Macho-1 regulates unequal cell divisions independently of its function as a muscle determinant

Gaku Kumano *, Narudo Kawai 1, Hiroki Nishida

Department of Biological Sciences, Graduate School of Science, Osaka University, 1-1 Machikaneyama-cho, Toyonaka, Osaka 560-0043, Japan

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ABSTRACT

The anterior–posterior (A–P) axis in ascidian embryos is established through the posteriorizing activities of a localized egg region known as the posterior vegetal cortex/cytoplasm (PVC). Here we describe a novel function of macho-1, a maternally-localized muscle determinant, in establishment of the A–P axis in the Halocynthia roretzi embryo. Macho-1, in addition to its known function in the formation of posterior tissue such as muscle and mesenchyme, and suppression of the anterior-derived notochord fate, acts independently of its transcriptional activity as a regulator of posterior-specific unequal cell divisions, in cooperation with β-catenin. Our results suggest that macho-1 and β-catenin regulate the formation of a microtubule bundle that shortens and pulls the centrosome toward a sub-cellular cortical structure known as centrosome-attracting body (CAB), which is located at the posterior pole of the embryo during unequal cell divisions, and act upstream of PEM, a recently-identified regulator of unequal cell divisions. We also present data that suggest that PEM localization to the CAB may not be required for unequal cleavage regulation. The present study provides an important and novel insight into the role of the zinc-finger-containing transcription factor and indicates that it constitutes a major part of the PVC activity.

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Introduction

One of the mysteries in animal development is how a single cell known as an egg develops into a body that is made of highly complex and well-arranged structures. To accomplish this, eggs contain a variety of information, among which that of maternal factors are used at the beginning of development. The mechanism by which maternal information contributes to generate complex body structures has been extensively studied for decades. Some developmentally important maternal factors are localized to certain portions of the egg and distributed into certain blastomeres over a series of subsequent cell divisions. The blastomeres then differentiate divergently from the other cells once the inherited maternal factors begin to exert an effect. Such localized factors include those that promote region-specific tissue formation or establish embryonic axes by distinguishing the location at which they reside from others. This localization strategy is not necessarily limited to pre-localized maternal factors but can also be used for any factors that, for example, could be localized to one side of a mother cell just before asymmetric (producing daughter cells with different fates) or unequal (producing unequal-sized daughter cells) cell divisions. This type of cell division is known to ensure that the localized factors are inherited by one daughter cell and not the other, thus contributing to the generation of cell diversity.

Ascidians are marine invertebrate chordates with a number of characteristics that are useful for studies of developmental mechanisms. Ascidian eggs are known to be highly organized with maternal factors localized at specific locations within the eggs. Those most studied are postplasmic/PEM RNAs, which become localized to the future posterior region of the egg known as the posterior vegetal cytoplasm/cortex (PVC) as a result of two series of post-fertilization cytoplasmic and cortical reorganizations (reviewed in Sardet et al., 2007; Prodon et al., 2007). The postplasmic/PEM RNAs are later concentrated at a sub-cellular structure known as the centrosome-attracting body (CAB), which is situated in the posterior cortex and inherited by the posterior-most blastomeres during the cleavage stages. The posterior-most blastomeres carry out three successive unequal cell divisions starting at the division to the 16-cell stage (Figs. 1A, F. K. P.), producing a smaller daughter cell on the posterior side at each division and ensuring that the posterior-most and smallest blastomeres at the 16- to 64-cell stages always contain the CAB and postplasmic/PEM RNAs.

The function of the postplasmic/PEM RNAs varies ranging from cell fate specification to cleavage pattern regulation. Since they are localized to the posterior region of the embryo, their function concerns posterior development or exclusion of anterior development, thus contributing to establishment of the anterior–posterior (A–P) axis. Among them, the function of macho-1, PEM and POPK1 in A–P axis determination is well documented (Nishida and Sawada, 2007; Prodon et al., 2007). The postplasmic/PEM RNAs are later concentrated at a sub-cellular structure known as the centrosome-attracting body (CAB), which is situated in the posterior cortex and inherited by the posterior-most blastomeres during the cleavage stages. The posterior-most blastomeres carry out three successive unequal cell divisions starting at the division to the 16-cell stage (Figs. 1A, F. K. P.), producing a smaller daughter cell on the posterior side at each division and ensuring that the posterior-most and smallest blastomeres at the 16- to 64-cell stages always contain the CAB and postplasmic/PEM RNAs.

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2001; Kobayashi et al., 2003; Yagi et al., 2004; Sawada et al., 2005; Yoshida et al., 1996, 1998; Negishi et al., 2007; Sasakura et al., 1998; Nakamura et al., 2005). Macho-1, originally isolated as a muscle determinant (Nishida and Sawada, 2001), is a zinc-finger-type transcriptional activator (Sawada et al., 2005) and serves also as an intrinsic competence regulator for mesenchyme induction (Kobayashi et al., 2003). It thus contributes to establishment of the A–P axis by promoting formation of the posterior-derived tissues (muscle and mesenchyme) and also by suppressing an anterior fate (Kobayashi et al., 2003). PEM characterizes the posterior by carrying out the posterior-specific unequal cell divisions mentioned above (Negishi et al., 2007). During these unequal cell divisions, a bundle of microtubules forms and establishes a connection between one of the centrosomes and the CAB. It then shortens and shifts the centrosome posteriorly, together with the nucleus, and the cleavage plane also forms posteriorly to produce unequal-sized daughter cells (Hibino et al., 1998; Nishikata et al., 1999). PEM protein is known to be localized to the CAB and to be involved in microtubule bundle formation (Negishi et al., 2007). PEM is also proposed to be involved in tilting the centrosome axes and thus regulating cleavage-plane orientation in the second and third cell divisions as its earlier function (Negishi et al., 2003). PEM-depleted embryos with POPK-1 MO show phenotypes similar to macho-1 and PEM-deficient embryos in that the muscle is absent and the unequal cell divisions are disrupted (Nakamura et al., 2005).

The A–P axis of the ascidian embryo is established through posteriorizing activities of the PVC. Removal of the PVC from the egg results in a symmetrical embryo along the A–P axis with its anterior developmental fates completely mirrored in the posterior half and its posterior-specific unequal cleavage pattern completely anteriorized (Nishida, 1994). This indicates that the PVC contains all the factors responsible for posterior development, and suggests that the postplasmic/PEM RNAs identified to date could together represent all of those factors (Negishi et al., 2007). Without PEM, the second and third divisions simply take place in parallel to the animal–vegetal (A–V) axis and perpendicular to it, respectively, resulting in the CAB position with respect to the third cleavage plane different from that in control embryos and appearing to be shifted more vegetally (Negishi et al., 2007). Recently, PEM has also been shown to contribute to A–P axis formation by regulating anterior- and posterior-derived tissue formation (Yoshida et al., 1996, 1998; Kumano and Nishida, 2009). Finally, POPK-1, an ascidian orthologue of SAD-1/SAD-A serine/threonine kinase (Sasakura et al., 1998), is proposed to transport maternal mRNAs and other materials including macho-1 and PEM mRNAs to the posterior pole of the embryo after fertilization (Nakamura et al., 2005). Knockdown embryos with POPK-1 MO show phenotypes similar to macho-1 and PEM-deficient embryos in that the muscle is absent and the unequal cell divisions are disrupted (Nakamura et al., 2005).

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the cleavage stages (Imai et al., 2000; Kawai et al., 2007), and regulates early gene expression that is required for the formation of tissues arising from the vegetal hemisphere (Imai et al., 2006; Kumano et al., 2006). The β-catenin protein is also found to be present at the CAB (Kawai et al., 2007).

In the present study, we found that macho-1, in cooperation with β-catenin, is involved in regulation of unequal cell divisions independently of its transcriptional activity and may act upstream of PEM in this process. Our results support the notion that multiple members of the postplasmic/PEM RNAs interact and function in the same pathways to ensure proper posterior development.

Materials and methods

Animals and embryos

Adults of Halocynthia roretzi were collected near the Asamushi Research Center for Marine Biology and the Otsuchi International Coastal Research Center, and kept in tanks during the spawning season. Eggs were spawned under temperature and light control, fertilized with a suspension of non-self sperm, and allowed to develop at 50°C. Eggs were spawned under temperature and light control, kept in tanks during the spawning season. Eggs were spawned under temperature and light control, and kept in tanks during the spawning season. Eggs were spawned under temperature and light control, fertilized with a suspension of non-self sperm, and allowed to develop at 50°C. Eggs were spawned under temperature and light control, kept in tanks during the spawning season.

Actinomycin D treatment

Embryos were developed in sea water containing 40 μg/ml actinomycin D (Sigma). Treatment of Ascidia nigra with 20 μg/ml actinomycin D has been shown to suppress 70% incorporation of uridine, with the unincorporated 30% being low-molecular-weight RNA (Smith 1967). This concentration is enough to inhibit the synthesis of actylcholinesterase, myosin heavy chain and an epidermis-specific antigen in Halocynthia embryos (Satoh, 1979; Nishikata et al., 1987a,b).

Morpholino oligonucleotides and RNA injection

Specific morpholino antisense oligonucleotides (Gene Tools) to knock down the expression of β-catenin, macho-1 and PEM were prepared as described previously (Kobayashi et al., 2003; Kumano et al., 2006; Negishi et al., 2007). As a control, the standard (c-MO) morpholino provided by Gene Tools was used. Plasmids for in vitro macho-1-EnR and full 3′UTR-containing macho-1 RNA synthesis were prepared as follows: PCR-amplifying fragments containing the entire ORF of the macho-1 gene and the Drosophila engrailed repressor domain [En5, kindly provided by Dr. H. Takahashi (NIBB, Okazaki)] and those containing the entire ORF and 3′UTR of the macho-1 gene were sub-cloned into the pBS-HTB and pBS-RN3 plasmids, respectively. Capped and poly(A)-tailed RNAs for macho-1, macho-1-EnR and PEM were synthesized as described previously (Kumano et al., 2006) using the above plasmids and the PEM plasmid (Negishi et al., 2007) as templates. Microinjection was carried out 45 min to 2 h after fertilization, as described previously (Miya et al., 1997). The injected amount was one fourth to one fifth of the diameter of the egg (about one hundredth of the volume). Results from at least two independent injections were combined for all the data presented in this study.

In situ hybridization

Detection of muscle actin (Kusakabe et al., 1991), PEM (Nishida and Sawada, 2001), and macho-1 (Nishida and Sawada, 2001) expression by in situ hybridization was performed according to the standard protocol (Miya et al., 1997) with the exception that hybridization was done at 50°C.

Preparation of an antibody against macho-1

A 1668-bp fragment containing the full length of macho-1 was sub-cloned in-frame into the pGEX-4T-3 expression vector (Amersham). Escherichia coli DH5α expressing glutathione S-transferase (GST)-macho-1 fusion protein after IPTG induction was solubilized in phosphate-buffered saline (PBS), and the extract was applied to glutathione-immobilized agarose beads to trap the fusion proteins. To release macho-1, GST-macho-1 was digested with thrombin protease (Amersham). The isolated macho-1 was emulsified in TiterMax Gold (CytRx Corporation) and injected subcutaneously into rabbits followed by six booster injections. The antisera was purified by affinity chromatography with a partial macho-1 protein (1–276 a.a. residues), and the immunoreactivity was verified by Western blotting of the GST-macho-1 fusion protein.

Immunostaining

Embryos were fixed according to Kawai et al. (2007) for detection of macho-1, and according to Negishi et al. (2007) for detection of PEM and α-tubulin. After incubation with the primary antibodies, either indirect immunofluorescence detection by standard methods using a TSA Fluorescein System (Perkin-Elmer Life Sciences) for macho-1, PEM and α-tubulin (Negishi et al., 2007) or direct immunofluorescence detection using an Alexa 488-conjugated anti-rat IgG antibody as the secondary antibody for α-tubulin (Negishi et al., 2007) was carried out. The staining methods for macho-1 and PEM has been slightly modified in that 2% goat serum was added in addition to 0.5% blocking reagent for the blocking solution and that 0.01%, instead of 0.1%, Triton-X was used for PBST. The anti-macho-1 antibody was used at 7.5 μg/ml. Embryos were extracted before fixation for anti-PEM and α-tubulin antibody staining, as described previously (Negishi et al., 2007). Fluorescent images shown in this paper were acquired with a BX61 fluorescence microscope (Olympus) or an Axiophot microscope (Carl Zeiss) equipped with a CSLU 10 confocal unit (Yokokawa).

Results

Macho-1 and β-catenin regulate unequal cell divisions

In the course of our previous studies attempting to show whether or not ectopic notochord formation in the posterior region caused by macho-1 knockdown was β-catenin-dependent by co-injecting β-catenin together with macho-1 MOs (Kumano and Nishida, 2009), we found that the double-knockdown also resulted in perturbation of the posterior-specific unequal cleavage at the division to the 16-cell stage (Fig. 1). Closer observation of the cleavage pattern of the MO-injected embryo from the 8-cell to 24-cell stages revealed that it was almost identical to that of an embryo injected with MO against PEM, which was recently shown to be involved in cleavage-plane orientation and unequal cell divisions in the Halocynthia embryo (Negishi et al., 2007).

First, the posterior–vegetal (B4.1) blastomere did not protrude as posteriorly as that of the control embryo at the 8-cell stage (Fig. 1C, D, G, H, M, N). These results suggest that
macho-1 and β-catenin play an essential role in the posterior-specific unequal cleavages, possibly via a pathway that involves PEM.

The unequal cleavages take place through formation of a microtubule bundle in the posterior-most blastomeres that connects one of the centrosomes and the CAB in the posterior-most position of the blastomere. The bundle then shortens, thus shifting the nucleus, the mitotic apparatus and the cleavage plane posteriorly (Hibino et al., 1998; Nishikata et al., 1999). In order to identify which step in these processes is disrupted by simultaneous knockdown of macho-1 and β-catenin, we stained microtubules with an anti-α-tubulin antibody, YL1/2. Whereas an intense signal was detected between the CAB (black arrowheads in Figs. 2, E, I) and the nucleus (red arrowheads in Figs. 2E, G, I) in the posterior-most blastomeres (B5.2) of the 16-cell-stage control embryo [100% (n = 11), white arrows in Figs. 2A, C, G], no concentrated signal was observed at the corresponding position in majority of the embryos injected with macho-1 and β-catenin MO [83% (n = 12)]. Figs. 2B, D, F, H, J]. Therefore, these embryos are unable to come together and form the bundle required to pull the mitotic apparatus. The same phenomenon was observed when PEM was knocked down (Negishi et al., 2007), again suggesting that macho-1 and β-catenin act in a pathway that involves PEM.

**Localization of macho-1 protein to the CAB**

Considering that the PEM and β-catenin proteins are present at the CAB in *Halocynthia* embryos (Negishi et al., 2007; Kawai et al., 2007) and that β-catenin and macho-1 act either redundantly or in a synergetic way on the microtubule bundle assembly, the macho-1 protein could also be present at the CAB, even though so far it has only been known as a transcriptional activator (Nishida and Sawada, 2001; Yagi et al., 2004; Sawada et al., 2005). In order to test this possibility, we developed an anti-macho-1 polyclonal antibody and used it for embryo staining (Fig. S1). As expected, a signal was observed at the CAB from the 4-cell to the 110-cell stages. However, a nuclear signal was not detected as predicted from the original function of macho-1, even though several different conditions for fixation and different concentrations of the antibody were utilized. This signal at the CAB is specific to macho-1, since it was no longer observed in embryos where macho-1 had been knocked down by MO injection [100% (n = 8) in Fig. S1H with comparison to control MO-injected embryos shown in Fig. S1G]. Taken together, co-localization of macho-1 with β-catenin and PEM proteins to the CAB may suggest that these proteins contribute to microtubule bundle formation, although it is still possible that they play that role in other locations besides the CAB.

**Macho-1 and β-catenin may act upstream of PEM on the unequal cleavage regulation**

In an attempt to clarify the relationship between macho-1/β-catenin and PEM in the process of unequal cleavage, we first examined whether injection of macho-1 RNA is able to rescue the abrogation of unequal cell division in PEM-knockdown embryos, or whether PEM RNA is able to eliminate the abrogation in macho-1/β-catenin-knockdown embryos. We found that co-injection of macho-1 RNA together with MO against PEM was unable to overcome the abrogation effect [95% (n = 19) showing equal cleavages, Fig. 3C, in comparison to embryos with only PEM MO-injected, Fig. 3B]. However, this result cannot be properly assessed as injection of the same macho-1 RNA did not overcome the abrogation in macho-1/β-catenin-knockdown embryos [79% (n = 28) showing equal cleavages, Fig. 3E, in comparison to embryos with only macho-1/β-catenin MOs injected, Fig. 3D]. This could be because the injection was carried out after fertilization, and thus too late for the injected RNA either to be translated and become a functional protein or to be trans-located to the CAB to be functional, although the macho-1 RNA that had been injected had the full length of its 3′UTR. This contrasts with results from co-injection of PEM RNA, where it was able to rescue the abrogation of unequal cell divisions in macho-1/β-catenin-knockdown [93% (n = 14) showing normal unequal cleavages, Fig. 3G, in comparison to embryos with only MOs injected, Fig. 3F] as well as PEM-knockdown embryos [100% (n = 24) showing normal unequal cleavages, Fig. 3L, in comparison to embryos with only PEM MO-injected, Fig. 3H] even though the injected PEM RNA did not contain its 3′UTR. In addition to the elimination of the abrogation of unequal cleavages at the division to the 16-cell stage, the B4.1 blastomeres also protruded posteriorly at the 8-cell stage when PEM RNA was co-injected [100% (n = 38), data not shown and Fig. 5K just like those of normal embryos (Fig. 1B). The difference in the rescuing activity by co-injection of PEM and macho-1 RNAs might reflect a difference in their protein amounts required to exert their influences or in their sub-cellular locations at which macho-1 and PEM might function. In any event, the above results at least suggest that macho-1 and β-catenin regulate microtubule bundle formation upstream of PEM.

RNA localization is not significantly perturbed in embryos whose unequal cell divisions are disrupted

In order to examine how macho-1/β-catenin might affect PEM in the process of unequal cleavage, we next examined PEM RNA localization by in situ hybridization in embryos that had been injected with MOs against macho-1 and β-catenin. In the β-catenin and macho-1 double-knockdown embryos, PEM RNA was detected in...
spots at the posterior ends of the B4.1 blastomeres [100% (n = 10), Figs. 4C, D], unlike c-MO-injected control embryos that showed a moustache-shaped area of RNA localization that constituted the CAB (Sardet et al., 2003; Negishi et al., 2007, Figs. 4A, B). Reduction in size of the area of RNA localization was also evident in single-knockdown embryos that had been treated with β-catenin MO (Figs. 4E, F). Given that knockdown of β-catenin alone did not affect the unequal cleavages (Figs. 1C, H, M, R), reduction in size and possibly the amount of localized RNA in the absence of (macho-1 and) β-catenin was unlikely to have been the cause of the abnormal unequal cleavages. In this sense, PEM RNA localization to the CAB can still occur even when both macho-1 and β-catenin are knocked down. Therefore, macho-1/β-catenin regulates the unequal cleavages via a pathway that does not involve PEM RNA localization to the CAB. In addition to the size change mentioned above, the PEM domain in the embryos injected with macho-1 and β-catenin MO was always shifted more vegetally with respect to the third cleavage plane (Fig. 4C) in comparison with control embryos (Fig. 4A). This vegetally-shifted localization pattern is reminiscent of that of other postplasmic/PEM RNAs, such as ZF-1 and PEM, in PEM MO-injected embryos (Negishi et al., 2007), thus suggesting that macho-1 and β-catenin together also play a role in determining the position of the second and third cleavage planes, as PEM does (Negishi et al., 2007).

PEM protein is decreased at the CAB in the absence of macho-1 but not of β-catenin

We next examined protein localization. Macho-1, β-catenin and PEM proteins are all localized to the CAB (Fig. S1, Kawai et al., 2007; Negishi et al., 2007). When macho-1 and β-catenin were knocked down simultaneously, PEM protein was not detected at the CAB in any of the cases examined [100% (n = 11) in Figs. 5D, H in comparison to the normal localization in the control shown in Figs. 5A, E]. Surprisingly, however, injection of macho-1 MO alone also resulted in elimination of the PEM signal from the CAB [100% (n = 10), Figs. 5C, G], while injection of β-catenin MO alone did not affect normal PEM localization (Figs. 5B, F in comparison to control shown in Figs. 5A, E). Considering that injection of macho-1 MO alone did not perturb unequal cell divisions (Figs. 1I, N, S), the above results suggest that PEM residing at the CAB may not be required for the regulation of unequal cell divisions and that macho-1/β-catenin regulates the unequal cleavages via a pathway that does not involve PEM protein localization to the CAB. The former notion that PEM at the CAB may not be required was surprising, but was further supported by the fact that PEM protein was not detected at the CAB in macho-1/β-catenin-knockdown embryos even when PEM RNA was injected and the
abrogation of unequal cleavage was overcome [100% (n = 16), Fig. 5K, also see Figs. 5L, J for comparison]. However, it is still possible that PEM protein is present at the CAB in macho-1-knockdown embryos under the detection level of the above antibody staining and that such a small amount of residual PEM protein could be important for executing unequal cell divisions. Therefore, we stained extracted embryos with anti-PEM antibody, which yields higher detectability (Figs. 5L, P, Negishi et al., 2007). Since the extent to which embryos are extracted with the procedure used in this study varies among embryos and thus the signal intensity also varies (data not shown), the extraction method was used only to find out whether there is even the slightest signal, that is, PEM protein, at the CAB. As a result, a weak signal was observed in embryos that had been injected with macho-1 MO [89% (n = 19), Figs. 5M, Q], with macho-1 and β-catenin MOs [71% (n = 24), Figs. 5N, R] and with macho-1/β-catenin MOs plus PEM RNA [43% (n = 35), Figs. 5O, S]. Therefore, although the majority of PEM protein residing at the CAB is dispensable for successful unequal cleavages, a small portion of it, which is independent of macho-1, could participate in the regulation of unequal cell divisions.

Fig. 5. PEM protein localization to the CAB is dependent on macho-1. (A–S) Immunostaining of PEM protein with anti-PEM antibody in 8-cell-stage embryos that have been uninjected (I) or injected with 3 μg/μl control (A, E, I, P), 1 μg/μl β-catenin (B, F), 1.75 μg/μl macho-1 (C, G, M, Q), 1 μg/μl β-catenin and 1.75 μg/μl macho-1 (D, H, J, N, R) MOs and with 1 μg/μl β-catenin, 1.75 μg/μl macho-1 and 0.5 μg/μl PEM RNA (K, O, S). Unextracted (A–K) and extracted (L–S) embryos were stained. Posterior views with animal pole up (A–D, L–O) and lateral views with anterior and animal pole to the left and top, respectively (E–K, P–S). White arrowheads indicate staining observed at the CAB. The number shown in the right-bottom corner of each image represents that of embryos with fluorescent signal at the CAB. Scale bars: 100 μm.
Regardless of whether or not PEM localization to the CAB is important, the above results indicate that macho-1 is required for PEM localization to the CAB and yet that this is possibly not an essential part of macho-1 function in regulating unequal cell divisions. What is an essential part, then, is that macho-1 in cooperation with β-catenin acts upstream of PEM on the unequal cleavage regulation.

Macho-1 regulates unequal cell divisions independently of its transcriptional activity

We finally found that the newly-found macho-1 activity of regulating unequal cell divisions is independent of its transcriptional activity, which presumably resides in the nucleus. Injection of RNA that encodes a dominant negative version of macho-1, in which the β-catenin acts upstream of PEM on the unequal cleavage regulation.

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Discussion

In the present study, we found that macho-1 and β-catenin regulate the posterior-specific unequal cleavages in the early Halocynthia embryo. Macho-1 has been shown to contribute to A–P patterning of the ascidian embryo by promoting posterior tissue fates such as muscle and mesenchyme and suppressing the anterior notochord fate (Nishida and Sawada, 2001; Kobayashi et al., 2003; Yagi et al., 2004; Sawada et al., 2005). Thus, the present findings suggest a novel function of macho-1 and indicate that it is a major component of the PVC's posteriorizing activity. After PEM, this is the second example of a factor that has been found to regulate both cell fate and cleavage pattern in the context of A–P patterning. PEM has recently been shown to suppress ectopic notochord formation in the posterior region of the embryo by negatively regulating the gene expression required for notochord induction (Kumano and Nishida, 2009). Importantly, the regulation of cell fate and cleavage pattern seems to be independent (this study, Kumano and Nishida, 2009).

In addition to the similar events in which these factors are involved, the RNAs for PEM and macho-1, both of which are postplasmidic/PEM RNAs (reviewed in Prodon et al., 2007), are similarly localized within the embryo during the early cleavage stages: their mRNAs and proteins are both detected at the CAB in the posterior-most blastomeres (this study, Yoshida et al., 1996; Nishida and Sawada, 2001; Negishi et al., 2007). Therefore, these two maternal factors could function in the same pathway and even interact with each other.

We propose that macho-1 has three independent functions in relation to PEM. First, macho-1 serves as a muscle determinant (Nishida and Sawada, 2001), possibly acting downstream of PEM (Kumano and Nishida, 2009). Second, macho-1 keeps PEM protein localized to the CAB, although the developmental consequence of this tethering activity remains to be seen. This activity is likely to be operated by the macho-1 protein residing at the CAB because these proteins are co-localized there. Third, macho-1 in cooperation with β-catenin regulates unequal cleavages, acting upstream of PEM. At this point, it is difficult to speculate on how macho-1 and PEM might interact or what they might do in the context of the cell cleavage patterning since we are not even sure whether the proteins residing at the CAB or in other places are important for executing unequal cleavage. Our antibody staining against either PEM (Negishi et al., 2007) or macho-1 (this study) gave us an exclusive signal only observed at the CAB, but it might not have been good enough to detect the proteins in other sub-cellular locations than the CAB. It would be important for the future to improve our antibody staining or to develop a way to target proteins of interest to the CAB for our rescue experiments.

The present study has cast uncertainty on the previous finding that localized PEM may play a role in anchoring microtubule ends to the CAB for successful unequal cleavages (Negishi et al., 2007). This assumption was premised by the fact that PEM protein was detected by our antibody staining only at the CAB (Negishi et al., 2007), as mentioned above, and that PEM-knockdown resulted in the loss of microtubule bundle formation and the abrogation of unequal cleavages (Negishi et al., 2007). Our argument that PEM localization to the CAB may not be required for successful unequal cleavages is based on the three results obtained in this study. First, injection of PEM RNA that is devoid of its 3′ UTR was able to rescue the abrogation of unequal cell divisions, although RNA localization to the CAB may not be a prerequisite for protein localization there. Second, PEM protein was not detected at the CAB when the abrogation of unequal

Fig. 6. Regulation of unequal cleavage by macho-1 independently of its function as a transcriptional activator. (A, B, E, F) Cell division patterns of 16-cell-stage embryos that have been injected with 1 μg/μl β-catenin MO together with either 1.75 μg/μl macho-1 MO (A) or 1 μg/μl macho-1-EnR RNA (B), or have been treated with DMSO (as a control) (E) or a transcription inhibitor, actinomycin D (F). Red lines link sister cells that would normally result from unequal cleavages. (C, D) Images of 110-cell-stage embryos stained in situ hybridization for muscle actin. Uninjected control (C) and macho-1-EnR RNA-injected (D) embryos. The number shown in the right-bottom corner of each image indicates that of embryos showing cleavage pattern (A, B, E, F) or staining (C, D) as appeared in the respective image. Scale bar: 100 μm.
cleavages was eliminated by injection of PEM RNA. Third, injection of MO against macho-1 alone resulted in substantial reduction in the PEM protein level at the CAB without affecting the cleavage pattern.

We found that macho-1 and β-catenin act either redundantly or in a synergetic manner in unequal cleavage regulation. Although how they have such a role remains elusive at this moment, it might be interesting to note that these proteins as well as PEM all have multiple functions: they at least regulate both cell fate specification and asymmetric or unequal cell divisions independently (Nishida and Sawada, 2001; Kobayashi et al., 2003; Negishi et al., 2007; Kuman and Nishida, 2009; Logan and Nusse, 2004; Clevers, 2006; this study). Armadillo, a Drosophila orthologue of β-catenin, is known to participate in tethering mitotic spindle to the cell cortex via adenomatous polyposis coli proteins (APCs) in cells such as male germine stem cells (Yamashita et al., 2003) and in embryonic cells (McCartney et al., 2001), thus contributing to the determination of cell division orientation during asymmetric cell divisions. Halocynthia β-catenin might also contribute to connect the microtubule bundle to the CAB during unequal cleavages since the β-catenin protein is present at the CAB (Kawai et al., 2007); however, this would be unlikely if PEM acting downstream of β-catenin (together with macho-1) does not reside at the CAB and not play a role in anchoring microtubule ends to the CAB, as discussed above.

Our result showing that β-catenin regulates the posterior-specific cleavage pattern has provided the first evidence that ascidian β-catenin is involved in A–P patterning of the early embryo. The A–P axis of the ascidian embryo sits perpendicular to the animal–vegetal (A–V) axis (Kumano and Nishida, 2007). Previously, ascidian β-catenin has been known to be required for the formation of tissues that arise from cells in the vegetal hemisphere (Imai et al., 2000, 2006; Kumano et al., 2006; Kawai et al., 2007) and, thus, to specify the A–V axis. The control of polarization along the A–V axis by β-catenin is also seen in embryos of other non-vertebrate deuterostomes such as sea urchin and amphioxus (reviewed in Petersen and Reddien, 2009). In contrast, β-catenin in vertebrates such as Xenopus and zebrafish is known to specify an axis (organizer to contra-organizer axis) that is perpendicular to the A–V axis (reviewed in Petersen and Reddien, 2009). Therefore, the ascidian embryo is the only one so far known to have its β-catenin used to specify two axes that are perpendicular to each other at the early stages, and embryos in other organisms might also have the same regulation.

While macho-1, β-catenin and PEM play essential roles in unequal cleavage, it is certain that not just these factors, but also others are involved in unequal cleavage regulation. For example, the aPKC protein, which is a common regulator of asymmetric cell division in different organisms (reviewed in Suzuki and Ohno, 2006), is known to be localized to the CAB in another ascidian species, Phallusia mammillata (Patalano et al., 2006). It has not yet been proved that ascidian aPKC protein is involved in unequal cell division; however, it could regulate microtubule tethering in a manner that has been reported in mammalian cells (Etienne-Manneville and Hall, 2003): aPKC facilitates accumulation of APCs at the plus end of microtubules. The microtubules may direct their plus ends toward the CAB in ascidian embryos (Nishikata et al., 1999). Future studies of such factors that are localized to the CAB or may not be localized but proved to be essential for unequal cleavages will be required for better understanding of the molecular mechanism by which unequal cell division takes place.

The present findings suggest that macho-1 acts as a cleavage pattern regulator as well as an anchoring factor to the CAB. Macho-1 was originally identified as a transcription factor, and thus has been thought to act in the nucleus. FLAG-tagged macho-1 protein has been detected in the nucleus when over-expressed (Nishida and Sawada, 2001). Therefore, macho-1 has multiple functions that may depend on its sub-cellular localization: a muscle determinant when present in the nucleus, an anchoring factor when located to the CAB and a cleavage pattern regulator in some yet-unidentified locations. A “double life” of a transcription factor has been reported previously: a general transcription factor known as TFII-I regulates the activity of a Ca2+ channel located at the cell surface independently of its regulation of gene expression (Caraveo et al., 2006). Thus, transcription factors acting both inside and outside the nucleus may be more common than currently believed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.05.013.

References


