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Expression of XNOA 36 in the mitochondrial cloud of *Xenopus laevis* oocytes

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Summary

In *Xenopus laevis* oocytes a mitochondrial cloud (MC) is found between the nucleus and the plasma membrane at stages I–II of oogenesis. The MC contains RNAs that are transported to the future vegetal pole at stage II of oogenesis. In particular, germinal plasm mRNAs are found in the Message Transport Organiser (METRO) region, the MC region opposite to the nucleus. At stages II–III, a second pathway transports Vg1 and VegT mRNAs to the area where the MC content merges with the vegetal cortex. Microtubules become polarized at the sites of migration of Vg1 and VegT mRNAs through an unknown signalling mechanism. In early meiotic stages, the centrioles are almost completely lost with their remnants being dispersed into the cytoplasm and the MC, which may contain a MTOC to be used in the later localization pathway of the mRNAs. In mammals, XNOA 36 encodes a member of a highly conserved protein family and localises to the nucleolus or in the centromeres. In the *Xenopus* late stage I oocyte, XNOA 36 mRNA is transiently segregated in one half of the oocyte, anchored by a cytoskeletal network that contains spectrin. Here we found that XNOA 36 transcript also localises to the nucleoli and in the METRO region. XNOA 36 protein immunolocalization, using an antibody employed for the library immunoscreening that depicted XNOA 36 expression colonies, labels the migrating MC, the cytoplasm of stage I oocytes and in particular the vegetal cortex facing the MC. The possible role of XNOA 36 in mRNA anchoring to the vegetal cortex or in participating in early microtubule reorganization is discussed.

Keywords: Cytoskeleton, Microtubules, Mitochondrial cloud, Oocyte, *Xenopus laevis*

Introduction

In *Drosophila* melanogaster, microtubule reorganisation induced by the early signal of the TGFα homologue Gurken underlies antero-posterior (A/P) polarisation in the oocyte and later its dorso/ventral (D/V) axis that will be inherited by the embryo (Schüpbach & Wieschaus, 1986; Neuman-Silberberg & Schüpbach, 1996; Roth, 2003). In *Xenopus*, the oocyte acquires a radial organisation along a primary coordinate, i.e. the animal–vegetal axis (A/V) that dictates the perspective A/P planning of the embryo. At early stages of oogenesis (stages I and II), a mitochondrial cloud (MC) is located between the germinal vesicle (GV) and the plasma membrane (Heasman *et al.*, 1984). The MC is rich in active mitochondria (Wilding *et al.*, 2001) and gathers nuclear messages destined to be transported within the MC to the future vegetal pole at late stage II of oogenesis (Kloc *et al.*, 1996). Germinal plasm RNAs are found in the message transport organiser (METRO) region, the MC endoplasmic reticulum rich region opposite to the nucleus. Other messengers are Xwnt11 mRNA implicated in the determination of D/V (Tao *et al.*, 2005) and part of Vg1 mRNA (Kloc *et al.*, 1996). Messengers destined to be transported to the vegetal cortex may first assemble with proteins in the nucleus (Kress *et al.*, 2004). The localisation of some mRNA into the MC occurs through a diffusion/entrapment mechanism into the MC endoplasmic reticulum (Chang *et al.*, 2004). Later, at stages II–III, a second pathway takes care of the transportation of other mRNAs including

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Vg1mRNA and VegT mRNA to the site where the MC content has merged to the vegetal cortex. Vg1 and Veg T are implicated in endoderm and mesoderm specification (Thomsen & Melton, 1993; Zhang *et al.*, 1998). A polarized organisation of microtubules and related kinesins initiates at the site of Vg1 and VegT mRNA migration to the cortex (Yisraeli *et al.*, 1990; and see Kloc & Etkin, 1998; King *et al.*, 2005). At present, the signal that causes the microtubule reorganisation is unknown. By stages III–IV, the microtubules become fully organised as an orderly crown that links the oocyte cortex to the germinal vesicle (Klymkowsky *et al.*, 1987; Gard *et al.*, 1995, 1997).

In the oogonia, a primordial MC is found that gives a polarised organisation to the cell. In this structure, centrioles, spectrin and the endoplasmic reticulum-rich fusome are located in addition to microtubules that radiate from the centrioles. The latter supposedly constitutes an interphase microtubule organisation centre (MTOC) active in the oogonia (Kloc *et al.*, 2004). In early meiotic stages, the centrioles are almost completely lost, their remnants are dispersed into the cytoplasm and the MC where γ-tubulin was localised (Kloc & Etkin, 1998), although this point remains controversial (Gard, 1999). It was hypothesised that the MC may contain a MTOC to be used in the late mRNAs pathway (Kloc & Etkin, 1998).

Previous data showed that, in the late stage I oocytes (250 μm oocytes), a transient XNOA 36 mRNA segregation occurs in a zone of the oocyte surrounding the MC and opposite to the ovarian hilum with the MC located between the XNOA 36 mRNA labelled region and the unlabelled region. The XNOA 36 mRNA is localised or anchored in the oocyte through a cytoskeletal network that contains spectrin. XNOA 36 encodes a member of a highly conserved protein family displaying characteristic CXXC motifs putatively involved in the formation of zinc-finger structures of the C2–C2 type (Bolivar *et al.*, 1999; Vaccaro *et al.*, 2010). NOA36 was first one to be isolated in *Homo sapiens* as an autoimmune antigen in a patient suffering from rheumatoid arthritis and was found in the nucleolus in G1/G2 or was associated with centromeres (Bolivar *et al.*, 1999). Recent data report that NOA36 (or ZN330) is a pro-apoptotic factor and associates dynamically to the outer mitochondrial membrane (de Melo *et al.*, 2009).

The *Xenopus laevis* NOA36 cDNA was isolated, using an anti-Gurken monoclonal antibody (anti-DrosGu), by immunoscreening of an expression library of stage I–III *Xenopus laevis* oocytes. The nucleotides sequence of the isolated XNOA 36 cDNA shows no homologies to Gurken cDNA, and the deduced amino acid sequences of Gurken and XNOA 36 do not share sequences longer than five amino acids (Vaccaro *et al.*, 2010).

In this paper we found that XNOA 36 transcript localises, in addition to its cytoplasmic localisation, in the nucleus and in a sector of the MC that eventually coincides with the METRO region facing the vegetal cortex. Moreover, we studied XNOA 36 protein immunolocalization, using the anti-DrosGu antibody employed for the library immunoscreening that depicted XNOA 36 expression colonies (Vaccaro *et al.*, 2010). We found that, in stage I oocytes, the antibody labels the nucleoli, the migrating MC and the peripheral cytoplasm, in particular the vegetal cortex facing the MC. The possible role of XNOA 36 is discussed.

Material and methods

Animals and oocytes

Adult *X. laevis* females were obtained from Rettili (Varese, Italy). They were kept and utilized at the Department of Structural and Functional Biology, the University of Naples Federico II, according to the guidelines and policies dictated by the University Animal Welfare Office and in agreement with European Community laws. Groups of oocytes at various stages of oogenesis were excised from the ovaries of females anaesthetized with MS222 (Sigma). The growth stages of the *X. laevis* oocyte are: stage I (50–300 μm), stage II (300–450 μm), stage III (450–600 μm), stage IV (600–1000 μm), stage V (1000–1200 μm) and stage VI (1200–1300 μm) (Dumont, 1972).

In situ hybridisation on sections

XNOA 36 cDNAs were used as templates to synthesise digoxigenin-labelled antisense and sense probes, using digoxigenin-labelled UTP and T3 and T7 RNA polymerase according to the manufacturer's recommendations (Roche RNA T3-SP6 transcription and labelling kit).

X. laevis oocytes were fixed in 2% PFA, 250 mM NaCl, 5% acetic acid for 50 min at room temperature, dehydrated in ethanol and processed for embedding in paraffin according to standard techniques. The 7-μm thick paraffin sections were placed on Superfrost Plus slides (Carlo Erba) and were hybridized overnight at 60 °C in 40% formamide, 1× Denhardt's solution, 5× SSC, 200 μg/ml tRNA (Sigma) and 100 ng of sense or antisense digoxigenin-labelled RNA on each slide. Slides were washed in 0.5× SSC, 20% formamide for 1 h at 60 °C, and exposed to RNase A at 37 °C for 30 min. After two washes in 0.5× SSC, 20% formamide, for 30 min at 60 °C, they were incubated overnight in anti-digoxigenin–alkaline phosphatase (AP)-conjugated antibody (Roche) diluted 1:2000 in 2% blocking reagent solution (Roche). After several washes in 1× PBS, 0.1% Tween 20, the detection was

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performed using BM purple (Roche) (for more details see Vaccaro *et al.*, 2001).

Confocal microscopy

Small clusters of stage I–II oocytes were fixed in 2–4% PFA in PBS and extracted in a Hepes buffer containing glycol hexylene according to Klymkowsky *et al.* (1987). The samples were then incubated in anti-DrosGu antibody. The antibody was purchased by the Developmental Studies Hybridoma Bank, maintained by the University of Iowa, developed under the auspices of the National Institute of Child Health and Human Development of NIH, Department of Biological Sciences, Iowa City, IA 52242. This antibody recognizes an epitope of *Drosophila melanogaster* Gurken encompassing amino acids 53–185 upstream of the EGF domain (Nilson & Schüpbach, 1999). Control samples were performed by omitting primary antibody in the experimental trial. The secondary antibodies were BODIPY and Texas Red (H+L) goat anti-mouse IgG (Molecular Probes). After washing in PBS, the samples were directly immersed in glycerol/PBS 1:9 (v/v). Optical sections 1-µm thick were obtained using an Olympus Fluoview confocal microscope based on Olympus IX70 inverted microscope equipped with an argon/krypton laser. Unless differently specified, each image was derived from an average of 10 optical sections at 1 µm intervals.

Results and Discussion

In situ hybridisation on sections

Extensive analysis of serial sections of oocytes derived from nine females showed a small amount of the XNOA 36 transcript in the GV and in the MC, in addition to the major cytoplasmic localisation previously described (Vaccaro *et al.*, 2010). In particular, in Fig. 1*a*, the hybridisation signal is observed in the nucleoli and around the nuclear envelope, at a site where the MC is associated with the GV. As some signal is also in the MC, Fig. 1*a* suggests a passage of XNOA 36 mRNA from the GV to the MC. Kress *et al.* (2004) showed the mRNAs destined to be transported to the vegetal cortex may assemble with proteins in the nucleus through *cis*-acting RNA sequences and that this event is essential for final localisation of the mRNA. The nucleolus localisation is in agreement with data of Bolivar *et al.* (1999), as well as its association with mitochondria (de Melo *et al.*, 2009). In later stages of growth (starting from 140 µm large oocytes) a little hybridisation signal is partially present at the periphery of the MC (Fig. 2b). This finding is also evidence in oocytes of about 200–250 µm (Fig. 1*c–e*), of XNOA 36 mRNA

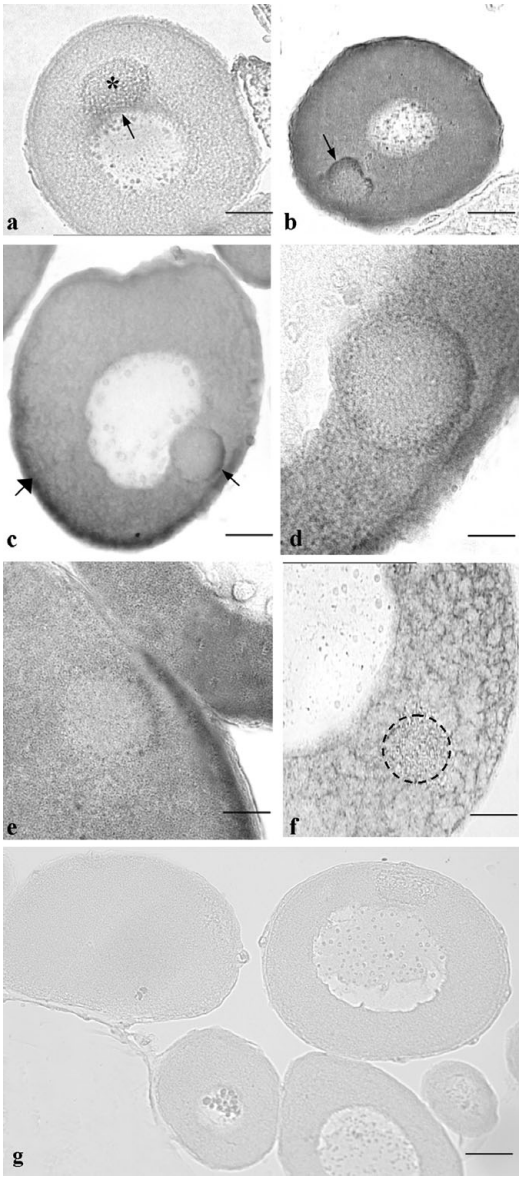

Figure 1 *In situ* hybridisation of oocyte sections. (*a*) A 130 µm oocyte is shown, where the hybridisation signal of XNOA 36 mRNA is located in the nucleoli, around the nuclear envelope and in the mitochondrial cloud (MC) (asterisk). In particular we observe a site where the MC is associated with the nuclear envelope (small arrow). As some signal is also in the MC, this figure suggests a passage from the germinal vesicle (GV) to the MC. Bar = 26 µm. (*b*) In this 140 µm-large oocyte some hybridisation signal is partially present at the periphery of the MC (small arrow). Bar = 30 µm. (*c–e*) A similar localisation is also evident in the oocytes of about 200–250 µm, being XNOA 36 mRNA located in the region recognisable as the METRO territory (small arrow). The large arrow indicates the mRNA localisation in the cortex, including the region facing the MC. (*d,e*) Magnification of (*c*). Bar in (*c*) = 35 µm, in (*d*) = 11 µm, in (*e*) = 17 µm. (*f*) The MC (bordered by interrupted lines) of this 250 µm oocyte is not labelled by the riboprobe Bar = 30 µm. (*g*) Section incubated with sense-probe showing absence of labelling. Bar = 40 µm.

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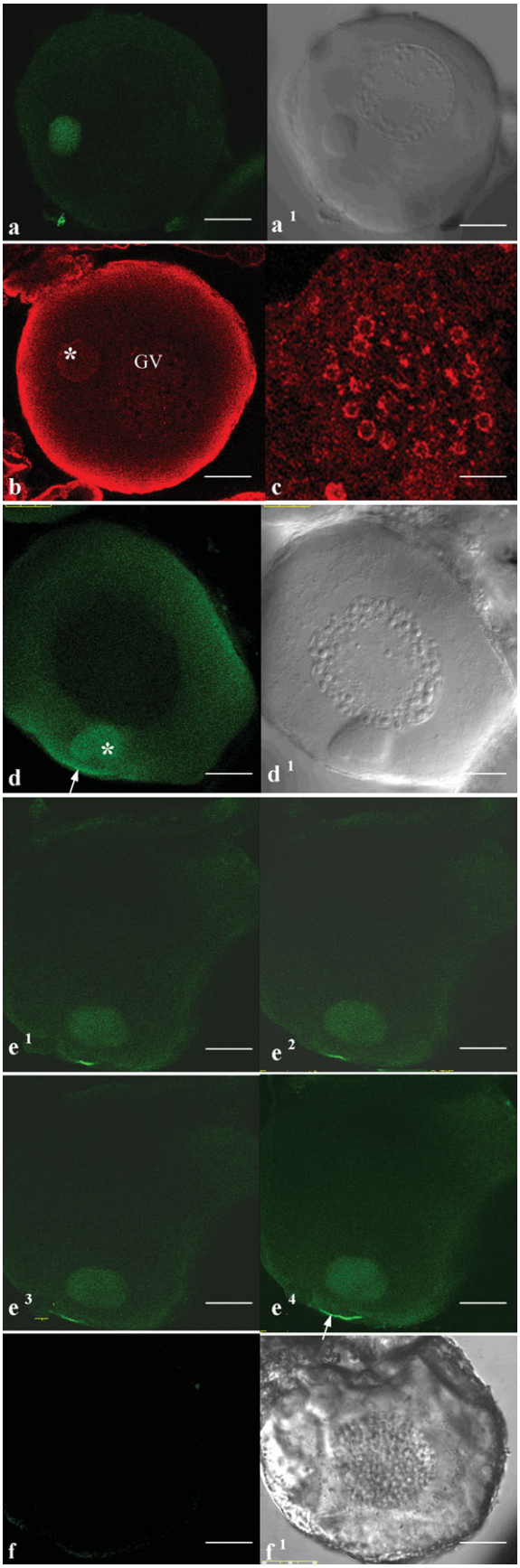

being located in the region recognisable as the METRO territory (Fig. 2*c–e*) as it faces the cortex where it will eventually collapse (Kloc *et al.*, 1996) and as it co-localises with a typically METRO-located mRNA, i.e. the Xcat-2 mRNA (Vaccaro, personal communication). The absence of the signal in most of the MC is shown in Fig. 1f showing an oocyte of 250 µm, about the same size as the oocyte in Fig. 1*e* (see also Vaccaro *et al.*, 2010). In Fig. 1*g* the section was incubated with the sense-riboprobe and the hybridization signal is absent. In the METRO region several germ-plasm mRNAs are located such as Xcat-2 (Mosquera *et al.*, 1993), Xdaz1 (Houston & King, 2000), DEADSouth (MacArthur *et al.*, 2000) as well as Xwnt11, (Ku & Melton, 1993) and Xlsirt (Kloc *et al.*, 1998) (see King *et al.*, 2005 for a review). Previous data indicated that XNOA 36 mRNA co-immunoprecipitates with spectrin that is notoriously present in the cytoplasm as well in the MC, in particular in the germ plasm (Kloc *et al.*, 1998; Vaccaro *et al.*, 2010), suggesting that this mRNA is anchored through the spectrin-related cytoskeleton in this MC zone.

Confocal microscopy

Anti-DrosGu immunofluorescence is present in the entire MC of oocytes of 190–200 µm, where the MC is halfway between the cortex and the GV (Fig. 2*a*,*a*^{1}). The association of the XNOA 36 protein with mitochondria

Figure 2 Confocal microscopy showing the immunofluorescence of anti-DrosGu-stained oocytes. (*a*) The immunofluorescence is present in the whole mitochondrial cloud (MC) of oocytes of 190–200 µm, whereas the MC is approximately halfway between the cortex and the germinal vesicle (GV). The immunofluorescence is mildly present at the periphery of the oocyte, in contrast to the bright signal present in the MC. Single optical sections of 3 µm. (*a*^{1}) Simultaneous differential interference contrast (DIC) image of the oocyte labelled in (*a*). Bar = 40 µm. (*b*) Oocyte of about 220 µm, in which several optical sections are pooled together in a single image (15 sections of 1 µm). A bright immunofluorescence is evident at the periphery MC = asterisk; GV = germinal vesicle. Bar = 44 µm. (*c*) The germinal vesicle contains nucleoli showing a rim of fluorescence at their periphery. Bar = 80 µm. (*d*) Oocyte of 250 µm, the MC is fluorescent (asterisk). The periphery of the oocyte is similarly fluorescent, however the cortex facing the migrating MC is particularly bright if compared with the rest of the oocyte cortex (small arrow). (*d*^{1}) Simultaneous DIC image of the oocyte in (*d*). Bar = 30 µm. (*e*^{1}–*e*^{3}) Single optical sections of an oocyte of about 270 µm, pooled together in (*e*^{4}), in which the cortex facing the MC is more fluorescent than the rest of the oocyte periphery. Bar = 25 µm. (*f*) Control section showing no immunofluorescence staining. (*f*^{1}) DIC of the oocyte shown in (*f*). Bar = 40 µm.

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can be also predicted by the PSORTII program (see Vaccaro *et al*., 2010). In Fig. 2*a* (one optical section of 1 μm) the anti-DrosGu immunofluorescence is mildly present at the periphery of the oocyte, in contrast to the bright signal present in the MC. The peripheral signal is evident in figures in which several optical sections are pooled together in a single image as in Fig. 2*b* (oocyte of about 220 μm, 15 optical sections of 1 μm), in agreement with previous data for XNOA 36 mRNA distribution indicating that this mRNA is highly concentrated at the oocyte periphery (Vaccaro *et al*., 2010). However, it was previously reported that XNOA 36 mRNA is located in the hemisphere of the 250/270 μm oocyte opposite to the hilum, while this situation does not appear to be the case for the immunofluorescence staining such as the one shown in Fig. 2*d*,*d*^{1},*e*, suggesting that diffusion of the newly translated protein has occurred. Nucleoli display immunofluorescence at their periphery (Fig. 2*c*), in agreement with the hybridisation signal in Fig. 1.

In oocytes of 250 μm, the MC is also quite fluorescent (Fig. 2*d*). The immunofluorescence distribution is conserved also in larger oocytes, as indicated in Fig. 2*e*^{1}–*e*^{4}, showing optical sections of an oocyte of about 270 μm. Interestingly, in our confocal micrographs the cortex facing the migrating MC is particularly fluorescent if compared with the rest of the oocyte cortex, suggesting that this localised enrichment of the protein may be due to diffusion of XNOA 36 from the MC (Fig. 2*e* and see also Fig. 2*d*).

Taken together these data indicate that XNOA 36 mRNA is located in the nucleoli, in agreement with Bolivar *et al.*'s (1999) findings and in the portion of the MC corresponding to the METRO region. The XNOA 36 protein is also found in the nucleoli, in addition to its location in the cytoplasm periphery. The MC contains an extensive network of endoplasmic reticulum that was suggested to contribute to RNAs to the MC region and maintains a close association with RNA (Heasman *et al*., 1984). Chang *et al.* (2004) showed that a particular endoplasmic reticulum (ER) domain is placed in the MC to provide support for mRNA immobilization and that this subdomain of ER is continuous with cytoplasmic and cortical networks. Accordingly, XNOA 36 mRNA translation might not necessarily occur within the MC, but be transported into the MC following translation in the ER cisternae penetrating into the MC. The fact that XNOA 36 protein is particularly concentrated in the oocyte cortex facing the MC is of specific interest. Prediction of XNOA 36 subcellular localisation, made with the PSORT II program, indicates the following: 73.9% nuclear, 17.4% cytoplasmic, 4.3% cytoskeletal and 4.3% mitochondrial, with the putative mitochondrial targeting sequence located in the N-terminal region (see also Vaccaro *et al*., 2010). As the cortex is notoriously rich in cytoskeletal proteins (Alarcon & Elinson, 2001) including spectrin (Vaccaro *et al*., 2010), it can be hypothesized that XNOA 36 interacts with the cytoskeleton, participating in the anchorage of the MC and its mRNAs in the future vegetal pole. Moreover, according to Kloc & Etkin (1998) the MC contains and releases residual centriole proteins such as γ-tubulin, which could be used in the late mRNAs pathway as a MTOC. As in mammals XNOA 36 associates with centromeres (Bolivar *et al*., 1999), we hypothesize that XNOA 36 may associate with residual centriole protein of the MC playing a role in early microtubule reorganization. This process starts at the vegetal cortex where the MC disaggregates and then spreads to the rest of the oocyte (Gard, 1999) cortex where XNOA 36 is also located (Vaccaro *et al*., 2010).

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