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Krüppel-like is required for nonskeletogenic mesoderm specification in the sea urchin embryo

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ABSTRACT

The canonical Wnt pathway plays a central role in specifying vegetal cell fate in sea urchin embryos. *SpKrl* has been cloned as a direct target of nuclear β–catenin. Using *Hemicentrotus pulcherrimus* embryos, here we show that HpKrl controls the specification of secondary mesenchyme cells (SMCs) through both cell-autonomous and non-autonomous means. Like *SpKrl*, *HpKrl* was activated in both micromere and macromere progenies. To examine the functions of HpKrl in each blastomere, we constructed chimeric embryos composed of blastomeres from control and morpholino-mediated *HpKrl*-knockdown embryos and analyzed the phenotypes of the chimeras. Micromere-swapping experiments showed that HpKrl is not involved in micromere specification, while micromere-deprivation assays indicated that macromeres require HpKrl for cell-autonomous specification. Transplantation of normal micromeres into a micromere-less host with morpholino revealed that macromeres are able to receive at least some micromere signals regardless of HpKrl function. From these observations, we propose that two distinct pathways of endomesoderm formation exist in macromeres, a Krl-dependent pathway and a Krl-independent pathway. The Krl-independent pathway may correspond to the Delta/Notch signaling pathway via GataE and Gcm. We suggest that Krl may be a downstream component of nuclear β–catenin required by macromeres for formation of more vegetal tissues, not as a member of the Delta/Notch pathway, but as a parallel effector of the signaling (Krl-dependent pathway).

Keywords: Krüppel-like, mesoderm specification, Delta/Notch signaling, effector

INTRODUCTION

In sea urchin embryos, the animal-vegetal (A-V) axis, which is established before fertilization, is evident at the 16-cell stage, and mesomeres, macromeres, and micromeres are aligned along the A-V axis. Classical experiments showed that the animal and vegetal halves of bisected eggs have different developmental potentials: the vegetal halves develop into pluteus-like larvae, while the animal halves form permanent blastulae consisting solely of ectoderm (Hörstadius, 1973; Maruyama et al., 1985). Recent reports have demonstrated the central role of the canonical Wnt pathway in specifying vegetal cell fate in sea urchin embryos (Wikramanayake et al., 1998; Emily-Fenouil et al., 1998; Logan et al., 1999; Vonica et al., 2000). The entry of β−catenin into embryonic cell nuclei begins in the micromeres and gradually spreads through the macromere progeny (Logan et al., 1999).

SpKrl (*Strongylocentrotus purpuratus Krüppel-like/Sp-z13*) and *micro1* are direct targets of nuclear β−catenin in sea urchin embryos, along with *Wnt8* and *Blimp1/Krox/z51*, which are included in the Gene Regulatory Network (GRN) model of endomesoderm specification (Howard et al., 2001; Nishimura et al., 2004; Oliveri and Davidson, 2004; Materna et al., 2006). *SpKrl* encodes a transcriptional repressor containing a Zn-finger DNA-binding domain similar to that in the *Drosophila* Krüppel protein (Howard et al., 2001). The gradual activation of *SpKrl* in the vegetal hemisphere coincides with the nuclear localization of β−catenin (Minokawa et al., 2004). In contrast, *micro1* encodes a transcriptional repressor with a homeodomain; its activation is restricted to the micromere lineage (Kitamura et al., 2002). Functional analyses of *SpKrl* and *micro1* have shown that these gene products are important mediators of the vegetalizing activity of nuclear β−catenin. Translational inhibition of SpKrl with

morpholino blocks archenteron formation but not primary mesenchyme cell (PMC) specification (Howard et al., 2001). Conversely, overexpression of *SpKrl* vegetalizes embryos by increasing the number of PMCs. *micro1* is sufficient for differential micromere specification, and its expression endows animal blastomeres with the ability to induce an animal cap for endoderm production (Yamazaki et al., 2005).

Endoderm and nonskeletogenic mesoderm are specified both cell-autonomously and non-autonomously. Although the microsurgical removal of micromeres significantly delays gastrulation, micromere-less embryos develop some secondary mesenchyme cells (SMCs) as well as endoderm (Sweet et al., 1999). These observations indicate that, unlike mesomeres, which exclusively give rise to ectoderm, macromeres are pre-specified for endomesoderm, and that micromere signals conditionally accelerate and complement the formation of endomesodermal tissues in macromere progeny. Micromeres require nuclear β−catenin for signal production, while macromeres require nuclear β−catenin in order to receive inductive signals, including Delta (Logan et al., 1999; McClay et al., 2000).

Morpholino-mediated *SpKrl*-knockdown in whole embryos delays archenteron formation (Howard et al., 2001). However, gastrulation is controlled by distinct mechanisms, including inductive signals emanating from micromeres, signal reception by macromere descendants, and cell-autonomous macromere specification. Here we examined the involvement of Krl in the specification of micromeres and macromeres. To evaluate the function of Krl in each blastomere type, we constructed several different chimeric embryos from control and experimental embryos. By analyzing the phenotypes of the chimeras, we found that Krl is required for the autonomous and conditional production of SMCs in macromeres. Finally, we propose

that two distinct pathways of SMC formation exist in macromeres, a Krl-dependent pathway and a Krl-independent pathway.

MATERIALS AND METHODS

Animals and embryos

Adult *Hemicentrotus pulcherrimus* were collected near Noto Marine Laboratory, Kanazawa University, Japan. The embryos were cultured at 15°C in Jamarin U artificial seawater (JSW; Jamarin Laboratory). For vegetalization, embryos were treated with JSW containing 66 mM LiCl for 4 hours beginning at the 16-cell stage.

Cloning of *HpKrl* cDNA

Degenerate PCR primers were designed that correspond to the first and fourth Zn-finger repeats of SpKrl: 5'-TGYAARTTYTGYCCNAA-3' (coding for CKFCPK) and 5'-ARRTGNGTNCKRTARTG-3' (coding for HYRTHL), respectively. PCR was performed under standard conditions with a 10-μM final concentration of each primer using cDNA from the cleavage-stage embryo as the template. Products of the expected size (~0.3 kb) were cloned into the p3T vector (Mo Bi Tec). The cDNA fragment was used to screen a cDNA library of *H. pulcherrimus* 16- to 60-cell-stage embryos, which was constructed using the lambda ZAP II vector (Stratagene). The sequence, which has been deposited in the DDBJ database as *HpKrl* (accession number AB300323), includes an in-frame ATG sequence 48 nucleotides upstream of the translation initiation site predicted in *SpKrl* (Howard et al., 2001). However, the sequence surrounding the first ATG did not match the consensus sequence reported by Kozak (1991) with respect to

the translation initiation site (a purine residue at nucleotide –3 and a guanine residue at +4 occur in 97% and 46% of vertebrate mRNAs, respectively). Therefore, we designated the second ATG as a putative initiation site since the surrounding sequence matched the consensus sequence.

RT-PCR

To estimate the expression level of several marker genes in embryos that had been injected with *HpKrl* mRNA or morpholino, semi-quantitative RT-PCR was carried out using ThermoStart *Taq* DNA polymerase (ABgene). cDNA was synthesized from total RNA using ReverTra ACE (Toyobo) and random 9-mer oligonucleotides. The primer sequences used for the marker genes were described by Yamazaki et al. (2005).

Constructs for *in vitro* transcription

Modified Bluescript RN3 (Nishimura et al. 2004) was used to make the expression constructs. For the HpKrl construct, full-length *HpKrl* cDNA containing the 5' and 3' UTRs was PCR-amplified using KOD Plus DNA Polymerase (Toyobo) and cloned between the *Eco*RI and *Not*I sites of the vector. The primer sequences and positions within the *HpKrl* cDNA were: *Eco*RI-Krl-forward, 5'-GGAATTCGTACGGCAAAGATCTATTTGG-3' (38 to 58); *Not*I-Krl-reverse, 5'-TAAAGCGGCCGCATGGGGTGCAAGAACATC-3' (1,242 to 1,259). To obtain an HpKrl construct without a morpholino target site, *Eco*RI-Krl-forward2 was used instead of *Eco*RI-Krl-forward (sequence [position]: 5'-GGAATTCCGTCCAATCGCAACGA-3' [123 to 138]). For an artificial HpKrl/GFP construct, the 5'UTR plus coding sequence (36 nucleotides) of *HpKrl* was PCR-amplified using *Eco*RI-Krl-forward and *Eco*RI-5Krl-reverse primers, and inserted into *Eco*RI site of the plasmid Bluescript

RN3'-GFP. The primer sequence and position within the HpKrl cDNA was: *Eco*RI-5Krl-reverse, 5'-GGAATTCAAGGGACAGGAGTGAAGAT-3' (166 to 184).

Synthetic mRNAs and antisense oligonucleotides

Capped RNA was transcribed from linearized constructs using the mMessage mMachine Kit (Ambion). The RNA was diluted to 0.1–2.5 pg/pl in 40% glycerol, and ~3 pl of the solution were injected into each egg as described by Gan et al. (1990). The morpholino oligonucleotides (MKrl-1 and MKrl-2) and a standard control oligo were obtained from Gene Tools. The sequences and positions of the morpholinos with respect to the translation initiation site were: MKrl-1, 5'-TGATGCCGAAAGGCAGTGGAGACAT-3' (-48 to -24); MKrl-2, 5'-ATGCCGCGTGTAAACGGTCCAT-3' (+1 to +25). The morpholinos were dissolved in 40% glycerol, and ~3 pl of a 1-mM solution was injected into fertilized eggs, giving a final concentration of ~5 μM in each egg.

Embryo manipulation

Chimeric embryos were produced according to the method of Amemiya (1996). Transplanted blastomeres were stained with rhodamine B isocyanate (Sigma) to trace the lineage of the cells. The chimeras were cultured in ASW containing 100 U/ml penicillin and 50 μg/ml streptomycin sulfate in dishes coated with 1.2% agar.

Antibody and phalloidin staining

Whole-mount immunostaining was performed using P4 monoclonal antibody as previously described (Yamazaki et al., 2005). For phalloidin staining, embryos were suspended in an extraction buffer (pH 6.7) containing 25 mM 2-[N-

morpholino]ethanesulfonic acid (MES), 10 mM ethyleneglycol-bis-tetraacetic acid (EGTA), 0.3 mM $MgCl_2$, 2% Triton X-100, 0.1 mM m-maleimidobenzoil-N-hydroxysuccinimide ester (MBS, Pierce), and 0.1 mM PMSF. MBS was added to cross-link the actin molecules within the muscle fibers. After extraction at room temperature for 1 hour, the embryos were fixed for 1 hour in phosphate-buffered saline (PBS) containing 3.7% formaldehyde. After washing three times with PBS, the embryos were incubated for 1 hour with 0.6 μM phalloidin-TRITC (Sigma) in PBS. The embryos were then washed three additional times with PBS and observed with epifluorescence optics.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out according to the method of Arenas-Mena et al. (2002) using a DIG-labeled probes derived from *HpKrl* cDNA.

RESULTS

Cloning, structure, and expression of *HpKrl*

Because SpKrl includes four Zn-finger motifs similar to those in the *Drosophila* Krüppel protein, we designed degenerate primers that correspond to the conserved sequences in the first and fourth Zn-finger repeats and amplified a ~0.3-kb *HpKrl* fragment by RT-PCR. Using the fragment as a probe, full-length *HpKrl* cDNA was isolated from a *H. pulcherrimus* library. The predicted peptide sequence, consisting of 336 amino acids, was 83% identical to that of SpKrl, which includes four highly conserved Zn-finger motifs (95% identity) at the C-terminus.

The expression of *HpKrl* was essentially identical to that of *SpKrl* (Howard et

al., 2001; Minokawa et al., 2004). *HpKrl* was activated zygotically; the transcript was first detected at the 16-cell stage, and the expression was maintained until the gastrula stage (Fig. 1A). The expression domain shifted from the vegetal pole as a vegetal-to-animal wave. Although the RNA was restricted to the micromere descendants until the 60-cell stage (Fig. 1B), expression of *HpKrl* began in the veg_2 region in the early blastula, concomitant with downregulation of the gene in the micromere progeny (Fig. 1C). Subsequently, expression faded from the veg_2 domain and appeared in the veg_1 region. Ultimately, expression was restricted to the veg_1 descendants encircling the blastopore in the gastrula (Fig. 1D).

HpKrl mRNA was restricted to micromeres at the 16-cell stage, while it was detected in the progeny of macromeres that had been fractionated at the 16-cell stage and cultured for 8 hours (Fig. 1A). This observation indicates that *HpKrl* was cell-autonomously activated in the macromere lineage, consistent with the results of Logan et al. (1999) showing that nuclear entry of β–catenin occurs cell-autonomously in macromere progeny.

HpKrl is required for SMC specification

Through morpholino-mediated *HpKrl*-knockdown assays, we confirmed the results of Howard et al. (2001). We injected a morpholino (MKrl-1 or MKrl-2) to a final concentration of ~5 μM in individual eggs. MKrl-1 and MKrl-2 are complementary to nucleotides –48 to –24 and +1 to +25, with respect to the translation initiation site in *HpKrl* mRNA, respectively. Embryos injected with either MKrl-1 or MKrl-2 developed normally to the mesenchyme blastula stage, and PMCs ingressed into the blastocoel at the same time as in control embryos that had been injected with a control morpholino

(Fig. 2A,F). However, archenteron formation was blocked in the MKrl-injected embryos, whereas the controls developed into late gastrulae; gastrulation occurred in the MKrl-1-injected embryos roughly 8 hours later than in the control embryos (Fig. 2B,G).

We cultured the embryos for 4 days to examine the differentiation of endomesodermal tissues. Guts were identified by morphology as well as by active staining for alkaline phosphatase, while spicules and pigment cells were examined as per Kominami (1998). Coelomic pouches were verified by morphology, while circumesophageal muscle cells were identified by phalloidin staining and/or contractions of the foregut. Embryos injected with the control morpholino developed into pluteus larvae with spicules, a three-part gut, and all of the SMC types examined (Fig. 2C,D,E; Table 1). In contrast, the embryos injected with MKrl-1 exhibited defects in development of the veg_2-derived endomesodermal tissues (Fig. 2H,I,J; Table 1). MKrl-2 injection produced similar results to those produced by MKrl-1 injection. However, the phenotypes varied between batches; severe defects, including a lack of foregut formation in addition to the nonexistence of some SMC types, dominated some batches, while less severe defects (i.e., greater SMC production) were dominant in others (Fig. 2K). Therefore, we extensively analyzed the phenotypes of the MKrl-1-injected embryos. Larvae containing MKrl-1 formed a complete skeleton (Fig. 2H,J). In fact, a majority of these larvae produced a three-part gut showing alkaline phosphatase activity and coelomic pouches (Fig. 2H). However, only 29% of the larvae generated muscle cells (Fig. 2I; Table 1). The muscle fibers were significantly decreased in number even in the muscle-positive larvae; on average, 5.0 fibers (s.d. = 2.6; n = 9) were detected in the MKrl-1-injected larvae, while 11.3 fibers (s.d. = 1.9; n = 9) occurred in the control larvae. Practically none of the MKrl-1-injected embryos

developed pigment cells (Fig. 2J; Table 1). Although four of the 106 larvae examined were pigment-positive, those larvae were derived from one of five experimental batches. In summary, defects evoked by MKrl-1 injection were through the veg2 endomesoderm, and occurred in a gradient along the A-V axis: pigment cells > muscle cells > coelomic pouches > foreguts.

To examine whether the phenotypes produced by MKrl-1-injection were due to defects in translation, we performed a rescue experiment using synthetic *HpKrl* RNA without the MKrl-1 target site. Embryos injected with MKrl-1 plus ~1.5 ng of the RNA developed into pluteus larvae with pigment cells, functional muscle, and coelomic pouches (Fig. 2O). Together with the observation that injection of a control morpholino did not alter development, and that two differently targeted MKrls induced similar phenotypes, we conclude that the phenotypes observed in the MKrl-1-injected embryos are due to *HpKrl*-knockdown. We also examined effectiveness of MKrl-1 on translational inhibition using an artificial mRNA, which consists of the 5'UTR plus coding sequence (first 36 nucleotides) of *HpKrl* and *GFP* sequence (Fig. 3). This target message (~1 x 10^7 copies) was co-injected into each fertilized egg with MKrl-1, and GFP signal was detected at 12 hours after fertilization. Intensities of the GFP signal in embryos including 4 μM, 2 μM, 1 μM, and 0.5 μM MKrl-1 were 1.36% (s.d. = 0.71, n = 5), 1.8%, 6.1%, and 8.5% of control without MKrl-1, respectively. Since more than 98% of translation of an excess amount of mRNA (more than 1,000 fold of endogenous *Krl* mRNA) was blocked at 2 μM MKrl-1, dose of 5 μM MKrl-1 in this study must be high enough to inhibit translation of *HpKrl* mRNA below 2% of control. Four day after fertilization, only larvae containing 1 μM or 0.5 μM MKrl-1 generated functional muscle (2/3 cases; 2/2 cases), suggesting that leaky expression of HpKrl over 6% of

control may release the block of muscle formation. This estimate supports the notion that muscle formation in ~30% of larvae including 5 μM MKrl-1 resulted not from residual expression of HpKrl, but from a distinct pathway(s) in which HpKrl is not involved.

To define the phenotype, we estimated the transcript levels of several marker genes in embryos injected with MKrl-1 by RT-PCR (Fig. 4). In the injected embryos upon hatching, *Endo16* was downregulated in two out of three batches by *HpKrl* perturbation, while *Krl* was upregulated; further quantitative analyses are required to confirm this result. At the mesenchyme blastula stage, *Endo16* and *SM50* appeared to be slightly downregulated. This observation is consistent with the result of quantitative analyses (*SpEndo16* and *SpSM50* are downregulated by *SpKrl* perturbation; http://sugp.caltech.edu/endomes/qpcr.html, QPCR Data Relevant to Endomesoderm Network, September 15, 2007).

To confirm that HpKrl is required for the specification of nonskeletogenic mesoderm rather than endoderm, we examined the development of endomesodermal tissue in embryos treated with lithium ions (Li^+). Control Li^+-treated embryos developed into typical exogastrulae with SMC-derived structures, including muscle cells and more pigment cells than in the controls (Fig. 2L,M,N; Fig.2E). Li^+ treatment also induced exogastrulation in MKrl-1-injected embryos, indicating that endoderm expansion is HpKrl-independent; however, those embryos did not develop muscle or pigment cells (Fig. 2P,Q,R; 4/4 cases). From these observations, we conclude that HpKrl is required for specification of the veg_2-derived endomesodermal tissues, particularly the development of muscle and pigment cells.

HpKrl is not required for micromere specification

HpKrl was activated in micromeres at the 16-cell stage (Fig. 1A). To examine the possible involvement of HpKrl in micromere specification, we constructed chimeric embryos using a micromere-less normal host and a micromere quartet from embryos that had been injected with MKrl-1 or the control morpholino (Fig. 5A,D). The chimeras with micromeres containing MKrl-1 developed in a manner that was essentially identical to that of the control chimeras; the micromere descendants ingressed as PMCs, and gastrulation occurred at the same time (Fig. 5B,E). We confirmed by rhodamine labeling that descendants of micromeres containing MKrl-1 differentiated to skeletogenic cells. Four days after fertilization, both types of chimeras developed into complete pluteus larvae with pigment cells and functional muscle (Fig. 5C,F; Table 1). These observations support the notion that HpKrl is not involved in micromere specification, when assayed using micromere-less embryos as a host.

Macromeres require HpKrl for cell-autonomous specification

Although micromere signals are required for normal endomesoderm development, micromere-deprived embryos partially generate endomesodermal tissues (Sweet et al., 1999; Ishizuka et al., 2001). To test whether HpKrl is required for cell-autonomous macromere specification, we removed micromeres from MKrl-1-injected embryos and compared the phenotype to that of control micromere-less embryos (Fig. 6A,E). Both micromere-less embryos underwent gastrulation without generating PMCs (Fig. 6B,C,F,G). Four days after fertilization, the micromere-less controls developed into pluteus-like larvae with skeletons, and the majority formed a three-part gut (Fig. 6C,D; Table 2). In addition, a minority of the micromere-less controls developed pigment cells and/or muscle cells, although the number of pigment cells was smaller than in the

untreated controls (Fig. 6D,I; Table 2). In contrast, the micromere-less embryos containing MKrl-1 remained gastrula-like (Fig. 6G). Although the minority formed small spicules and three-part guts, none of the larvae developed SMC-derived tissues (Fig. 6G,H; Table 2). Since *HpKrl* activation occurred cell-autonomously in the macromere progeny (Fig. 1A), we conclude that HpKrl is required for cell-autonomous macromere specification, and especially for SMC development in the absence of micromere signals.

Macromeres can receive micromere signals in part without Krl function

Macromeres require nuclear β–catenin in order to receive micromere signals, including Delta (Logan et al., 1999; McClay et al., 2000). To test whether HpKrl is a downstream component required for signaling, we transplanted a normal micromere quartet into a micromere-less embryo containing MKrl-1 (Fig. 6J). The micromere descendants formed a complete skeleton in the hosts; thus, the recombinants developed into pluteus-like larvae in terms of their shape. In addition, three-part guts and muscle cells were restored in a minority of the recombinants (Fig. 6L,M; Table 2). Using rhodamine-tagged micromeres, we confirmed that the muscle was not derived from the transplanted micromeres. These observations indicate that the macromere progeny can receive micromere signals, at least in part, without Krl function; the inductive rescue was restricted to foreguts and muscle cells.

Animal halves require HpKrl to generate SMCs in response to micromere signals

We confirmed the result of Sweet et al. (1999), which showed that mesomeres are less responsive to micromere signals than are macromeres (i.e., fewer SMCs are induced in the animal halves than in the micromere-less hosts following micromere

transplantation). Chimeras composed of an animal half and a micromere quartet produced roughly ten pigment cells and four muscle fibers (Fig. 6P,Q), while sham controls, composed of a micromere-less host and a micromere quartet, formed about 60 cells and ten fibers, respectively (data not shown). As described, HpKrl controlled macromere specification autonomously and conditionally, and the expression was restricted to the vegetal hemisphere of the embryos (Fig. 1B). These observations imply that the decreased responsiveness of the mesomeres to micromere signals may be due to less *HpKrl* expression in the mesomere. To test this possibility, we constructed chimeric embryos composed of animal cap mesomeres from MKrl-1-injected embryos and a micromere quartet from normal embryos (Fig. 6R). The chimeras developed into egg-shaped larvae with nearly normal skeletal rods (Fig. 6T,U), indicating that an oral-aboral axis was established in the animal half. However, the chimeras developed no SMCs, although they formed small one- or two-part guts (Fig. 6T,U; Table 2). This observation indicates that animal halves require HpKrl to generate veg_2-derived tissues in response to micromere signals and suggests that, in the control chimeras, the micromeres induced SMC development through ectopic *Krl* activation in the animal halves.

DISCUSSION

Through morpholino-mediated *HpKrl*-knockdown, we confirmed the results of Howard et al. (2001) and discovered novel features of SMC development in *H. pulcherrimus* embryos. Previous experiments did not indicate which cells require Krl and for what functions, since *Krl* is expressed in both micromere and macromere progeny, and the

morpholino is present ubiquitously. To sort out the roles of HpKrl, we conducted blastomere transplantation experiments.

HpKrl affects the Delta/Notch signaling

Sweet et al. (2002) showed that *Delta* expression by micromere descendants is necessary and sufficient for the development of pigment cells and blastocoelar cells in macromere progeny. McClay et al. (2000) predicted the existence of additional components downstream of nuclear β−catenin in macromeres that are required for SMC specification via the Delta/Notch signaling, since expression of activated Notch fails to rescue β−catenin-deficient micromere-less embryos from SMC-less conditions.

In this study, practically no pigment cells developed in embryos that ubiquitously expressed MKrl-1 or in recombinants of a micromere-less host containing MKrl-1 with normal micromeres (Fig. 2J; Fig. 6L; Table 1,2). This situation resembles the pigment-less condition of micromere-less embryos rather than that of PMC-deprived embryos, because the embryo/recombinant did not generate pigment cells despite the presence of skeletogenic PMCs in the blastocoel (Fig. 2J; Fig. 6L). This observation supports the view that pigment cells did not differentiate in the embryo/recombinant (Sweet et al., 1999). Together with observations that nuclear entry of β−catenin and the activation of *HpKrl* occur cell-autonomously in macromere progeny (Logan et al., 1999; Fig. 1A), we suggest that Krl may be one of the downstream regulators of nuclear β−catenin in macromeres, which enhances the Delta/Notch signal transduction (see Discussion).

Macromeres use Krl-dependent and Krl-independent pathways of SMC formation

Defects evoked by MKrl-1 injection were through the veg_2 endomesoderm, and occurred in a gradient along the A-V axis: pigment cells > muscle cells > coelomic pouches > foreguts. As shown in Fig. 6, macromeres require HpKrl for both autonomous and conditional SMC specification. Even in the absence of micromeres, macromere progeny generated pigment cells and/or muscle cells at low frequency as well as three-part guts (Fig. 6C). In contrast, micromere-less embryos containing MKrl-1 did not develop SMC-derived tissues, most of which developed only one/two-part guts (Fig. 6G). The poor gut in morphology showed the alkaline phosphatase activity (Yamazaki and Yamaguchi, unpublished), indicating that mid/hindgut differentiation had occurred. These observations indicate that HpKrl is required cell-autonomously by macromeres for specification (i.e., formation of SMCs and foreguts depends on HpKrl function). When micromeres were transplanted to the host containing MKrl-1, however, the macromere progeny generated three-part guts and muscle cells in ~60% and ~30% of the recombinants, respectively, but no pigment cells (Fig. 6L,M; Table 2). It is plausible to consider that the foregut and muscle were specified by micromere signals, via a pathway(s) in which HpKrl is not involved, since 5 μM Mkrl-1 in the macromere must keep blocking more than 98% of translation (Fig. 3). Alternatively, micromere signals may enhance HpKrl affinity to the target sites in muscle/foregut determining genes, via an unknown Krl-independent mechanism(s). In either case, this phenotype indicates that macromeres can receive micromere signals, at least in part, without Krl function, and that induced tissues were graded in frequency along the A-V axis: foreguts > muscle cells. This may explain greater rescue in gastrulation; formation of the veg_2 endoderm (foreguts) and a SMC type (bottle cells) by micromere signals drove

morphogenetic movements, i.e., invagination of differentiated mid/hindguts.

Animal cap mesomeres include only Krl-dependent pathway

Animal cap mesomeres require HpKrl function for formation of the veg_2-derived tissues, since animal halves containing MKrl-1 generated no SMCs or foreguts when combined with normal micromeres (Fig. 6T; Table 2). This observation suggests that mesomeres may include only the Krl-dependent pathway for SMC formation, and that the pathway must be activated ectopically by micromere signals. Alternatively, mesomeres may require ectopic *HpKrl* expression for activation of the Krl-independent pathway in response to micromere signals. In either case, this model may explain the previous observation that mesomeres are less responsive to micromere signals than are macromeres (Sweet et al., 1999).

Krl-independent pathway corresponds to Delta/Notch pathway, while Krl-dependent pathway enhances the signaling as a parallel effector

Although the Delta/Notch pathway plays a central role in endomesoderm specification, macromeres require downstream components of nuclear β−catenin to respond to micromere signals, including Delta. In the *S. purpuratus* embryo, *Krl* (*z13*) expression in macromeres begins at the early blastula stage, which is chronologically comparable to the activation of components of the Delta/Notch pathway, including *Delta*, *GataE*, and *Gcm*. Since *Krl* perturbation does not affect either of the genes, the pathway appears to drive through macromere descendants, regardless of Krl function (Fig. 7A). However, loss-of-Krl-function downregulates pigment cell differentiation genes, *SuTx*, *Dpt*, and *Pks*, the expression of which is driven by Gcm and GataE

(http://sugp.caltech.edu/endomes/qpcr.html, QPCR Data Relevant to Endomesoderm Network, September 15, 2007; Ben-Tabou de-Leon and Davidson, 2007).

Based on the current version of the GRN model and our observations in this study, we suggest that a Krl-independent pathway may correspond to the Delta/Notch pathway, and that Krl may be a downstream component of nuclear β–catenin required by macromeres for the Delta/Notch signaling, not as a member of the pathway, but as a parallel effector (Fig. 7A). Since the activation of *HpKrl* occurs cell-autonomously in macromere descendants (Fig. 1A), HpKrl appears to endow the descendant with a regulatory state for endomesoderm differentiation. This Krl-dependent state may contribute to cell-autonomous endomesoderm specification in micomere-less conditions, and enhance the Delta/Notch signaling to generate more vegetal tissues in normal development. It is plausible to consider that this Krl-dependent state (pathway) functions by repressing negative effectors for endomesoderm differentiation, including SoxB1, since SoxB1 opposes vegetalizing effects of β–catenin, while β–catenin-dependent gene products, SpKrl and Pmarl, downregulate SoxB1 (Kenny et al., 1999, 2003; Angerer et al., 2004).

Fig. 7 shows a hypothetical GRN diagram of veg_2 endomesoderm specification. Delta/Notch signaling specifies endomesoderm via GataE and Gcm. On the other hand, Krl enhances the signaling as a parallel effector to generate more vegetal tissues (Fig. 7A). In micromere-less embryos, cell-autonomous activation of *Krl* and *Krox/blimp1* contributes to partial formation of endomesodermal tissues in the absence of Delta input. Formation of the SMC types and a part of foreguts depends on Krl function (Fig. 7B; Fig, 6C,D; Table 2), because micromere-less embryos including MKrl-1 generate no SMC types and few foreguts (Fig. 7C; Fig. 6G,H; Table 2). When

micromeres are transplanted, the micromere-less host with MKrl-1 restored foreguts and a part of muscle cells. This rescue occurs via Delta/Notch pathway regardless of Krl function, that is, Krl-independently (Fig. 7D; Fig. 6L,M; Table 2). However, the chimera generates no pigment cells; formation of the pigment cell and a majority of muscle cells depends on Krl function. Therefore, the Krl-dependent pathway is used in both cell-autonomous (micromere-less embryos) and conditional (control embryos) specification. On the other hand, Krl-independent induction of foreguts and muscle cells is evident only when are compared the chimera shown in Fig. 7D and the micromere-less embryo with MKrl-1 shown in Fig. 7C. However, this Krl-independent induction is used in normal development, since a phenotype of the chimera is practically identical to that of Krl-knockdown whole embryos (Fig. 2H-J; Table 1).

Krl-dependent SMC formation shown in Fig. 7D suggests that Krl is required for differentiation of SMC types in addition to Gcm and GataE, and also implies that Krl permits multiple interpretations of the Delta/Notch signaling in the GRN. The Krl-dependent pathway in this study provides an insight into a general feature of GRNs in which the same signaling pathway generates distinct differential states according to the regulatory state of signal receiving cells.

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FIGURE LEGENDS

Fig. 1. *HpKrl* is activated zygotically, and its expression domain shifts from the vegetal pole as a wave during development. (A) RT-PCR using cDNA from unfertilized eggs (UE) and 2-cell (2c), 4-cell (4c), 8-cell (8c), 16-cell (16c), 32-cell (32c), 60-cell (60c), early blastula (eBl), hatched blastula (hBl), mesenchyme blastula (mBl), gastrula (Gs), prism (Pr), and pluteus (Pl) stage embryos. Mesomeres (m), macromeres (M), and micromeres (μ) were fractionated by elutriation (Yamaguchi et al., 1994). Total RNA was extracted from the blastomeres immediately after (16c) or 8 hours (eBl) after fractionation. (B-D) *HpKrl* expression. *HpKrl* expression is restricted to the micromere descendant at the 32-cell stage (B). Upon hatching, expression shifts from the micromere progeny to the veg_2 region (C). At the gastrula stage, *HpKrl* expression is restricted to the veg_1 descendants encircling the blastopore (D).

Fig. 2. Loss of *HpKrl* function blocks SMC formation. (A-E) Embryos injected with a

control morpholino (Mcont). The embryo developed normally, like uninjected embryos. (F-J) Embryos injected with MKrl-1. In the injected embryos, PMCs ingressed at the same time as in the controls (F), but gastrulation was delayed (G). At 4 days, the injected embryos developed into pluteus-like larvae, most of which formed three-part guts with alkaline phosphatase (AP) activity (H). Approximately 70% of the injected larvae lacked circumesophageal muscle cells (I), whereas the larvae injected with Mcont developed phalloidin-positive muscle fibers (arrowhead in D). Almost none of the MKrl-1-injected embryos developed pigment cells (J). (K) Embryos injected with MKrl-2 at 4 days. The phenotype was similar to that of embryos injected with MKrl-1; this larva lacks a foregut in addition to SMCs. (L-N) Li^+-treated embryos. At 4 days, the embryo developed into exogastrulae with muscle (arrowhead in M) and additional pigment cells (N). (P-R) Li^+-treated embryos with MKrl-1. At 4 days, the embryo developed into exogastrulae without muscle or pigment cells (Q,R; 4/4 cases). (O) Embryos co-injected with *HpKrl* mRNA lacking the MKrl-1 target site. The embryo developed pigment cells (arrows), coelomic pouches (arrowheads), and functional muscle.

Fig. 3. Effectiveness of MKrl-1 on translational inhibition. Target message (~1 x 10^7 copies) with *HpKrl* leader sequence followed by *GFP* sequence was co-injected into each fertilized egg with MKrl-1, and GFP signal was detected at 12 hours after fertilization using LAS-3000. (A) Uninjected control. (B) Embryo injected with *Krl/GFP* mRNA. (C,D,E,F) Embryo co-injected with MKrl-1. More than 99% of GFP is blocked at 4 μM MKrl-1 (C,D). Approximately 91% and 94% of GFP are blocked at 0.5 μM (E) and 1 μM MKrl-1 (F), respectively.

Fig. 4. Expression of marker genes in embryos injected with MKrl-1. Total RNA was extracted from control embryos and embryos injected with MKrl-1 at the hatched blastula stage (hBl) and the mesenchyme blastula stage (mBl). mRNA expression of marker genes was analyzed by RT-PCR, using RNA from one embryo. The numbers below indicate the number of PCR cycles. In the injected embryo at 20 hours (hBl), the number of ectoderm marker (*Ars*) and presumptive PMC marker (*Tbr*) transcripts were comparable to those in the control embryos. An endomesoderm marker (*Endo16*) was downregulated in two out of three batches, while *HpKrl* was upregulated in the injected embryos. In the injected embryos at 24 hours (mBl), the transcript number of *Endo16* and *SM50* appeared to be slightly downregulated.

Fig. 5. HpKrl is not required for micromere specification. Development of chimeric embryos composed of a micromere-less host from normal embryos and a micromere quartet from embryos injected with MKrl-1. (A-C) Control chimeras with micromeres containing Mcont. (D-F) Chimeras with micromeres containing MKrl-1. Development of the chimeras was identical to that of the control chimeras; the chimeras began gastrulation at 30 hours (arrowhead), and developed into complete pluteus larvae with pigment cells and functional muscle.

Fig. 6. Macromeres require HpKrl for cell-autonomous and non-autonomous SMC specification, but can receive micromere signals in part without HpKrl function. On the other hand, mesomeres require HpKrl function to generate ectopic SMC. (B,F,K,O,S)

Embryos at 24 h. (C,D,G-I, L,M,P,Q,T,U) Embryos at 4 days. (A-D,I) Control micromere-less embryos. The embryo developed into prism-like larvae (C) without generating PMCs (B). The minority developed pigment cells (D) and/or circumesophageal muscle cells (arrowhead in I) between the mouth (m) and stomach (st). (E-H) Micromere-less embryos containing MKrl-1. The embryos remained gastrula-like with a small gut (G) and no pigment cells (H). (J-M) Chimeric embryos composed of a micromere-less host containing MKrl-1 and normal micromeres. The chimeras developed into pluteus-like larvae. In some embryos, vegetal structures were restored by micromere transplantation, including a three-part gut (L) and muscle cells (arrowhead in M), but not pigment cells. (N-Q) Control chimeras composed of animal cap mesomeres and a micromere quartet. The chimera developed into small but complete pluteus larvae with muscle (arrowhead in inset of P) and pigment cells (Q). (R-U) Chimeras composed of animal halves including MKrl-1 and a micromere quartet. The chimeras developed into egg-shaped larvae with almost normal skeletal rods and a one-/two-part gut (T); however, the chimeras did not develop SMCs, including pigment cells (U).

Fig. 7. Krl-independent pathway corresponds to Delta/Notch pathway, while Krl-dependent pathway enhances the signaling as a parallel effector. (A) Control embryo. Delta/Notch signaling specifies endomesoderm via GataE and Gcm, while cell-autonomous Krl endows the macromere progeny with a regulatory state for endomesodermal differentiation. This Krl-dependent state (pathway) enhances Delta/Notch signal transduction to generate SMC types. (B) Micromere-less embryo. Cell-autonomous activation of *Krl* as well as *Krox/blimp1* contributes to partial

formation of endomesodermal tissues. (C) Micromere-less embryos including MKrl-1. Few vegetal structures are formed due to lack of the Krl-dependent pathway. (D) Chimera composed of micromere-less host including MKrl-1 and normal micromeres. Transplanted micromeres drive Delta/Notch pathway, restoring endomesodermal tissues without Krl function. Curiously, the chimera generates muscle cells instead of pigment cells (see text).

Table 1. Endomesoderm development following the injection of the morpholinos

Morpholino Injection	No. of larvae with each tissue/No. of larvae examined (%)				
	Spicules	Pigment cells	Muscle cells	Coelomic pouches	Three-part guts
Mcont	22/22 (100)	22/22 (100)	22/22 (100)	22/22 (100)	22/22 (100)
MKrl-1	106/106 (100)	4/106 (4)	25/86 (29)	44/86 (51)	81/106 (76)

Table 2. Endomesoderm development following the elimination of HpKrl function in micromere-less embryos or aninal cap mesomeres, and the transplantation of micromeres

Blastomeres	Meso/macro	Meso/macro (MKrl)	Meso/macro (MKrl) + micro	Meso + micro	Meso (MKrl) + micro
Tissues	No. of larvae with each tissue/No. of larvae examined (%)				
Spicules	15/15 (100)	3/12 (25)	13/13 (100)	10/10 (100)	15/15 (100)
Pigment cells	4/15 (27)	0/12 (0)	0/13 (0)	9/10 (90)	0/15 (0)
Muscle cells	2/15 (13)	0/12 (0)	4/13 (31)	9/10 (90)	0/15 (0)
Three-part guts	9/15 (60)	4/12 (33)	8/13 (61)	9/10 (90)	0/15 (0)

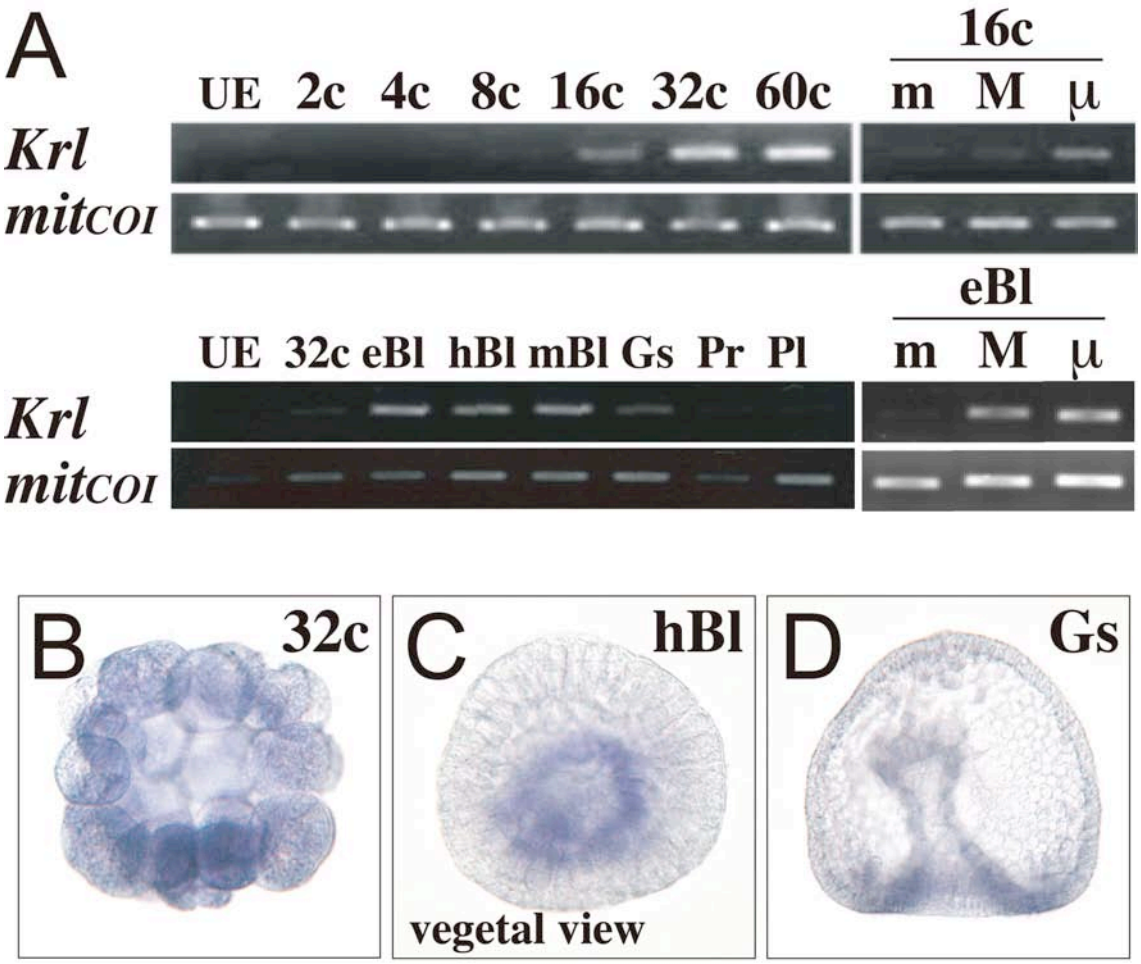
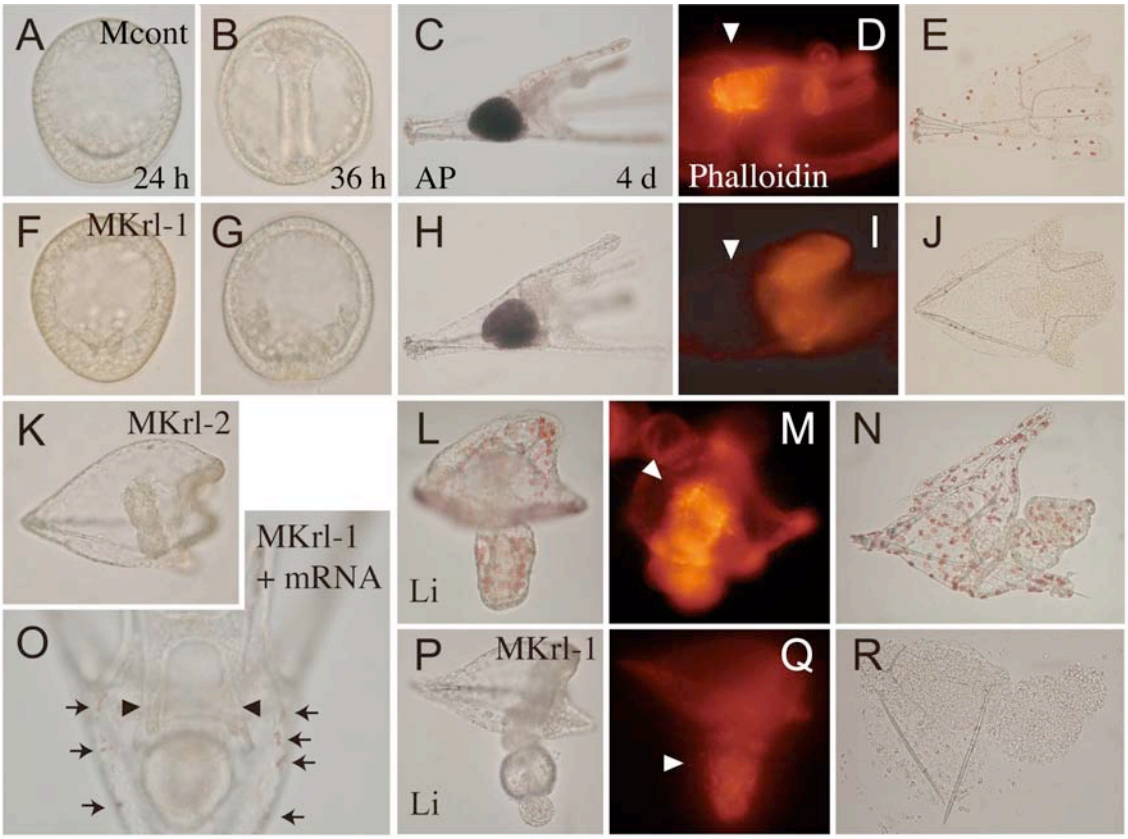

A
16c
UE 2c 4c 8c 16c 32c 60c
m M μ
Krl
mitCOI
eBl
UE 32c eBl hBl mBl Gs Pr Pl
m M μ
Krl
mitCOI
B 32c
C hBl
vegetal view
D Gs

Figure 1


A
Mcont
24 h
B
36 h
C
AP
4 d
D
Phalloidin
E
F
MKrl-1
G
H
I
J
K
MKrl-2
MKrl-1
+ mRNA
L
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M
N
O
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MKrl-1
Li
Q
R

Figure 2

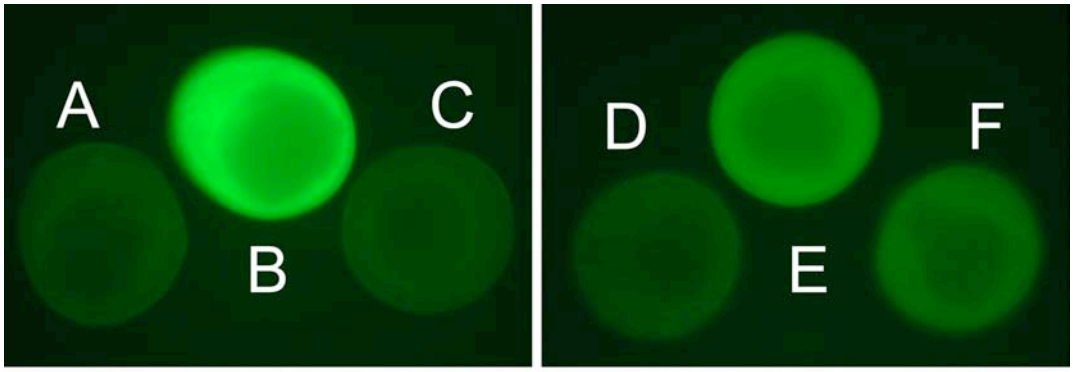


Figure 3

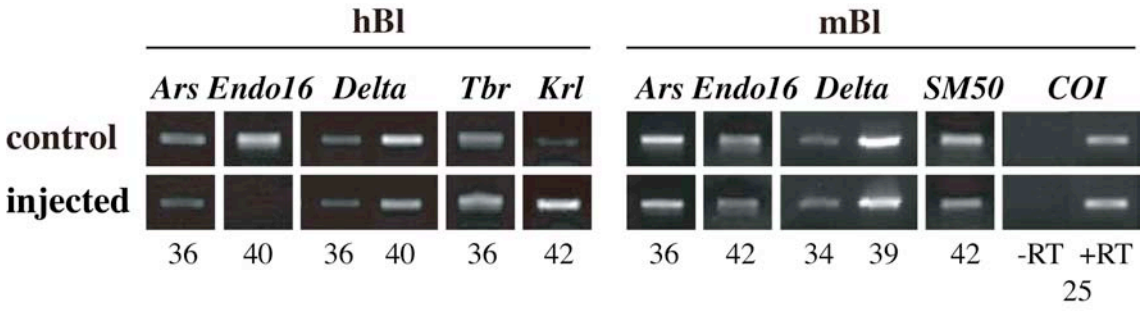

hBl
mBl
Ars Endo16 Delta Tbr Krl
Ars Endo16 Delta SM50 COI
control
injected
36 40 36 40 36 42
36 42 34 39 42 -RT +RT
25

Figure 4

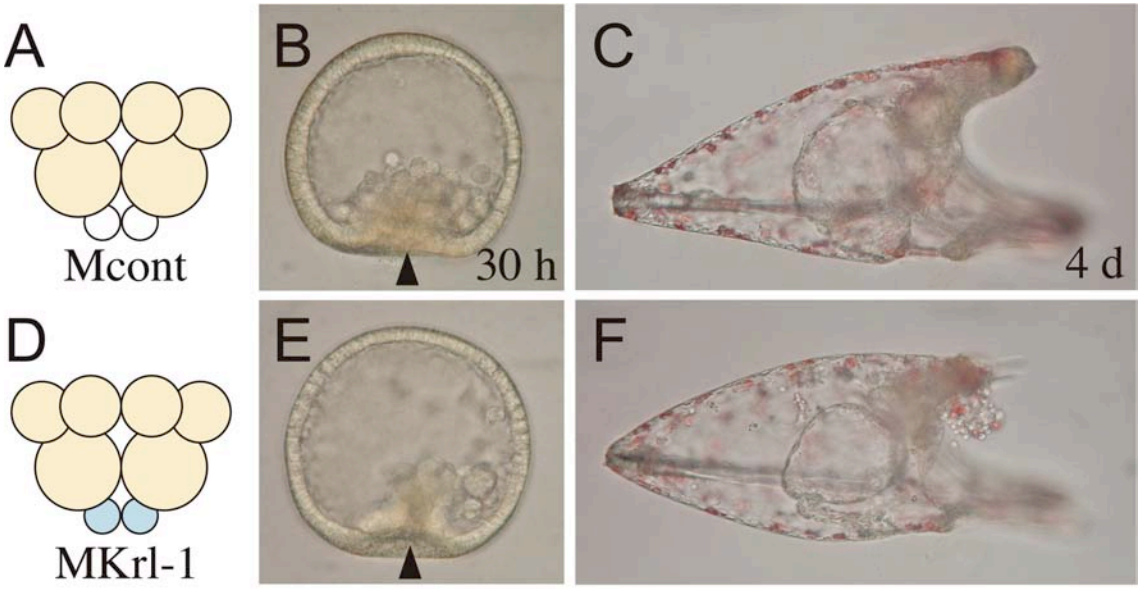


Figure 5

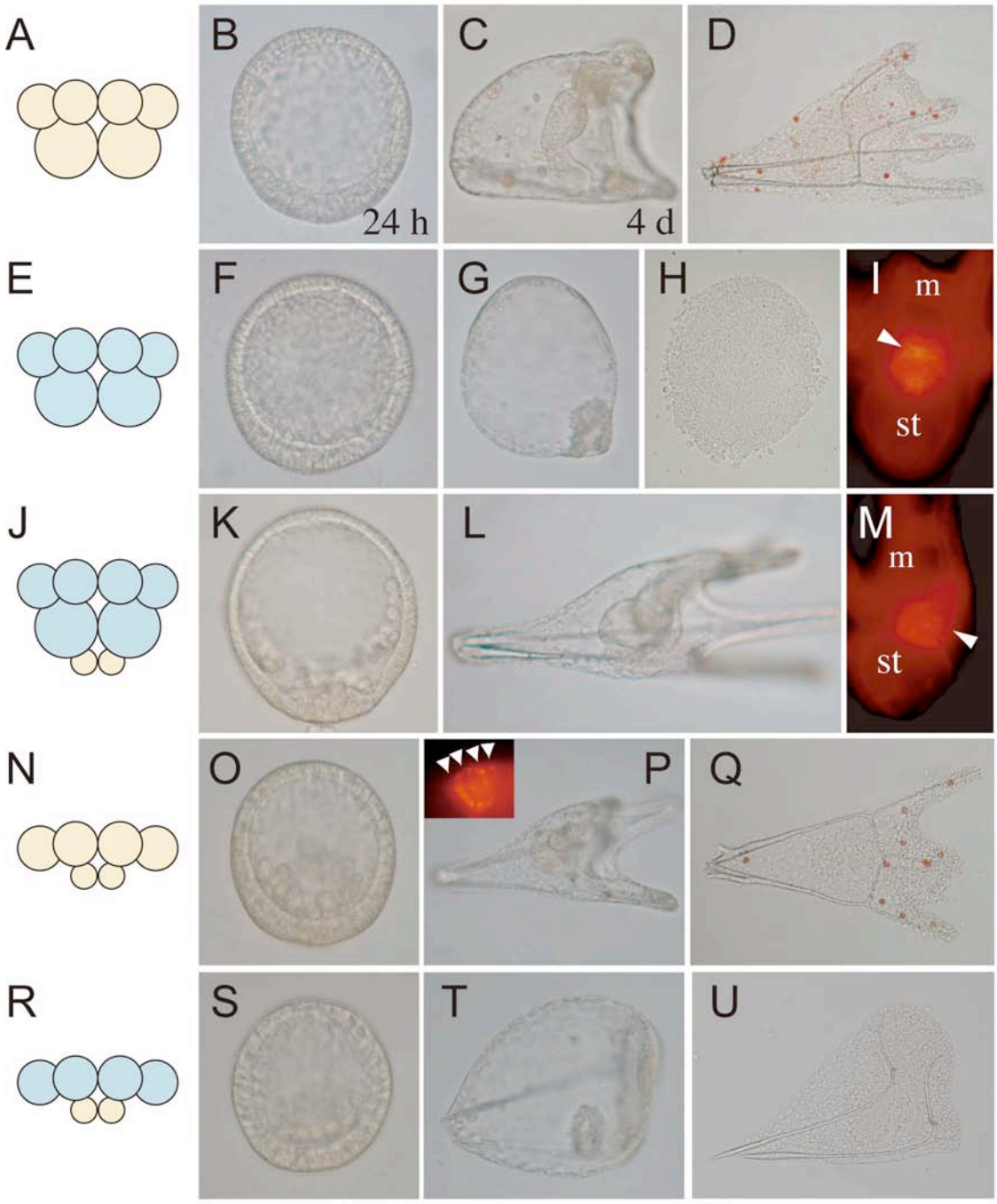
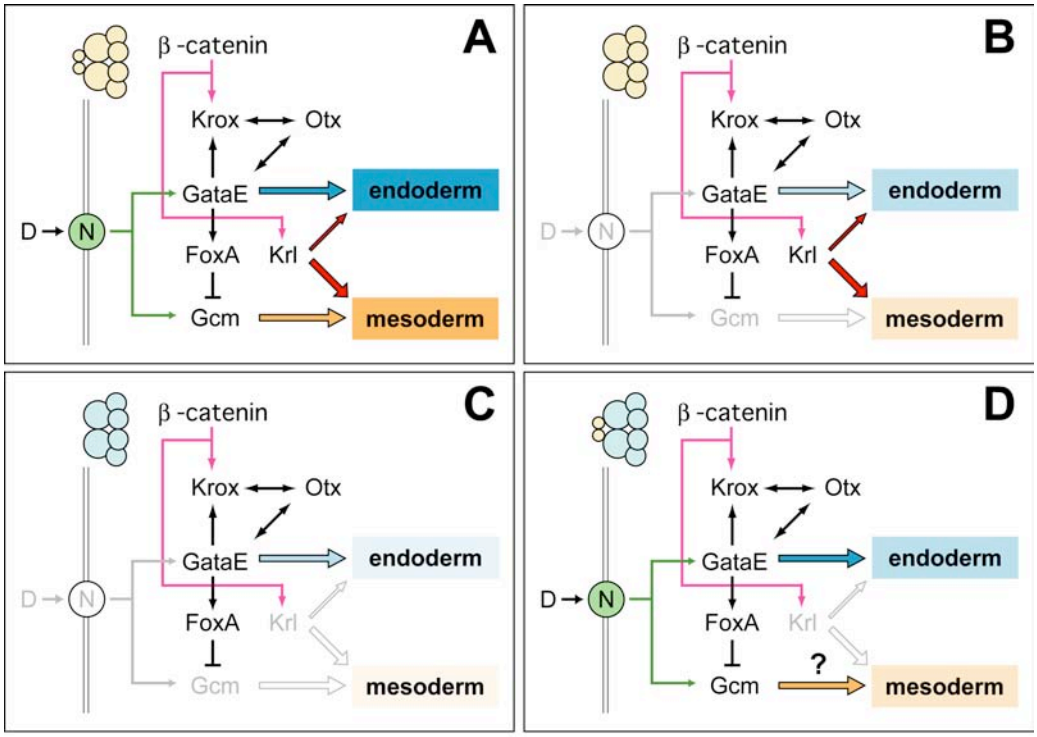


Figure 6


A
β -catenin
Krox
Otx
GataE
endoderm
D
N
FoxA
Krl
Gcm
mesoderm
B
β -catenin
Krox
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GataE
endoderm
D
N
FoxA
Krl
Gcm
mesoderm
C
β -catenin
Krox
Otx
GataE
endoderm
D
N
FoxA
Krl
Gcm
mesoderm
D
β -catenin
Krox
Otx
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Gcm
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Figure 7