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Three distinct RNA localization mechanisms contribute to oocyte polarity establishment in the cnidarian *Clytia hemisphærica*

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ABSTRACT

Egg animal–vegetal polarity in cnidarians is less pronounced than in most bilaterian species, and its normal alignment with the future embryonic axis can be disturbed by low-speed centrifugation. We have analyzed the development of oocyte polarity within the transparent and autonomously functioning gonads of *Clytia* medusae, focusing on the localization of three recently identified maternal mRNAs coding for axis-directing Wnt pathway regulators. Animal–vegetal polarity was first detectable in oocytes committed to their final growth phase, as the oocyte nucleus (GV) became positioned at the future animal pole. In situ hybridization analyses showed that during this first, microtubule-dependent polarization event, CheFz1 RNA adopts a graded cytoplasmic distribution, most concentrated around the GV. CheFz3 and CheWnt3 RNAs adopt their polarized cortical localizations later, during meiotic maturation. Vegetal localization of CheFz3 RNA was found to require both microtubules and an intact gonad structure, while animal localization of CheWnt3 RNA was microtubule independent and oocyte autonomous. The cortical distribution of both these RNAs was sensitive to microfilament-disrupting drugs. Thus, three temporally and mechanistically distinct RNA localization pathways contribute to oocyte polarity in *Clytia*. Unlike the two cortical RNAs, CheFz1 RNA was displaced in fertilized eggs upon centrifugation, potentially explaining how this treatment re-specifies the embryonic axis.

Introduction

In many animal species, a polarized distribution of "determinants" within the egg is crucial to direct normal development of the embryonic body plan. Localized or graded distributions of organelles and macromolecules commonly become established along the primary animal–vegetal (a–v) egg axis during oogenesis. The animal pole corresponds to the site of polar body emission during meiosis, and is often predicted by the position of the GV prior to meiotic maturation. The timing and mechanisms by which oocyte polarity is established have been well studied in classical models from the Bilateria, notably *Drosophila*, *Xenopus* and ascidians. In these cases, the decisive maternal localized determinants have been shown to consist of mRNAs positioned at different sites with respect to the a–v axis (Di Carlo, 2004; King et al., 2005; Nishida, 2005; van Eeden and St Johnston, 1999).

In the Cnidaria and Ctenophora, experimental evidence has indicated that pre-localized maternal factors may be less important for directing embryo organization than in the Bilateria (Freeman, 1976, 1981a). Thus in ctenophore embryos, the embryonic "oral" pole always forms at the site of first mitosis but does not necessarily relate to egg a–v polarity, as seen following physiological migration of the female nucleus away from the animal pole or experimental displacement of the zygote nucleus by centrifugation (Freeman, 1976, 1977; Houliston et al., 1993). Similarly in cnidarians, the single "oral–aboral" embryonic axis can be re-oriented from its usual alignment with the egg a–v axis by experimental displacement of the zygote nucleus (Freeman, 1981a). The sensitivity of embryonic axis specification to low-speed centrifugation in cnidarians and ctenophores distinguishes them from bilaterian models such as sea urchins, in which localized determinants are thought to be anchored tightly to the cortex of the egg along its a–v axis (Horstadius, 1953).

In cnidarians, despite the sensitivity of embryonic axis orientation to nuclear displacement, there is considerable evidence that in undisturbed eggs a–v polarity provides polarity cues for axial development. Firstly, the animal pole of the undisturbed egg has been shown to predict reliably the oral (=posterior) pole of the larva and the mouth end of the adult polyp in a number of cnidarians including the hydrozoans *Clytia (=Phialidium)*, *Dynamena*, *Amphisbetia* and *Hydractinia*, and the anthozoan *Nematostella*, (Freeman, 1981a; Fritzenwanker et al., 2007; Lee et al., 2007; Teissier, 1931). Secondly, while many cnidarian oocytes and eggs appear visibly uniform, signs of polarity have been noted in some cases, including

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the eccentric position of the Germinal Vesicle (GV) at the animal pole, as well as polarized localization of pigment in *Amphisbetia* eggs and of symbiont dinoflagellates in the coral *Pocillopora* (Marlow and Martindale, 2007; Teissier, 1931). Thirdly, embryo manipulation studies in the hydrozoan *Podocoryne* and in *Nematostella* have provided experimental evidence for animally-localized axis-determining factors (Fritzenwanker et al., 2007; Lee et al., 2007; Momose and Schmid, 2006). In these two distantly-related cnidarians, bisection perpendicular to the a–v axis at the 8 cell-stage yields normal planula larva from the animal half but non-polarized ectodermal balls (lacking endodermal tissue) from the vegetal half, implying that determinants for both polarity and endoderm formation are excluded from the vegetal half of the early embryo. The formation of normally patterned larvae conserving the original polarity from animal fragments in these species, and from both animal and vegetal fragments in other species including *Clytia gregarium*, likely reflects the exceptional regulative ability of cnidarians at all stages of their life cycles (Freeman, 1981b; Galliot and Schmid, 2002).

Recent studies have shown that animally localized axis determinants in cnidarians act as regulators of the canonical Wnt signaling pathway, promoting stabilization and nuclear localization of the transcriptional co-factor β catenin on the future oral side of the early embryo (Momose and Houliston, 2007; Wikramanayake et al., 2003). Maternally directed Wnt pathway activation is thought to be an evolutionary ancient embryo patterning mechanism, functioning not only in cnidarians but also in many deuterostomes, including sea urchins, ascidians, amphibians and fish (Imai et al., 2000; Schneider et al., 1996; Wikramanayake et al., 1998) as well as in some protostomes (Henry et al., 2008). In *Clytia hemisphaerica* the localized maternal determinants upstream of Wnt pathway activation have been identified as mRNAs coding for two frizzled family receptor proteins, CheFz1 and CheFz3, found concentrated in the animal cytoplasm and at the vegetal cortex of the egg respectively (Momose and Houliston, 2007). CheFz1 mediates canonical Wnt pathway activation and directs the development of oral fate, while CheFz3 acts negatively to down-regulate this pathway in the future aboral territory. mRNA for the Wnt family ligand CheWnt3 is also maternally localized and essential for embryonic polarity development, exhibiting a third distinct localization pattern at the animal cortex (Momose et al., 2008). In other cnidarians, localized Wnt pathway activation may be directed by alternative or additional determinants: In *Nematostella*, no maternal RNA localization has been detected yet for Wnt ligands or receptors, but the cytoplasmic regulator protein Disheveled is localized maternally at the egg animal pole (Lee et al., 2007), while in *Hydractinia* maternal RNAs for both Wnt3 and the downstream transcription factor Tcf are localized (Plickert et al., 2006).

It is remarkable that unfertilized *Clytia* eggs, despite their lack of visible polarity, contain axis-directing mRNAs with three distinct distributions along the animal–vegetal axis: CheFz1 mRNA exhibiting a declining animal–vegetal gradient in the cytoplasm, CheWnt3 mRNA localized at the animal cortex and CheFz3 mRNA at the vegetal cortex. This provides an opportunity to examine the extent to which the cellular and molecular mechanisms responsible for generating egg and embryo polarity via mRNA localization are common between evolutionary distant phyla. Taking advantage of the exceptional transparence and developmental autonomy of the *Clytia* female gonad we have characterized by various techniques of microscopy the development of polarity during oocyte growth and during oocyte maturation, the light induced process that triggers meiosis completion and spawning of unfertilized eggs. Our study has defined distinct temporal characteristics and cytoskeletal requirements for the localization of each of these three key maternal mRNAs. We also performed centrifugation experiments to assess the anchoring of these localized RNAs in fertilized and unfertilized eggs, allowing us to address the basis of embryonic axis re-specification in *Clytia* upon nuclear displacement.

Materials and methods

Medusae, gonads and oocytes

C. hemisphaerica medusae were obtained from laboratory colonies (Chevalier et al., 2006). Oocytes and gonads were cultured in 0.2 μm Millipore Filtered Sea Water (MFSW). Gonads were dissected from the underside of the bell of mature medusae using surgical scissors. Oocytes were liberated from dissected gonads by treatment with 1% Thioglycolate/0.05% Pronase/0.3 M NaCl for fixation, or without enzymatic treatment using fine forceps for maturation experiments. Maturation was triggered by incubation of dissected gonads or isolated fully-grown oocytes for 5–15 min in 2–4 mM bromo adenosine 3′5′cyclic MonoPhosphate (Br-cAMP) in MSFW, diluted just before use from a 20 mM aqueous stock, then washed in MFSW. All experiments were performed at 18–20 °C. We confirmed that Br-cAMP-matured *C. hemisphærica* oocytes can fertilize and develop normally, as reported previously for *C. gregarium* (Freeman and Ridgway, 1988). Time-lapse recordings of oocyte maturation were made using DIC optics on a Zeiss Axiovert microscope equipped with a motorized stage and CCD camera driven by MetaMorph software.

Immunofluorescence

For immunofluorescence, isolated gonads or oocytes were fixed and permeabilized in 0.1 M Hepes pH6.9/50 mM EGTA/10 mM $MgSO_4$/0.5 M Maltose/4% paraformaldehyde (Methanol+RNase free/Electron Microscopy Science)/0.2% Triton X-100 for 2 h at room temperature, followed by washing in PBS/Triton X-100 0.2%. Microtubules were visualized by incubation in anti-tubulin antibody DM1A (Sigma diluted in 3% BSA/PBS) overnight at 4 °C, followed by a FITC coupled anti-mouse Ig (Sigma, diluted 1/500 in PBS) for 2 h at room temperature, with Hoechst dye 33258 (1 μg/ml) included to stain DNA and rhodamine-phalloidin (1 μg/ml; Sigma) to stain polymerized actin. PBS was used for washes between antibodies. Specimens were mounted in Citifluor (Citifluor Ltd) and imaged using a Leica SP2 confocal microscope.

In situ hybridization

In situ hybridization was performed exactly as described previously (Chevalier et al., 2006) using DIG-labeled antisense RNA probes, synthesized from linearized Express1 plasmid using T7 or T6 or SP6 polymerase and the RNA DIG-labeling mix (Roche). Following in situ hybridization, each egg and oocyte was examined under the microscope and the distribution of the signal assessed in relation to the position of the nucleus, visualised using with Hoechst dye. Separate localization criteria were established for each RNA: CheFz1, which in normal unfertilized eggs shows a declining animal–vegetal gradient in the cytoplasm (Momose and Houliston, 2007), was considered to be localized if its concentration of the RNA signal was higher in the hemisphere containing the nucleus than in the opposite hemisphere. The two cortical RNAs were considered to be localized if the majority of the staining was located in the half of the cortex opposite (for CheFz3) or centered on (for CheWnt3) the nucleus. Note that when assessing RNA localization we took into consideration the natural variation in intensities and distributions of the in situ signal between eggs.

Cytoskeletal inhibitor treatments and centrifugation

Dissected gonads, maturing oocytes and unfertilized eggs were treated with 10 μM nocodazole to depolymerize microtubules, or 10 μg/ml cytochalasin B or lantrunculin B to disrupt microfilaments, diluted into MFSW just before use from stock solutions in DMSO. Equivalent results were obtained using cytochalasin B and