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Xenopus Dead end mRNA is a localized maternal determinant that serves a conserved function in germ cell development

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Abstract

Germ plasm formation is considered to define the first step in germ cell development. *Xenopus Dead end* represents a germ plasm specific transcript that is homologous to the previously characterized zebrafish dead end, which is required for germ cell migration and survival. *XDead end* mRNA localizes to the vegetal pole of *Xenopus* oocytes; in contrast to all other known germ plasm associated transcripts in *Xenopus*, *XDead end* is transported via the late transport pathway, suggesting a different mode of germ plasm restriction. Vegetal localization in the oocyte is achieved via a localization element mapping to a 251 nucleotide element in the 3′-UTR. This RNA sequence binds to a set of proteins characteristic for the late localization pathway and to one additional protein of 38 kDa. Inhibition of XDead end translation in *Xenopus* embryos results in a loss of primordial germ cells at tadpole stages of development. Early specification events do not seem to be affected, but the primordial germ cells fail to migrate dorsally and eventually disappear. This phenotype is very similar to what has been observed in the zebrafish, indicating that the role of XDead end in germ cell development has been conserved in evolution.

Keywords: XDead end; Germ cell development; Primordial germ cells

Introduction

RNA localization is an evolutionarily conserved mechanism to generate cell asymmetry. In *Xenopus* oocytes, mRNA molecules that localize to the vegetal pole fall into two major subgroups, as defined by characteristic differences in their localization pathways. Late localizing mRNAs are uniformly distributed in early stages of oogenesis and become localized to the entire vegetal cortex in a microtubuli-dependent manner during intermediate stages of oocyte development (Forristall et al., 1995; King et al., 2005, Kloc and Etkin, 1995, 1998; Yisraeli et al., 1990). The mRNAs of the second group, referred to as early or METRO pathway RNAs, are localized to the mitochondrial cloud during early stages of oogenesis and become anchored to the tip of the vegetal cortex thereafter (Houston et al., 1998; Hudson and Woodland, 1998; Mosquera et al., 1993). A third minor group of mRNAs share characteristic features of both early and late pathway and are therefore referred to as intermediate transport pathway mRNAs (Chan et al., 1999; Zearfoss et al., 2004).

Cis-acting elements, referred to as localization elements (LEs), mediate the directed migration of RNA molecules. A detailed analysis of the *Xenopus* Vg1 LE has resulted in the hypothesis that relatively short redundant RNA sequence motifs act as targets for *trans*-acting factors (Cote et al., 1999; Deshler et al., 1998; Gautreau et al., 1997; Havin et al., 1998; Kwon et al., 2002; Lewis et al., 2004; Mowry, 1996). These clustered repeats have also been found in other late and early localizing mRNAs, suggesting a common mechanism for RNA localization in *Xenopus* oocytes (Betley et al., 2002; Kwon et al., 2002). Different LE-binding proteins have been described; Vg1RBP/Vera (Deshler et al., 1998; Havin et al., 1998; Lewis et al., 2004) and VgRBP60 (Cote et al., 1999; Lewis et al., 2004) have been described as repeat motif interacting factors. Both proteins interact with *Vg1* mRNA in the nucleus of *Xenopus* oocytes, suggesting a role in the initiation of the RNA localization process (Kress et al., 2004). Directional movement of the *Vg1* mRNA localization complex could involve the function of *Xenopus* Staufen, mediating the

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association with a conventional kinesin (Yoon and Mowry, 2004). Notably, the heterotrimeric kinesin II has also been implicated in the transport process (Betley et al., 2004). Other proteins, such as Prrp and VgRBP71/KSRP, may serve in anchoring mRNAs to the vegetal cortex (Kress et al., 2004; Kroll et al., 2002; Zhao et al., 2001). As judged on the basis of cross-linking experiments, early and late localizing mRNAs bind to overlapping but non-identical sets of oocyte proteins (Claussen et al., 2004).

Several of the late localizing mRNAs have been found to be involved in early embryonic patterning events (Tannahill and Melton, 1989; Wessely and De Robertis, 2000; Zhang et al., 1998), while early localizing mRNAs have been implicated in aspects of germ cell specification, migration and differentiation. As in many other organisms, the development of primordial germ cells in *Xenopus* is based on the asymmetrical inheritance of the germ plasm (Ikenishi et al., 1974; Zust and Dixon, 1975). The germ plasm is a structure enriched in fibrillar, electron dense germinal granules, mitochondria and ribosomes; it localizes to the vegetal pole of the egg in a form of large discrete islands (Nieuwkoop and Sutasurya, 1976). After fertilization, these islands aggregate to form characteristic patches at the tip of the vegetal blastomeres. Until gastrulation, the germ plasm is segregated unequally before it becomes perinuclear and divided equally among daughter cells (Whitington and Dixon, 1975). During subsequent stages of embryogenesis, germ plasm containing primordial germ cells (PGCs) remain localized to the endoderm. In tailbud stage *Xenopus* embryos, PGCs first migrate ventrally and then dorsally, assembling in the dorsal crest of the posterior endoderm before becoming incorporated into the lateral plate mesoderm that forms the dorsal mesentery during early tadpole stages. In later stages, PGCs migrate to the dorsal body wall and then laterally to the developing genital ridges (Wylie and Roos, 1976). To date, five different early and one intermediate pathway mRNA have been described to be associated with the germ plasm and primordial germ cells in *Xenopus* (Berekelya et al., 2003; Chan et al., 2001; Houston et al., 1998; Hudson and Woodland, 1998; MacArthur et al., 1999, 2000), but only one, namely *Xdazl*, has been tested for its function in PGC development; in the absence of Xdazl, PGCs are impaired in their ability to migrate from the ventral to the dorsal endoderm (Houston and King, 2000).

In the present study, we describe the first example for the identification of novel vegetally localized mRNAs in *Xenopus* by the use of a microarray-based screening procedure. *Dead end* mRNA encodes a putative RNA binding protein and was previously described as a germ plasm component in the zebrafish as well as in cleavage stage *Xenopus* embryos; knockdown of zebrafish *dead end* had resulted in defective migration and death of PGCs in fish embryos (Weidinger et al., 2003). We report that maternal *Xenopus Dead end* localizes via the late pathway in the oocyte and is germ cell specific during embryogenesis. We have further identified a 251 bp localization element, which resides in the 3′-UTR and exhibits protein binding characteristics very similar to other late localizing mRNAs in *Xenopus* oocytes. Effects observed upon XDead end knockdown during embryogenesis suggest a conserved function during germ cell development in fish and frog.

Materials and methods

Library screening

We made use of a cDNA library enriched in cortically and vegetally localized transcripts (Claussen and Pieler, 2004; Claussen et al., 2004). Bacterial suspensions of single colonies of the SMART cDNA phage library (Clontech) served as templates for insert amplification. Single cDNA clone inserts were amplified by PCR using the following vector specific primers: 5′λTriplEx2-LD, CTCGGGAAGCGCGCCATTGTGTTGGT and 3′λTriplEx2Seq, TAATACGACTCACTATAGGGC. PCR products were purified by use of a Biomac™ pipetting robot (Biorad) and spotted onto coated glass slides (Quantifoil) using a Chipwriter Pro robot (Biorad). The targets for hybridization were produced with detergent insoluble and soluble RNA fractions from stage VI *Xenopus* (Zhang and King, 1999) as starting material by using the SMART cDNA Fluorescent Probe Amplification kit (Clontech) and the LabelStar Array kit (Qiagen) according to the manufacturer's instructions. After hybridization of arrays following the swap design (Kerr and Churchill, 2001), the arrays were analyzed with a scanner (Affymetrix), data were normalized (Yang et al., 2002) and contrast, as well as significance calculated as described (Landgrebe et al., 2004). Candidate clones were sequenced to correct for redundant and previously known mRNAs; subcellular localization was verified by whole mount in situ hybridization. To obtain full-length cDNA clones, plaque filter screening of a *Xenopus laevis* oocytes phage cDNA library (Claussen and Pieler, 2004) by use of the ECL™ direct nucleic acid labeling and detection system (Amersham Biosciences) was performed according to the manufacturer's protocol.

Preparation of antisense RNA probes and whole mount in situ hybridization

Oocytes used for whole mount in situ hybridization and microinjection experiments were obtained from albino *X. laevis* as described previously (Claussen et al., 1999).

PCR products, amplified by PCR from single clone bacterial suspensions, served as templates for the subsequent in vitro transcription of labeled whole mount in situ antisense probes (see Hollemann et al., 1999 and Claussen and Pieler, 2004 for a more detailed description). Alternatively, the antisense transcripts were generated from linearized plasmids containing either the full-length sequence or fragments of the corresponding cDNAs. The Xpat antisense probe for in situ hybridization was generated by *Eco*RI digestion of the plasmid pBKXpat (Hudson and Woodland, 1998) and T7 transcription. The Xdazl antisense probe was generated by *Eco*RI digestion of the plasmid pTriplEx-velo61 and T7 transcription. Whole mount in situ hybridization was carried out as described previously (Harland, 1991; Hollemann et al., 1999). After the color reaction, embryos were fixed in MEMFA, and pictures were taken directly or after a clearing procedure (in 2:1 benzylbenzoate:benzylalcohol) using a digital video imaging system. Vibratome sections (30 μm) were prepared as described previously using the Leica VT1000S vibratome (Hollemann et al., 1998).

Cloning procedures

The vector pBK-CMV (Stratagene) was used for the subcloning of XDead end cDNA fragments. For the detection of injected RNAs, cDNAs were fused to a 320 nt fragment of the open reading frame of the bacterial *lac*Z gene. Subfragments of the XDead end cDNA were generated by PCR and ligated into *Bam*HI/*Xho*I sites of the pBK-CMV-*lac*Z.

Embryo microinjection and antisense depletion

The XDead end specific antisense morpholino complementary to a region of the cDNA spanning the start codon (5′CTGCTCGTCT-GACAGCTTCATTTTC) and standard control antisense morpholino

(5′CTTCTTACCTCAGTTACAATTTATA) were from Gene-Tools. Up to 0.5 pmol antisense morpholino oligonucleotide either alone or together with different amounts of *zDead end globinUTR* RNA (Weidinger et al., 2003) was injected vegetally into albino embryos at the 2-cell stage into both blastomeres. To estimate the number of primordial germ cells, control and injected embryos were fixed at stage 31/32 and stained for *Xpat* RNA (Hudson and Woodland, 1998) by whole mount in situ hybridization.

RT-PCR analysis

Total RNA from embryos and tissues was isolated as described (Hollemann et al., 1998). The RT-PCR was carried out using the Gene Amp RT-PCR kit (Perkin-Elmer) following the manufacturer's instructions. Amplification reactions were done with XDead end specific primers (5′AAAGAGACCCTGCTAAGTGCTGTC and 5′CTTGCAGTGA-CGTCTCCCAGTGAG), resulting in a PCR product of 300 bp. Histone 4 primers served as a control (5′CGGGATAACATTCAGGGTATCACT and 5′ATCCATGGCGGTAACTGTCTTCCT) and gave rise to a product of 200 bp in length.

Synthesis, injection and detection of lacZ-tagged RNAs

Using the T3 MESSAGE mMASCHINE kit (Ambion) according to the manufacturer's protocol, capped *lac*Z-tagged RNAs for injection into stage III–IV oocytes were prepared. Oocytes were obtained from albino female *X. laevis* and staged by using nylon sieves with different mesh sizes. Depending on the oocyte size, 0.1–0.15 ng RNA in a volume of 2 to 3 nl was injected into the oocyte nuclei. Injected oocytes were incubated at 18°C for 2 to 3 days in vitellogenin-enriched L-15 medium as described previously (Kloc and Etkin, 1999; Wallace et al., 1980; Yisraeli and Melton, 1988). Injected RNAs were detected by whole mount in situ hybridization using a digoxygenin-labeled *lac*Z antisense probe.

UV cross-linking assays and coimmunoprecipitation experiments

Xenopus oocyte S100 extract was prepared from collagenase-treated oocytes (stage I–IV) as described (Claussen et al., 2004). Radioactively labeled RNA probes were synthesized by in vitro transcription using the Stratagene T7 transcription kit, according to the standard reaction protocol. For each labeling reaction, 5 μl of 20 μCi/μl [α^{32}P] UTP (Amersham Biosciences) was used. Unincorporated nucleotides were removed by using RNeasy columns (Qiagen). In vitro UV cross-linking reactions were performed as described previously (Claussen et al., 2004). The UV cross-linked proteins were separated by 10% SDS-PAGE and analyzed by phosphoimaging (Amersham Biosciences). For coimmunoprecipitation experiments, myc-tagged Vg1RBP, Prrp, VgRBP71 and L5 were in vitro translated using the T_NT coupled transcription and translation system (Promega) and incubated with labeled RNA and coimmunoprecipitated as described (Claussen et al., 2004). Extracted supernatant containing unbound or bound RNA was analyzed by denaturing 8% urea PAGE and phosphoimaging.

Results

Vegetal localization of XDead end mRNA in X. laevis oocytes

To identify novel RNAs, which localize to the vegetal cortex of *X. laevis* oocytes, we made use of a cDNA library enriched in RNAs associated with the vegetal cortex (Claussen and Pieler, 2004; Claussen et al., 2004). Approximately 10,000 individual cDNA clones were screened using a microarray-based technique. In addition to the complete set of all previously known vegetally localized RNAs, a large number of novel mRNAs exhibiting subcellular distribution patterns typical for late, early and intermediate localization pathways respectively were isolated. One of the novel late localizing RNAs was isolated in 18 independent copies. A corresponding full-length cDNA 1905 bp in length, consisting of 53 nt of 5′-UTR, 1064 nt of coding region and 786 nt of 3′-UTR, was isolated from a total oocyte cDNA library (Accession Number: AY971581). The coding sequence was found to be identical with the previously published complete coding sequence from *X. laevis* (AY321494), which had been proposed to represent the frog homologue of Zebrafish Dead end (Weidinger et al., 2003). The coding sequence of the isolated cDNA clone differs only in 2 nucleotides, but not on the protein level, from the previously published one.

The subcellular distribution of *XDead end* transcripts during oogenesis was analyzed by whole mount in situ hybridization (Figs. 1A to C). In stage I and early stage II oocytes, *XDead end* mRNA is detected throughout the cytoplasm, while from late stage II onwards, transcripts become enriched at the vegetal cortex. In stage III–IV oocytes, the mRNAs are found to be distributed along the entire vegetal cortex, where they remain until stage VI. According to this localization pattern, *XDead end* mRNA belongs to the group of late localizing RNAs.

XDead end becomes restricted to the germ plasm and is expressed in migrating primordial germ cells

After the first cell division, *XDead end* RNA is found to be distributed in a granular pattern at the vegetal tip of both blastomeres, a situation which is characteristic for germ plasm associated transcripts (Figs. 1D, E). Similarly, in the 8-cell stage, *XDead end* RNA localizes to the germ plasm islands at the vegetal pole (Fig. 1F). A similar distribution was reported by Weidinger et al. (2003) for a 16-cell stage *Xenopus* embryo. In gastrula stage embryos (stage 11), *XDead end* mRNA is detected in isolated scattered cells within the yolk plug (Fig. 1G). During neurulation, expression is not visible from the outside of the embryo. However, sections of a neurula stage (stage 15) embryo reveal that *XDead end* is expressed in isolated cells lining the future gut (Fig. 1H). In tailbud stage embryos, transcripts are weakly detected in the migrating primordial germ cells (Fig. 1I). Thus, while *XDead end* RNA localizes via the late pathway, it becomes specifically restricted to the germ plasm/PGCs only after fertilization.

To determine the temporal pattern of *XDead end* expression during embryogenesis, as well as its expression in adult tissues, RT-PCR analysis was performed (Fig. 1J). Starting with the oocyte, a relatively constant level of *XDead end* mRNA is detected until stage 15 of embryogenesis, from whereon expression drops to a very low basal level and eventually disappears. In adult tissues, *XDead end* transcripts are entirely specific to the ovary.

The 3′-UTR of the XDead end mRNA contains a vegetal localization signal

To identify RNA signal sequences, which are responsible for the transport of the *XDead end* mRNA to the vegetal pole,

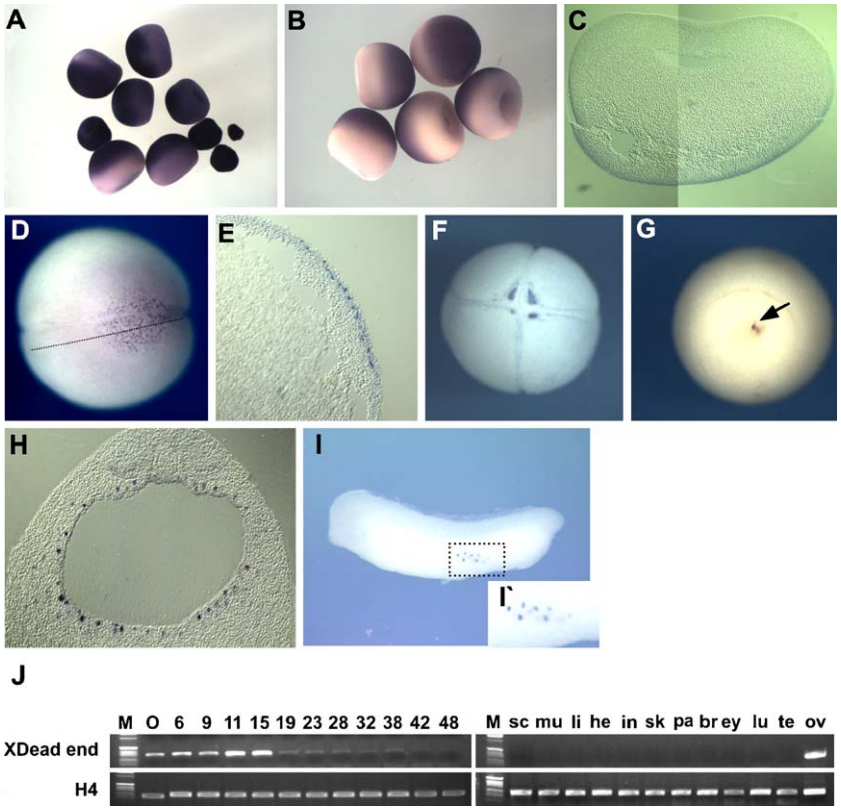


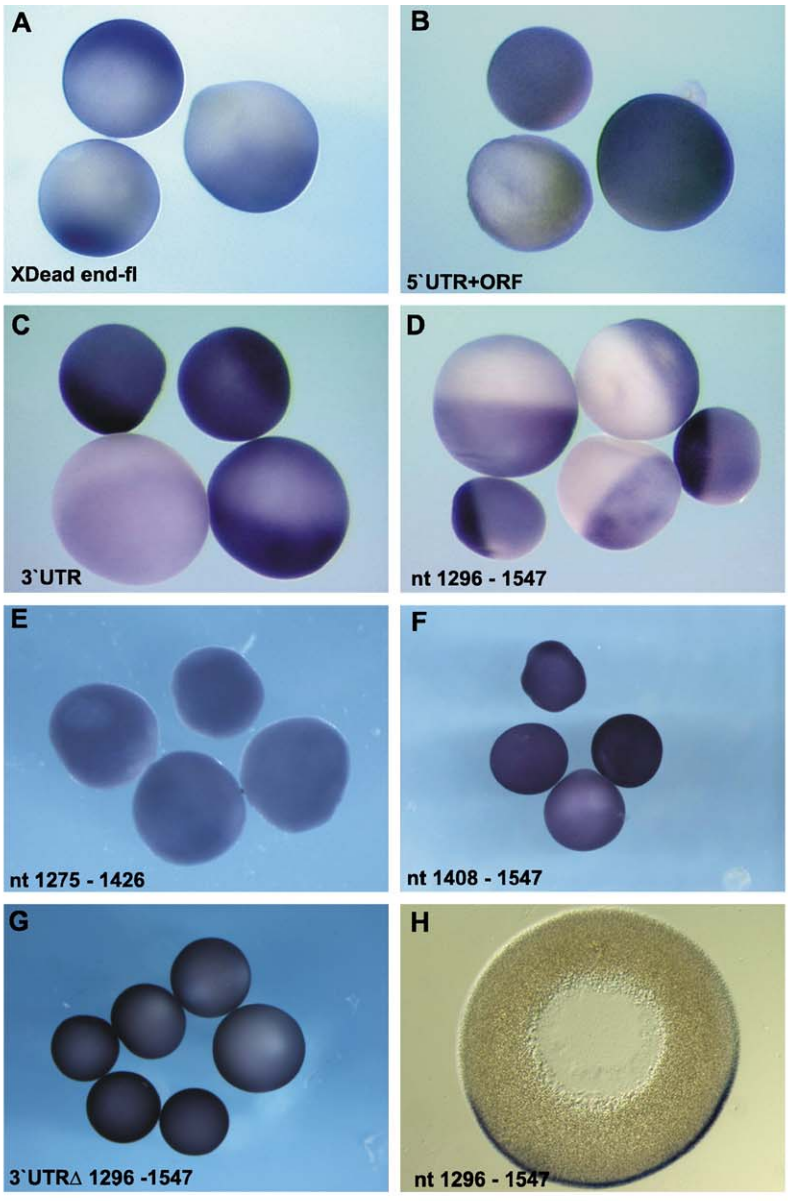
Fig. 1. Localization of XDead end mRNA in *Xenopus* oocytes and embryos. The subcellular distribution of the *XDead end* mRNA was analyzed using albino oocytes or embryos of different stages for whole mount in situ hybridization. In stage I and II oocytes, *XDead end* RNA is homogeneously distributed (A). In later stages (early and late stage III, stage IV) (A) and stage V and VI (B), the *XDead end* RNA becomes enriched at the vegetal cortex. (D, E) Two-cell stage embryo exhibiting a granular structure at the vegetal tip of both blastomeres. (F) Vegetal view of an 8-cell stage embryo (G) Vegetal view of a gastrula embryo with *XDead end* positive cells in the center of the yolk plug (marked by an arrow). (H) Transverse section of a neurula stage embryo exhibiting *XDead end* expression in scattered cells linking the future gut. (I, I′) *XDead end* expression in migrating germ cells of a tailbud stage embryo. (J) Temporal expression during embryogenesis and tissue expression of *XDead end*. RT-PCR analysis with RNA preparations from staged embryos (according to Nieuwkoop and Faber, 1967) and adult tissue respectively (sc, spinal cord; mu, muscle; li, liver; he, heart; in, intestine; sk, skin; pa, pancreas; br, brain; ey, eye; lu, lung; te, testis; ov, ovary). Control RT-PCR reactions with primers specifically for Histone 4 (H4).

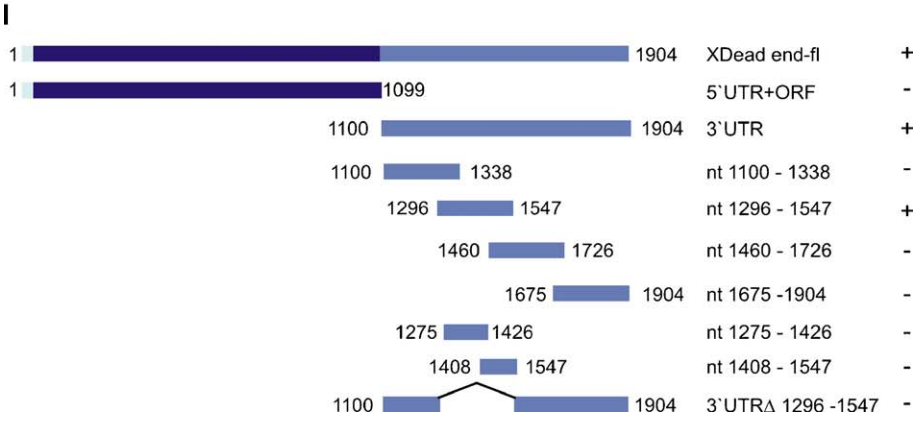
microinjection experiments were performed using different constructs corresponding to subfragments of the *XDead end* mRNA fused to a *lac*Z reporter sequences (Fig. 2). In vitro transcribed capped RNAs generated from these constructs were then injected into nuclei of stage III–IV oocytes, and localization was detected by whole mount in situ hybridization using a *lac*Z specific antisense probe. Localization is evident from preferential staining in the vegetal half of injected oocytes, while failure to localize results in diffuse ubiquitous staining. Full-length *XDead end* mRNA fused to *lac*Z localizes to the vegetal pole of the injected oocyte (Fig. 2A). The 3′-UTR fragment is equally capable of localizing to the vegetal pole, while the coding region plus 5′-UTR fragment is not (Figs. 4B, C). To narrow down the region of the 3′-UTR mediating vegetal localization, four overlapping fragments of the 3′-UTR were employed. A fragment spanning nucleotides 1296 to 1547 showed efficient vegetal localization (Fig. 2D). Cortical enrichment of the same injected RNA is revealed by sectioning (Fig. 2H). Internal deletion of nucleotides 1296 to 1547 within the 3′-UTR leads to a loss of localization activity, revealing that this portion is, not only sufficient, but also required for localization (Fig. 2G). Subfragments covering either the 5′- or 3′-portion of the 3′-UTR were not capable to mediate efficient vegetal localization (Figs. 2E, F). Thus, in summary, these experiments identify a 251 nt region which serves as a vegetal localization element in *XDead end* mRNA.

Fig. 2. The 3′-UTR of XDead end contains a vegetal localization element. *lacZ*-tagged RNAs were injected into stage III–IV albino oocytes and visualized by whole mount in situ hybridization after culturing for 3 days. Vegetal localization was analyzed in oocytes injected with transcripts containing the complete RNA (A), with a fragment containing the 3′-UTR (B), with a fragment containing the open reading frame and 5′-UTR (C), a fragment containing nucleotides 1296–1547 (D), a fragment containing nucleotides 1274–1426 (E), a fragment containing nucleotides 1408–1547 (F) and a fragment containing an internal deletion spanning nucleotides 1296–1547 (G). Section (H) shows the cortical staining of a stage III oocyte that had been injected with a transcript containing the nucleotides 1296–1547 as shown in panel (D). (I) Schematic illustration of the constructs used for *XDead end* localization element mapping experiments. 5′-UTR, ORF and 3′-UTR are marked with light blue, dark blue and normal blue respectively. Corresponding nucleotide positions of the XDead end cDNA are indicated. The capability of vegetal localization is marked (+), absence of vegetal localization is indicated by (−).

Binding of the XDead end localization element to specific oocyte proteins

To identify proteins that interact in a specific manner with the XDead end localization element, UV cross-linking experiments were performed. The radioactively labeled XDead end and Vg1 localization elements, as well as the *lac*Z probe serving as a negative control, were incubated with S100 oocyte extract, subjected to UV cross-linking. All RNAs tested contain the *lac*Z-tag and bind strongly to 54/56 kDa proteins, which





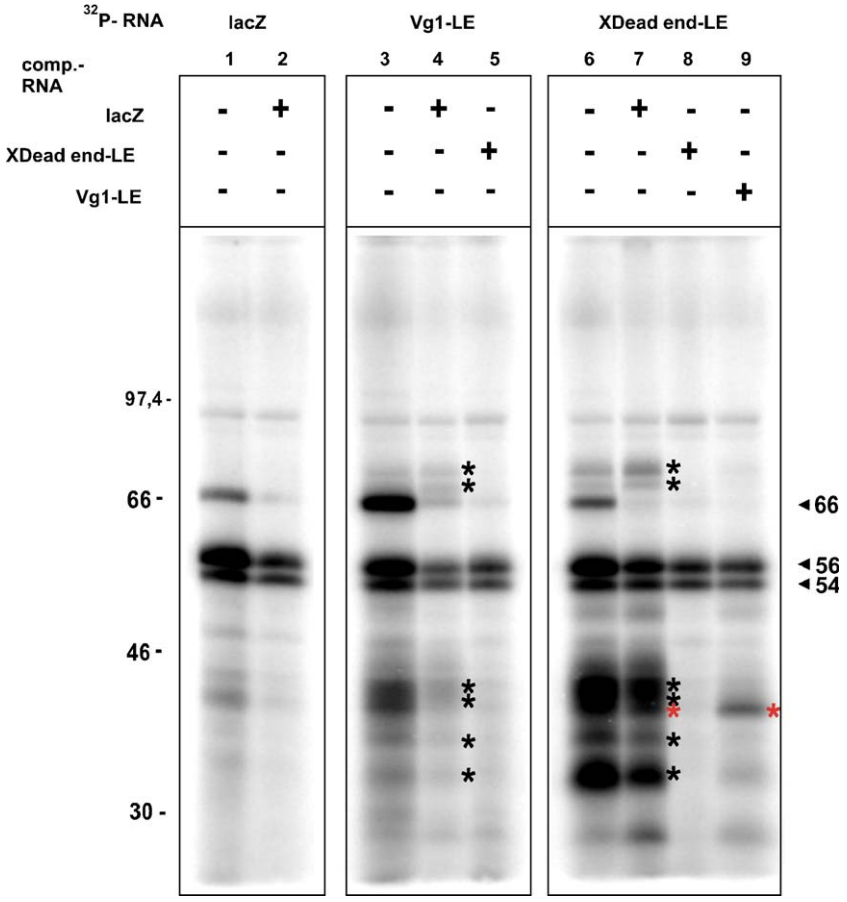


Fig. 3. Identification of protein binding to XDead end-LE by UV cross-linking. RNA binding was assayed in vitro by UV cross-linking. RNA-binding reactions contained ^{32}P-labeled *lac*Z-tag RNA (lane 1 and 2), *lac*Z-tag-Vg1-LE (3, 4 and 5) and *lac*Z-tag-XDead end-LE (nt 1296–1568) (lanes 6, 7, 8 and 9) transcripts and S100 extracts supplemented with tRNA competitor alone (1, 3 and 6), tRNA in combination with nonspecific *lac*Z competitor RNA (lanes 2, 4 and 7) or sequence specific competitor RNAs (lane 5, 8 and 9). Cross-linked proteins were analyzed and detected by 10% SDS-PAGE and phosphoimaging. Unspecific proteins are labeled with arrows. Proteins specifically cross-linked to both Vg1-LE and XDead end-LE are marked by black asterisk. The XDead end-LE specific protein is marked by a red asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

may represent the FRGY proteins (Bouvet and Wolffe, 1994; Cote et al., 1999; Marello et al., 1992), as well as to a 66 kDa protein of unknown identity (Fig. 3, lane 1 and 2). Patterns of proteins cross-linking with XDead end-LE on the one hand, and with Vg1-LE on the other, are similar and identify at least six different polypeptides, which qualify a specific binding partners for both RNAs since they can be competed in the presence of either XDead end-LE or Vg1-LE RNA (Fig. 3, lanes 3–8). These proteins migrate with an estimated size of 78, 69, 42, 40, 36 and 33 kDa respectively, similar to what had been described for the Vg1-LE previously (Bubunenko et al., 2002; Mowry, 1996. The previously described 60 kDa protein VgRBP60 (Cote et al., 1999) migrates close to the very abundant 54/56 kDa proteins and is not detected here. XDead end-LE specifically binds two additional proteins with an estimated size of 38 and 25 kDa respectively. The 38 kDa protein seems to be specific for the XDead end-LE since the corresponding cross-link is insensitive towards an excess amount of unlabeled Vg1-LE. Similarly, only weak competition was observed for the 25 kDa protein (Fig. 3, lane 9). Furthermore, the cross-linking efficiency of the XDead-LE to proteins of an estimated size of 42, 40 and 33 kDa is much

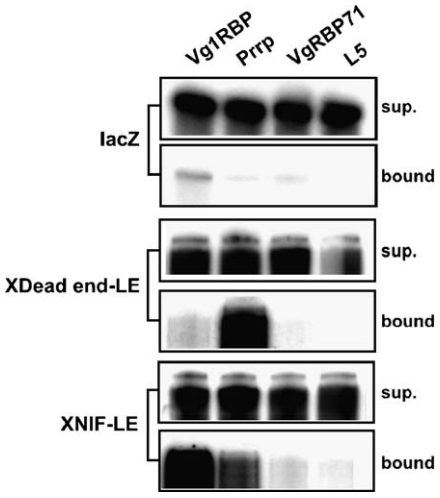


Fig. 4. The XDead end-LE interacts with Prrp. *lacZ*-RNA, XDead end-LE (nt 1296–1568) and XNIF-LE were radioactively labeled by in vitro transcription. Vg1RBP, Prrp, VgRBP71 and L5 proteins were produced by a transcription and translation reaction and incubated with indicated labeled RNAs. Proteins of the binding reaction were immunoprecipitated via their myc epitope tags, and unbound (sup.) and coimmunoprecipitated (bound) RNAs were analyzed by denaturing PAGE and phosphoimaging.

stronger than interaction of Vg1-LE to the same set of proteins. We conclude that the XDead end-LE interacts with all of the previously described proteins binding to the Vg1-LE (78, 69, 33, 36 and 40 kDa), and it binds to at least two additional proteins with an estimated molecular weight of 25 and 38 kDa respectively.

To assign proteins from the UV cross-linking experiment to known LE-interacting factors, we analyzed binding of individual proteins to the XDead end-LE by making use of coimmunoprecipitation assays. Based on the size of the proteins identified by UV cross-linking, the 69 and 40 kDa proteins might correspond to Vg1RBP/Vera (69 kDa) and Prrp (39 kDa). Vg1RBP, Prrp, VgRBP71 and the 5S rRNA binding ribosomal protein L5 serving as a negative control, all containing an N- or C-terminally located myc epitope, were translated in vitro and incubated with ^{32}P-labeled *lac*Z XDead end-LE RNA, as well as with XNIF-LE RNA serving as internal positive control (Claussen and Pieler, 2004; Claussen et al., 2004). From their use in oocyte injection experiments, these RNAs are fused to a *lac*Z tag, and this *lac*Z sequence alone served as a negative control. The immunoprecipitation of tagged proteins was tested by precipitation of proteins labeled with ^{35}S-methionine in vitro (data not shown). The nonspecific *lac*Z control RNA can be found only in the supernatant of the binding reactions and shows only weak background interaction with Vg1RBP (Fig. 4). The RNA fragment containing the localization element of XDead end is strongly bound by Prrp but shows only a very weak interaction with Vg1RBP,

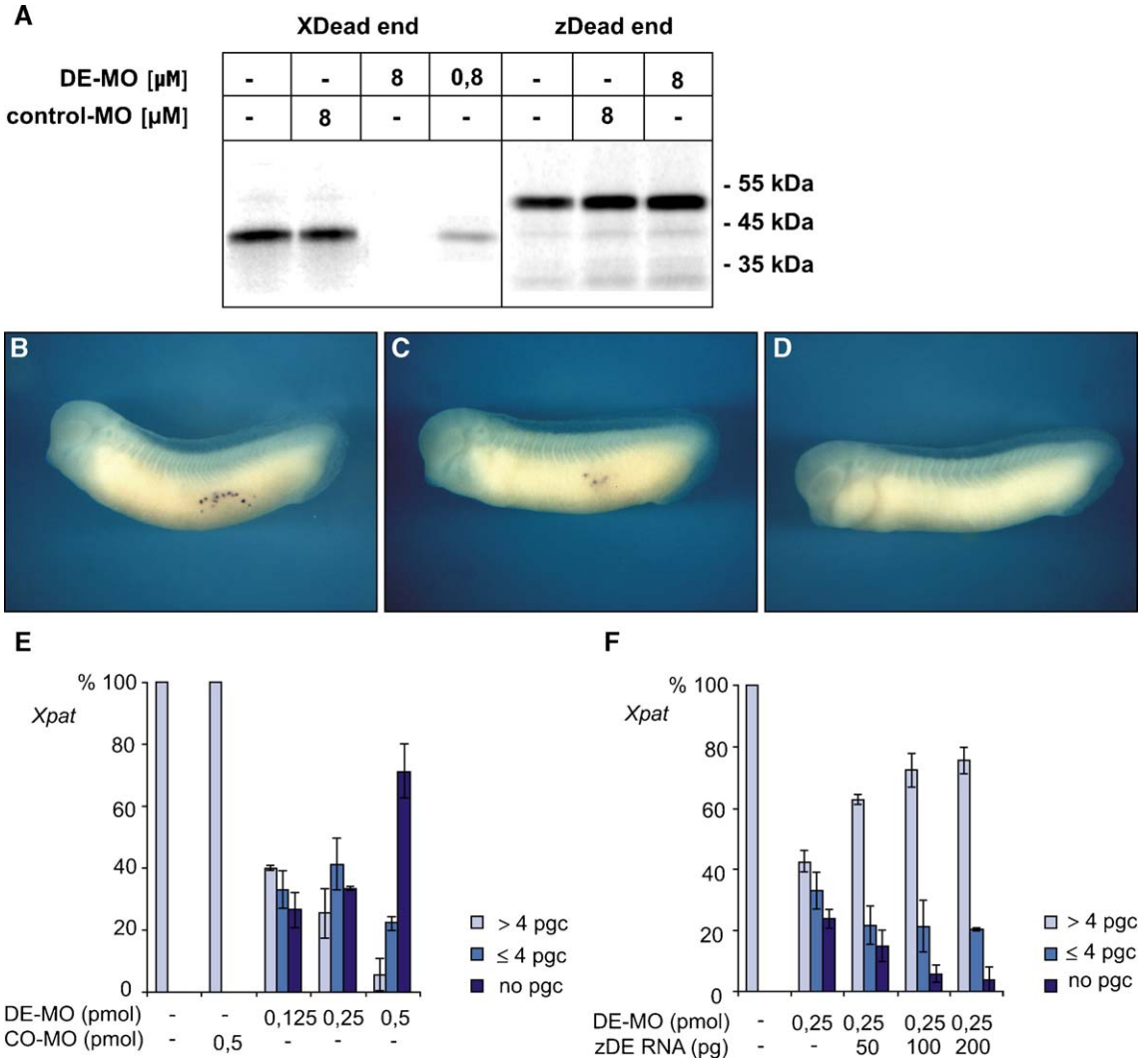


Fig. 5. XDead end protein is important for primordial germ cell development. (A) In the coupled in vitro transcription/translation system (Promega), XDead end and zDead end respectively were translated with or without different amounts of XDead end specific morpholino antisense oligonucleotide (DE-MO) or with control morpholino as indicated. Two-cell stage albino embryos were injected vegetally into both blastomeres with XDead end specific morpholino antisense oligonucleotide (DE-MO) or control morpholino (CO-MO). Stage 31/32 embryos were fixed and analyzed by whole mount in situ hybridization using a digoxigenin labeled antisense RNA probe for *Xpat*. Visible *Xpat* positive primordial germ cells (PGCs) were counted. (B) An uninjected stage 31 embryo showing an average number of *Xpat* expressing primordial germ cells. (C) Example of a stage 31 embryo injected with DE-MO resulting in a reduced number of *Xpat* containing primordial germ cells. (D) Example of a DE-MO injected stage 31 embryo with a total loss of *Xpat* positive primordial germ cells. (E) Percentage of embryos with a normal number of PGC marker positive cells (light blue), less or at least 4 PGC marker positive cells (blue) and no PGC marker expressing cells (dark blue) after DE-MO or control MO injection. Usage of *Xpat* or *Xdazl* respectively is indicated above the chart. Amount of injected DE-MO or control MO are indicated below the chart. (F) Partial rescue of the DE-MO effect by coinjection of increasing amounts of zebrafish *Dead end-globinUTR* mRNA.

comparable to the *lac*Z background intensity. No interaction with VgRBP71 and L5 can be detected. The binding pattern observed is very similar to the one previously reported for the Vg1-LE and also for the Xvelo1-LE using the same type of assay (Claussen and Pieler, 2004). We conclude that the XDead end localization element strongly interacts with Prrp, but not with Vg1RBP under the in vitro conditions used here.

XDead end is required for germ cell migration

To investigate a possible function of *XDead end* in the context of germ cell development in *Xenopus* embryos, we employed a loss of function approach. A *XDead end* specific antisense morpholino oligonucleotide (DE-MO) was found to specifically repress XDead end protein translation in vitro (Fig. 5A). Injection of the same DE-MO into the vegetal region of both blastomeres of 2-cell stage *Xenopus* embryos resulted in a concentration-dependent decrease in respect to the number of *Xpat* positive primordial germ cells (PGCs) at stage 31/32 (Figs. 5B to E). Similar results were also obtained with DE-MO injected embryos analyzed for *Xdazl* expression (data not shown). The number of PGCs detected in uninjected control embryos varies significantly, but it was always more a total of four PGCs visible from external inspection of stage 31/32 embryos. Therefore, experimental embryos with more than four PGCs were considered as unaffected. At the highest concentration of DE-MO employed, the majority of the embryos obtained was without *Xpat* positive cells at stage 31/32. The absence of PGCs in such DE-MO injected embryos was verified by sectioning to exclude the existence of such cells in the inner mass of the embryo (data not shown).

These experiments do not reveal at which developmental stage PGC development is impaired. Indeed, no significant difference in the distribution or number of cells expressing the PGC marker *Xpat* was detected in a comparison of control and DE-MO injected embryos at stage 11 and at stage 17 (Figs. 6A to C). At stages 24 and 27 of embryonic development, a minor but significant portion of the DE-MO injected embryos analyzed are already devoid of *Xpat* expressing cells or they tend to form clusters which are not observed in control embryos (Figs. 6D, D′, E and E′). These observations suggest that XDead end function is required for proper germ cell migration and, perhaps as a consequence of this, for their differentiation and/or survival.

A

	Xpat positive cells	stage 11	stage 17	stage 24	stage 27	stage 31
control	> 4	15/15	10/10	11/11	10/10	24/24
	≤ 4	0/15	0/10	0/11	0/10	0/24
	none	0/15	0/10	0/11	0/10	0/24
DE-MO injected (0,5 pmol)	> 4	13/13	15/15	8/10	7/10	2/28
	≤ 4	0/13	0/15	0/10	0/10	7/28
	none	0/13	0/15	2/10	3/10	19/28

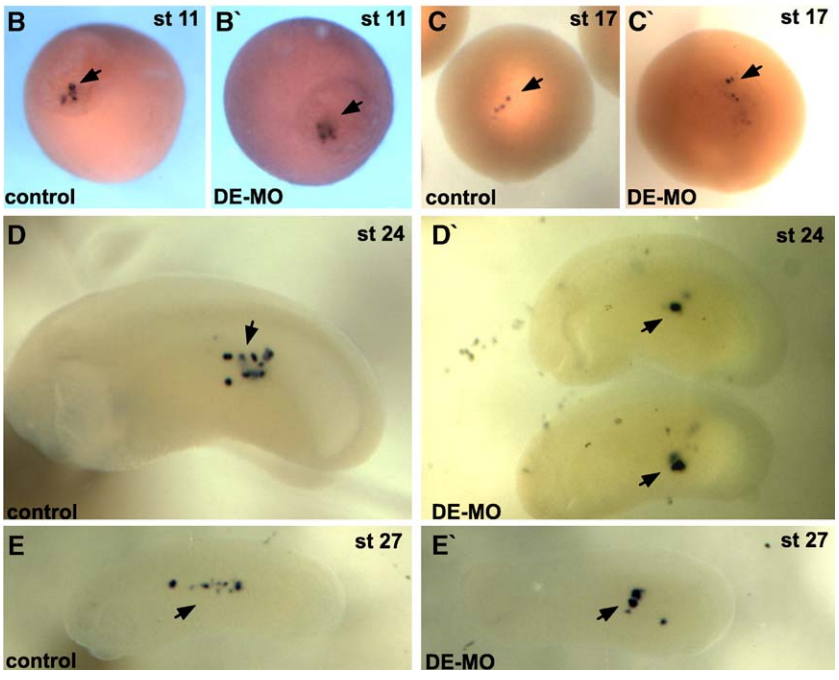


Fig. 6. Xpat expression in DE-MO injected embryos. (A) DE-MO injected embryos were analyzed for *Xpat* expression at the indicated stages. Data are shown as the number of embryos with the indicated expression out of the total number tested. (B, B′) Control and DE-MO injected stage 11 embryos analyzed for *Xpat* expression showing normal *Xpat* expression located in the posterior endoderm. (C, C′) Control and DE-MO injected stage 17 embryos showing *Xpat* stained PGC in the endoderm. (D) Control embryo (stage 24) showing normal PGC distribution and (D′) DE-MO injected embryos with tightly clustered PGCs. (E) Control stage 27 embryo with spaced distribution of *Xpat*-labeled PGCs and (E′) in the DE-MO injected embryo PGCs appear slightly clustered.

To confirm the specificity of the phenotype observed, we made use of zebrafish *Dead end* mRNA flanked by *Xenopus* β-globin 5′and 3′-UTRs in an attempt to rescue the DE-MO effect. The *XDead end* specific morpholino does not affect in vitro translation of the same zebrafish Dead end mRNA (Fig. 5A). Microinjection of a constant amount of DE-MO along with increasing amounts of the zebrafish *Dead end* mRNA resulted in a gradual restoration of normal numbers of PGCs (Fig. 5F). This experiment not only confirms the specificity of the DE-MO for the *XDead end* mRNA in *Xenopus* embryos, but it also suggests that Dead end function in the context of germ cell development is conserved across different species.

Discussion

A microarray-based screen for novel mRNAs localizing to the vegetal pole of *X. laevis* oocytes resulted in the identification of the Dead end homologue. *XDead end* mRNA is transported along the late pathway. An RNA localization element (LE) was mapped to a 251 nucleotide region in the 3′-UTR and found to interact with a set of proteins typical for late localizing mRNAs, but containing one additional, specific protein of 38 kDa molecular weight. During embryogenesis, *XDead end* is found to be associated with the germ plasm and specifically expressed in the primordial germ cells (PGCs). Knockdown of XDead end function in the *Xenopus* embryo resulted in a loss of primordial germ cells in tailbud/tadpole stages of development.

A number of different maternal *Xenopus* mRNAs have been described to be associated with the germ plasm; these include the early localizing *Xcat2*, *Xdazl*, *DEADSouth*, *Xpat* and *germes*, as well as the intermediate pathway mRNA *fatvg* (Berekelya et al., 2003; Chan et al., 2001; Houston et al., 1998; Hudson and Woodland, 1998; MacArthur et al., 1999). Consequently, *XDead end* defines the first example for a late localizing transcript that becomes specific to the germ plasm after fertilization. In contrast to those mRNAs localizing via the early pathway and involving structures of the germ plasm already in earliest stages of oogenesis (Kloc et al., 2001), *XDead end* mRNA follows the late pathway and is therefore not transported via the germ plasm containing material during early oocyte development. However, we cannot exclude the possibility that *XDead end* mRNA is everywhere in the cortex of later stage oocytes, including the germ plasm islands. This situation implies a mechanism, which allows restriction of *XDead end* RNA to the germ plasm afterwards. In the context of our screen, we have identified several other late localizing mRNAs, which follow a similar pattern of localization. It will be interesting to search for signal structures that these RNAs have in common as a first step towards elucidation of the underlying mechanism, which could also imply selective RNA stabilization/degradation.

In search of the *cis*-acting signal that is responsible for the vegetal localization of *XDead end* mRNA in *Xenopus* oocytes, we were able to define a 252 nt element within the 3′-UTR that is sufficient and necessary for proper vegetal translocation. A putative consensus signal that directs vegetal localization has been proposed and described to contain tandem repeats of what is referred to as the E2 (UUCAC or closely related) and VM1 (YYUCU) elements (Bubunenko et al., 2002; Kwon et al., 2002). The XDead end localization element (XDead end-LE) contains no VM1 motif, and multiple E2 elements are found both within (five copies) and flanking (four copies) the XDead end-LE. Furthermore, it was postulated that clusters of short CAC-containing motifs could be found in the localization element of many mRNAs (Betley et al., 2002). Application of the REPFIND program (Betley et al., 2002) to test for the presence of such clusters in the 3′-UTR of the *XDead end* mRNA did not reveal a statistically relevant correlation.

A comparative analysis of proteins binding to either the Vg1-LE or the XDead end-LE by UV cross-linking reveals almost identical protein patterns; there was also no difference in the binding activities in respect to different isolated proteins. These observations are in support of the notion that different late localizing mRNAs make use of the same protein machinery for their directional migration. Interestingly, the binding of the XDead end-LE to the 33, 40 and 42 kDa proteins appears to be much stronger when compared to the Vg1-LE. Furthermore, an additional specific XDead end-LE binding protein of 38 kDa was identified. It remains to be analyzed if this protein is involved in the association of *XDead end* mRNA with the germ plasm at the beginning of embryogenesis.

Xenopus Dead end expression during embryogenesis is germ cell specific. This principal finding had previously been reported in the context of a study focusing on the characterization of zebrafish *dead end*; expression of *XDead end* was analyzed at the 16-cell stage and found to be restricted to the germ plasm (Weidinger et al., 2003). In extension of this observation, we find that *XDead end*, similar to what has been reported for zebrafish *dead end*, is already restricted to the germ plasm at the two-cell stage and persists to be expressed in PGCs also in later stages of embryogenesis. In the adult, *XDead end* is specific to the female gonad. Inhibition of XDead end translation by injection of the appropriate antisense morpholino oligonucleotide (DE-MO) results in a severe reduction to total loss of PGCs in tadpole stage embryos. Interestingly, PGC formation and migration seem not to be affected in these experiments before organogenesis; stage 24/27 embryos exhibit formation of atypical clusters of PGCs in the endoderm. These findings correlate with some, but not all, aspects of the situation observed in the zebrafish; while, similar to *Xenopus*, PGC specification as well as their initial localization was not affected, dead end knockdown resulted in a marked mislocalization of PGCs in the fish (Weidinger et al., 2003). Interestingly, dead end knockdown zebrafish PGCs often remained in groups of cells with close cell–cell contacts (Weidinger et al., 2003), reminiscent of the clustering observed in stage 24/27 dead end knockdown frog embryos. The loss of PGCs in the zebrafish is probably a result of apoptosis; we have no experimental information on the cause of PGC disappearance in frog embryos upon XDead end knockdown. It is interesting to note that zebrafish dead end can substitute for XDead end activity. Taken together, these findings argue

strongly for a highly conserved function of Dead end in fish and frog PGC development.

Previous work had identified another RNA binding protein from *Xenopus* with a function in germ cell development and referred to as Xdazl. Different from *XDead end*, *Xdazl* mRNA localizes via the early pathway in the oocyte, but, similar to *XDead end*, it is also associated with the germ plasm as well as the primordial germ cells during embryogenesis (Houston et al., 1998). Furthermore, a function for Xdazl in the context of PGC differentiation has been demonstrated (Houston and King, 2000). Antisense oligonucleotide-mediated depletion of maternal *Xdazl* mRNA results in a phenotype comparable to the one that we have described for XDead end knockdown in this communication. *Xdazl*-depleted embryos exhibit a reduced number or absence of PGCs in stage 35 or 39/40 embryos, as well as PGC clustering in stage 26 embryos. The rather striking similarity in respect to the phenotypic effects as induced by knockdown of either Xdazl or XDead end suggests that both proteins may be functionally linked. What exactly such a function might be remains to be determined.

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