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ORIGINAL ARTICLE

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The protein encoded by the germ plasm RNA *Germes* associates with dynein light chains and functions in *Xenopus* germline development

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Abstract Germ plasm plays a prominent role in germline formation in a large number of animal taxons. We previously identified a novel maternal RNA named *Germes* associated with *Xenopus* germ plasm. In the present work, we addressed possible involvement of Germes protein in germ plasm function. Expression in oocytes followed by confocal microscopy revealed that the EGFP fused to Germes, in contrast to the free EGFP, co-localized with the germ plasm. Overexpression of intact Germes and Germes lacking both leucine zipper motifs (GermesΔLZs) resulted in a statistically significant reduction of the number of primordial germ cells (PGCs). Furthermore, the GermesΔLZs mutant inhibited PGC migration and produced abnormalities in germ plasm intra-cellular distribution at tailbud stages. To begin unraveling biochemical interactions of Germes during embryogenesis, we searched for Germes partners using yeast two-hybrid (YTH) system. Two closely related sequences were identified, encoding *Xenopus* dynein light chains dlc8a and dlc8b. Tagged versions of Germes and dlc8s co-localize in VERO cells upon transient expression and can be co-immunoprecipitated after injection of the corresponding RNAs in *Xenopus* embryos, indicating that their interactions occur *in vivo*. We conclude that Germes is involved in organization and functioning of germ plasm in *Xenopus*, probably through interaction with motor complexes.

Key words cytoskeletal motors · yeast two-hybrid system · primordial germ cells · immunoprecipitation · confocal microscopy · *Xenopus* · dynein · dlc8 · germ plasm

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Introduction

The germline is established very early in animal development by induction signals from surrounding tissues or by inheriting cytoplasmic determinants localized in the egg. The latter mechanism is common among various animal taxons (Extavour and Akam, 2003), including anurans, like *Xenopus*, as well as a number of other prominent model organisms such as the zebrafish, chicken, *Drosophila melanogaster* and *Caenorhabditis elegans*. The determinants are contained in a structure called germ plasm, which consists of maternal RNAs, proteins, and organelles. The organization of germ plasm is fundamentally similar between organisms and includes dense granules called P-granules in *C. elegans*, polar granules in *Drosophila*, and germinal granules in *Xenopus*, mitochondria, endoplasmic reticulum (ER), cytoskeletal, and other proteins and specifically localized RNAs. The formation of the precursor structures of germ plasm can be traced back to the very early

stages of oocyte development. In *Xenopus*, as in *Drosophila*, the oocytes derive from the 16-cell germline cyst, representing a syncytium formed by four synchronous divisions from the germline stem cell in *Drosophila* (de Cuevas et al., 1997; Pepling et al., 1999) or from the oogonium in *Xenopus* (Coggins, 1973). These 16 cells remain interconnected through ring canals and a microtubular structure called a fusome, which traverses all ring canals and consists of dense material rich in spectrin and ER-like vesicles (de Cuevas et al., 1996). In *Drosophila*, the fusome itself originates from the spectrosome, the spectrin-rich spherical structure of the germline stem cell (Lin and Spradling, 1995; Deng and Lin, 1997).

In the pre-vitellogenic oocyte the germ plasm develops from the mitochondrial cloud (MC), a dense mass of mitochondria, ER, Golgi, RNAs, and proteins (Kloc and Etkin, 2005). It also contains mitochondrial cement that has been reported to be a precursor of germinal granules (Heasman et al., 1984). At fusome-like stages, *Xenopus* oocytes contain multiple aggregates of mitochondria around the nucleus (pre-clouds) and a centrosome (Kloc et al., 2004). The aggregate that contains the centrosome forms the single MC close to the nucleus on the future vegetal side of the oocyte. From the earliest oogenesis stages (prestage I and stage I) the MC becomes the anchoring point for germ plasm-localized RNAs (Kloc et al., 1998, 2000). They are concentrated by a diffusion-entrapment mechanism in the MC (Chang et al., 2004), which then translocates them to the vegetal pole where it disaggregates into germ plasm islands anchored at the vegetal cortex (Kloc et al., 2001; Wilk et al., 2005). This localization pathway of the germ plasm-associated RNAs is termed as the METRO or early pathway, to distinguish it from the late pathway used by other RNAs specifying polarity and germ layers formation (Forristall et al., 1995; Kloc and Etkin, 1995; Joseph and Melton, 1998; Zhang et al., 1998).

Following fertilization, germ plasm islands aggregate into larger complexes (Kloc et al., 2002) in a process dependent on surface contraction waves (Quaas and Wylie, 2002). These aggregates remain in close contact with the vegetal membranes of blastomeres until the early blastula stage, being unequally inherited by about five vegetal cells (Whitington and Dixon, 1975), where they are localized along the basolateral membranes of primordial germ cell (PGC) precursors. In the late blastula germ plasm is decompacted and translocated from the plasma membrane to the perinuclear regions of 10–15 PGC precursors (Blackler, 1958). Subsequent mitoses are accompanied by uniform distribution of germ plasm among daughter cells, which may be regarded as *bona fide* PGCs from that moment.

With the onset of gastrulation, PGCs are translocated into the posterior, internal endodermal mass of the embryo by morphogenetic movements. At late tailbud stages (stage 31) they start active migration toward the developing gonads (Kamimura et al., 1980). They move first laterally and then dorsally to quit the endoderm, migrating through the dorsal mesentery and dorsal body wall to enter the gonads by stages 43–45 (Kalt and Gall, 1974; Whitington and Dixon, 1975; Wylie and Heasman, 1976). During this time they undergo several waves of synchronous divisions and about 40–50 PGCs reach the gonads (Dziadek and Dixon, 1977).

Germ plasm-specific RNAs include non-coding transcripts with a structural function (Kloc et al., 1993; Kloc and Etkin, 1994; Zearfoss et al., 2003), those encoding RNA-binding proteins (Mosquera et al., 1993; Houston et al., 1998; MacArthur et al., 2000; Zearfoss et al., 2004), and others encoding the novel proteins such as Xpat and Germes, which are unique to *Xenopus* (Hudson and Woodland, 1998; Berekelya et al., 2003). Despite our growing knowledge of the formation of germ plasm, information on the functions of the proteins encoded by germ plasm-specific RNAs is still insufficient to understand how germ plasm establishes germline fate in cells inheriting it.

The present study is focused on the protein encoded by the *Germes* transcript. *Germes* mRNA has been shown to be a constituent of *Xenopus* germ plasm in oocytes and embryos, where it is detectable until neurulation. The predicted protein possesses two leucine zippers and a calcium binding EF-hand domain. We show that an EGFP fusion of this protein concentrates in germ plasm in the oocyte and overexpression of normal and mutant forms of Germes in embryos specifically alters PGC structure and behavior. Germes interacts with light chains of dynein, suggesting that it may function as an adaptor protein linking germ plasm components to the cytoskeleton.

Methods

Molecular cloning

The expression construct encoding wild-type (WT) Germes was prepared by insertion of the Germes ORF into the vector pCS2+. This construct, pCS2+Germes, was used as a template to prepare Germes mutants by inverse PCR (Hemsley et al., 1989). The circular plasmid was nicked with DNase I as described (Greenfield et al., 1975) and used as a template in seven cycles of PCR amplification with the Advantage HF polymerase mix (Clontech, Mountain View, CA). To delete the first leucine zipper sequence (L[356]SFRPIGLFSTTSLLESNDSFL[377]) or the second one (L[424]QGIEDRLKYTEAILKEEETAL[445]), the following pairs of primers were used, respectively: 5′-CTCAGTTTGAACACCA CAT-3′ and 5′-AGCAGTTACAGTGAACCAA-3′ or 5′-TTC ATTTTGTTCCTTCAC-3′ and 5′-AGAGAAGAACAGGCAC TGG-3′. The GermesΔLZs mutant (with deletion of both leucine zippers) was produced sequentially as described above. To produce the GermesΔEFh mutant, the EF-hand consensus sequence (D[469]VNKGNYLQNQDI[482]) was deleted by inverse PCR with following primers: 5′-ACCATTAAATATGCAGTCC-3′ and 5′-CAGAAGACTATGCAGAATAA-3′. The amplified plasmids were gel-purified, recircularized, and verified by sequencing.

To create plasmid with Germes bait for the yeast two-hybrid (YTH) screen, Germes cDNA was sub-cloned from the pCS2+Germes plasmid into the pBTM116 vector (LexA-based YTH system).