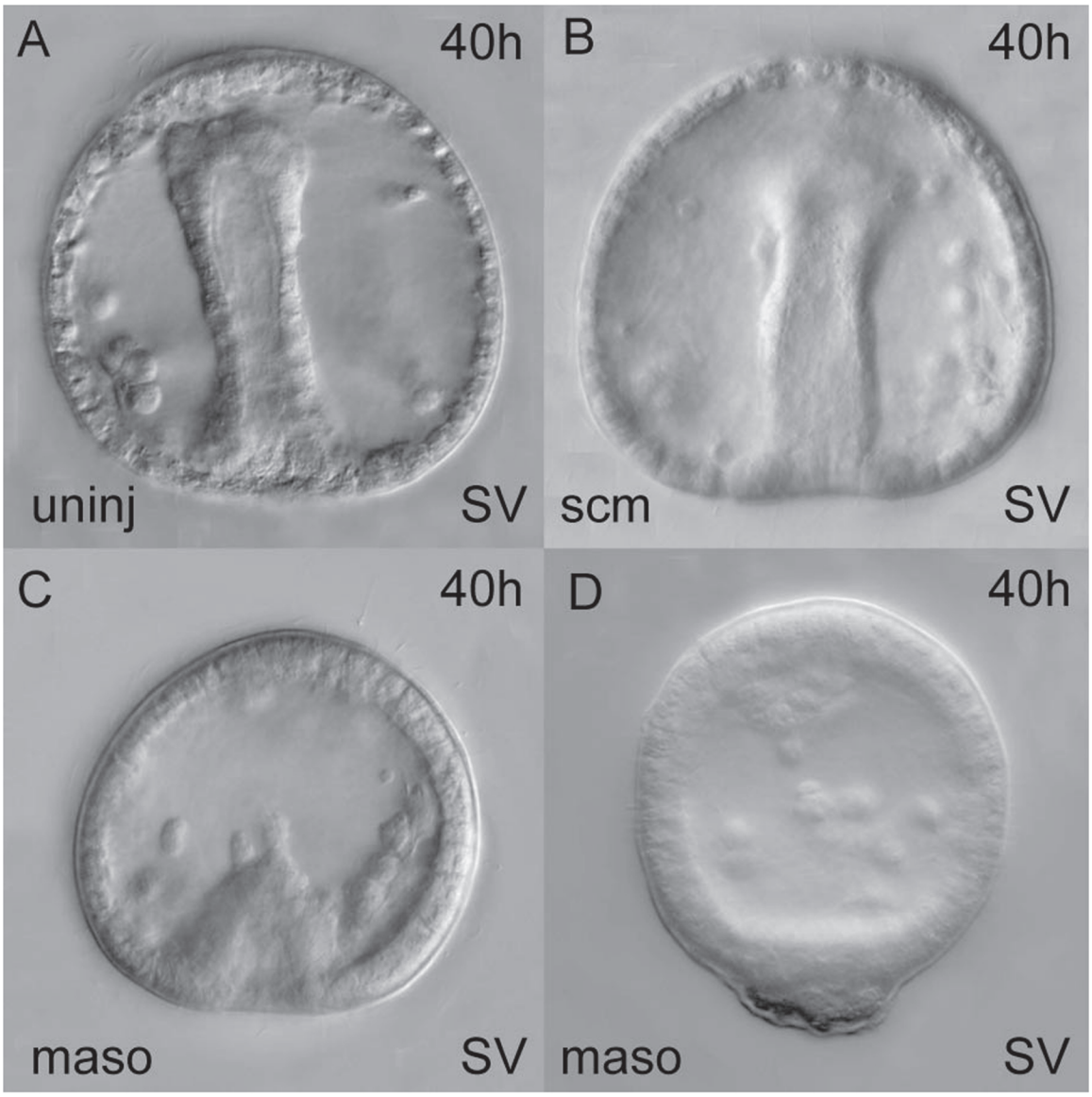


Fig. 8. Negative blimp1/krox spatial autoregulation in veg2 mesodermal cells. Embryos were injected with 2 to 4 pl of a 100 μM of anti-blimp1/krox1b MASO and 200 μM of anti-blimp1/krox1a MASO, and WMISH performed using blimp1/krox probes. (A, B) uninjected embryos (uninj); (C) standard control morpholino (scm); and (D–H) blimp1/krox MASO. In (A–C), arrows point to boundaries of mesodermal region cleared of signal. In the MASO-treated embryos, staining is expanded to the center of the vegetal plate (arrow in E), displaying the ectopic expression of *blimp1/krox* message in the mesodermal veg2 descendents. An ingressed skeletogenic mesenchyme cell expressing *blimp1/krox* is indicated by the arrow in (D).

Figure 8

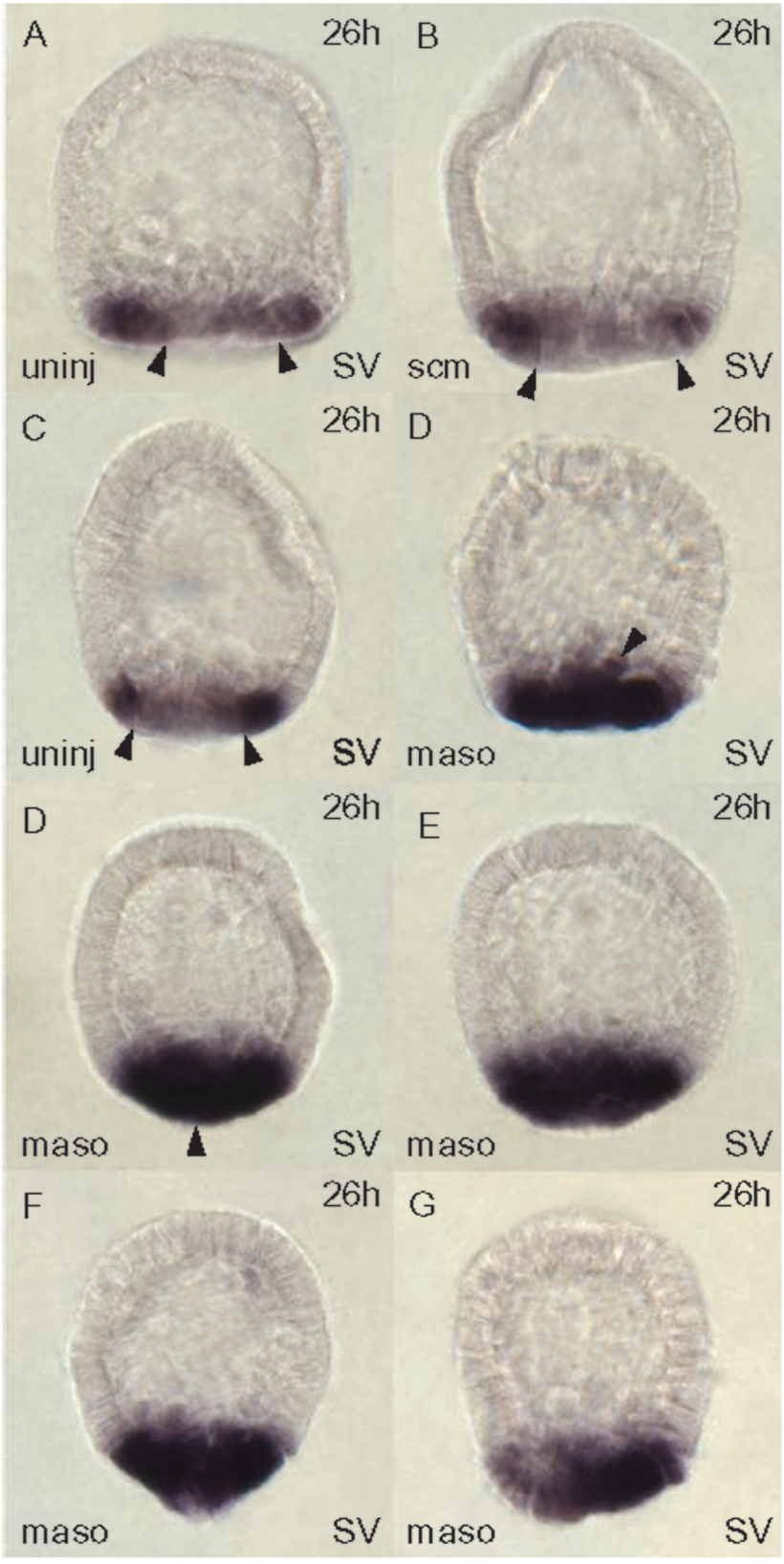
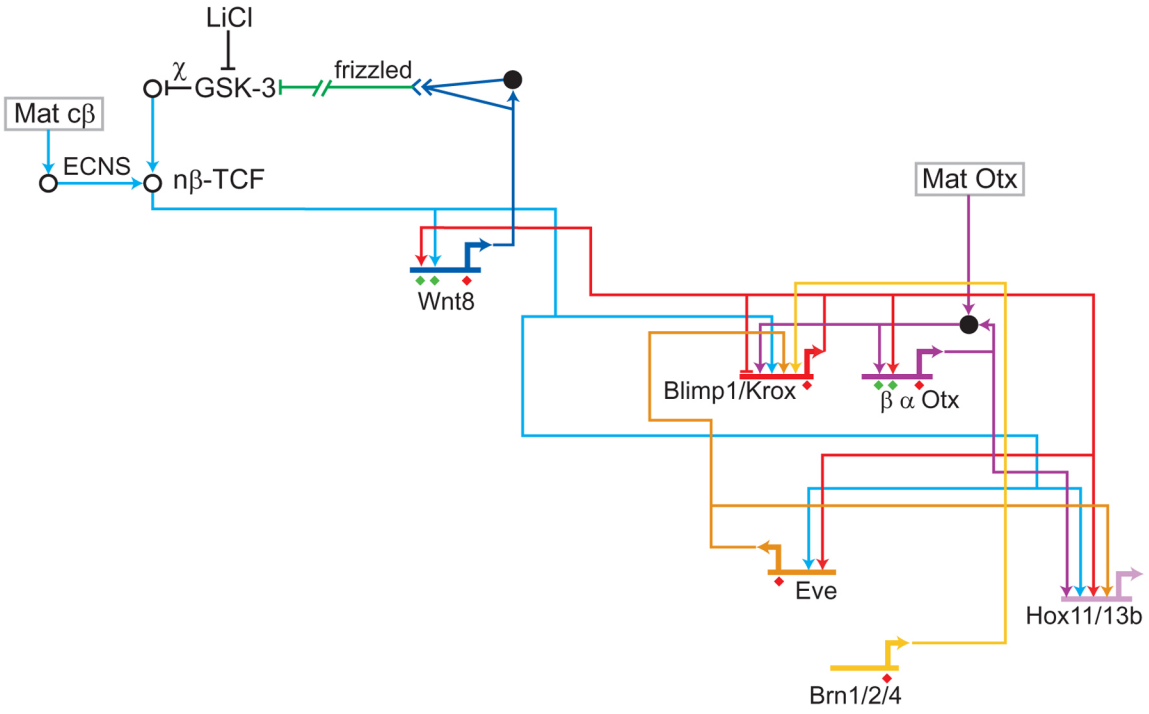

A
26h
uninj
SV
B
26h
scm
SV
C
26h
uninj
SV
D
26h
maso
SV
D
26h
maso
SV
E
26h
maso
SV
F
26h
maso
SV
G
26h
maso
SV

Fig. 9. Linkages of the blimp1/krox gene in the endomesoderm gene regulatory network. A view from the genome network diagram (Bolouri and Davidson, 2002 and Longabaugh et al., 2005) highlights the upstream and downstream connections of blimp1/krox. This view shows all functional connections, independent of when and where these interactions are taking place. There are five unique known inputs into blimp1/krox (including both the 1a and 1b regulatory systems). Outputs from the blimp1/krox gene are shown in red. There are three known downstream target genes encoding transcription factors (aside from itself) that are likely to be directly regulated by blimp1/krox, i.e., otx, hox11/13b, and eve, plus the wnt8 gene. Note the negative autoregulatory loop of blimp1/krox by which it represses its own transcription. Other important features displayed are the cross-regulatory loops formed with otx and eve.

Figure 9


LiCl
χ
GSK-3
frizzled
Mat cβ
ECNS
nβ-TCF
Wnt8
Mat Otx
Blimp1/Krox
β α Otx
Eve
Hox11/13b
Brn1/2/4

Supplemental Material Figure Legends:

Supp. Table 1. ΔCt QPCR table. Table with examples of cycle differences (ΔCt) from QPCR measurements in Blimp1/Krox-En (Bl1/K-En) and blimp1/krox MASO (Bl1/K MASO) injected embryos compared to control MASO (SCM). Numbers are normalized to the amount of ubiquitin mRNA in each sample. ΔCt values correspond to the difference in number of cycles required to attain threshold in SHAM and Bl1/K-En (Davidson et al., 2002a, b) or SCM and Bl1/K MASO embryos (see materials and methods for details). The complete data set on which the EM-GRN is based on can be found at http://sugp.caltech.edu/endomes/qpcr.html. Positive values indicate an increase in transcripts by the perturbation; negative values indicate a decrease in transcripts. Note that Bl1/K-En and Bl1/K MASO have an effect of the same polarity on *eve*, but affect *blimp1/krox* expression differently. This clearly indicated that while blimp1/krox is a positive input into *eve* regulation, it negatively regulates itself.

Supporting Table 1

	Eve 18h	Blimp1/krox1b 12h
Blimp1/Krox-En	-4.4, -4.3	-4.0, -4.8
blimp1/krox MASO	-1.6, -1.7, -2.7, -2.3	+2.1, +1.8

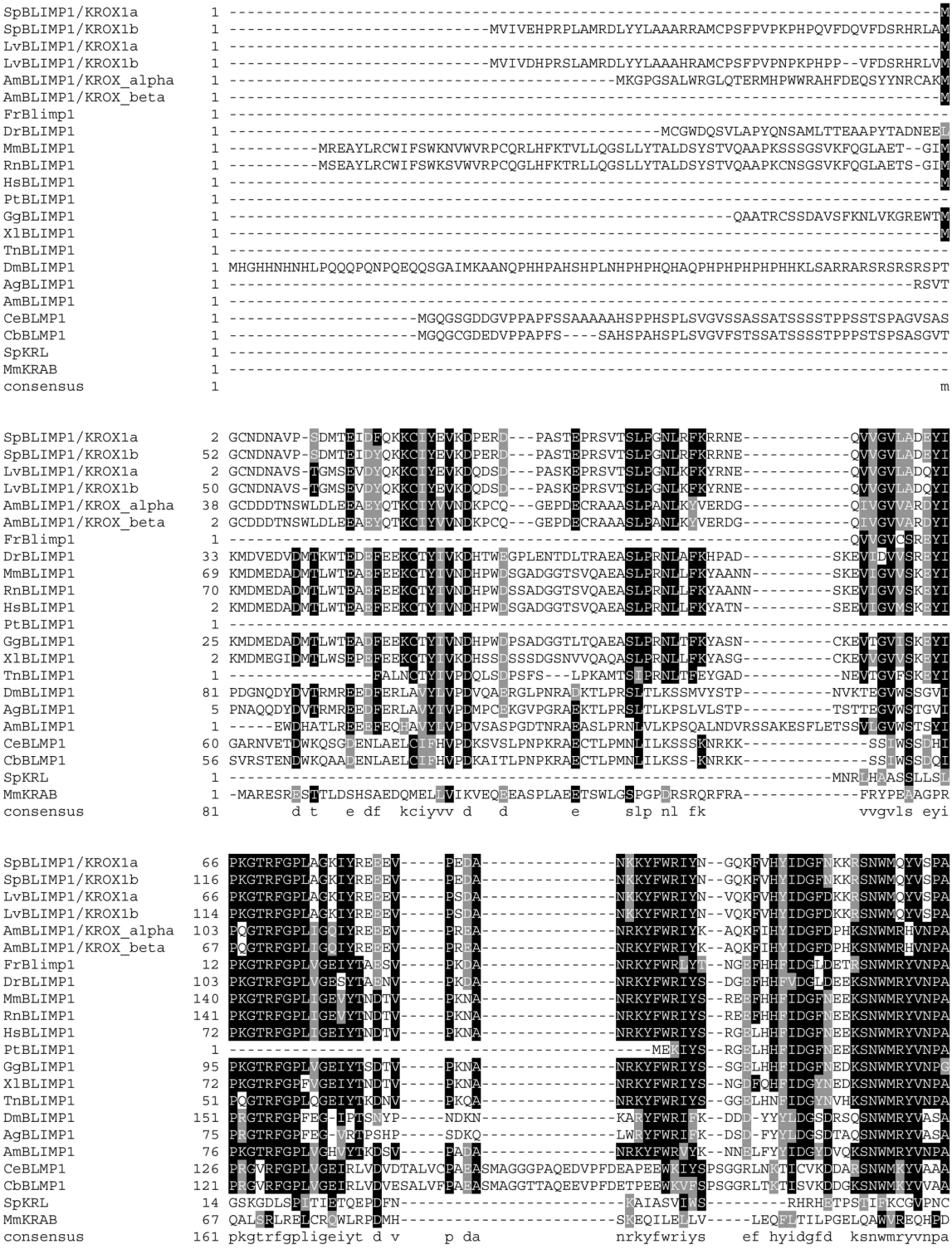
Supp. Fig. 1. Nucleotide alignment of cDNA and BAC sequences for blimp1/krox exon 1a, and exon 1b with part of exon 2. (A), *Spblimp1/krox1a* cDNA from +1 to +116, and exon 1a BAC sequences, corresponding to the previously-cloned late form. This exon is spliced with exon 2, common to both isoforms described here. See sequence after bar in bottom alignment for part of exon 2 sequence. (B), *Spblimp1/krox1b* cDNA from +1 to +226, and exon 1b BAC sequences, corresponding to the newly-cloned early form, and the first 74 nucleotides of *Spblimp1/krox* exon 2 from cDNA and BAC sequences. *Spblimp1/krox1b* was cloned from a 10h 5' race cDNA library. The cloned fragment contained sequence for exon 1b as well as part of exon 2, where the sequence for the primer utilized in the amplification was located (see Materials and methods for details).

Supporting Figure 1

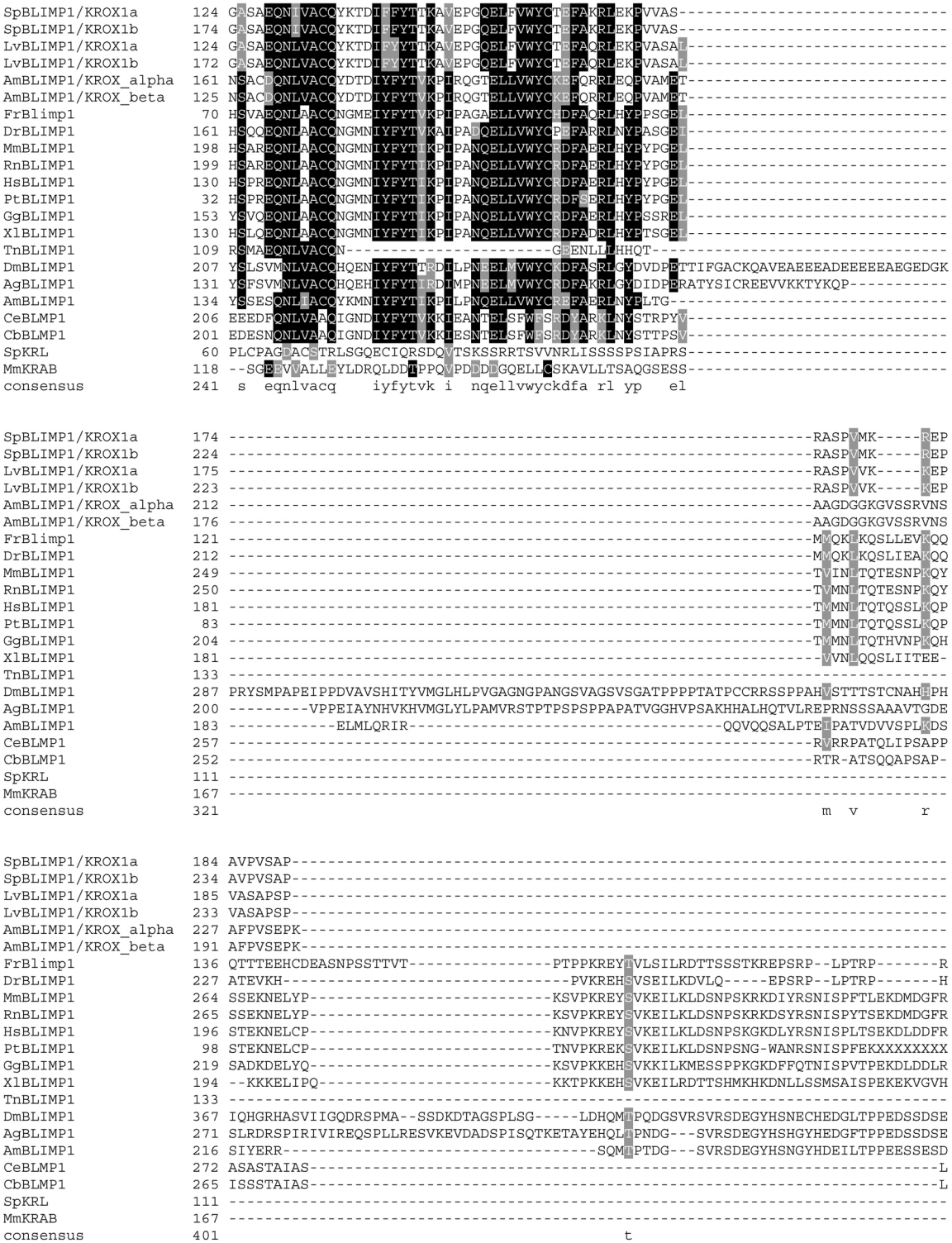
```
(A)
SpBlimp1/Krox1a alignment
(exon 1a cDNA X exon 1a BAC sequences)

Bl/K1a   1-tcaagttggaaagcacttgcttgctgttaccaccacagaaaagagaaaaa-50
1a BAC     tcaagttggaaagcacttgcttgctgttaccaccacagaaaagagaaaaa

Bl/K1a  51-gcgaaaaaa-gaagtctaaagaaattccccgattgaaaccgcaagctatt-100
1a BAC     gcgaaaaaaagaagtctaaagaaattccccgattgaaaccgcaagctatt

Bl/K1a 101-ttttctacgcttcaacg-116
1a BAC     ttttctacgcttcaacg

(B)
SpBlimp1/Krox1b alignment
(exon 1b+2 cDNA X exon 1b+2 BAC sequences)

5'race   1-atagtacacgttctancgatcaaggatagcggacactcatctactttcgc-50
1b BAC     atagtacacgttctagcgatcaagaatagcggacactcatctactttcgc

5'race  51-tgccagnactcccccgattagtttgttgngattttgtaccgcggtgttnt-100
1b BAC     tgccagtactcccccgattagtttgttgtgattttgtaccgcggtgtttt

5'race 101-tcaagcgaaagggagaaatggttatcgttgaacaccctcgcccgctagca-150
1b BAC     tcaagcgaaagggagaaatggttattgttgaacaccctcgcccgctcgct

5'race 151-atgcgggatctctactacctagcggcggctaggcgagcgatgtgcccgag-200
1b BAC     atgcgggatctctactacctagcggcggctaggcgagcgatgtgcccgag

5'race 201-cttccccgtgcctaagcctcatccac|aggtcttcgatcaggtcttcgaca-250
Sp BAC     cttccccgtgcctaaacctcatccac|aggtcttcgatcaggtcttcgaca

5'race 251-gccgccatcgcctggccatggggtgcaacgacaacgccgtgccgtctgac-300
Sp BAC     gccgccaccgcctggccatggggtgcaacgacaacgccgtgccgtccgac
```

Supp. Fig. 2. Global alignment of orthologous sequences. This full-length alignment includes *S. purpuratus*, *L. variegatus*, and *A. miniata* Blimp1/krox sequences for both isoforms differing in their N-terminal regions (the sequence in the SET domain and zinc finger regions is identical as far as we know, and thus, isoform sequences are not shown in Fig. 2). Phylogenetic tree shown in Fig. 3 is based on this alignment. The alignment was built using Clustal X and formatted using BOXSHADE (see Materials and Methods). Conserved residues are shaded with black and gray. List of species and accession numbers for sequences used are listed in the Materials and methods section of the paper.

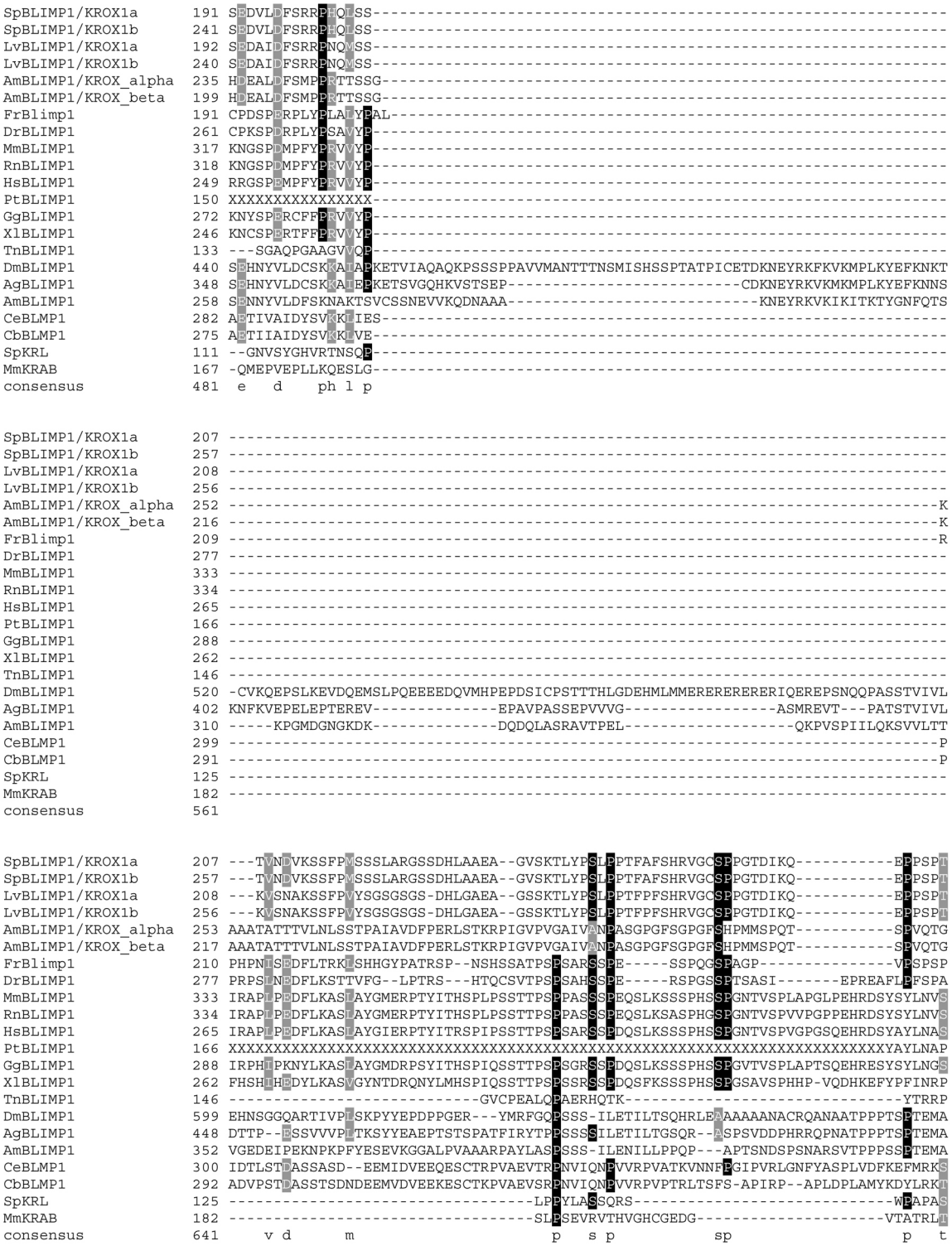
Supporting Figure 2 - Page 1



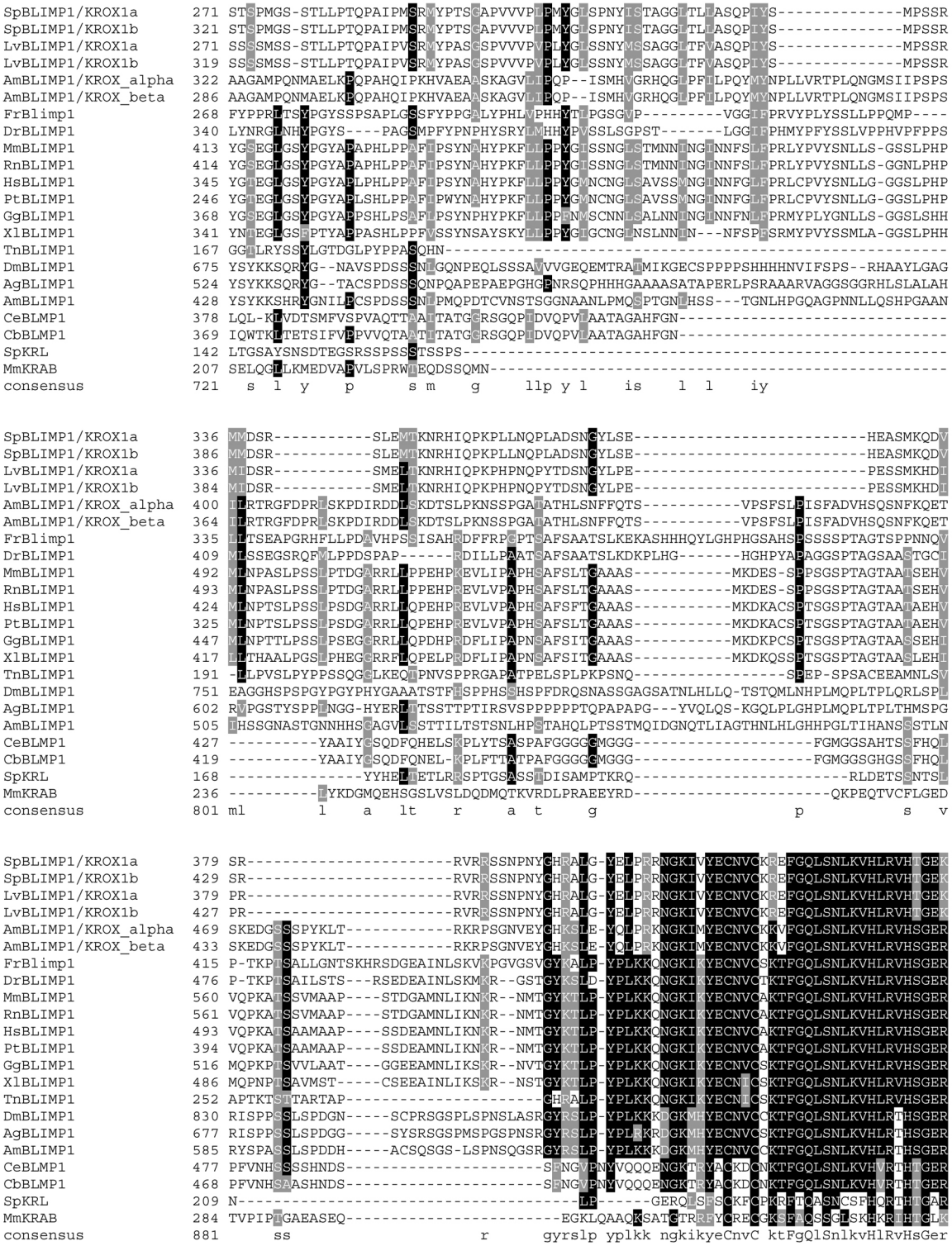
Supporting Figure 2 - Page 2



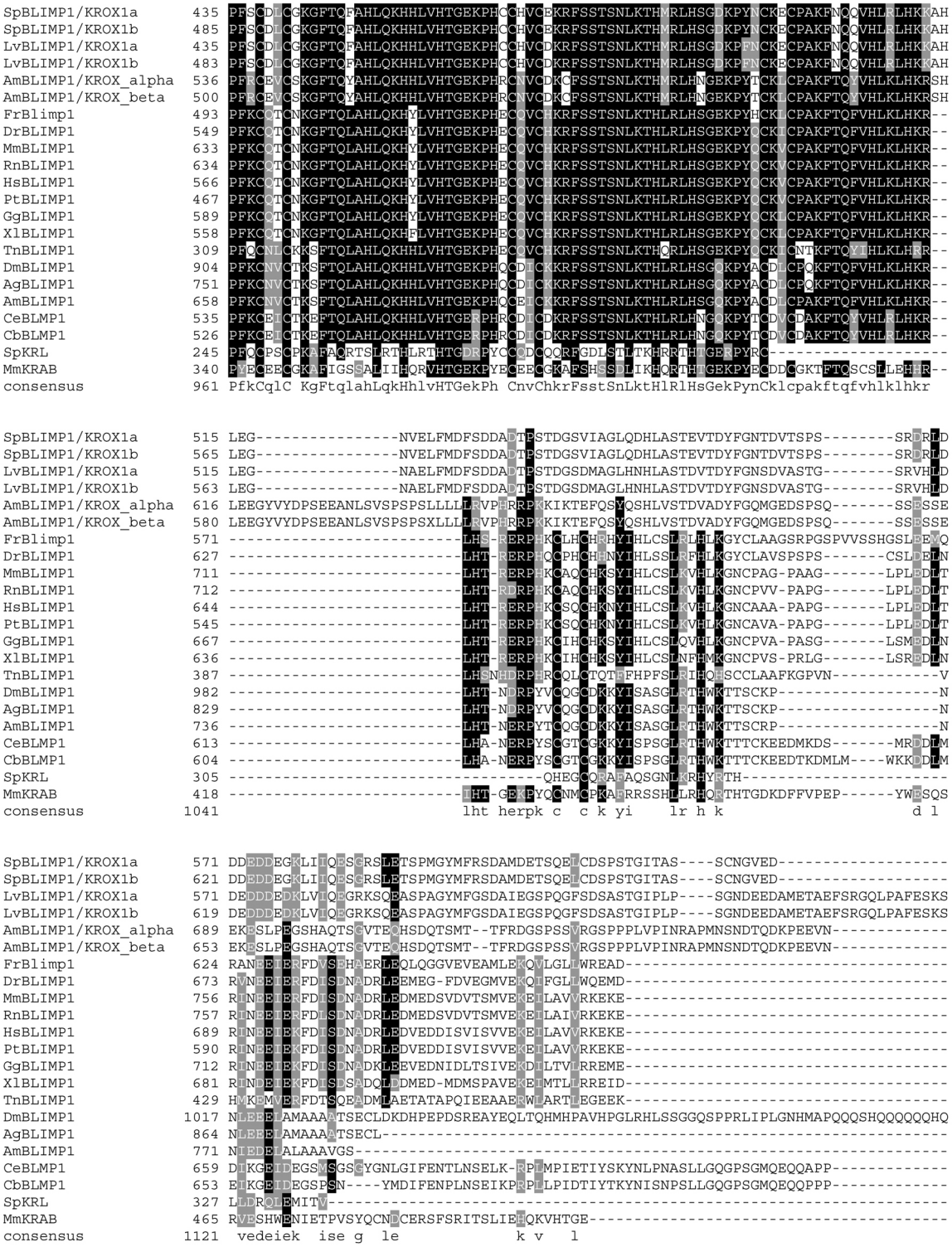
Supporting Figure 2 - Page 3



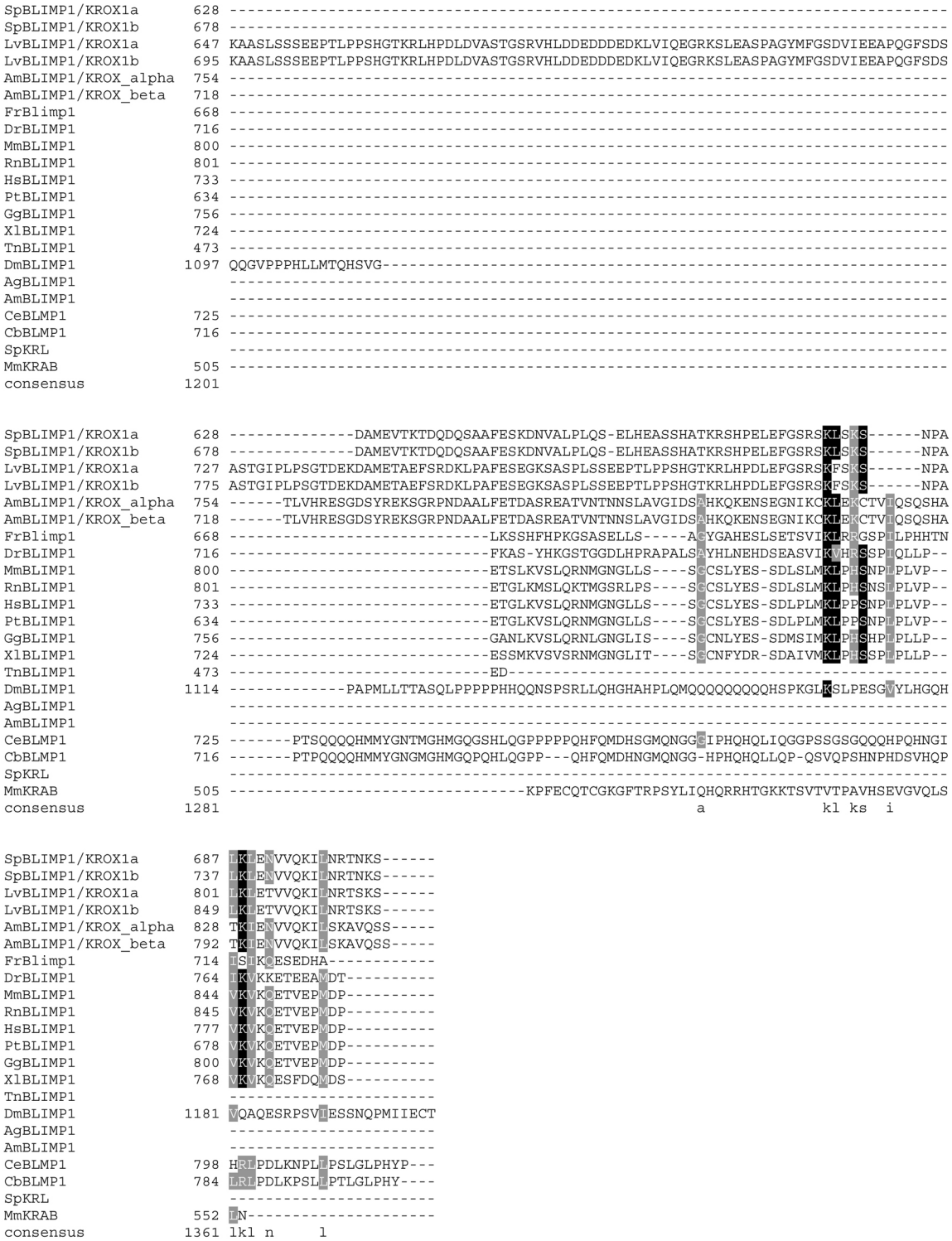
Supporting Figure 2 - Page 4



Supporting Figure 2 - Page 5



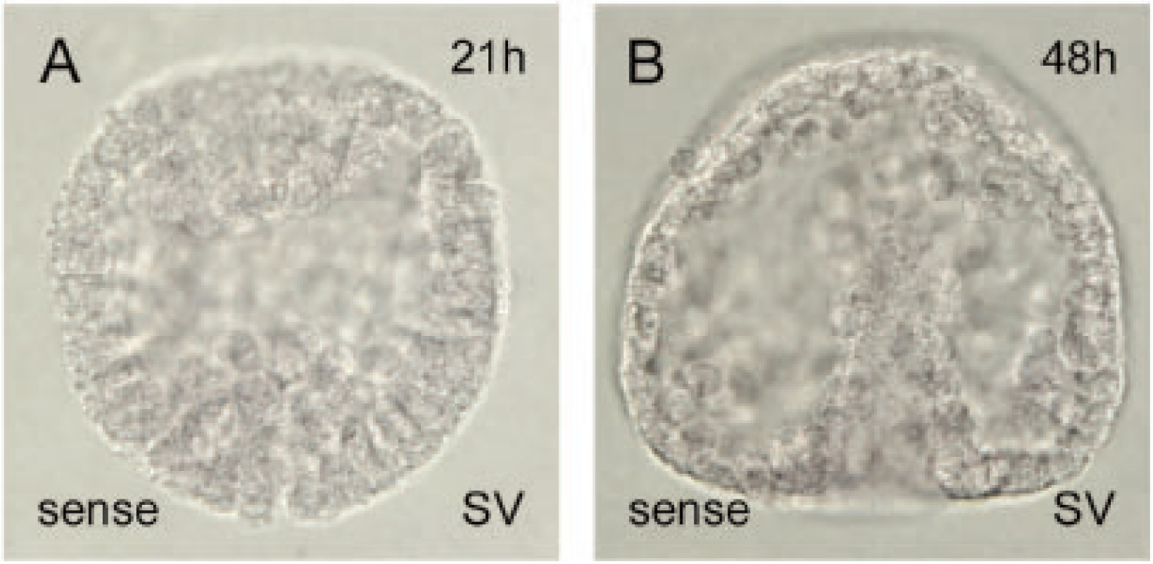
Supporting Figure 2 - Page 6



Supp. Fig. 3. Negative control for *blimp1/krox* WMISH. Embryos processed through WMISH protocol using sense blimp1/krox probe. Embryos hybridized with sense probe made from the same vector as blimp1/krox probe originated but linearized 5' of message and in vitro transcribed using the opposing promoter. No background staining was observed using sense probes (see Materials and methods for details).

Supporting Figure 3

WMISH using sense blimp1/krox probe



Supp. Fig. 4. Gel shifts using oligo 54.5. (left), Blimp1/krox site 54.5 from blimp1/krox promoter used as probe and oligo 54.5 or inespecific oligos corresponding to other Blimp1/krox sites from blimp1/krox promoter used as competitors in the presence of 22 hpf nuclear extract. (center), gel shift using oligo 54.5 as probe in the presence of nuclear extracts from different stage embryos (12, 18, 22, and 36 hpf from left to right respectively). (right), gel shift from CH Yuh using an oligo containing a Blimp1/krox site from the otx promoter which forms a complex of similar mobility to the complexes found when using oligos containing Blimp1/krox sites from the blimp1/krox promoter. Radioactively-labeled probes and unlabeled competitors were designed based on sites present on the putative blimp1/krox or otx promoter sequences. Gel shifts were carried out as described by Yuh et al., 2004. Oligo sequences are listed below, with the putative Blimp1/krox binding sites underlined.

39.3_5': AAATGAGGGTATCGATTTCACTTCCTAAAACTACG

39.3_3': CATTTCGTAGTTTTAGGAAGTGAAATCGATACCCT

41.5_5': CAATTTCTATTTCTATTTCACTTAAATGTTTCT

41.5_3': AATTGAGAAACATTTAAGTGAAATAGAAATAGA

43_5': GACATATTAGTAGATTTCACTTATCGGGAGAGTG

43_3': ATGTCCACTATCCCGATAAGTGAAATCTACTAAT

53.1_5': CATATCCCTCTTTTTCACTTTATTCATTG

53.1_3': ATATGCAATGAATAAAGTGAAAAAGAGGG

54_5': TCGGTAATTGTTCTGTATTTTTCACTTTCGCAGTACCGTTA

54_3': ACCGATAACGGTACTGCGAAAGTCAAAAATACAGAACAATT

54.5_5': GGATAATAAAAATATGAAAGGGAAAGCAGGAAGGTA

54.5_3': TATCCTACCTTCCTGCTTTCCCTTTCATATTTTTAT

62.7_5': CTTAAAATCATATGGTTTCACTTAAACCTATTT

62.7_3': TTAAGAAATAGGTTTAAGTGAAACCATATGATT

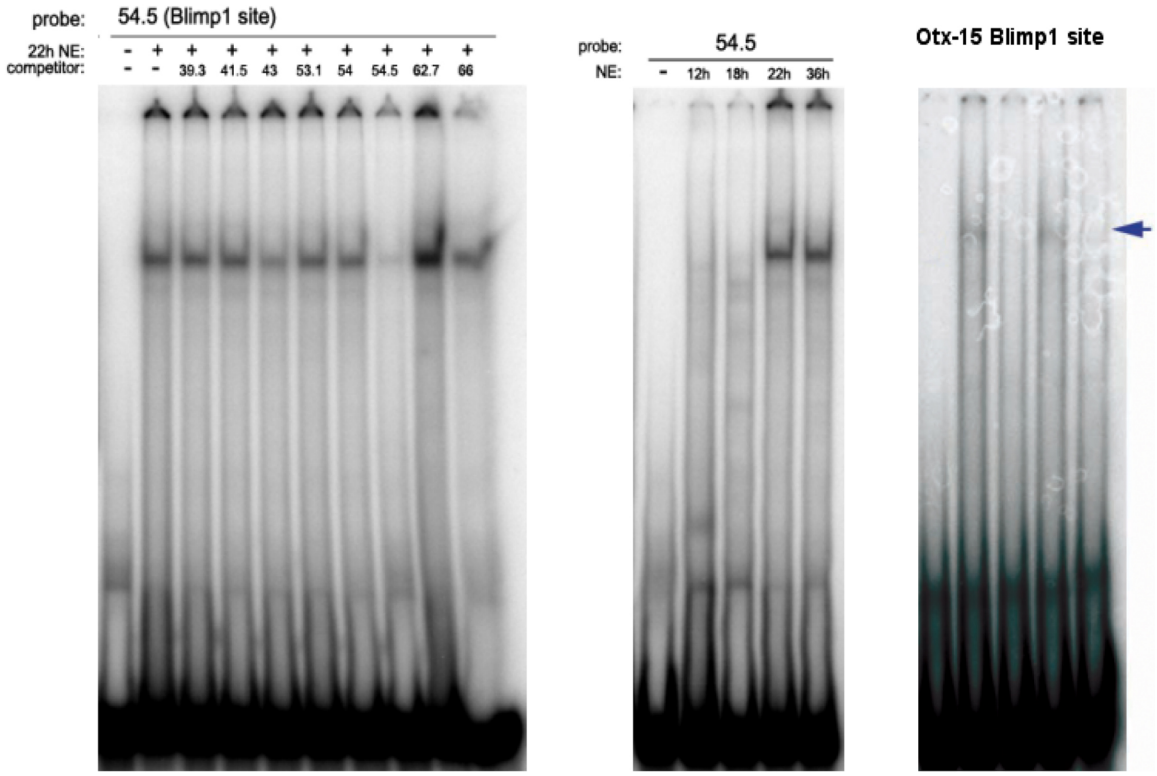
66_5': ATAATTATGAATGGACTAATTTCACTTTCAGTGATTTGACA

66_3': ATAATTATGAATGGACTAATTTCACTTTCAGTGATTTGACA

otx15_5': CATTAGGCCGATAGAGCTCGCTGAGAAGGGAAAAAAC

otx15_3': ATGGTTTTTTCCCTTCTCAGCGAGCTCTATCGGCCTA

Supporting Figure 4



Supp. Fig. 5. Gel shift using oligo 43. Blimp1/krox site from blimp1/krox promoter used as probe and specific and inspecific oligos used as competitors in the presence of nuclear extract (ne) from different stages (lanes 15-16 = 7 h ne; lanes 2-6 = 12 h ne; lanes 17-18 = 18 h ne, lanes 8-12 = 22 h ne; and lanes 19-20 = 36 h ne). One of the Blimp1/krox site from the otx promoter (otx5) effectively competes with one of the Blimp1/krox sites from the blimp1/krox promoter (43). Radioactively-labeled probes and unlabeled competitors were designed based on sites present on the putative blimp1/krox or otx promoter sequences. Gel shifts were carried out as described by Yuh et al., 2004. Oligo sequences are listed below, with the putative Blimp1/krox binding sites underlined.

43_5': GACATATTAGTAGATTTCACTTATCGGGAGAGTG

43_3': ATGTCCACTATCCCGATAAGTGAAATCTACTAAT

54_5': TCGGTAATTGTTCTGTATTTTCACTTTCGCAGTACCGTTA

54_3': ACCGATAACGGTACTGCGAAAGTCAAAAATACAGAACAATT

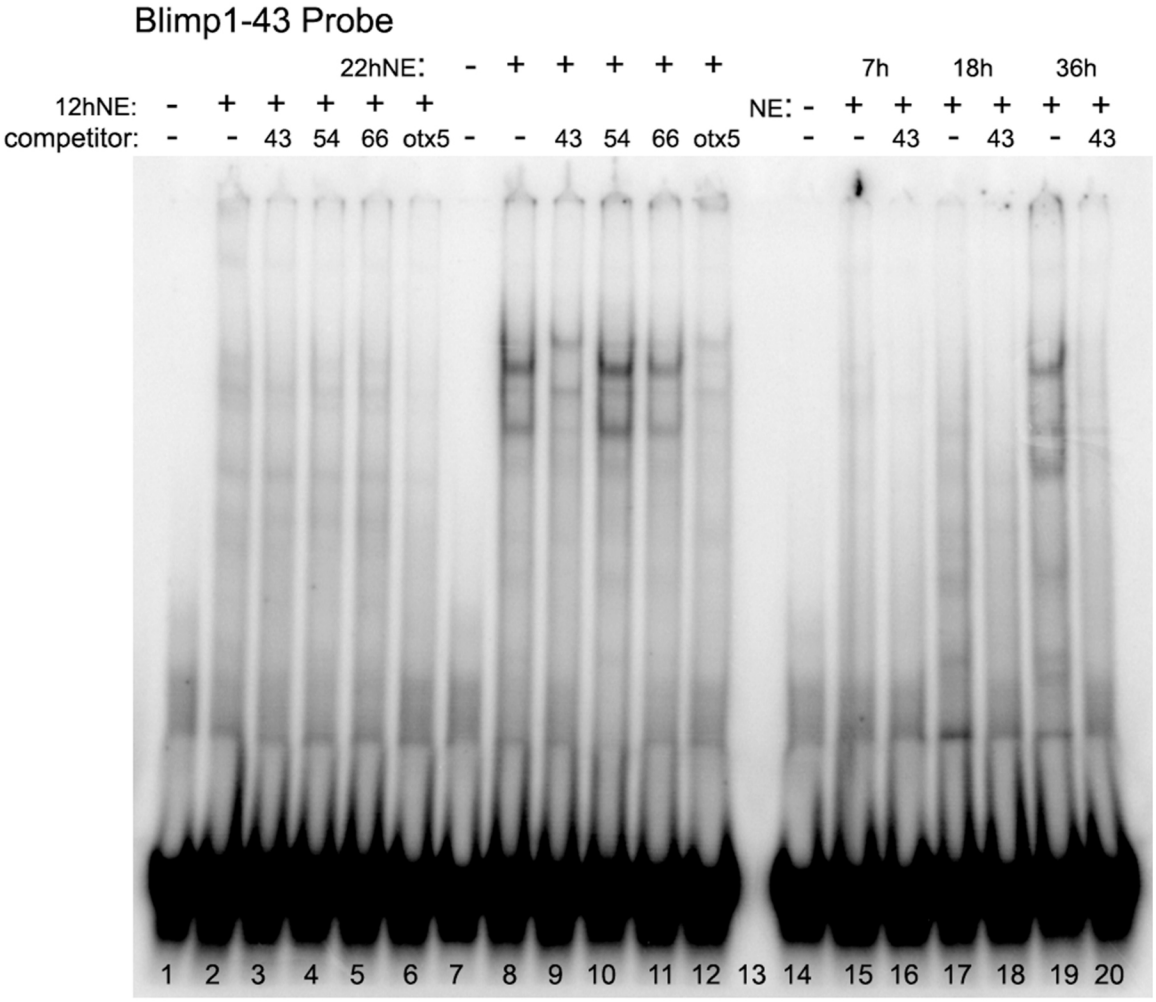
66_5': ATAATTATGAATGGACTAATTTCACTTTCAGTGATTTGACA

66_3': ATTATTGTCAAATCACTGAAAGTGAAATTAGTCCATTCATA

otx5_5': TCTTATCCTATCTTTCAGTTCGTGAGGCGG

otx5_3': GAACCGCCTCACGAACTGAAAGATAGGATA

Supporting Figure 5



Supplemental Material References:

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.H., et al., 2002a. A provisional regulatory gene network for specification of endomesoderm in the sea urchin embryo. Dev. Biol. 246, 162-190.

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.H., 2002b. A genomic regulatory network for development. Science 295, 1669-1678.

Yuh, C.H., Dorman, E.R., Howard, M.L., Davidson, E.H., 2004. An otx *cis*-regulatory module: a key node in the sea urchin endomesoderm gene regulatory network. Dev. Biol. 269, 536-551.