Int. J. Dev. Biol. 51: 701-706 (2007)
doi: 10.1387/ijdb.072293mk

Original Article

Centroid, a novel putative DEAD-box RNA helicase maternal mRNA, is localized in the mitochondrial cloud in *Xenopus laevis* oocytes

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ABSTRACT In *Xenopus* species, the early stages of oogenesis take place in the developing tadpole ovary when the oocytes are in a period critical for the organization of the germ plasm (believed to be a determinant of germ-cell fate) and the initial stages of localization of RNAs involved in germ plasm functions. We constructed a cDNA library from the ovaries of stage 64 *Xenopus* tadpoles with the idea that it will be enriched for oogonia and pre-stage I and stage I oocytes and thus, RNAs involved in oocyte development and germ plasm formation and function. From this cDNA library, we cloned a new maternal localized mRNA which we named centroid. This RNA codes for the protein belonging to the DEAD-box RNA helicase family. Some of the members of this protein family are components of the messenger ribonucleoprotein (mRNP) particles stored in the germ plasm in oocytes of *Xenopus, Drosophila* and *Caenorhabditis* species and are believed to play a role in translational activation of stored mRNPs and sorting of mRNPs into the germ plasm. We found that *centroid* mRNA is localized in *Xenopus* oocytes by a combination of early and late pathways, a pattern of localization that is very similar to the intermediate pathway localization of *fatvg* mRNA, another germ-plasm-localized RNA in *Xenopus* oocytes. Also, *centroid* mRNA is present in the mitochondrial cloud and in the germ plasm at the surface of germinal granules. This suggests that centroid is involved in the regulation of germ plasm-stored mRNPs and/or germ plasm function.

KEY WORDS: *DEAD-box RNA helicase, localized RNA, germ plasm, oocyte, Xenopus*

Introduction

Localized RNAs are known to play important roles in the establishment of asymmetry in a wide variety of systems from yeast to mammals (Bashirullah *et al*., 1998; Jansen, 2001; King *et al*., 1999; Kloc *et al*., 2001a, 2002b; Palacios and Johnston, 2001). In the frog *Xenopus laevis*, subsets of RNAs are localized to the animal and vegetal poles of oocytes (Forristall *et al*., 1995; King *et al*., 1999; Kloc *et al*., 2001a, 2002b; Kloc and Etkin, 1995). RNAs are localized to the vegetal pole of *Xenopus* oocyte by three different pathways. First, the early or METRO pathway uses the mitochondrial cloud (Balbiani body) to deliver RNAs such as Xlsirts, Xcat2 (related to the Nos/Vasa DEAD-box family; Asp-Glu-Ala-Asp, D-E-A-D; hence the family name), Xpat, Xwnt 11, XdazI, the DEAD-box RNA helicase DEADSouth and germinal granules (collectively called the germ plasm and believed to be a germ-cell determinant) to the vegetal pole in early oogenesis. Second, the late pathway operates in late oogenesis and uses microtubules and molecular motors to deliver RNAs such as Vg1 and VegT to the vegetal pole of the oocyte (Forristall *et al*., 1995; Kloc and Etkin, 1995). Third, the intermediate pathway uses a combination of early and late pathways to deliver RNAs such as fatvg to the vegetal pole of the oocyte (Chan *et al*., 1999, 2001).

Studies have shown that vegetally localized mRNAs Vg1, VegT and Xwnt 11 are determinants of mesoderm and endoderm fate as well as the left-right axis in the embryo (Joseph and Melton, 1998; Rebagliati *et al*., 1985; Stennard *et al*., 1996; Xanthos *et al*., 2001; Zhang *et al*., 1998; Zhang and King, 1996). Most recently, we showed that the localized RNAs Xlsirts and VegT in *Xenopus* play a structural role in maintaining the integrity of the cytoskeleton of the vegetal cortex (Kloc *et al*., 2005; Kloc *et al.,* 2007). Other vegetally localized mRNAs in *Xenopus* species, such as Xcat2, Xdazl and fatvg, are believed to play roles in germ-cell

Abbreviations used in this paper: mRNP, messenger ribonucleoprotein; ORF, open reading frame; UTR, untranslated region.

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0214-6282/2007/$30.00
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determination or migration (Houston and King, 2000a, 2000b; Chan *et al.*, 2001, 2007), whereas Xpat may be involved in the organization of the germ plasm, perhaps playing a role similar to that of oskar in *Drosophila* species (Hudson and Woodland, 1998). Thus, a substantial number of localized transcripts clearly play critical roles in a wide variety of cellular and developmental processes and the discovery of novel localized transcripts will undoubtedly lead to a greater understanding of how oogenesis, development and many other cellular processes are regulated.

Results

We constructed a cDNA library from the ovaries of stage 64 *Xenopus* tadpoles with the idea that it will be enriched for oogonia and pre-stage I and stage I oocytes. In *Xenopus*, the early stages of oogenesis take place in the developing tadpole ovary. Each oogonium undergoes four mitotic divisions with incomplete cytokinesis, giving rise to a cluster (nest) of 16 pre-stage I oocytes (connected by cytoplasmic bridges) that enter the prophase of meiosis (Kloc *et al.*, 2004). Subsequently, in the ovaries of froglets, the oocytes become separated and surrounded by ingrowing follicular cells and cytoplasmic bridges connecting the oocytes disintegrate; in the ovaries of adult frogs, the oocytes enter the phase of growth and accumulation of yolk (stage I-VI oocytes).

The prominent structure in pre-stage I and stage I-II oocytes is the mitochondrial cloud, which is located in the vicinity of the oocyte nucleus (Kloc *et al.*, 2004). The main body of the mitochondrial cloud is composed of mitochondria and its vegetal apex contains germ plasm. In the tadpole ovary, the oocytes are in a period critical for the organization of the germ plasm and the initial stages of localization of germ plasm RNAs such as Xcat2 (Kloc *et al.*, 1998, 2002a, 2004). In pre-stage I and early stage I oocytes, the germ plasm contains the mitochondrial cement, which is located between mitochondria and originates from the perinuclear nuage. The mitochondrial cement is the immediate precursor of granulofibrillar material (GFM), which ultimately forms the “mature” germinal granules present in stage I and older oocytes (Bilinski *et al.*, 2004; Kloc *et al.*, 2004). Therefore, we constructed the cDNA library described above with the intent of identifying RNAs critical for these processes. After isolation of individual clones, we determined the patterns of localization of their cognate RNAs in different-stage oocytes (pre-stage I to stage VI) using *in situ* hybridization.

We isolated a total of 91 individual clones. Of these clones, 27 were positive by *in situ* hybridization in nest-stage (pre-stage I) oocytes: 8 clones gave a positive signal in the mitochondrial cloud and 19 clones gave a positive signal in the cytoplasm. In stage I-VI oocytes, we found 27 positive clones: 11 clones, including the clone named centroid described below, gave a positive signal in both the mitochondrial cloud and vegetal cortex and 16 clones gave a positive signal in the cytoplasm. From the screen we identified 10 new localized transcripts. We sequenced all of the clones showing definite localization patterns and analyzed them for homology using database searches (unpublished data).

A

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taagctaccc attccattag cacccacatc tacccaatcc ttcacacctg cacccatttc tccctcacca
tctgtagcca cctccactcc atccatatct tgtgctgcca aggctgcttc tccctccaaa tctgtcaaac
tcccaccgtt agtcactcct acagaggatg ctgaggaaga agcagtcaag tcatacagta aaagtcaacg
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gaccatgacg tgtgcagctt agagtgcaaa gccatggaca ttttgcaggc ttctggggca gccagcaccc
cacttgtttt cccagacaag gccactaact catcaccttt attgtattca actgctggcc ctttgagtaa
ttcagttgtc accagtgata tatcctcatg ccaaaatatt gaagaagata gttcacaact gacaacacac
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gcagcatttc tgcttccagc tataattcga tgccttgaga agaaggattc tccagctgca ctgattctca
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aagaacagaa gaaagttgat gagagagggc ggcgggacaa agagaaagtg gtaacaggag accaaattct
tgatctcatc cgaaaacatg acaggaggaa atctcaaaaa tgactgtcgg catggtctct atttttctct
ctttctacgg ataatgaccg tttacaagga ctctgactgc tttaaaatgt ctctgttata gaaatatata
ttattaaata tatatgcttc aaaaaaaaaa
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5!UTR 614bp; ORF 765bp; 3!UTR 611bp Total 1,990bp

B

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M E F D H C Q F P P V L S S N I K A A G Y E V P T P I Q M Q M I P V G L M E R D I L A S A D T G S G K
T A A F L L P A I I R C L E K K D S P A A L I L T P T R E L A V Q I E G Q A K E L M R G I P H M R T A L
L V G G M P L P P Q I H R L K Q G V Q V I I A T P G R L L E I I N Q D C V N L G D L K I L I V D E A D T
M L K M G F Q Q Q V L D I L E H A S H D H Q T I L V S A T I P A G I E A F T K Q L L Q D P V R I A V G E
K N Q P C S N V R Q I V L W V E E P S K K K K L F E I L N D S K L F Q P P V L V L G L P P W C
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C

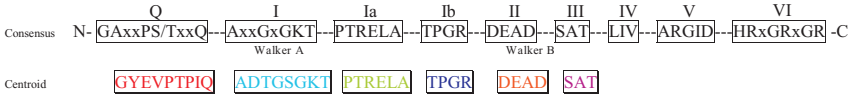

Fig. 1. Centroid nucleotide sequence and deduced amino acid sequence. (A) *Nucleotide sequence of the* centroid *cDNA clone. The whole clone was 1,990 bp long with a 614-bp-long 5′ untranslated region (UTR), 765-bp-long open reading frame (ORF, marked in red) and 611-bp-long 3′ UTR.* **(B)** *Deduced amino acid sequence of centroid protein showing motifs (boxed) common to DEAD-box RNA helicases.* **(C)** *Comparison of the amino acid composition of DEAD-box RNA helicase consensus motifs and centroid. Motifs Q, I (Walker A), II (Walker B) and VI are involved in ATP binding and hydrolysis; motifs Ia, Ib, IV and V are involved in RNA binding; and motif III is involved in RNA-induced conformational changes (Cordin* et al.*, 2006; Heung and Del Poeta, 2005).*

Centroid is a member of the DEAD-box RNA helicase protein family

One of the cDNA clones that we isolated from the tadpole ovary cDNA library was 1,990 bp long (GenBank accession number

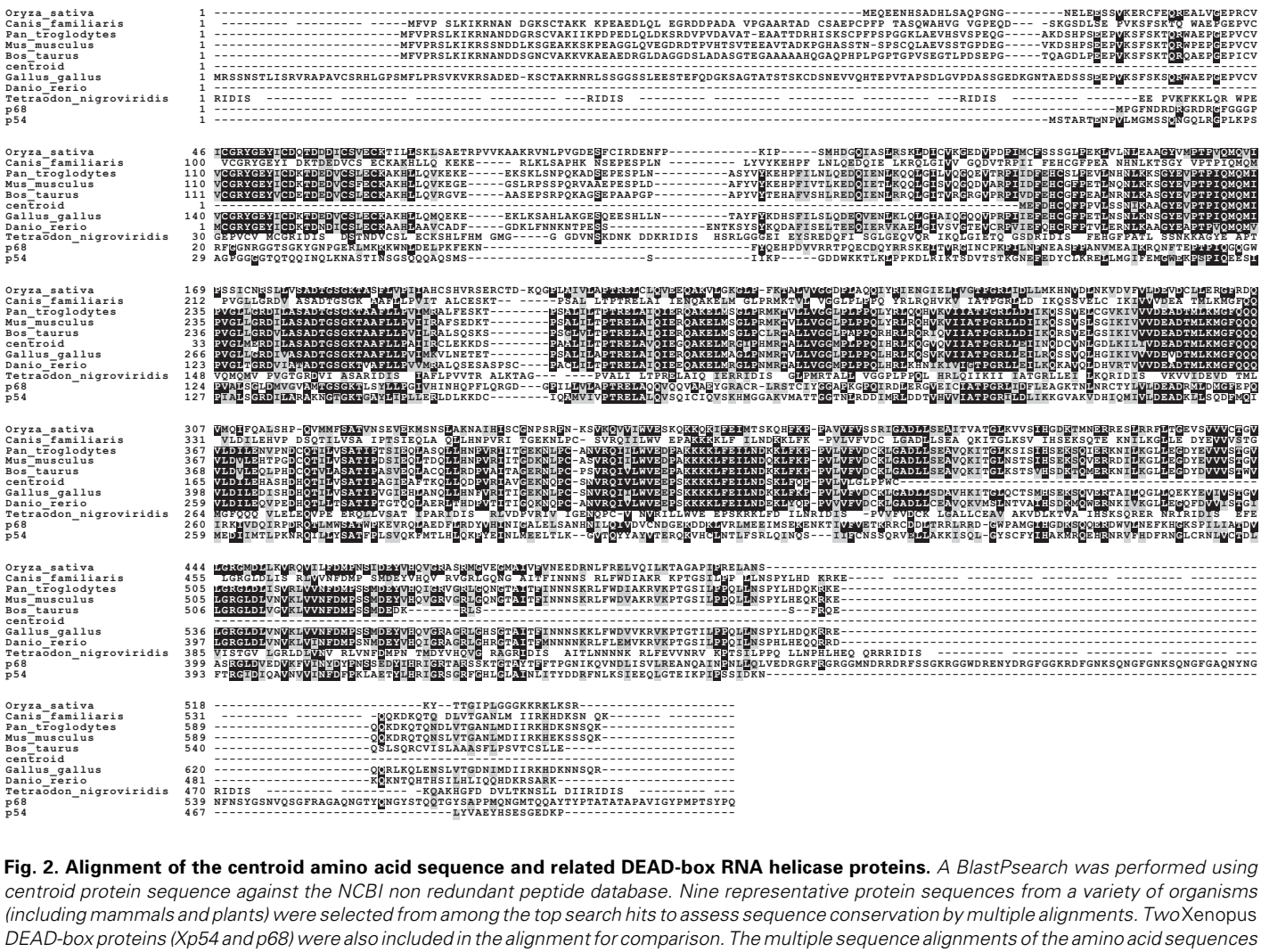

Fig. 2. Alignment of the centroid amino acid sequence and related DEAD-box RNA helicase proteins. *A BlastPsearch was performed using centroid protein sequence against the NCBI non redundant peptide database. Nine representative protein sequences from a variety of organisms (including mammals and plants) were selected from among the top search hits to assess sequence conservation by multiple alignments. Two* Xenopus *DEAD-box proteins (Xp54 and p68) were also included in the alignment for comparison. The multiple sequence alignments of the amino acid sequences were generated using ClustalW (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html) followed by BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). The black and gray shaded boxes indicate identical and similar amino acid residues, respectively, in a given column.*

843804; Fig. 1A) and contained a 765-bp-long open reading frame (ORF). Translation of the ORF showed a conceptual protein of 254 amino acids containing a DEAD-box motif (Figs. 1B and 1C). We performed a database search using the Basic Local Assignment Search Tool (National Center for Biotechnology Information) that showed extensive homology between this clone and other vertebrate and invertebrate DEAD-box RNA helicases (Fig. 2). Specifically, we found 75% identity of this clone with *Gallus gallus* (GenBank accession number XM-422189; unpublished data), 73% identity with *Canis familiaris* (GenBank accession number NW-876323; unpublished data), 72% identity with *Bos taurus* (GenBank accession number XM-592818.2; unpublished data) and *Mus musculus* (Carninci and Hayashizaki, 1999), 68% identity with *Danio rerio* (Strausberg *et al.*, 2002), 37% identity with chordate p68 (Seufert *et al.*, 2000) and 36% identity with *Xenopus* p54 (Ladomery *et al.*, 1997) DEAD box RNA helicases (Fig. 2). Analysis of the centroid protein sequence showed the presence of six of nine conserved motifs—Q, I, Ia, Ib, II and III—characteristic of DEAD-box RNA helicases (Fig. 1C). Motifs Q, I and II are known to function in ATP binding and hydrolysis, motifs Ia and Ib are known to function in RNA binding and motif III is known to function in ATP-induced conformational changes (Cordin *et al.*, 2006, Heung and Del Poeta, 2005).

Centroid *mRNA is localized by the intermediate pathway and is a component of germ plasm*

To determine the localization pattern of centroid RNA, we performed whole mount *in situ* hybridization and *in situ* hybridization on sections of different-stage oocytes for light and electron microscopy. Light microscopy showed that in pre-stage 1 oocytes, centroid RNA was dispersed throughout the cytoplasm but was not present in the main mitochondrial cloud and secondary clouds (Fig. 3). In stage I oocytes, centroid RNA was present in the center of the mitochondrial cloud; starting at late stage I, it co-localized with the germ plasm first at the vegetal tip of the mitochondrial cloud and then in the mitochondrial cloud fragments at the oocyte vegetal cortex (Fig. 3). This indicated that centroid mRNA is localized by a combination of early and late

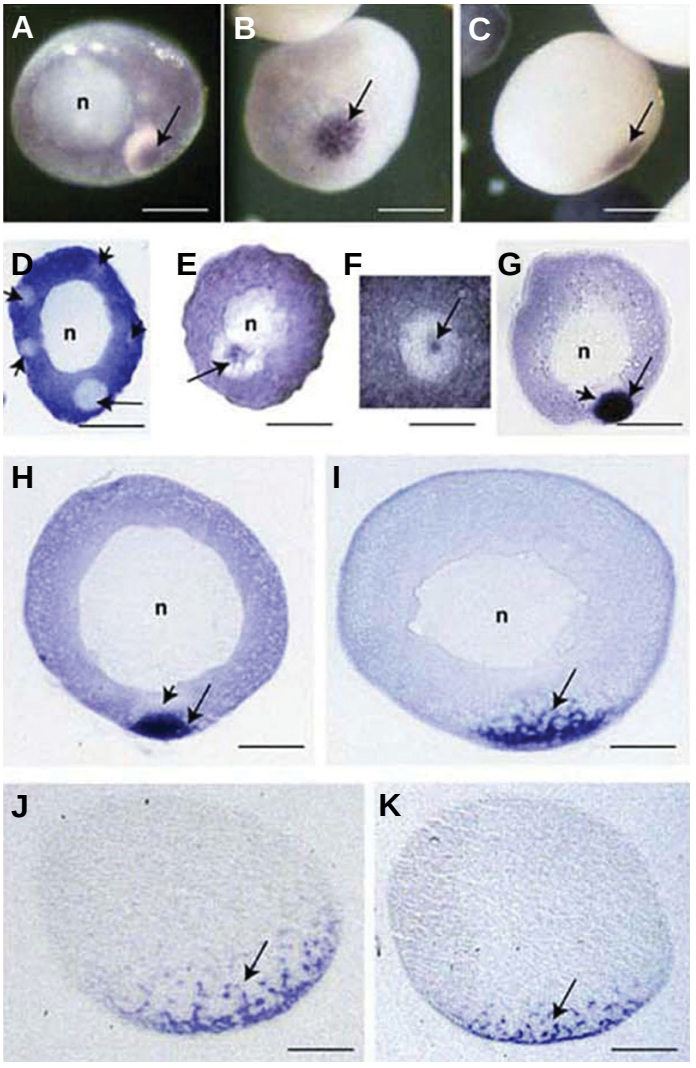

Fig. 3. *Centroid* mRNA localization in *Xenopus* oocytes. (A-C) *Whole mount* in situ *hybridization showing localization of* centroid *mRNA (arrows) in the mitochondrial cloud in a stage 1 oocyte* **(A)** *and in the apex of the vegetal cortex in a stage II oocyte* **(B)** *and a stage III oocyte* **(C)**. **(D-J)** *Sections of whole mount* in situ *hybridization showing localization of* centroid *mRNA in oocytes at different stages.* **(D)** *In a pre-stage I oocyte,* centroid *mRNA is uniformly dispersed in the cytoplasm but excluded from the mitochondrial cloud (long arrow) and secondary clouds (short arrows).* **(E,F)** *In a stage I oocyte,* centroid *mRNA (arrow) is located in the center of the mitochondrial cloud. Panel (F) shows the high magnification of the mitochondrial cloud (white sphere) with centrally located* centroid *mRNA (arrow).* **(G,H)** *In late stage I/early stage II and stage II oocytes,* centroid *mRNA is limited to the vegetal tip of the mitochondrial cloud (co-localizing with the germ plasm [long arrow]) and is excluded from the apical part of the mitochondrial cloud (short arrows).* **(I-K)** *Stage III, early stage IV and stage IV oocytes showing localization of* centroid *mRNA in the islands of a dispersing mitochondrial cloud (arrows). n, nucleus. Scale bars are equal to 56 μm in (A), 90 μm in (B,G), 100 μm in (C), 70 μm in (D), 75 μm in (E), 65 μm in (F), 86 μm in (H), 80 μm in (I) and 100 μm in (J,K).*

pathways, a pattern of localization that is very similar to the intermediate pathway localization of fatvg mRNA, another germ-plasm-localized RNA in *Xenopus* oocytes (Chan *et al.*, 1999, 2001, 2007).

Electron microscopy analysis of centroid mRNA localization showed that centroid mRNA is absent from the mitochondrial cement in pre-stage I oocytes (Fig. 4). We calculated the number of silver grains present in mitochondrial cement and surrounding cytoplasm in 20 samples. We found no (zero) grains in the mitochondrial cement and on average 9.75 (s. d. 3.45) grains in the matrix region of mitochondrial cloud. In early stage I oocytes centroid mRNA was visible in the vicinity of but not on the germinal granules (on average 2.7 grains with s. d. = 2.1 in the vicinity of germinal granules) and subsequently starting at late stage I/ early stage II oocytes it was present at the periphery of the germinal granules (on average 3.2 grains with s. d. =1.58 on the periphery of each granule; Fig. 4).

Discussion

We cloned a new maternal localized mRNA that belongs to the DEAD-box RNA helicase family of proteins. These proteins are ATP-dependent enzymes involved in many aspects of RNA metabolism such as transcription, RNA splicing, ribosome biogenesis, translation initiation and RNA transport and degradation and are found in all eukaryotes and most prokaryotes (Cordin *et al.*, 2006; Heung and Del Poeta, 2005). Members of this family share conserved motifs that play a role in ATP binding and hydrolysis, RNA binding and RNA-induced conformational changes. Comparison of the amino acid composition of DEAD-box RNA helicase consensus motifs and centroid showed that centroid contains motifs Q, I (Walker A), II (Walker B), which are involved in ATP binding and hydrolysis, motifs Ia, Ib involved in RNA binding and motif III which is involved in RNA-induced conformational changes (Cordin *et al.,* 2006; Heung and Del Poeta, 2005). The presence in the centroid of the motifs Q and I-III suggests that this protein is involved in the ssRNA binding, ATP hydrolysis and it may possess helicase activity. Interestingly, centroid lacks the motif IV, V and VI, which are present in DEAD-box RNA helicase consensus sequence. So far the function of motif IV is poorly understood but it was suggested that it may be involved in ssRNA binding and that has a functional connection to motif V involved in ATP hydrolysis (Cordin *et al.,* 2006). Motif VI has been shown to participate in RNA binding and ATPase activity (Cordin *et al.,* 2006). The DEAD-box RNA helicases are the multifunctional molecules and their activities depend on the communication and interaction between multifunctional motifs. Only future functional studies will be able to show how the lack of motif IV-VI influences the centroid function in comparison with other known DEAD-box helicases. Some of the members of this protein family, such as the DEAD-box RNA helicase p54 (Ladomery *et al.*, 1997; Weston and Sommerville, 2006), are components of the messenger ribonucleoprotein (mRNP) particles stored in the germ plasm in oocytes of *Xenopus*, *Drosophila* and *Caenorhabditis* species and are believed to play a role in translational activation of stored mRNPs and sorting of mRNPs into the germ plasm (Bilinski *et al.*, 2004; Cordin *et al.*, 2006; Weston and Sommerville, 2006).

Previously, we described the presence of two DEAD-box RNA helicases DEADSouth (Bilinski *et al.*, 2004; Komiya *et al.*, 1994; MacArthur *et al.*, 2000) and vasa-like XVLG1 in the germ plasm in *Xenopus* oocytes (Bilinski *et al.*, 2004). DEADSouth mRNA is

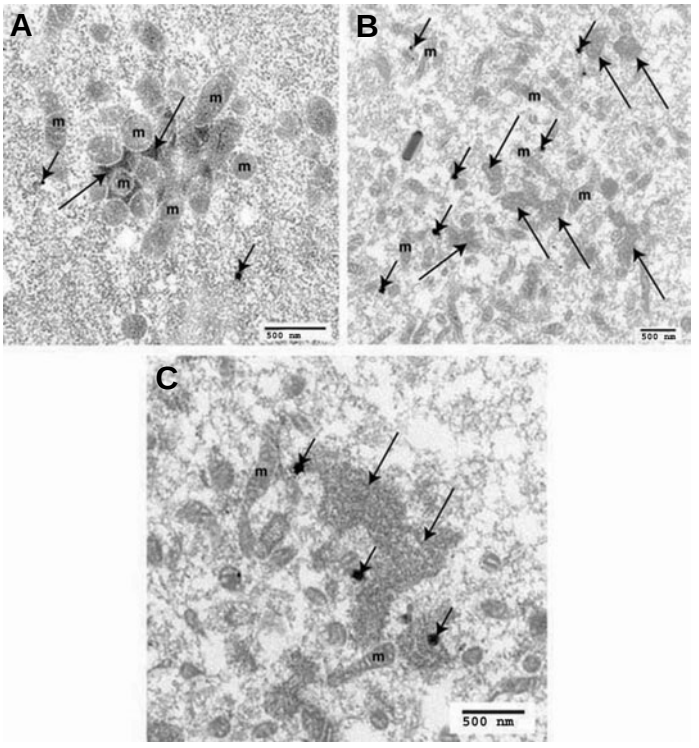

Fig. 4. Electron microscopy analysis of *centroid* mRNA localization in the germ plasm of *Xenopus* oocytes. (A) *Fragment of the mitochondrial cloud in a pre-stage I oocyte hybridized with a* centroid *anti-sense RNA probe, labeled with nanogold and silver-enhanced showing the mitochondrial cement (long arrows) located between the mitochondria.* Centroid *mRNA (short arrows) is present in the mitochondrial cloud but excluded from the mitochondrial cement.* **(B,C)** *Fragment of the mitochondrial cloud from stage 2 oocytes showing* centroid *mRNA (short arrows) located in the vicinity* **(B)** *and at the periphery* **(C)** *of the germinal granules (long arrows). m, mitochondria.*

present at the surface of GFM and germinal granules but absent from nuage and mitochondrial cement. In contrast, XVLG1 mRNA is absent from germinal granules and GFM, but XVLG1 protein is present in nuage and mitochondrial cement (Bilinski *et al.*, 2004). The fact that centroid mRNA is also present at the surface of germinal granules in *Xenopus* oocytes suggests its involvement in the regulation of germ plasm-stored mRNPs and/or germ plasm function. In addition, the fact that different DEAD-box RNA helicases are present in germinal granules at different stages of formation (nuage, GFM, mature germinal granules) suggests that their function is temporarily regulated during the formation and "maturation" of the germ plasm. However, determination of the role of centroid and the precise role of other germ-plasm-localized DEAD-box RNA helicases in germ plasm function will require further functional study.

Materials and Methods

Construction of the stage 64 tadpole cDNA library (nest library)

Several dozen ovaries (300 mg of ovarian tissue) were collected from 4 cm-long froglets into RNAlater solution (Ambion). Total RNA was prepared using an RNAqueous kit (Ambion) and poly(A+) RNA was isolated using an Oligotex RNA mini kit (Qiagen) according to the manufacturer's protocol. A directional cDNA library was prepared using poly (A+) RNA and the SuperScript plasmid system with pSPORT1 plasmid (Gibco BRL). In short, 4 μg of poly (A) RNA, TTTTTT *Not*I primer adapter and SuperscriptII reverse transcriptase were used to introduce directionality and to make first-strand cDNA. Subsequently, the *Escherichia coli* ligase, DNA polymerase I and T4 polymerase were used to make second-strand and double-strand cDNA. The resulting double-strand cDNA was *Sal*I-adapted with T4 DNA ligase. After subsequent digestion with *Not*I, the cDNA with *Not*I/*Sal*I termini was size-fractionated using column chromatography. Fractions 1-12 were pooled and precipitated and the cDNA was ligated to a *Not*I/*Sal*I-cut pSPORT1 vector. Vector-ligated cDNA was introduced by transformation into *E. coli* (Xl blue) cells. Transformed bacteria were plated on ampicillin plates and colonies from 10 plates were scraped into LB medium and frozen in glycerol at -80°C. This served as a library stock for further screening using *in situ* hybridization.

Screening of the nest library

The nest library was plated on LB ampicillin plates. Plasmid DNA from single colonies was purified using a plasmid purification kit (Qiagen). DNA from each colony was linearized with *Sal*I and antisense digoxigenin-labeled RNA probes were synthesized *in vitro* using Sp6 RNA polymerase. Froglet ovaries containing nest-stage and early pre-stage I oocytes and stage I-VI oocytes from large frog ovaries were defolliculated with collagenase, fixed in MEMFA and hybridized whole mount with RNA probes according to a protocol described previously (Kloc and Etkin, 1995). Anti-digoxigenin antibody conjugated with alkaline phosphatase and a BCIP/NBT substrate was used to detect (by color reaction) the hybridization signal.

Oocytes that showed positive signal were photographed as whole mounts and subsequently embedded in paraplast and sectioned at 10 μm. The sections were deparaffinated in HistoClear (National Diagnostics), mounted in Permount (Sigma) and photographed under a Nikon microscope.

Whole mount in situ *hybridization for electron microscopy*

Whole mount *in situ* hybridization for electron microscopy was performed exactly as described previously (Kloc *et al.*, 2001b). In short, oocytes were fixed in 4% formaldehyde, 0.1% glutaraldehyde, 100 mM KCl, 3 mM $MgCl_2$, 10 mM HEPES, 150 mM sucrose and 0.1% Triton X-100, pH 7.6. After fixation and washing, oocytes were treated for 7 min with 10 μg/ml proteinase K in PBS-0.1% Tween 20 and hybridized overnight at 50°C with a digoxigenin-labeled antisense RNA probe (see above). After washing, oocytes were incubated overnight at 4°C with a 1:30 dilution of anti-DIG 0.8 nm gold (Roche) in G2 buffer (Roche). After intensive washing in PBS-Tween 20, oocytes were silver-enhanced and processed for embedding and sectioning for electron microscopy as described previously (Kloc *et al.*, 2001b). The sections were examined in a JEOL 1200EX transmission electron microscope.

Acknowledgements

EM analysis was supported by Cancer Center Support (Core) Grant CA 16672 from the National Institutes of Health for The University of Texas M. D. Anderson Cancer Center High Resolution Electron Microscopy Facility We would like to thank Kenneth Dunner, Jr., for his electron microscopy work.

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Published Online: 4th October 2007

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