Chapter II

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

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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

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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 μ M of this morpholino when indicated, in conjunction with a MASO targeting the late form, antiblimp1/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 μ M concentrations. In all experiments, as a negative control, embryos injected with 300 μ M of the standard control morpholino (SCM) were included. All morpholinos were acquired from Gene Tools (Corvallis, OR).

Morpholino oligos were resuspended in water to a concentration of 500 μ M or 1 mM. A working solution of 100 to 300 μ M 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'CTGCCCATTCATCACATTTTCAACAATCTGAGTCGACAGATGACTCGAAGA 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 werealculated 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

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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 = Musmusculus; 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 timecourse 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 18h 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 longlived 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

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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.

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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 1bspecific 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 1/20tx 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 farestablished, 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

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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

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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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Figure Legends:

Fig. 1. Structure of blimp1/krox gene and splicing isoforms. (A) Annotation of BAC 169019 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).





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

п

A	1a protein		Zinc	Fingers		
	SET	domain		500		
	10 MGCNDNA	DO 200 300 VPSDMTEVDYQKK	400	500	600 70	00
В	1b protein		Zinc	Fingers		
	SET o	lomain				_
	100 MVIVEHPRPLAM	200 300 RDLYYLAAARRAMCPSFP\	400 500 PKPHPQVFDQV) 600 /FDSRHRLA	700 IGCNDNAVPSI	OMTEVDYQKK
С	SET Domain /	Alignment				
	SpBL1/K 1 LvBL1/K 1 AmBL1/K 1 AmBL1/K 1 MmBL1MP1 1 PtbLIMP1 1 RDBUMP1 1 SGBLIMP1 1 ThBLIMP1 1 DrBLIMP1 1 DrBLIMP1 1 AmBLIMP1 1 CeBLMP1 1 CbBLMP1 1 SpRL1/K 58 SpBL1/K 58		V D V I PKGTREG V D V I PKGTREG V D V I PKGTREG V S S S V I PKGTREG V S S S S S S S S S V S S V S S V S			-N KYEWRIYN -N KYEWRIY -NRKYEWRIY DACSTRIS- SEQILELUY
	SJEJI/K Se LVBL1/K Se AmBL1/K Se AmBL1/K Se AmBL1/K Se MmBL1MP1 61 PtBL1MP1 61 GgBL1MP1 60 GgBL1MP1 60 X1BL1MP1 60 DrBL1MP1 60 DrBL1MP1 60 MBL1MP1 59 AmBL1MP1 59 AmBL1MP1 60 CeBLMP1 68 CbBLMP1 67 SpKRL 41 MmKRAB 50			TI FYTTKI, EP TI FYTTKI, EP TI FYTTKI FI PA MI YFYT KFI PA MI YFYT KFI PA MI YFYT KFI PA MI YFYT KFI PA MI YFYT FI PA EI YFYT FI PA EI YFYT FI FI EI YFYT FI FI FI YFYT FI FI FI YFYT FI FI YFYT KFI FA MI YFYT KFI FI FI YFYT KFI FI MI YFYT FI M	CELEVANCE PAC NOELLAWC DEAD NOELLAWC DEAD NOELLAWC DEAD NOELLAWC DEAD NOELLAWC DEAD NOELLAWC DEAD NOELLAWC DEAD NOELLAWC DEAD NUC DELAWC DEAD NUC	ERSVASA IECOVANCT IECOVANCT IECYPS IEVYPS IEVYPS IEVYPS IEVPTS
D	Zinc Finger Al	ignment				
	SpBL1/K 1 LvBL1/K 1 AmBL1/K 1 RnBLIMP1 1 PtBLIMP1 1 GgbLIMP1 1 XIBLIMP1 1 FrBLIMP1 1 TrBLIMP1 1 DrBLIMP1 1 DmBLIMP1 1 AgBLIMP1 1 CeBLMP1 1 CbBLMP1 1 SpKRL 1 MmKRAB 1	CH ALG_TEP PRGKTVYECNVCK GY LE-YPLKKORKT YECNVCA S MC ENVOQOENGKT YECNVCA S MC ENVOQOENGKT YECNVCA S MC ENVOQOENGKT YEKKONA S MC ENVOQOENGKT YEKKONA S MC ENVOQOENGKT YEKKONA S MC ENVOQOENGKT YEKKONA S ME ENVOQOENGKT YEKKONA S ME ENV	EFGQLSNLKVHLEVE FGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE (TFGQLSNLKVHLEVE)		SPTC RALLOK HLW SPTC RALLOK HLW SPTC VAHLOK HLW SPTC VAHLOK YLW SPTC VAHLOK YLW SPTC VAHLOK YLW SPTC VAHLOK YLW SPTC VAHLOK HLW SPTC VAHLOK VAHLOK HLW SPTC VAHLOK VAHLOK HLW SPTC VAHLOK VAH	TGEK PLC CHVC TGEK PLC CHVC TGEK PLC VC TGEK PLC VC TGE PLC VC
	SpBL1/K 80 LvBL1/K 80 AmBL1/K 80 MmBL1MP1 80 RnBLIMP1 80 GgBLIMP1 80 GgBLIMP1 80 DrBLIMP1 80 DrBLIMP1 80 DrBLIMP1 80 DrBLIMP1 80 DrBLIMP1 80 CbBLMP1 80 CeBLMP1 80 CbBLMP1 81 CbBLMP1 81 SpKRL 74 MmKRAB 80	EKRESSTSNLKTH RLHSG KPY CKRESSTSNLKTH RLHSG KPY CKGESSTSNLKTH RLHSG KPY CKRESSTSNLKTH RLHSG KPY CKRESSTSNLKT LRLHSG KPY CCRESSTSNLKT LRLHSG K	CPAKENCOVILLIA CORAKTOVILLIA CORAKTOVILLIA CORAKTOVILLIA CORAKTOVILLIA CRAKTOPVILLIA CRAKTOPVILLIA CRAKTOPVILLIA CORAKTOPVILLIA CORAKTOVILLIA CORAKTOVILLIA CORAKTOVILLIA CORAKTOVILLIA CORAKTOVILLIA CORAKTOVILLIA CORAKTOVILLIA CORAKTOVILLIA CORAKTOVILLIA CORAKTOVILLIA CORAKTOVILLIA CORAKTOVILLIA	ALLEGNVELFM ALLEGNAELFM RELEGN	DFSDDADTPS DGSV DFSDDADTPS DGSL DPSDEANS PSPE AC HESTH CSV SC HENYTH CSV SC HENYTH CSV SC HENYTH CSV LH HESTH CSDV LH HESTH CSDV LH HESTH CSDV LH HESTH CSDV LH HESTH CSDV CC CKKYSA CV CC	IAGLQDHL MAGLHNNL IJLLRVPHR GN GGN GGN GGN GGN GGY GGY GGY GGY GSCLAAF MGTTSCKP- MGTSCKP- MGTSCKP

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.





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.





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

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- μ M 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.





Fig. 8. Negative blimp1/krox spatial autoregulation in veg2 mesodermal cells. Embryos were injected with 2 to 4 pl of a 100 μ M of anti-blimp1/krox1b MASO and 200 μ M 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



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.





Supplemental Material Figure Legends:

Supp. Table 1. \triangle Ct QPCR table. Table with examples of cycle differences (\triangle Ct) 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. \triangle Ct 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 <u>http://sugp.caltech.edu/endomes/qpcr.html</u>. 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

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(A)
SpBlimp1/Krox1a alignment
(exon 1a cDNA X exon 1a BAC sequences)
Bl/K1a
                       1-tcaaqttqqaaaqcacttqcttqctqttaccaccacaqaaaaqaqaaaaa-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-tqccaqnactcccccqattaqtttqttqnqattttqtaccqcqqtqttnt-100
1b BAC
                             tgccagtactcccccgattagtttgttgtgattttgtaccgcggtgtttt
5'race 101-tcaagcgaaagggagaaatggttatcgttgaacaccctcgcccgctagca-150
1b BAC
                             t c a a g c g a a a g g g a g a a a t g g t t a t t g t t g a a c a c c c t c g c c c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t c g c t 
1b BAC
                             5'race 201-cttccccgtgcctaagcctcatccac|aggtcttcgatcaggtcttcgaca-250
                            cttccccgtgcctaaacctcatccac aggtcttcgatcaggtcttcgaca
Sp BAC
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.



SpBLIMP1/KROX1a SpBLIMP1/KROX1a LvBLIMP1/KROX1a LvBLIMP1/KROX1b AmBLIMP1/KROX_alpha AmBLIMP1/KROX_beta FrBlimp1 DrBLIMP1 MmBLIMP1 MmBLIMP1 MmBLIMP1 SdBLIMP1 SlBLIMP1 DmBLIMP1 AgBLIMP1 AmBLIMP1 CeBLMP1 CeBLMP1	124 174 124 172 161 125 70 161 198 199 130 32 153 130 109 207 131 134 206	G SAEONIVACOYKTDI FYTTKA EPGOELFVWYCT FAKRLEKEVVAS G SAEONIVACOYKTDI FYTTKA EPGOELFVWYCT FAKRLEKEVVAS G SAEONIVACOYKTDI FYTTKA EPGOELFVWYCT FAKRLEKEVVAS G SAEONIVACOYKTDI FYTTKA EPGOELFVWYCT FAORLEKEVASA SAEONIVACOYKTDI FYTTKA EPGOELFVWYCT FAORLEKEVASA NSACIONIVACOYTDI YYTVKPI ROGTELLVWYCK FORLECEVAMET SACIONIVACOYTDI YYTVKPI ROGTELLVWYCK FORLECEVAMET SAREONIAACONGMI YYTVKPI ROGTELLVWYCK FORRIECEVAMET SAREONIAACONGMI YYTVKPI ROGTELLVWYCK FORRIECEVAMET SAREONIAACONGMI YYTVR I PAOELLVWYC ROFARLHY PYSGE SAREONIAACONGMI YYTI KPI PANOELLVWYC ROFARLHY PYSGE SAREONIAACONGMI YYTI KPI PANOELLVWYC ROFARLHY PYSGE SPREONIAACONGMI YYTI KPI PANOELLVWYC ROFARLHY PYSGE SPREONIAACONGMI YYTI KPI PANOELLVWYC ROFARLHY PYSGE SVCBONIAACONGMI YYTI KPI PANOELLVWYC ROFARLHY PYSGE SVCBONIAACONGMI YYTI KPI PANOELLVWYC ROFARLHY PYSGE SLOVONIAACONGMI YYTI KPI PANOELLVWYC ROFARLHY PYSGE SLOVONIAACONGMI YYTI KPI PANOELLVWYC ROFARLHY PYSGE SLOVONIAACONGMI YYTI KPI PANOELLVWYC ROFARLHY PYSGE SSONI AACONGMI YYTI ROTI PI
SpKRL	60	LCPAG AC TLLSGOECIORSDOVTSKSSRTSVVNRLISSSSPSIAPRS
MmKRAB	118	- SGERVALLEYLDROLDD PPOVPD D GOELLCSKAVLLTSAQGSESS
consensus	241	s egnlvacg iyfytvk i ngellvwyckdfa rl yp el
SpBLIMP1/KROX1a SpBLIMP1/KROX1b LvBLIMP1/KROX1a	174 224 175	
Ampi IMD1 /KPOX alpha	243	
AMBLIMPI/KROA_aipha	176	
FrBlimp1	121	MOR KOSLIEVIOO
DrBI.IMP1	212	
MmBLTMP1	249	
RnBLIMP1	250	
HsBLIMP1	181	
PtBLIMP1	83	
GaBLIMP1	204	
XIBLIMP1	181	
TnBLIMP1	133	
DmBLIMP1	287	PRYSMPAPEIPPDVAVSHITYVMGLHLPVGAGNGPANGSVAGSVSGATPPPPTATPCCRRSSPPAH
AqBLIMP1	200	
AmBLIMP1	183	
CeBLMP1	257	RVRRPATOLIPSAPP
CbBLMP1	252	RTR-ATSQQAPSAP
SpKRL	111	
MmKRAB	167	
consensus	321	m v r
SABLIMD1 /KROX12	184	۵،۷۵۷۹۵
SDBLIMP1 /KROX1b	234	
LVBLIMP1/KROX1a	185	VASAPSP
LVBLIMP1/KROX12	233	
AmBLIMP1/KROX alpha	227	AFPV/SEPK
AmBLIMP1/KROX beta	191	AFPVSEPK
FrBlimp1	136	OTTTEEHCDEASNPSSTTVTPTPPKREY VLSILRDTTSSSTKREPSRP-LPTRPR
DrBLIMP1	227	ATEVKHEPSRP-LPTRP
MmBLIMP1	264	SSEKNELYPRSVPKREYSVKEILKLDSNPSKRKDIYRSNISPFTLEKDMDGFR
RnBLIMP1	265	SSEKNELYPKSVPKREYSVKEILKLDSNPSKRKDSYRSNISPYTSEKDMDGFR
HsBLIMP1	196	STEKNELCPKNVPKREYSVKEILKLDSNPSKGKDLYRSNISPLTSEKDLDDFR
PtBLIMP1	98	STEKNELCPTNVPKREKSVKEILKLDSNPSNG-WANRSNISPFEKXXXXXXX
GgBLIMP1	219	SADKDELYQKSVPKKEHSVKKILKMESSPPKGKDFFQTNISPVTPEKDLDDLR
XIBLIMP1	194	KKKELIPQSKTPKKEHSVKEILRDTTSHMKHKDNLLSSMSAISPEKEKVGVH
TnBLIMP1	133	
DmBLIMP1	367	IQHGRHASVIIGQDRSPMASSDKDTAGSPLSGLDHQM PQDGSVRSVRSDEGYHSNECHEDGLTPPEDSSDSE
AgBLIMP1	271	${\tt SLRDRSPIRIVIREQSPLLRESVKEVDADSPISQTKETAYEHQL _ {\tt PNDGSVRSDEGYHSHGYHEDGFTPPEDSSDSE}$
AmBLIMP1	216	SIYERRSQM PTDGSVRSDEGYHSNGYHDEILTPPEESSESD
CeBLMP1	272	ASASTAIASL
CbBLMP1	265	ISSSTAIASL
SPKRL	111	
MINKRAB	167	
consensus	401	t

SpBLIMP1/KROX1a	191	
SpBLIMP1/KPOX1b	241	
LARDI THE TARGET	192	
LTDI IMD1 /VDOV1b	240	
LVBLIMP1/KROAID	240	
AMBLIMPI/KROX_alpha	235	
AMBLIMPI/KROx_beta	199	HEAL FSMPPRIISSG
FrBlimpi	191	CPDSP® RPLYPLA PAL
DrBLIMP1	261	CPKSPDRPLYPSAVYP
MmBLIMP1	317	KNGSP MPFYPRV YP
RnBLIMP1	318	KNGSPDMPFYPRVVYP
HsBLIMP1	249	RRGSPEMPFYPRVVYP
PtBLIMP1	150	XXXXXXXXXXXXXXXXX
GqBLIMP1	272	KNYSPERCFFPRVVYP
XIBLIMP1	246	
TnBI.IMP1	133	
DmBI IMD1	140	
	240	
AGBLIMPI	348	SHNIVLDCSNA EKEISVGQHAVSISEP
AMBLIMPI	258	S NNYVLDFSKNAKTSVCSSNEVVKQDNAAAKNEYRKVKIKITKTYGNFQTS
CeBLMP1	282	ABTIVAIDYSVKKTIES
CbBLMP1	275	AETIIAIDYSVKKUVE
SpKRL	111	GNVSYGHVRTNSQ P
MmKRAB	167	-QMEPVEPLLKQESLG
consensus	481	e d phlp
00110011000		
CODI IMDI /KDOVI -	207	
SPBLIMPI/KROXIA	207	
SDRTIMDI/KKOXID	257	
LvBLIMP1/KROX1a	208	
LvBLIMP1/KROX1b	256	
AmBLIMP1/KROX alpha	252	К
AmBLIMP1/KROX beta	216	К
FrBlimp1	209	R
DrBI.IMP1	277	
Mmpi IMpi	222	
	224	
RUBLIMPI	334	
HSBLIMP1	265	
PtBLIMP1	166	
GgBLIMP1	288	
XlBLIMP1	262	
TnBLIMP1	146	
DmBLIMP1	520	- CVKOEPSLKEVDOEMSLPOEEEEDOVMHPEPDSICPSTTTHLGDEHMLMMERERERERERIOEREPSNOOPASSTVIVL
AGBLIMP1	402	KNEKVEDELEDTEREV
AmBLIMD1	310	
	200	
CEBLMPI	299	
CDBLMP1	291	р
SpKRL	125	
MmKRAB	182	
consensus	561	
SOBLIMP1 /KROX1a	207	
SpBLIMD1 /KDOV1b	257	
LADI IMD1 /KDOV1 -	200	
LVBLIMPI/KROXIA	208	
LVBLIMP1/KROX1b	256	KVSNAKSSFPVYSGSGSGS-DHLGAEAGSSKTLYPSLPPTFPFSHRVGCSPPGTDIKQBPPSP
AmBLIMP1/KROX_alpha	253	AAATATTTVLNLSSTPAIAVDFPERLSTKRPIGVPVGAIVANDASGPGFSGPGFSHPMMSPQTSDVQTG
AmBLIMP1/KROX_beta	217	AAATATTTVLNLSSTPAIAVDFPERLSTKRPIGVPVGAIV@NPASGPGFSGPGFSHPMMSPQTSPVQTG
FrBlimp1	210	PHPN S DFLTRK SHHGYPATRSPNSHSSATPS SARSSESSPQGSEAGPVESPSP
DrBLIMP1	277	PRPS N®DFLKSTTVFGLPTRSHTOCSVTPSPSAHSSPERSPGSSPTSASIEPREAFLPFSPA
MmBLIMP1	333	IRAP PODFLKAS AYGMERPTYITHSPLPSSTTPS PASSEROSI KSSSPHSECONTUSPI. APGL PEHEDSYSYI MVS
PnBI.TMD1	334	TRADI DEDELKA SI AVGMED DTVTTHSDI. DSSTTDSDDA SSSDEGSI. KSASDHCSDGNTUSDIA/DCDDEHDDSVSVI. NVS
HeBLIMD1	265	TDAD DODDELKA CIAVCEADDTVTTDCDTDCCTTDCCADCCDCCDCCCCAMUCODVCACCOBUDDCVAVVIA
	200	
PLDLIMPI	төө	
GGRFIW51	000	IRPH PKNYLKAS AYGMDRPSYITHSPIQSSTTPSPSGRSSDDQSLKSSSPHSSPGVTVSPLAPTSQEHRESYSYLNGS
X1BLIMP1	288	
	288 262	FHSHIHDDYLKASVGYNTDRQNYLMHSPIQSSTTPS <mark>P</mark> SSR <mark>S</mark> SPDQSFKSSSPHS <mark>SP</mark> GSAVSPHHP-VQDHKEFYPFINRP
TnBLIMP1	288 262 146	FHSH_H_DVLKAS_GYNTDRQNYLMHSPIQSSTTPSPSSRSSEDQSFKSSSPHSSEGSAVSPHHP-VQDHKEFYPFINRP
TnBLIMP1 DmBLIMP1	288 262 146 599	FHSH HEDYLKAS GYNTDRQNYLMHSPIQSSTTPS SSRSSEDQSFKSSSPHSSEGSAVSPHHP-VQDHKEFYPFINRP
TnBLIMP1 DmBLIMP1 AgBLIMP1	288 262 146 599 448	FHSH HEDYLKASVGYNTDRQNYLMHSPIQSSTTPSPSSRSSEDQSFKSSSPHSSEGSAVSPHHP-VQDHKEFYPFINRP
TnBLIMP1 DmBLIMP1 AgBLIMP1 AmBLIMP1	288 262 146 599 448 352	FHSH HEDYLKASVGYNTDRQNYLMHSPIQSSTTPSESSRSSEDQSFKSSSPHSSEGSAVSPHHP-VQDHKEFYPFINRP
TnBLIMP1 DmBLIMP1 AgBLIMP1 AmBLIMP1 CeBLMP1	288 262 146 599 448 352 300	FHSH H DYLKASVGYNTDRQNYLMHSPIQSSTTPSESSRSSEDQSFKSSSPHSSEGSAVSPHHP-VQDHKEFYPFINRP
TnBLIMP1 DmBLIMP1 AgBLIMP1 AmBLIMP1 CeBLMP1 CbBIMP1	288 262 146 599 448 352 300 282	FHSH HEDYLKAS'GYNTDRQNYLMHSPIQSSTTSSESSEDQSFKSSSPHSSEGSAVSPHHP-VQDHKEFYPFINRP
TnBLIMP1 DmBLIMP1 AgBLIMP1 AmBLIMP1 CeBLMP1 CbBLMP1	288 262 146 599 448 352 300 292	FHSH HE DYLKASVGYNTDRQNYLMHSPIQSSTTPSESSRSSEDQSFKSSSPHSSEGSAVSPHHP-VQDHKEFYPFINRP
TnBLIMP1 DmBLIMP1 AgBLIMP1 CmBLMP1 CmBLMP1 CbBLMP1 SpKRL SpKRL	288 262 146 599 448 352 300 292 125	FHSH HE DYLKASVGYNTDRQNYLMHSPIQSSTTPS SSRSSEDQSFKSSSPHSSEGSAVSPHHP-VQDHKEFYPFINRP
TnBLIMP1 DmBLIMP1 AgBLIMP1 CBLMP1 CBLMP1 CDBLMP1 SpKRL MmKRAB	288 262 146 599 448 352 300 292 125 182	FHSH HEDVLKASVGYNTDRQNYLMHSPIQSSTTPSESSRSSEDQSFKSSSPHSSEGSAVSPHHP-VQDHKEFYPFINRP

SpBLIMP1/KROX1a SpBLIMP1/KROX1a LvBLIMP1/KROX1a LvBLIMP1/KROX1b AmBLIMP1/KROX_alpha AmBLIMP1/KROX_beta FrBlimp1 DrBLIMP1 MmBLIMP1 MmBLIMP1 HsBLIMP1 PtBLIMP1	271 321 271 319 322 286 268 340 413 414 345 246	STSPMGS-STLLPTOPAIPMSR.YPTS APVVVP EMIG SPNYTSTAGG TLASOPTYS	MPSSR MPSSR MPSSR RTPLQNGMSIIPSPS RTPLQNGMSIIPSPS JYSSLUPPQMP YSSLUPPHVPPPPS JYSNLLS-GSSLPHP VYSNLLG-GGSLPHP VYSNLLG-GGSLPHP
GgBLIMP1 XIBLIMP1	368 341	YGSEGLGSYPGYAPPSHLPSAFT PSYNPHYPKFLLPPFNMSCNNLSALNNUNGTNNFNLFPRMYPJ	YGNLLS-GGSLSHH
TnBLTMD1	167		
DmBLTMD1	675	VSVKKCODWCNAVSDCSSN CONDEOLSSSAWW/CEOEMTDA MIKCECSDDDSSUUUUWVI	FODG DUNAVICAC
AGBLIMP1	524	YSYKKSOPYC - TACSDSSS ON GONFEGISSBAUV VGEGEMIKA MIKGECSFFFFSIMININVI	ACCSCCPHI.SI.AI.AH
Ambi.TMD1	428	YSYKKSPRYCHTLDCSDDSSCH DMODDTCVNSTSCCNAAN.DMOSDTCN HSSTCNLHDCO	AGDNNI.I.OGHDGAAN
CertMP1	378		
CbBLMP1	369	LOWTKITETSI FUDPVVOTAATTTATGCRSGOPTDVOPVLAATAGAHEGN	
SpKRL	142	LTGSAYSNSDTEGSRSSPSSSTSSPS	
MmKRAB	2.07	SELOGILKMEDVAPVLSPRW EODSSOMN	
Consensus	721	slvp sm a llpvl is l iv	
consensus	/21	5 i y p 5 m g iipyi is i i iy	
SpBLIMP1/KROX1a	336	MMDSRSLEMTKNRHIQPKPLLNQPLADSNCYLSE	HEASMKQDV
SpBLIMP1/KROX1b	386	MMDSRSLEMTKNRHIQPKPLLNQPLADSNCYLSESLEMTKNRHIQPKPLLNQPLADSNCYLSE	HEASMKQDV
LvBLIMP1/KROX1a	336	MIDSRSME <mark>L</mark> TKNRHIQPKPHPNQPYTDSN <mark>G</mark> YLPESME <mark>L</mark> TKNRHIQPKPHPNQPYTDSN	PESSMKHDI
LvBLIMP1/KROX1b	384	MIDSRSMELTKNRHIQPKPHPNQPYTDSNCYLPESMELTKNRHIQPKPHPNQPYTDSNC	PESSMKHDI
AmBLIMP1/KROX_alpha	400	ILRTRGFDPRISKPDIRDDISKDTSLPKNSSPGATATHLSNFFQTSVPSFSLPI	SFADVHSQSNFKQET
AmBLIMP1/KROX_beta	364	ILRTRGFDPR SKPDIRDDSKDTSLPKNSSPGATATHLSNFFQTSVPSFSLPI	3FADVHSQSNFKQET
FrBlimp1	335	LLTSEAPGRHFLLPDAVHPSSISAHRDFFRPGPTSAFSAATSLKEKASHHHQYLGHPHGSAHSES	SSPTAGTSPPNNQV
DrBLIMP1	409	MLSSEGSRQFMLPPDSPAPRDILLPAATSAFSAATSLKDKPLHGHGHPYARAG	GSPTAGSAASTGCI
MmBLIMP1	492	MLNPASLPSSLPTDGARRLEPPEHPKEVLIPAPHSAFSLTCAAASMKDES-SEPS	3GSPTAGTAATSEHV
RnBLIMP1	493	MLNPASLPSSLPTDGARRLEPPEHPREVLVPAPHSAFSLTCAAASMKDES-SEPS	3GSPTAGTAATSEHV
HsBLIMP1	424	MINPTSLPSSIPSDGARRIQPEHPREVLVPAPHSAFSFTCAAASMKDKACSPTS	3GSPTAGTAA AEHV
PtBLIMP1	325	MUNPTSLPSS PSDGARRL QPEHPREVLVPAPHSAFSLTCAAASMKDKACSPTS	3GSPTAGTAA AEHV
GGBLIMP1	447	MENPTTLPSS PSEGGRRL QPDHPRDFLIPAPNSAFSITCAAASMKDKPCSPTS	3GSPTAGTAASSEHI
XIBLIMP1	417	THAALPGS PHEGERRFTQPELPROFLIPAPNSAFSITGAAASMKDKQSSTS	3GSPTAGTAASLEHI
TnBLIMP1	191	-ULPVSLPYPPSSQGelkEQ PNVSPPRGAPATPELSPLPKPSNQSPEI	?-SPSACEEAMNLSV
DmBLIMPI	751	EAGGHSPSPGYPGYPHYGAAATSTFISPPHSHSPFDRQSNASSGAGSATNLHLLQ-TSTQMLNHI	PLMQPLTPLQRLSP
AGBLIMPI	602	RVPGSTYSPP NGG-HYERITTSSTTPTTRSVSPPPPPPTQPAPAPGYVQLQS-KGQLPLGH	PLMQPLTPLTHMSPG
AMBLIMPI	505	HSSGNASTGNNHHS AGVUSSTTILTSTSNLHPSTAHQLPTSSTMQIDGNQTLIAGTHNLHLGH	1PGLTTHANSSSTLN
CEBLMPI	427		CMGGSAHTSSFHQL
CDBTWLI	419		GMGGSGHGSSFHQL
SPARL	168		RLDETSSNTSL
MMKRAB	236		QKPEQTVCFLGED
consensus	801	mi i aitratg p	s v
SpBLIMP1/KROX1a SpBLIMP1/KROX1b	379 429		LSNLKVHLRVHTGEK LSNLKVHLRVHTGEK
LvBLIMP1/KROX1a	379	PRRVRRSSNPNYCHRALG-YELPRRNGKTVYECNVCKREEGO	SNLKVHLRVHTGEK
LvBLIMP1/KROX1b	427	PRPRRSSNPNYCHRALG-YELPRRNCKIVYECNVCKREFCO	LSNLKVHLRVHTGEK
AmBLIMP1/KROX alpha	469	SKEDGSSSPYKLTRKRPSGNVEYCHKSLE-YQLPRKNGKIMYECNVCKKVFGQ	LSNLKVHLRVHSGER
AmBLIMP1/KROX beta	433	SKEDGSSSPYKLTRKRPSGNVEYGHKSLE-YQLPRKNGKIMYECNVCKKVFGQ	LSNLKVHLRVHSGER
FrBlimp1	415	P-TKPTSALLGNTSKHRSDGEAINLSKVKPGVGSVGYKALP-YPLKKQNGKIKYECNVCSKTFGQI	LSNLKVHLRVHSGER
DrBLIMP1	476	P-TKPTSAILSTSRSEDEAINLSKMKRGSTGYKSLD-YPLKKQNGKIKYECNVCTKTFGQI	LSNLKVHLRVHSGER
MmBLIMP1	560	VQPKATSSVMAAPSTDGAMNLIKNKRNMTGYKTLP-YPLKKQNGKIKYECNVCAKTFGQI	LSNLKVHLRVHSGER
RnBLIMP1	561	VQPKATSSVMAAPSTDGAMNLIKNKRNMTGYKTLP-YPLKKQNGKIKYECNVCAKTFGQI	LSNLKVHLRVHSGER
HsBLIMP1	493	VQPKATSAAMAAPSSDEAMNLIKNKRNMTGYKTLP-YPLKKONGKIKYECNVCAKTFGQ	LSNLKVHLRVHSGER
PtBLIMP1	394	VQPKATSAAMAAPSSDEAMNLIKNKRNMTGYKTLP-YPLKKQNGKIKYECNVCAKTFGQI	LSNLKVHLRVHSGER
GgBLIMP1	516	MQPKPISVVLAATGGEEAMNLIKSKRNVTGYKILP-YPLKKONGKIKYECNVCSKTFGQ	LSNLKVHLRVHSGER
XIBUIMPI	486	MQPNPISAVMSTCSEEAINLIKSWRNSTGYKILP-YPLKKQNGKIKYECNICSKTFGQ	SNLKVHLRVHSGER
TNBLIMP1	252	APTKTS TTARTAPGHRALP-YPLKKQNGKI KYECNI CSKTFGQ	SNLKVHLRVHSGER
DmBLIMP1	830	RISPPSSLSPDGNSCPRSGSPLSPNSLASRGYRSLP-YPLKKKDGKMHYECNVCCKTFGQ	SNLKVHLRTHSGER
AGBLIMP1	677	RISPPSSLSPDGGSYSRSGSPMSPGSPNSRGYRSLP-YPLRKRDGKMHYECNVCSKTFGQ	SNLKVHLRTHSGER
AMBLIMPI	585	RISPASSLSPDDHACSQSGS-LSPNSQGSRGYRSILP-YPLKKKDGKMHYECNVCCKTFGQ	SNLKVHLRTHSGER
CERTWAT	477	PFVNHOSSSHNDSSINGVPNYVQQQENGKT MACKDONKTFGQ	SNLKVHVRTHTGER
CDBLMPI	468	Prvnhstashnus	SNLKVHVRTHTGER
MWKDYB WWKDYB	209 201		SONCOPINQRI HIGAR
CONSENSIIS	204 881	ss r aureln unlik nakikuaChuc ktean	Sn]kvH]PvHaCor
0011001000	00T		

SpBLIMP1/KROX1a SpBLIMP1/KROX1b LvBLIMP1/KROX1b AmBLIMP1/KROX1b AmBLIMP1/KROX_alpha AmBLIMP1/KROX_beta FrB1imp1 DrBLIMP1 MmBLIMP1 HsBLIMP1 HsBLIMP1 GgBLIMP1 X1BLIMP1 DmBLIMP1 AgBLIMP1 AgBLIMP1 AgBLIMP1 SpKRL MmKRAB consensus	435 485 485 500 493 549 633 634 566 467 558 309 904 751 558 309 904 751 558 535 526 2450 961	PFSCDLCGKGFTGFAHLQKHHLVHTGEKPHCGHVCEKRFSSTSNLKTHMRLHSGLKPYNCKECPAKENQCVHLKLHKKAH PFSCDLCGKGFTGFAHLQKHHLVHTGEKPHCGHVCEKRFSSTSNLKTHMRLHSGLKPYNCKECPAKENQCVHLKLHKKAH PFSCDLCGKGFTGFAHLQKHHLVHTGEKPHCGHVCEKRFSSTSNLKTHMRLHSGLKPFNCKECPAKENQCVHLKLHKKAH PFSCDLCGKGFTGFAHLQKHHLVHTGEKPHCGHVCEKRFSSTSNLKTHMRLHSGLKPFNCKECPAKENQCVHLKLHKKAH PFRCEVCSKGFTGVAHLQKHLVHTGEKPHCGVCHKGFSSTSNLKTHMRLHSGKPYTCKLCPAKETQVHLKLHKKAH PFRCEVCSKGFTQAHLQKHLVHTGEKPHCGVCHKRFSSTSNLKTHMRLHSGKPYTCKLCPAKETQFVHLKLHKRSH PFRCEVCSKGFTQLAHLQKHYLVHTGEKPHCGVCHKRFSSTSNLKTHMRLHSGEKPYCKLCPAKETQFVHLKLHKRSH PFKCTCNKGFTQLAHLQKHYLVHTGEKPHECQVCHKRFSSTSNLKTHLRLHSGEKPYCKLCPAKETQFVHLKLHKR PFKCTCNKGFTQLAHLQKHYLVHTGEKPHECQVCHKRFSSTSNLKTHLRLHSGEKPYCKUCPAKETQFVHLKLHKR PFKCTCNKGFTQLAHLQKHYLVHTGEKPHECQVCHKRFSSTSNLKTHLRLHSGEKPYCKUCPAKETQFVHLKLHKR PFKCTCNKGFTQLAHLQKHYLVHTGEKPHECQVCHKRFSSTSNLKTHLRLHSGEKPYCKUCPAKETQFVHLKLHKR PFKCTCNKGFTQLAHLQKHYLVHTGEKPHECQVCHKRFSSTSNLKTHLRLHSGEKPYCKUCPAKETQFVHLKLHKR PFKCTCNKGFTQLAHLQKHYLVHTGEKPHECQVCHKRFSSTSNLKTHLRLHSGEKPYCKUCPAKETQFVHLKLHKR PFKCTCNKGFTQLAHLQKHYLVHTGEKPHECQVCHKRFSSTSNLKTHLRLHSGEKPYCKUCPAKETQFVHLKLHKR PFKCTCNKGFTQLAHLQKHYLVHTGEKPHECQVCHKRFSSTSNLKTHLRLHSGEKPYCKUCPAKETQFVHLKLHKR PFKCTCNKGFTQLAHLQKHYLVHTGEKPHECQVCHKRFSSTSNLKTHLRLHSGEKPYCKUCPAKETQFVHLKLHKR PFKCTCNKGFTQLAHLQKHLVHTGEKPHECQVCHKRFSSTSNLKTHLRLHSGEKPYCKUCPAKETQFVHLKLHKR PFKCTCNKGFTQLAHLQKHLVHTGEKPHECQVCHKRFSSTSNLKTHLRLHSGKPYQCKUCPAKETQFVHLKLHKR PFKCTCNKGFTQLAHLQKHLVHTGEKPHCQVCHKRFSSTSNLKTHLRLHSGKPYQCKUCPAKETQYVHLKLHKR PFKCNVCTKSFTQLAHLQKHLVHTGEKPHQCDICKKRFSSTSNLKTHLRLHSGKPYQCKUCPAKETQFVHLKLHKR PFKCNVCTKSFTQLAHLQKHLVHTGERPHQDICKKRFSSTSNLKTHLRLHSGKPYAQDLCPQKFTQFVHLKLHKR PFKCNVCTKSFTQLAHLQKHLVHTGERPHQDICKKRFSSTSNLKTHLRLHNGCKPYTQVQUQAKETQYVHLKLHKR PFKCNVCTKSFTQLAHLQKHLVHTGERPHQDICCKKRFSSTSNLKTHLRLHNGCKPYTQVQUQAKETQYVHLKLHKR PFKCDICTKBFTQLAHLQKHLVHTGERPHCDICCKKRFSSTSNLKTHLRLHNGCKPYTQVQUQAKETQYVHLKLHKR PFKCDICTKBFTQLAHLQKHLVHTGERPHCDICCKKRFSSTSNLKTHLRLHNGCKPYTQVQUCAKFTQYVHLKLHKR PFKCDICTKBFTQLAHLQKHLVHTGE
GEDI IND1 (VDOV1 -	F 1 F	
SpBLIMPI/KROAIA	515	LEG
LVBLIMP1/KROX1a	515	LEGNAELFMDFSDDAT TESTDGSDMAGLMHHJASTDVTDVFGNSDVASTGSRVHUD
LvBLIMP1/KROX1b	563	LEGNAELFMDFSDDA TESTDGSDMAGLHNHLASTDVTDYFGNSDVASTGSRVH
AmBLIMP1/KROX_alpha	616	LEEGYVYDPSEEANLSVSPSPSLLLLERVPRRFKIKTEFQSYQSHLVSTDVADYFGQMGEDSPSQSSESE
AmBLIMP1/KROX_beta	580	$\label{eq:legyvydpseeanlsvspspsylllevprox} Legyvydpseeanlsvspspsylllevproxes to the state of t$
FrBlimp1	571	LHS-RERPHKCLHCHRHYIHLCSLRLHLKGVCLAAGSRPGSPVVSSHGSLDEVQ
DrBLIMP1	627	
MMBLIMPI	711	
HeBLIMPI	644	
PtBLIMP1	545	
GgBLIMP1	667	LHT-RERPHKCIHCHKSYIHLCSLQVHLKGNCPVA-PASGLSMEDLN
X1BLIMP1	636	LHT-RERPIKCIHCHKSYIHLCSLNFHMKGNCPVS-PRLGLSREDIN
TnBLIMP1	387	LHSNHDRPIR QLCTQTFHPFSLRIHQSCCLAAFKGPVN
DmBLIMP1	982	N
AGBLIMPI Ambi.tMp1	829	
CeBLMP1	613	
CbBLMP1	604	LHA-NERPYSCGTCGKKYISPSGLRTHWKTTTCKEEDTKDMLMWKKDDLM
SpKRL	305	QHEGCORA AQSGNEKRHYRTH
MmKRAB	418	WESQS
consensus	1041	lht herpk c c k yi lr h k d l
SpBLIMP1/KROX1a	571	DDDDDEGKLINOSERSHETSPMGYMFRSDAMDETSOE
SpBLIMP1/KROX1b	621	DDEDDEGKLIIQESCRSIETSPMGYMFRSDAMDETSQELCDSPSTGITASSCNGVEDSCNGVED
LvBLIMP1/KROX1a	571	DEDDDEDKLVIQEGRKSQEASPAGYMFGSDAIEGSPQGFSDSASTGIPLPSGNDEEDAMETAEFSRGQLPAFESKS
LvBLIMP1/KROX1b	619	DEDDEDEEMKUVQEGRKSQEASPAGYMFGSDAIEGSPQGFSDSASTGIPLPSGNDEEDAMETAEFSRGQLPAFESKS
AmBLIMP1/KROX_alpha	689	EK®SLPEGSHAQTSCVTECHSDQTSMTTFRDGSPSSVRGSPPLLVPINRAPMNSNDTQDKPEVN
AmbLIMPI/KROX_Deta	653	EKSLPEGSHAQISCITE HSDQISMI-IFRDGSPSSVRGSPPLVPINRAPMNSNDTQDRPEEVN-
DrBLIMP1	673	
MmBLIMP1	756	RINEE BRFDISINADRIEDMEDSVDVTSMVERE LAVVRKEKE
RnBLIMP1	757	RINBELERFOLSONADRLEDMEDSVDVTSMVEKELAIVRKEKE
HsBLIMP1	689	RINEELEKFDISONADRIEDVEDDISVISVVEKEILAVVRKEKE
PtBLIMP1	590	RINEELEKFDISONADR <mark>DE</mark> DVEDDISVISVVEKEILAVVRKEKE
GGBLIMP1	712	
TDRI IMPI	120	R NDE ENFLIS SAUGE DMED-MDMSPAVENE MIL RREID-
DmBLTMP1	+∠9 1017	NUBER AMAAAATSECLDKDHPEPDSREAYEOLTOHMHPAVHDGLRHLSSCGOSPDRI.TPLGNHMAPOOOSHOOOOOOO
AqBLIMP1	864	NIEDE AMAAAATSECL
AmBLIMP1	771	NIEDELALAAAVGS
CeBLMP1	659	DIKGEIDEGSMSGSCYGNLGIFENTLNSELK-RPIMPIETIYSKYNLPNASLLGQGPSGMQEQQAPP
CbBLMP1	653	EIKGEIDEGSPSNYMDIFENPLNSEIKPRPLLPIDTIYTKYNISNPSLLGQGPSGMQEQQPPP
SpKRL	327	
MMKRAB	465	RVDSHWENIETPVSYQCNDCERSFSRITSLIENQKVHTGE
CONSENSUS	TTST	vedetek ise g te k v i

CODI IMDI /KDOVI -	620	
SPELIMPI/KROXIA	628	
LVBLIMP1/KROX1a	647	KAASI.SSSEEDTI.DDSHCTKEI.HDDI.DVASTGSEVHI.DDEDADEDKI.VI.OFGEKSI.EASDACKMEGSDVIEEADOGESDS
LVBLIMP1/KROX12	695	KAASUSSEEPTUPPOIGTKELEDI. VASTGSRVHI. DEEDDEDKUVU VEGRKSLEASPAGVMEGSDVIEEAPOGSDS
AmBLIMP1/KROX alpha	754	
AmBLIMP1/KROX beta	718	
FrBlimp1	668	
DrBLIMP1	716	
MmBLIMP1	800	
RnBLIMP1	801	
HsBLIMP1	733	
PtBLIMP1	634	
GgBLIMP1	756	
X1BLIMP1	724	
TnBLIMP1	473	
DmBLIMP1	1097	QQGVPPPHLLMTQHSVG
AgBLIMP1		
AmBLIMP1		
CeBLMP1	725	
COBLMPI	716	
SPARL	FOF	
MMKRAB	1201	
consensus	1201	
SpBLTMP1 /KPOX1a	628	
SpBLIMP1/KROX1b	678	
LvBLIMP1/KROX1a	727	ASTGIPLOSGTDEKDAMETAEFSRDKLPAFESEGKSASPLSSEEPTLPSHGTKRLHPDLEFGSRSKFSNPA
LVBLIMP1/KROX1b	775	ASTGIPLPSGTDEKDAMETAEFSRDKLPAFESEGKSASPLSSEEPTLPPSHGTKRLHPDLEFGSRSKFSKSNPA
AmBLIMP1/KROX alpha	754	TLYHRESGDSYREKSGRPNDAALFETDASREATVNTNNSLAVGIDSEHKOKENSEGNIKCK EKCTVIOSOSHA
AmBLIMP1/KROX beta	718	TLVHRESGDSYREKSGRPNDAALFETDASREATVNTNNSLAVGIDSAHKOKENSEGNIKCKEKCTVIOSOSHA
FrBlimp1	668	ACYGAHESLSETSVIKLRGSPILPHHTN
DrBLIMP1	716	FKAS-YHKGSTGGDLHPRAPALSAYHLNEHDSEASVIKVHRSSPIQLLP
MmBLIMP1	800	SCCSLYES-SDLSLMKLPHSNPUPLVP-
RnBLIMP1	801	SCCSLYES-SDLSLMKLPSSCCSLYES-SDLSLMKLPS
HsBLIMP1	733	S ^C CSLYES-SDLPLM <mark>KL</mark> PP <mark>S</mark> NPIPLVP
PtBLIMP1	634	SCCSLYES-SDLPLM <mark>KL</mark> PP <mark>S</mark> NPLPLVP
GgBLIMP1	756	SCCNLYES-SDMSIM <mark>KL</mark> PHSHPIPLLP-
X1BLIMP1	724	SCCNFYDR-SDAIVMKLPISSMKVSVSRNMGNGLITSCCNFYDR-SDAIVMKLPISSPIPLLP
TnBLIMP1	473	ED
DmBLIMP1	1114	PAPMLLTTASQLPPPPPHHQQNSPSRLLQHGHAHPLQMQQQQQQQQSHSPKGL K SLPESG W YLHGQH
AgBLIMP1		
AmBLIMP1		
CEBLMPI	725	PTSQQQQHMMYGNTMGHMGQGSHLQGPPPPPQHPGMDHSGMQNGGGTPHQHQLLQGGPSSGSGQQQHPQHNGI
CDBLMPI	716	htbddddynwianawehwadadtdardabadhrdwnhuawduae-hbhdhdrrdb-dsadashuhndsahdb
SPARL	FOF	
CORSORGUS	1291	
consensus	1201	
SDBLIMP1 /KROX1a	687	K RNV/OKINPTNKS
SpBLIMP1/KROX1b	737	K ENVVOKI NRTNKS
LVBLIMP1/KROX1a	801	K ETVVOKI NRTSKS
LvBLIMP1/KROX1b	849	K ETVVOKI NRTSKS
AmBLIMP1/KROX alpha	828	TKIENVVOKI SKAVOSS
AmBLIMP1/KROX beta	792	TKTENVVQKILSKAVQSS
FrBlimp1	714	S K ESEDHA
DrBLIMP1	764	K KKETEEAMDT
MmBLIMP1	844	VKVK©ETVEPMDP
RnBLIMP1	845	VKVKCETVEPMDP
HsBLIMP1	777	VKVKCETVEPMDP
PtBLIMP1	678	VKVK0ETVEP#DP
GgBLIMP1	800	VKVK0ETVEP#DP
XIBLIMP1	768	WWWK@ESFDQWDS
TnBLIMP1	1107	
DMBLIMP1	1181	MÖYÖR2KA2AMPR2NŐBUTIECI.
AGRLIMPI		
	700	
CEBLMP1 Chrimp1	798	
SpKRI.	/84	
MmKRAB	552	IN
consensus	1361	lkl n l
	-	

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': AAATGAGGGTATCGA<u>TTTCACTTCC</u>TAAAACTACG 39.3_3': CATTTCGTAGTTTTA<u>GGAAGTGAAA</u>TCGATACCCT 41.5_5': CAATTTCTATTTCTA<u>TTTCACTT</u>AAATGTTTCT 41.5_3': AATTGAGAAACATTT<u>AAGTGAAA</u>TAGAAATAGA 43_5': GACATATTAGTAGA<u>TTTCACTT</u>ATCGGGAGAGTG 43_3': ATGTCCACTATCCCGAT<u>AAGTGAAA</u>TCTACTAAT 53.1_5': CATATCCCTCTT<u>TTTCACTT</u>TATTCATTG 53.1_3': ATATGCAATGAATA<u>AAGTGAAA</u>AAGAGGG 54_5': TCGGTAATTGTTCTGTATT<u>TTTCACTT</u>TCGCAGTACCGTTA 54_3': ACCGATAACGGTACTGCGA<u>AAGTCAAA</u>AATACAGAACAATT 54.5 *5*': GGATAATAAAAATAT<u>GAAAGGGGAAA</u>GCAGGAAGGTA 54.5_3': TATCCTACCTTCCTGC<u>TTTCCCTTTC</u>ATATTTTAT 62.7_5': CTTAAAATCATATGG<u>TTTCACTT</u>AAACCTATTT 62.7_3': TTAAGAAATAGGTTT<u>AAGTGAAA</u>CCATATGATT 66_5': ATAATTATGAATGGACTAA<u>TTTCACTT</u>TCAGTGATTTGACA 66_3': ATAATTATGAATGGACTAA<u>TTTCACTT</u>TCAGTGATTTGACA otx15_5': CATTAGGCCGATAGAGCTCGCTG<u>AGAAGGGAAA</u>AAAC otx15_3': ATGGTTT<u>TTTCCCTTCT</u>CAGCGAGCTCTATCGGCCTA



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 $15 \cdot 16 = 7$ h ne; lanes $2 \cdot 6 = 12$ h ne; lanes $17 \cdot 18 = 18$ h ne, lanes $8 \cdot 12 = 22$ h ne; and lanes $19 \cdot 20 = 36$ 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. $43_5'$: GACATATTAGTAGA<u>TTTCACTT</u>ATCGGGAGAGTG $43_3'$: ATGTCCACTATCCCGAT<u>AAGTGAAA</u>TCTACTAAT

54_5': TCGGTAATTGTTCTGTATT<u>TTTCACTT</u>TCGCAGTACCGTTA 54_3': ACCGATAACGGTACTGCGA<u>AAGTCAAA</u>AATACAGAACAATT 66_5': ATAATTATGAATGGACTAA<u>TTTCACTT</u>TCAGTGATTTGACA 66_3': ATTATTGTCAAATCACTGA<u>AAGTGAAA</u>TTAGTCCATTCATA otx5_5': TCTTATCCTATC<u>TTTCAGTTCG</u>TGAGGCGG otx5_3': GAACCGCCTCACGAACTGAAAGATAGGATA





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.

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