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RhoA/ROCK pathway activity is essential for the correct localization of the germ plasm mRNAs in zebrafish embryos

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ABSTRACT

Zebrafish germ plasm is composed of mRNAs such as *vasa* and *nanos* and of proteins such as Bucky ball, all of which localize symmetrically in four aggregates at the distal region of the first two cleavage furrows. The coordination of actin microfilaments, microtubules and kinesin is essential for the correct localization of the germ plasm. Rho-GTPases, through their effectors, coordinate cytoskeletal dynamics. We address the participation of RhoA and its effector ROCK in germ plasm localization during the transition from two- to eight-cell embryos. We found that active RhoA is enriched along the cleavage furrow during the first two division cycles, whereas ROCK localizes at the distal region of the cleavage furrows in a similar pattern as the germ plasm mRNAs. Specific inhibition of RhoA and ROCK affected microtubules organization at the cleavage furrow; these caused the incorrect localization of the germ plasm mRNAs. The incorrect localization of the germ plasm led to a dramatic change in the number of germ cells during the blastula and 24 hpf embryo stages without affecting any other developmental processes. We demonstrate that the Rho/ROCK pathway is intimately related to the determination of germ cells in zebrafish embryos.

1. Introduction

The distribution and localization of RNA is a highly organized process and is conserved through evolution. Multiple examples of the specific localization of mRNAs have been described in organisms from yeast to human (reviewed by Martin and Ephrussi (2009)). The process of mRNA localization is widespread, as nearly 70% of mRNAs in *Drosophila* embryos showed a specific pattern of distribution in the embryo (Lécuyer et al., 2007). Maternal mRNAs play critical roles in oocyte development and in the earliest steps of embryogenesis such as cell division and embryonic patterning; a clear example of the importance of RNA segregation is the determination of primordial germ cells (reviewed by Zhou and King (2004)).

Germ cells are specified by two possible modes: either by a zygotic cell-cell inductive signaling during embryogenesis or by maternally inherited germ plasm (Gilbert, 2010). Germ plasm (GP) is a collection of specific mRNAs and proteins encoded by the maternal genome and deposited into a specific location in the oocyte (reviewed in Wylie (1999)). Segregation of GP is essential during determination of the germ line in *Drosophila melanogaster*, *Caenorhabditis elegans*, *Xenopus laevis* and *Danio rerio* (zebrafish), among others. In zebrafish, the GP localization is particularly intricate, involving several steps: during early oogenesis, GP mRNAs localize with perinuclear mitochondria and actin fibrils inside the Balbiani body, a membrane-less organelle whose functions include the establishment of oocyte polarity and mRNA transport (Kosaka et al., 2007; Marlow and Mullins, 2008; Bontems et al., 2009; Gupta et al., 2010). Following Balbiani body disintegration, individual mRNAs localize in different parts of the oocyte cytoplasm along the animal-vegetal axis (Kosaka et al., 2007). After fertilization of the zebrafish egg, some GP mRNAs such as *vasa* (Yoon et al., 1997) and *nanos* (Koprunner et al., 2001) move from the egg yolk to the first blastomere, located at the animal pole. During the first two cell divisions, *vasa* and *nanos* mRNAs are recruited to the cleavage furrows as large aggregates; after a series of events, they exhibit a stereotypic localization in the distal ends of cleavage furrows of the four-cells stage embryo. Furthermore, there is a second group of GP mRNAs termed vegetal, such as *dazl*, that migrate later than animal GP (Maegawa et al., 1999), presumably by different pathways; these mRNAs end up overlapping with animal mRNAs at the distal ends of cleavage furrows (Theusch et al., 2006). These data indicate that the mechanisms of assembly and transport of the different mRNAs is regulated in a spatio-temporal manner. The removal of the cytoplasm

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from the distal end of the cleavage furrows causes the loss of all germ cells in the larvae without affecting any other developmental process (Hashimoto et al., 2004); these results demonstrate that the localization of GP is necessary for germ cell determination.

In zebrafish embryos, there is an intimate interaction between the GP granules and the cytoskeleton during the first cell division. This initial interaction regulates the segregation of the GP components to the distal furrows. Animal GP is associated with actin filaments in one-cell embryos, and it is moved to the periphery by astral microtubules during the first cell division (Theusch et al., 2006). The interaction between the microtubules and actin filaments is necessary in order to displace the GP, and Birc5b protein regulates this interaction (Nair et al., 2013). Birc5b is the zebrafish homologue of the mammalian Chromosomal Passenger Complex (CPC) protein Survivin, a member of the inhibitor of apoptosis protein family, which also promotes mitosis and cell proliferation (reviewed in Schimmer (2004)). In *motley/birc5b* mutant embryos, the correct segregation of the chromosomes during meiosis and mitosis and the organization of the microtubules and of the actin filaments are altered early in development, leading to impaired GP aggregation (Nair et al., 2013). Furrow microtubules (FMA) participate in the compaction of the GP to the external part of the furrow. Although the primordial function of the FMA seems to be the transport of vesicles to the membrane during cell division, it has been proposed that this mechanism is utilized also for the aggregation of the GP (Lindeman and Pelegri, 2010). Mutants that produce maternal defects in the FMA, such as *nebel*, or experimental disturbance of microtubules with nocodazole treatment cause defects in *vasa* mRNA localization (Pelegri et al., 1999). In contrast, *acytokinetic* mutant embryos, which are defective in generating contractile rings, are capable of aggregating *vasa* mRNA along the first mitotic plane even in the absence of cytokinesis (Kishimoto et al., 2004). Recently, Campbell et al. (2015) demonstrated that Kinesin-1 is necessary for the correct localization of the GP into the furrows independently of any furrow associated cytoskeletal abnormalities. Myosin accumulates during maturation of the furrow, and inhibition of Myosin activity affects GP compaction at the distal ends without affecting furrow formation (Urven et al., 2006).

Rho-GTPases are a family of small proteins that participate as molecular switches during the regulation of multiple cellular processes, mainly by regulating cytoskeletal dynamics. Rho-GTPases cycle between an inactive state bound to GDP and an active state bound to GTP; GEF (guanine nucleotide exchange factors) proteins maintain the Rho-GTPases in the active state, while GAP (GTPase-activating proteins) proteins catalyze their return to the inactive state. Proteins known as GDIs (guanine nucleotide dissociation inhibitors) sequester the inactive forms of the Rho-GTPases away from the membrane (Etienne-Manneville and Hall, 2002). In the active state, Rho-GTPases can interact with various effectors to generate a cellular response. There are 32 Rho-GTPase genes in the zebrafish genome, as indicated by their homology to 17 human Rho-GTPase genes (Salas-Vidal et al., 2005); nevertheless, Rac1, Cdc42 and RhoA have been studied most often. Cdc42, Rac1 and RhoA have been implicated in mRNA distribution. Cdc42 and Rac1 can join protein complexes with cytoskeletal proteins such as Tubulin, Tau, Actin, Internexin and Staufen (Villace et al., 2004). RhoA has also been implicated in the subcellular localization of various mRNAs. Disruption of the RhoA interferes with *β-actin* mRNA localization in fibroblasts *in vitro* (Latham et al., 2001); meanwhile, activation of the RhoA/ROCK/myosin II pathway regulates the targeting of mRNA to particular cell domains (Stuart et al., 2008), and the RhoA/ROCK pathway actively participates in the formation of mRNA containing stress granules (Tsai and Wei, 2010).

The correct assembly, migration and segregation of the mRNAs of the GP during the first cell divisions are intimately connected to the cytoskeleton and cytokinesis; nevertheless, the mechanism that regulates this process remains poorly characterized. In the present study we evaluated the participation of RhoA as a key regulator of GP localization during the first two cell division cycles in zebrafish embryos. Here, we report the localization of the most studied Rho GTPases, RhoA, Rac1 and Cdc42, during the first division cycles, when the GP localizes. Our observations show that only RhoA is distributed dynamically at the cell division furrows; we also show that the RhoA effector ROCK is localized to the furrows but is confined to the distal ends, resembling GP components localization. Finally, we demonstrate that pharmacological inhibition of RhoA and ROCK induced incorrect localization of GP mRNAs, causing a significant decrease in the germ cell population later in development. These findings suggest that the RhoA/ROCK pathway has a direct role in GP establishment.

2. Materials and methods

2.1. Fish maintenance and strains

AB-Tu-WIK hybrid line and wild-type zebrafish (*Danio rerio*) embryos were obtained from natural crosses and raised at 28 °C based on standard procedures (Westerfield, 2000). Embryo stages were determined by morphological criteria according to Kimmel and collaborators (Kimmel et al.,1995). Zebrafish were handled in compliance with local animal welfare regulations, and all protocols were approved by the Comité de ética (Instituto de Biotecnología, UNAM).

2.2. Immunofluorescence

Whole-mount immunostaining in zebrafish embryos was used to determine the localization patterns of RhoA, RhoA-GTP (active form of RhoA), Rac1, Cdc42, ROCK-2α, the phosphorylated myosin light chain and α-tubulin as previously described (Mendieta-Serrano et al., 2013) with slight modifications. Freshly collected embryos at different stages of development were fixed overnight (o.n.) at 4 °C in 4% PFA in PBS. Afterwards, the samples were washed 3 times in blocking buffer (PBS, BSA 0.1%, triton X100 1%), manually dechorionated and blocked for at least 5 h in blocking buffer at room temperature with constant shaking. For detection of active RhoA (RhoA-GTP) fixation was done with 10% trichloroacetic acid instead of paraformaldehyde. For α-tubulin detection embryos were fixed in 4% PFA, 0.25% glutaraldehyde, 5 mM EGTA, and 0.2% Triton X-100 in PBS 1X for 6 h at room temperature and overnight at 4 °C. After treatment with 0.5 mg/ml sodium borohydrate, embryos were manually deyolked (Theusch et al., 2006). The primary antibodies used were: rabbit IgG anti-RhoA (119, sc-179 Santa Cruz Biotechnology); mouse IgG2b anti-RhoA-GTP (26904 NewEast Biosciences); rabbit IgG anti-Rac1 (C14, sc-217 Santa Cruz Biotechnology), rabbit IgG anti-Cdc42 (P1, sc-87 Santa Cruz Biotechnology); rabbit IgG anti-ROCK-2α, (CT, Z – FISH; AS-55431s Anaspec), rabbit IgG anti-Phospho-Myosin Light Chain 2 (Ser19) (3671, Cell signalling technology) and mouse IgG anti-αtubulin (T9026, Sigma) were diluted 1:100 in blocking buffer and the embryos were incubated in this solution at 4 °C o.n. Subsequently, the embryos were washed in blocking buffer and were incubated in the secondary antibody goat anti-rabbit Alexa Fluor 647 (A-21244, Molecular Probes, Invitrogen) or secondary antibody goat anti-mouse IgG Alexa Fluor488 (A-11001, Molecular Probes, Invitrogen) diluted 1:100 in blocking buffer (final concentration 20 mg/ml) for 8 h at 4 °C. The embryos were washed in blocking buffer and treated with 0.4 mg/ml RNase (Roche) for 1 h at 37 °C, washed with blocking buffer and stained with either Sytox Green (s7020, Molecular Probes, Invitrogen) or DAPI (Molecular probes) at a dilution of 1:2000 in blocking buffer for 1 h at room temperature to visualize the DNA and nuclei. For actin filaments detection we used Alexa 488-conjugated phalloidin (Molecular Probes, A12379) at 1:100 dilution in PBS for 30 min at room temperature. The embryos were then washed 3 times and mounted in low melting point agarose in PBS for confocal laser scanning microscopy.