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Identification of 3′ UTR sequence elements and a teloplasm localization motif sufficient for the localization of *Hro-twist* mRNA to the zygotic animal and vegetal poles

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Abstract

The early localization of mRNA transcripts is critical in sorting cell fate determinants in the developing embryo. In the glossiphoniid leech, *Helobdella robusta*, maternal mRNAs, such as *Hro-twist,* localize to the zygotic teloplasm. Ten 7 nucleotide repeat elements (AAUAAUA) called ARE2 and a predicted secondary structural motif, called TLM (teloplasm localization motif), are present in the 3′UTR of *Hro-twist* mRNA. We utilized site-directed mutagenesis, deletions, and microinjection of labeled, exogenous transcripts to determine if ARE2 elements, and the TLM, play a role in *Hro-twist* mRNA localization. Deleting the poly-A tail and the cytoplasmic polyadenylation element (CPE) had no effect on *Hro-twist* mRNA localization. Site-directed mutagenesis of nucleotides that altered ARE2 element sequences or the TLM suggest that the ARE2 elements and the TLM are important for *Hro-twist* mRNA localization to the teloplasm of pre-cleavage zygotes. Hro-Twist protein expression data suggest that the localization of *Hro-twist* transcripts in zygotes and stage two embryos is not involved in ensuring mesoderm specification, as Hro-Twist protein is expressed uniformly in most cells before gastrulation. Our data may support a shared molecular mechanism for leech transcripts that localize to the teloplasm.

Keywords

teloplasm; leech; 3′UTR motif; mRNA localization; ARE2 sites; stem-loop structure

INTRODUCTION

Role of mRNA localization during early development

The localization of mRNA to specific sites in a cell generates cell polarity, which is critical in distributing cell fate determinants (Du *et al*. 2007). Asymmetric mRNA localization is also important for the specification of the germ cell lineage in many organisms including the fruit fly, nematode, zebrafish, and frog (Zhou and King, 2004). Major advantages of localizing mRNA to a discrete site include the controlled synthesis of proteins at targeted locations (Kloc *et al*. 2002; Paquin and Chartrand, 2008) and the production of a morphogen gradient (Du *et al*. 2007).

mRNA localization is crucial for the development of several organisms and it is widely used across the evolutionary spectrum. In the fruit fly, localization of the *bicoid* mRNA to the

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anterior portion of the developing embryo determines its anterior axis (Kloc *et al*. 2002). The anterior localization of the ortholog of *giant* is important for the proper formation of the head and thorax in developing wasp embryos (Brent *et al*. 2007). In the frog, *vg1* mRNA is localized in the egg vegetal hemisphere (Dale *et al*. 1993). *Vg1* induces mesodermal fates in cells that would normally become ectodermal, if it is mislocalized to the animal hemisphere (Dale *et al*. 1993). In ascidians, localization of the *posterior end mark (PEM)* mRNA to the posterior region of the embryo is required for unequal cell divisions in the early embryo (Negishi *et al*. 2007).

Two mechanisms of mRNA localization

There is support for two mechanisms of mRNA localization, both arising from the importance of *cis*-acting elements and secondary structural motifs (Jansen, 2001; Svoboda and Di Cara, 2006; Jambhekar and Derisi, 2007; Claußen and Pieler, 2010). In many organisms studied, the localization signal primarily resides in the 3′UTR (Jansen, 2001; Betley *et al*. 2002; Kwon *et al*. 2002; Lewis *et al*. 2004; Czaplinksi and Mattaj, 2006; Claußen and Pieler, 2010). For instance, the pair-rule segmentation mRNAs of *Drosophilia melanogaster* localize via signals in their 3′UTRs (Bullock *et al*. 2003). However, fruit fly oocytes (Capri *et al*. 1997; Thio *et al*. 2000), yeast (Chartrand *et al*. 1999; Gonzalez *et al*. 1999), and nerve cells (Prakash *et al*. 1997), contain a few mRNAs that have localization signals in the 5′UTR and/or coding region.

RNA secondary structural motifs have been shown to be involved in mRNA localization. These motifs consist of stem loop structures that are recognized by RNA-binding proteins, and are usually found in the 3′UTR of localized transcripts (Svoboda and Di Cara, 2006). For instance, the bcd localization element 1 (BLE1) is a localization signal in the 3′UTR that forms a complex secondary structure containing a stem-loop (Snee *et al*. 2005). *BLE1* mutations, as well as stem-loops III/IV, block localization of *bcd* (Macdonald and Kerr, 1998). Other examples include the gurken localization signal (GLS), a stem loop structure involved in *gurken* mRNA localization (Van De Bor *et al*. 2005), and the transport localization signal (TLS), a stem loop structure found in the transcripts of *K10* and *orb.* The TLS is sufficient for proper RNA localization, regardless of its sequence or position within the *K10* and *orb* transcript (Cohen *et al*. 2005). In addition, Santos *et al*. (2008) showed that a stem loop structure in the *wingless* mRNA 3′UTR called the WLE3, was required for the apical localization of the *wingless* transcript. The WLE3 motif is conserved among several other apically localized transcripts. The *c-myc* and *c-fos* transcripts have also been shown to localize via recognition of an AU-rich stem loop structure (Chabanon *et al*. 2005). *C-myc* and *c-fos* transcripts localize to the perinuclear cytoplasm and associate with the cytoskeleton.

mRNA localization in the glossiphoniid leech

Glossiphoniid leech embryos provide an alternative model to study questions of mRNA localization. Zygotes are large, and methods for microinjecting *in vitro*-transcribed mRNAs are established. In leeches, maternal mRNAs localize with the teloplasm of zygotes to the cortex prior to the first cleavage (Holton *et al*. 1994). The teloplasm, which consists of a large number of mitochondria, ribosomes, protein, and mRNA, appears as a clear cytoplasmic area devoid of yolk platelets near the vegetal and animal poles during the first cell cycle (Astrow *et al*. 1987; Fernandez *et al*. 1987; Astrow *et al*. 1989; Holton *et al*. 1994).

Early transcripts that localize to the teloplasm have been identified. Most of these are maternally inherited and include: *Hro-nos, Le-msx, Hau-pax3/7a,* and *Hau-pax beta1. Hro-nos* mRNA is expressed in cells that give rise to the mesodermally-derived gonadal tissue (Kang *et al*. 2002), and *Le-msx*, after multiple cell divisions, becomes concentrated in cells

giving rise to the mesoderm and ectoderm (Master *et al*. 1996). *Hau-pax3/7a* and *Hau-pax beta1* maternal transcripts localize to the zygotic teloplasm and segregate to early ectodermal and mesodermal precursors (Woodruff *et al*. 2007; Schmerer *et al*. 2009). Later on, zygotic *Hau-pax3/7a* mRNA is present in mesodermally-derived cells that give rise to the nephridia (Woodruff *et al*. 2007). Zygotic *Hau-pax beta1*mRNA is present in mesodermal derivatives during gastrulation, and during organogenesis is found in the central nervous system (CNS) and eye primordial (Schmerer *et al*. 2009).

We previously isolated a leech homolog (*Hro-twist*) to the fruit fly *Dm-Twist* (Soto *et al*. 1997). *twist* mRNA is found in other invertebrates such as the jellyfish, nematode, and lancelet along with vertebrates such as fish, mouse, chick, frog, and humans (Castanon and Baylies, 2002). In the leech, *Hro-twist* mRNA is detected in oocytes and during cleavage stages (Soto *et al*. 1997).

In this study, we characterized two types of mRNA localization recognition elements in the 3′ UTR of *Hro-twist* transcripts. The first one is a 7 nucleotide element repeat (AAUAAUA), termed ARE2, of which there are ten in the 3′UTR. The second type is a predicted secondary structural motif called the Teloplasm Localization Motif (TLM). The predicted TLM consists of a 20 unpaired nucleotide bulge with one major and one minor stem loop structure arising from it, along with a stem structure in continuum with the rest of the transcript. We used site-directed mutagenesis to alter ARE2 elements and the predicted TLM structure to determine their role in localizing *Hro-twist* transcripts in the one-celled stage. Mutation of the ARE2 elements affected the localization of exogenous transcripts. Furthermore, we showed that reducing the unpaired nucleotide bulge from 20 nucleotides to 10 nucleotides (in the predicted TLM) resulted in a higher level of abnormal RNA localization than altering the major stem loop structure. We determined the protein expression of Hro-Twist protein. Endogenous *Hro-twist* mRNA and Hro-Twist protein expression patterns match during the zygote and two-cell stages.

MATERIALS AND METHODS

Embryos

Helobdella sp. leeches were collected from a sturgeon farm at Galt, CA, and maintained in the laboratory using 1% ocean water. Embryos were manually removed from the cocoons deposited on the ventral side of the leech immediately after zygote deposition (AZD) and transferred to sterile HL saline (4.8 mM NaCl, 8.0 mM $CaCl_2$, 2.0mM $MgCl_2$, 1.2 mM KCl, and 1.0 mM Tris-HCl, pH 6.6) media until they were ready for microinjection.

Bioinformatics

A bioinformatics database called REPFIND (http://zlab.bu.edu/repfind/) was used to reveal conserved probable localization elements ($p = 0.0001$) in the *Hro-twist* 3′ UTR and coding region. The *Hro-twist* 3′ UTR sequence was also analyzed using the UTR Blast database (http://bighost.ba.itb.cnr.it/BIG/Blast/BlastUTR.html) to confirm the significance of these elements (Altschul et al.,1997). In addition, we used the UTRd and UTRSite website (ftp://bighost.ba.itb.cnr.it/pub/Embnet/Database/UTR/) that contains characterized functional elements in the 3′ UTR of diverse mRNAs (Pesole et al., 2002).

Predicted secondary structures of leech transcripts that localize to the one-celled teloplasm were obtained by submission of mRNA sequences to the Rensselaer bioinformatics web server: (http://mfold.bioinfo.rpi.edu/), with the temperature set to 16°C (the incubation temperature of leeches in the lab). These transcripts included: *Hro-twist, Hro-sna1, Hro-dl, Lzf2, Le-msx, Hro-nos, Hro-hes, Hau-pax 3/7a,* and *Hau-pax beta1. Hro-twist* (accession number AF410867), *Hro-sna1* (accession number AF410864), *Lzf2* (accession number

X91395), *Le-msx* (accession number U61846), *Hro-hes* (accession number AY144625), *Hau-pax 3/7a* (accession number DQ858213.1), and *Hau-pax beta1* (accession number EF554314.2) sequences were obtained from the NCBI, GenBank database (http://www.ncbi.nlm.nih.gov/). The complete *Hro-dl* and *Hro-nos* sequences were obtained from the Joint Genome Institute's *Helobdella* genome database (http://genome.jgi-psf.org/Helro1/Helro1.home.html).

Slot blot analysis

Fifteen zygotes, 4 h after zygote deposition (AZD), were frozen at −80 °C and stored until used. Zygotes were homogenized in 50 μl of 3 M guanidinium. The entire zygotic homogenate was placed in a slot under vacuum. One hundred nanograms of Torula total RNA were placed in a slot and used as a negative control. The nylon filter was dried at 65 °C and pre-hybridized with *in situ* pre-hybridization solution for 3 h. The filter was hybridized overnight in a solution containing a ^{32} P-UTP-labeled *Hro-twist* riboprobe (1×10^{6}cpm/ml) at 55 °C. The filter was washed at room temperature for 20 min with 2X SSC/0.1% SDS, and for 20 min at 65°C with 2X SSC/0.1% SDS. Radioactive intensity was quantified using a phosphorimager. Each experiment was repeated three times and a t-test was performed to determine significance.

Detection of endogenous Hro-twist mRNA

Antisense and sense *Hro-twist* DIG-labeled mRNA probes were synthesized from a construct containing the entire CDS. A probe concentration of 2ng/μl was used for each probe. *Helobdella sp*. zygotes were fixed 4h AZD in 4% with 4% paraformaldehyde (Electron Microscope Sciences), 0.25 X PBS, and 100 mM cacodylic acid, for 1 h at room temperature with constant rocking. After fixation, the embryos were dehydrated in 100% methanol and stored overnight at 4°C. Then, the procedure described by Rivera et al. (2005) was used to complete the *in situ* hybridization experiments.

A 3′ UTR PCR deletion, 3′UTR and coding region cloning

A fragment containing the complete *Hro-twist* 3′ UTR, and one deletion of this sequence, were generated by PCR, using p1.2 as a template. The cDNA clone p1.2 contains the 3′ UTR, and codes for the last 60 amino acids of Hro-Twist (Soto et al., 1997). The coding region was excised from pG10 (an *Hro-twist* expression construct cloned into a pGEX-KG vector) with *EcoRI/HindIII*, separated on a 1.2% low-melt agarose gel, purified and ligated into *EcoRI/HindIII* cloning sites of Stratagene's pBluescript (SK+). A 279 bp deletion lacking the poly-A tail, the cytoplasmic polyadenylation element (CPE), and three ARE2 sites was subcloned directionally into pBluescript (SK+). The plasmids with the correct size insert, and free of vector concatamers, were sequenced using vector-specific primers (M13 forward and M13 reverse universal primers) by Sequetech, in Mountain View, California.

Site-directed mutagenesis of ARE2 sites and the predicted TLM

Primers were designed to introduce point mutations among the ARE2 elements, AATAATA, to AGGAATA. The goal was to produce single ARE2 and multiple ARE2 mutations. Primer design was partially accomplished using the Primer3 program (http://frodo.wi.mit.edu/) (Rozen and Skaletsky, 2000). Mutagenic primers were also determined from Stratagene's web-based QuickChange Primer Design Program, which allowed for the option of setting mismatches (http://www.stratagene.com/qcprimerdesign). The primers used for site-directed mutagenesis of the predicted TLM were designed based on the predicted Mfold structures obtained from the Rensselaer bioinformatics web server. The wild-type 3′UTR was used as a template to produced TLM mutations. Two criteria were

considered for primer design: 1) mutations only altered the structure of the TLM without altering the rest of the 3′UTR structure, and 2) the length of the 3′UTR was not altered.

The QuikChange Multi Site-Directed Mutagenesis Kit was used to induce the mutations following the manufacturer's (Stratagene) instructions. Mutated *Hro-twist* 3′UTR constructs were transformed in XL-10 supercompetent cells, plasmid DNA was purified, and sequenced by Tocore DNA Sequencing LLC. BioEdit Sequence Alignment Editor Version 7.0.5.3 (Hall, 1999) was used to identify mutant sequences.

In-vitro Transcription of DIG-labeled mRNA

Hro-twist 3′ UTR mutants were transcribed *in vitro* with the addition of a 7-methyl guanosine 5′-cap analog (Ribo m^7G Cap Analog, Promega) and labeled with digoxigenin-11-UTP (DIG-11-UTP, Roche) for microinjection experiments. *In vitro* co-transcriptional capping and DIG-labeling was carried out with the following reaction mixture: 1X transcription optimized buffer (Promega), 1 μg of linearized template, 10 mM Ribo m^7G Cap Analog (Promega), 1X DIG RNA labeling mix (Roche), 10 mM DTT (Promega), 80 U Rnasin (Promega), and 80 U T3 RNA polymerase (Promega) in a final volume of 20 μl. The reaction was incubated for 3 h at 37°C, then for another 30 min with 4 U of Rnase-free Dnase I (Ambion). It was stopped using 0.8 μl of Rnase-free 0.5 M EDTA (pH 8.0) and then heat inactivated for 10 min at 65°C. RNA precipitation was performed using 2.5 μl of 4 M LiCl, 75 μl pre-chilled 100% ethanol, and 2 μl of 15 mg/ml glycoblue (Ambion) for 30 min at -20°C. The labeled transcripts were recovered by centrifugation at 10,000 × g at 4°C. The RNA pellet was re-suspended in nuclease-free water (Ambion) for 30 min at 37°C.

Microinjection of mutant mRNA transcripts in Helobdella sp. zygotes

Eppendorf's FemtoJet microinjector, and InjectMan 2 micromanipulator were used to pressure-inject zygotes under a Nikon Type 102 Dissecting Microscope. In order to normalize the amount of transcripts injected, solution was injected into a droplet of mineral oil on a micrometer slide. The injected solution beaded up into a perfectly round, spherical droplet and the diameter was measured. The volume of the sphere was calculated using the following equation: $V = 4/3\pi r^3$. The injection pressure (P_i) and time (T_i) was varied accordingly on the Femtojet for each microinjection needle to achieve a consistent injection volume of 0.5 nl.

Freshly-laid zygotes, within 25 minutes AZD, were injected with 12.5 pg of capped digoxigenin-labeled sense mRNA (25 ng/μl)/sterile fast green (0.4%) solution in 0.2 N KCl, prior to the extrusion of the first polar body.

Detection of exogenous transcripts

Only normal embryos were used for transcript detection. Normal embryos were those that formed teloplasm 4 h AZD. Embryos were incubated with HL saline, where they were allowed to develop for 3–4 h until the teloplasm was formed. Upon teloplasm formation the embryos were fixed with 4% paraformaldehyde (Electron Microscope Sciences), 0.25 X PBS, and 100 mM cacodylic acid for 1 h at room temperature with constant rocking. After fixation, the embryos were dehydrated in 100% methanol and stored overnight at 4°C.

The next day, the embryos were gradually rehydrated with increasing concentrations of PBS and devitellinized manually under a dissecting microscope. The embryos were then incubated for 3 h at room temperature in (0.22 μm filtered) blocking solution (10% goat serum, 25% bovine serum albumin in PBS, 0.1% Tween-20). After the 3 h incubation time, anti-DIG Fab (Roche Diagnostics) was added at a concentration of 1:200 and the mixture

was incubated overnight at 4°C, and then 4 h at room temperature. The embryos were washed three times for 15 min, and six times for 1 h with PBT, after which coloration buffer (100mM Tris-HCl, 1.0mM NaCl, and 0.1% Tween-20, pH 9.5) was added to them. The embryos were washed twice (with 5 min incubations at room temperature with constant rocking) in the coloration buffer, after which they were transferred to a 9-well staging dish.

NBT/BCIP reagent (Roche Diagnostics) was added to the wells, and the embryos were incubated in the dark for 10–15 min. After staining was visible, the embryos were rinsed with PBS, slowly dehydrated with increasing concentrations of ethanol, cleared with methyl salicylate (Sigma), and viewed as whole mounts using a Zeiss Axiophot 2 microscope.

Hro-Twist Protein Expression in Helobdella sp. embryos

The *Hro-twist* coding sequence was obtained by PCR from *Helobdella robusta* genomic DNA, and cloned into the expression vector pGEX-KG. Synthesis of full-length GST/Hro-twist fusion proteins were induced in BL21 *E. coli* cells (Stratagene), with 1 mM IPTG. After induction, cells were sonicated, and GST/Hro-Twist fusion proteins purified, using the Bulk GST Purification Module (Amersham Pharmacia). Polyclonal antibody serum was obtained from injecting three rabbits with purified recombinant Hro-Twist protein (Babco labs, Berkeley, California). Initially, 500 μg of purified, recombinant Hro-Twist protein were injected in Freund's adjuvant. Boost injections were performed at weeks 3, 6 and 9, with 250 μg of recombinant Hro-Twist protein in Freund's. The polyclonal antibodies used for this experiment were obtained from the second bleed. To purify the polyclonal serum, approximately 1 mg of enriched, recombinant Hro-twist protein in HEPES buffer, was incubated with 0.8 ml Affi-gel 10 (Bio-Rad), for 4 h at 4 C. Polyclonal antibody sera were added to the Hro-Twist protein affinity column. Non-specific proteins were washed off the column before adding 100 mM glycine buffer (pH 2.5), to elute anti-Hro-Twist antibodies.

Embryos were fixed and stained using a modification of protocols from Goldstein *et al.* (2001). Goat anti-rabbit-IgG antibodies conjugated to HRP were used as secondary antibodies (Bio-Rad). Embryos were viewed and photographed under a Zeiss Axioskop 2 microscope.

RESULTS

Mapping of probable functional localization elements

The bioinformatics database, REPFIND, identified ten elements containing a 7-nucleotide repeat (AAUAAUAA) termed ARE2 ($p = 0.0001$) in *Hro-twist* 3′ UTR (Fig. 1A). REPFIND was developed to find common localization element clusters in *Xenopus* mRNAs (Betley *et al.* 2002). Thus, we hypothesized that these sites could be potential candidates for functional 3′ UTR elements used in the localization of *Hro-twist* mRNA.

Endogenous and exogenous DIG-labeled Hro-twist localize similarly

We performed *in situ* hybridization experiments to examine localization patterns of endogenous *Hro-twist* mRNA at 4 h AZD. A slot blot was performed to corroborate the expression of endogenous *Hro-twist* mRNA at this stage (Fig. 1B). Prior to the first cleavage, endogenous *Hro-twist* mRNA localizes only to the clear cytoplasmic area (teloplasm), but not to the yolk (Fig. 1C).

For functional *in vivo* studies, exogenous digoxygenin (DIG)-labeled transcripts were microinjected into early zygotes, to determine patterns of localization. Montana *et al.* (1998) used a similar approach to successfully study *bep 3* mRNA localization sequences in the sea urchin. The amount of microinjected *Hro-twist* transcripts was normalized for all injected

transcripts to 12.5 pg, to ensure that the results obtained were not due to differences in the quantity of injected exogenous transcripts.

Prior to the first cleavage, exogenous DIG-labeled *Hro-twist* 3′ UTR transcripts localized similarly to endogenous *Hro-twist* mRNA. The localization of DIG-labeled 3′ UTR only-transcripts was restricted to the teloplasm (Fig. 1D). The equatorial view shows discrete localization of the transcripts to the animal and vegetal poles. This result demonstrates that DIG-labeled transcripts are capable of localizing normally in the leech embryo.

Localization patterns of exogenous Hro-twist transcripts in pre-cleavage zygotes and two-celled embryos

We examined the localization of exogenous *Hro-twist* open reading frame (ORF) only, and 3′ UTR transcripts in pre-cleavage zygotes (4.25 h AZD) and two-celled embryos, to determine if the separate transcripts segregated differently to the AB and CD cells. The exogenous transcripts containing the *Hro-twist* ORF localized asymmetrically to the teloplasm during the first cleavage (Fig. 2A and B). When the first cleavage was complete, the *Hro-twist* ORF transcripts were also present in the yolk, when viewed axially (Fig. 2C). At this stage, most of the transcripts containing the *Hro-twist* ORF segregated to the animal and vegetal poles of CD (Fig. 2D). By contrast, *Hro-twist* 3′ UTR transcripts localized symmetrically to the teloplasm (Fig. 2E and F). Unlike the ORF transcripts, the 3′ UTR transcripts remained localized to the teloplasm boundary at the two-celled embryo. In addition, most of the exogenous *Hro-twist* 3′ UTR transcripts localized to the CD cell and some segregated to the AB cell, when viewed in the animal (Fig. 2G) and equatorial (Fig. 2H) orientations. The exogenous *Hro-twist* 3′ UTR transcripts localization pattern is similar to the endogenous pattern of *Hro-twist* mRNA, as detected by *in situ* hybridization (Fig. 2I–J).

The 279 nucleotides at the 3′ end of the transcript are not necessary for Hro-twist mRNA localization

The poly-A tail, the CPE, and the ARE2 elements 7–10 are not required for mRNA localization to the teloplasm, prior to the first cleavage. Removal of these elements in a single 279-nucleotide deletion, did not affect the localization of the exogenous transcript to the teloplasm (Fig. 3A–C).

ARE2 elements are required for Hro-twist localization

Localization differences were observed in transcripts containing mutations in multiple ARE2 elements (Fig. 4). The first seven ARE2 elements (ARE2 elements 1–7) appeared to be sufficient for restricting transcripts to the teloplasm, as mutating ARE2 elements 8–10 had no effect in altering localization to the teloplasm (Fig. 4A–C). Mutations of ARE2 elements 6–10 affect the localization of some of the exogenous transcripts that localize to the vegetal pole (Fig. 4D–F). Similar patterns of mislocalization are observed with exogenous transcripts containing mutations in ARE2 elements 1–2 and 6–10. Together, this suggests that ARE2 elements 1–2 are not required for *Hro-twist* mRNA localization (Fig. 4G–I). Data for the 279-nucleotide deletion (lacking ARE2 elements 7–10) demonstrate that these ARE2 elements are not required for *Hro-twist* localization (Fig. 3). In addition, data for the exogenous transcripts containing mutations on elements 8–10 corroborate the deletion results (for elements 8–10). These results suggested the possibility that ARE2 element 6 is required for localization of some of the exogenous transcripts.

We were unable to mutate ARE2 elements 3, 4 and 5 by site-directed mutagenesis. Therefore, we produced a 26-nucleotide deletion removing ARE2 elements 2–5 from the construct that already contained mutations in ARE2 elements 1–2 and 6–10. Functional

analysis of these exogenous transcripts indicates that localization patterns were affected at both poles (Fig. 4J–L). However, failure to localize was not equal at both poles; none of the exogenous transcripts localized to the vegetal teloplasm, while some transcripts were detected in the animal teloplasm.

Deleting a region containing ARE2 elements 2–5 and mutating ARE2 element 6 altered two structural motifs of Hro-twist predicted secondary structure

The bioinformatics program Mfold (Zucker, 2003) was used to obtain predicted secondary mRNA structures of the complete *Hro-twist* mRNA and the *Hro-twist* 3′UTR. The 3′UTR secondary structure was similar in both the predicted, complete *Hro-twist* mRNA and *Hro-twist* 3′UTR transcript (Fig. 5). ARE2 elements were mapped out on the predicted 3′UTR structure (Fig. 6). ARE2 elements 7–9 clustered within a hairpin loop at one end of the predicted structure (Fig. 6A). ARE2 elements 3–5 were also clustered within a predicted hairpin loop (Fig. 6B). ARE2 element 6 makes up half of a predicted larger loop of a stem-loop (Fig. 6B). ARE2 element 2 is located within two nucleotides of the predicted 3–5 region (Fig. 6B), and ARE2 elements 1 and 10 are both within predicted small loop structures (Fig. 6B–C).

Site-directed mutagenesis and deletion data suggest that ARE2 elements 3–6 are important for normal *Hro-twist* mRNA localization. Mfold was then used to obtain predicted structures of the *Hro-twist* 3′UTR containing different ARE2 mutations. The predicted structure of the transcript containing mutations in ARE2 elements 8–10 showed that the predicted secondary structural motifs that contain ARE2 elements 3–5 and 6 were not affected (Fig. 7A). However, transcripts containing mutated ARE2 elements 6–10 resulted in the lengthening of the hairpin loop containing ARE2 elements 3–5, by one base pair, and the removal of the predicted structural motif that contains ARE2 element 6 (Fig. 7B). The structural motif (named Teloplasm Localization Motif or TLM) containing ARE2 element 6 is composed of a 20 unpaired nucleotide bulge with one major and one minor stem loop structure projecting from it, and one stem structure in continuum with the rest of the 3′UTR (Fig. 5). Transcripts containing mutations in ARE2 elements 1–2 and 6–10 resulted in the shortening of the predicted hairpin loop containing ARE2 elements 3–5, by 5 base pairs. Predicted structures of transcripts containing mutations in ARE2 elements 1 and 6–10 and a deletion of ARE2 elements 2–5 showed that both the predicted hairpin loop containing ARE2 elements 3–5 and the TLM were removed (Fig. 7C–D).

It is possible that the ARE2 elements 3–6 and the secondary structural motifs (hairpin loop and TLM) play independent roles in the localization of *Hro-twist* mRNA. Therefore, altering the structural motifs without changing the nucleotide sequence of ARE2 elements was necessary in order to test for the contribution of each, independently. However, we were unable to distinguish independent localization contributions of the hairpin loop containing ARE2 elements 3–5 and their nucleotide sequence because these elements overlap. Thus, it was not possible to alter the predicted hairpin loop structural motif without altering the nucleotide sequence of ARE2 elements 3–5. However, we were able to examine the contribution to mRNA localization of the TLM and the sequence of ARE2 element 6, since it was feasible to introduce mutations that altered the predicted TLM without changing the nucleotide sequence of ARE2 element 6.

Alteration of the predicted TLM in Hro-twist 3′UTR

If the TLM has a functional role in early localization, mutations in the TLM should affect the localization of *Hro-twist* transcripts in the one-celled embryo. In order to test this hypothesis, mutations were designed that altered only the predicted TLM, and not the ARE2 element 6. The wild-type 3′UTR was used as a template for TLM mutations. Mutations

were analyzed with Mfold to ensure that the remaining secondary structure was not altered. Three mutations were made. TLM mutation A converted the predicted major stem loop structure into two smaller loops. TLM mutation D increased the size of the predicted major stem loop by one nucleotide and decreased the distance between the adjacent stem loops. TLM mutation F resulted in the complete distortion of the predicted structure.

Altering the predicted TLM affects the localization of exogenous Hro-twist transcripts

Figure 8A–C shows localization patterns of exogenous *Hro-twist* transcripts with the predicted, unaltered TLM. Figure 8D shows the predicted structure of the *Hro-twist* transcript containing TLM mutation D. In the axial view (Fig. 8E), the exogenous *Hro-twist* transcript containing TLM mutation D localized to three small areas including the animal teloplasm. The equatorial view shows that the *Hro-twist* transcript containing TLM mutation D failed to localize to the teloplasm of the vegetal pole (Fig. 8F).

Converting the major stem loop into two minor loops (TLM mutation A) is a greater alteration of the predicted TLM structure (Fig. 8G). The exogenous *Hro-twist* transcript containing TLM mutation A localized to a smaller area in the teloplasm of the animal pole (Fig. 8H). This localization pattern is different than the one observed with TLM mutation D. The equatorial view shows that TLM mutation A also prevents the exogenous transcripts from localizing to the vegetal pole (Fig. 8I). Instead, localization is observed in the animal pole and as well as in the central region of the embryo.

TLM mutation F showed the highest degree of alteration to the predicted TLM structure relative to the other mutations (Fig. 8J). The resulting predicted structure contained only one stem loop and the bulge is smaller in size. The axial and equatorial views show that exogenous *Hro-twist* transcript containing TLM mutation F failed to localize to the teloplasm of both poles (Fig. 8K–L). The exogenous transcripts localized to some of the edges of the zygote (when viewed axially, Fig. 8K). Thus, the exogenous transcripts are localized outside of the teloplasm of both poles (Fig. 8L).

Conservation of ARE2 elements and TLM structure

ARE2 elements were conserved in four of the nine leech transcript sequences analyzed (Table 1). These include *Hro-twist, Hro-nos, Hro-hes,* and *Le-msx.* However, only three transcripts contained a predicted TLM structure (Fig. 9). *Hro-twist* and *Le-msx* contain one TLM structure each, and *Lzf2* contain two structures.

Hro-Twist protein expression in early leech embryos

We examined Hro-Twist protein expression in early leech development. We hypothesized that *Hro-twist* mRNA localization to the cell CD may ensure that its protein product is expressed in the mesodermal precursor (cell DM) and pro-mesodermal cells (M teloblasts). Before the first cell division Hro-Twist protein is present in the teloplasm. In the two-celled embryo, it is detected mostly in the teloplasm of the CD blastomere (Fig. 10A–B). These expression data demonstrate that *Hro-twist* mRNA (Figs. 1C and 2I–J) and protein are the same for stages 1 and 2. However, our hypothesis was refuted as Hro-Twist protein was detected in the micromeres, and in cells A’ (pro-endodermal), DNOPQ’ (ectodermal precursor), and DM (mesodermal precursor) at stage 4b. Furthermore, in all of the cleavage stages and during gastrulation Hro-Twist protein is found in all prospective ectodermal and mesodermal cells (Fig. 10C–E). Hro-Twist protein expression was detected in a subset of cells only by the end of gastrulation (Fig. 10F).

DISCUSSION

Hro-twist 3′UTR and mRNA localization to the animal and vegetal teloplasm

A localization signal is required to transport mRNAs to correct subcellular compartments. These signals can be in the form of nucleotide sequences (zip codes), secondary structures, or a combination of both (Jansen, 2001). *Trans*-acting factors (RNA-binding proteins and non-coding RNAs) bind to these *cis*-acting regions and form tertiary structural motifs.

The *Hro-twist* CPE and poly-A tail are not required for mRNA localization, as exogenous transcripts lacking these sequences localize normally to the animal and vegetal teloplasm (Fig. 3). The presence of a CPE in the 3′UTR is consistent with the previous finding that *Hro-twist* mRNA is present as a maternal transcript (Soto *et al*. 1997), since CPEs have been identified in the 3′ UTR of quiescent, maternal mRNAs (Vasalli and Stutz, 1996; Verrotti *et al*. 1996; Wickens *et al*. 1996).

A 3′UTR, AU-rich element is used in the localization of Hro-twist mRNA to the vegetal pole

Localization of exogenous *Hro-twist* transcripts demonstrates that its 3′UTR contains functional *cis-acting* elements that are used for normal mRNA localization. The region containing ARE2 elements 3–5 have a strong role in *Hro-twist* localization, as the deletion of this nucleotide fragment resulted in complete failure of exogenous transcripts to localize to the vegetal pole and incomplete localization to the animal pole. ARE2 element 6 appears to have a weak role in the transport to the vegetal pole because most exogenous mRNA containing mutations in this domain were able to migrate to the teloplasm of both animal and vegetal poles (Fig. 4). However, we cannot exclude the possibility that the role of ARE2 elements in mRNA localization to the teloplasm is only observed when most of these elements are mutated or removed, as a consequence of a progressive effect.

Importantly, the functional ARE2 elements 3–5 are found in an overlapping cluster at a predicted hairpin loop. Functional clusters have been shown to be important in localizing transcripts in the frog. For example, the *Vg1* localization element (VgLE), which is mapped to a 360 nucleotide region in the *Xenopus Vg1* 3′UTR, contains repeated sub-elements that produce weak localization when acting alone. Together, they are able to target *Vg1* transcripts to the vegetal pole of oocytes (Deshler *et al*. 1998; Mowry and Cote, 1999; Yaniv and Yisraeli, 2001). Similarly, *Fatvg* mRNA relies on multiple redundant localization elements to direct its transcripts to the vegetal pole of *Xenopus* oocytes during the METRO and late pathways (Chan *et al*. 1999).

Localization is affected by alterations in the predicted TLM motif

We identified a predicted mRNA secondary structural motif (TLM) that is critical for *Hro-twist* mRNA localization. Our results indicate that different levels of TLM alterations result in varying degrees of abnormal localization (Fig. 8). Subtle changes in the predicted TLM (mutation D) resulted in transcripts that still localized to the animal teloplasm. However, a drastic alteration in the motif (TLM mutation F) completely abolished normal localization of the *Hro-twist* mRNA. Furthermore, our data suggest that each individual component of the motif has a different influence in the localization of the transcript. For example, TLM mutations A and D affected only the major stem loop structure causing a reduction in the level of proper RNA localization, as some of the exogenous transcripts localized to a smaller area in the animal teloplasm. TLM mutation F affected both stem loop structures and the unpaired bulge. These changes caused most of the exogenous transcripts to localize abnormally.

Conservation of TLM structure and ARE2 elements

We analyzed the predicted secondary structure of early leech transcripts in order to examine if a common secondary structural motif is found in all of the mRNAs that localize to the teloplasm in the one-celled embryo. Of the structures examined, *Hro-sna1* and *Hro-dl* mRNA expression patterns have not been reported, but their proteins are detected in the teloplasm before the first cell division (Goldstein *et al*. 2001). Furthermore, most of the other mRNAs folded for this study have a maternal component of expression, with the exception of *Hro-hes*, and all localize to the teloplasm of uncleaved zygotes (*Lzf2,* Savage and Shankland, 1996*; Le-msx,* Master *et al*. 1996; *Hro-twist,* Soto *et al*. 1997; and this study; *Hro-nos*, Kang *et al*. 2002; *Hro-hes,* Song *et al*. 2004; *Hau-pax 3/7a,* Woodruff *et al*. 2007; *Hau-pax beta1*, Schmerer *et al*. 2009). Moreover, our analysis showed that only of two of these mRNAs (*LzF2* and *Le-msx)* contain a predicted secondary structural motif similar to the TLM found in *Hro-twist* (Fig. 9). ARE2 elements were also found in three other leech mRNAs that localize to the teloplasm (one ARE2 element in *Hro-nos*; two ARE2 elements in *Hro-hes*; and four ARE2 elements in *Le-msx*, two of which are overlapping) suggesting a conserved functional role for this element in the leech.

It is possible that a RNA-binding protein that recognizes ARE2 elements or the TLM is involved in teloplasm localization of leech transcripts sharing this sequence and structural motifs. Three types of RNA-binding proteins involved in mRNA localization during development and in some adult cells have been identified (Kiebler and DesGroseillers, 2000). These include heterogeneous nuclear ribonucleoproteins (hnRNPs), zipcode-binding proteins (ZBPs), and double-stranded RNA-binding proteins. Some of these proteins are evolutionarily conserved and bind to different mRNAs from different species. Squid hnRNP have been implicated in the mRNA localization of *fushi tarazu* (Lall *et al*. 1999) and *gurken* (Norvell *et al*. 1999) in fruit fly embryos and oocytes, respectively. Frog Vg1 ZBP localizes *vg1* mRNA by recognizing an AU-rich hexanucleotide sequence in the vegetal localization element of *vg1*transcripts (Havin *et al.* 1998). While, fruit fly *bicoid* 3′UTR secondary structures are recognized by Staufen protein, a double stranded RNA binding protein that is also involved in *prospero* mRNA localization in neurons (Broadus *et al.* 1998). Investigating the possibility of a common RNA-binding protein involved in teloplasm mRNA localization will require a careful biochemical analysis of potential ARE2 or TLM binding protein candidates and a similar mutation/deletion analysis of leech transcripts that localize to the teloplasm.

Are ARE2 elements 3–6 and the TLM sufficient to ensure proper localization of Hro-twist mRNA?

Mutations in ARE2 elements and in the predicted TLM alone, do not adequately explain the element/structure requirements by which *Hro-twist* mRNA localizes to the animal and vegetal teloplasm. For instance, exogenous transcripts containing mutations in ARE2 elements 6–10 lacked the predicted TLM structure (Fig. 7B). However, those mutations (as well as those in ARE2 elements 1–2, 6–10) affected only the localization of some of the exogenous transcripts that localize to the vegetal pole (Fig. 4D–I). Localization was greatly affected only when the hairpin loop containing elements 3–5 was deleted (Fig. 4J–L). However this hairpin loop alone is not sufficient to ensure normal localization, as the length or sequence of the hairpin loop containing ARE2 elements 3–5 was not altered in TLM mutants.

Spatial orientation of a functional RNA structural motif may affect a protein's ability to bind to it. A spatial orientation requirement has been proposed in the mechanism used for transcriptional attenuation by the RNA binding protein TRAP and its binding to *trp*-leader RNA (McGraw *et al.* 2009). Three-dimensional (3D) molecular modeling of the transcripts

containing mutations in ARE2 elements 1–2 and 6–10 and the TLM may provide a possible explanation of our results. However, the available bioinformatics programs that are used for 3D modeling of RNA structures have size limitations and only provide models of short sequences. The *Hro-twist* 3′UTR is 957 nucleotides long. Investigating the interesting possibility that the spatial orientation of the hairpin loop containing elements 3–5 and the TLM are important for proper *Hro-twist* mRNA localization will require the development of 3D modeling software that can predict structures of longer RNA sequences.

Hro-twist mRNA localization in pre-cleavage zygotes is not involved in ensuring mesoderm formation

RNA localization provides a way for cells to control protein synthesis regionally, by targeting mRNAs to specific sub-cellular regions. RNA localization also provides a route for polarized cells to transport RNAs to specific sites (Mohr and Richter, 2001). Overall, RNA localization serves three roles. Oocytes/early embryos localize their mRNAs to establish a body axis, ensuring the proper organization and cellular differentiation of germ layers. Polarized metazoan cells and budding yeast localize their mRNAs to sub-cellular regions for cell maintenance. Coding and non-coding RNAs have a structural role in maintaining the actin microfilament network in the cytoskeleton (Kloc, 2009).

The *Hro-twist* ORF is able to direct almost all of the transcripts to the CD blastomere. The results of injected 3′UTR-only transcripts suggest that *Hro-twist* 3′ UTR may contain structural elements (either nucleotide sequences or secondary structural motifs) that may keep transcripts sequestered within the teloplasm boundary in the CD blastomere of stage two embryos (Fig. 2). In the absence of the 3′UTR, the *Hro-twist* ORF transcripts segregated to the correct cell (CD blastomere) but could not anchor to the teloplasm region of the CD blastomere, which resulted in complete diffusion (when viewed axially). Teloplasm that segregates to the CD blastomere after the first cleavage is distributed to stem cells that give rise to ectodermally- and mesodermally-derived tissues. Furthermore, studies by Astrow *et al*. (1987) and Nelson and Weisblat (1992) demonstrated that teloplasm has a role in specifying the fate of these stem cells.

Hro-Twist protein expression patterns were examined in order to test the possibility that *Hro-twist* mRNA localization to the cell CD may have a role in specifying mesodermal fate. Our data suggest that the localization of *Hro-twist* transcripts in pre-cleavage zygotes and stage two embryos does not ensure mesodermal differentiation, as Hro-Twist protein expression becomes restricted only after gastrulation. Hro-Twist protein is found uniformly in endodermal precursors (during early cleavage stages), micromeres (at stage 7), and ectodermal and mesodermal precursors during cleavage stages and early gastrulation (Fig. 9).

A few of the Twist orthologs are expressed before the mesodermal layer is specified. These include orthologs in the fruit fly (Thisse *et al*. 1987), the Brazilian fly (Carvalho *et al*. 2005), the beetle (Handel *et al*. 2005), and the purple sea urchin (Wu *et al*. 2008). However, there is functional evidence for Twist's role in gastrulation only for Dm-Twist (fruit fly). Homozygous null *Dm-twist* mutants fail to undergo gastrulation and the embryos lack mesoderm (Simpson, 1983). The leech *twist* ortholog is the only lophotrochozan mRNA that is expressed before gastrulation takes place. In the polychaete, *Ca-twist 1* and *Ca-twist 2* mRNA are not expressed before gastrulation (Dill *et al*. 2007). *Ca-twist 1* mRNA is expressed in developing head muscle fibers and in muscles around the stomodeum. *Cap-twist 2* mRNA is expressed in all of the segmental mesoderm (at stages 6 and 7). In the mollusk, *Pv-twist* mRNA is expressed in the ectomesoderm but not in the endomesoderm (Nederbraght *et al*. 2002).

Conclusions

Teloplasm localization signals used for the correct localization of *Hro-twist* mRNA are found within a 100-nucleotide span that contains two primary functional structures. Our data suggest that nucleotide elements called ARE2 and a secondary structural element called TLM have a role in the localization of transcripts to the teloplasm of pre-cleavage zygotes. Although our protein expression data do not suggest a role of *Hro-twist* mRNA localization in mesoderm specification, our data may support a shared mechanism for leech transcripts that localize to the zygotic teloplasm.

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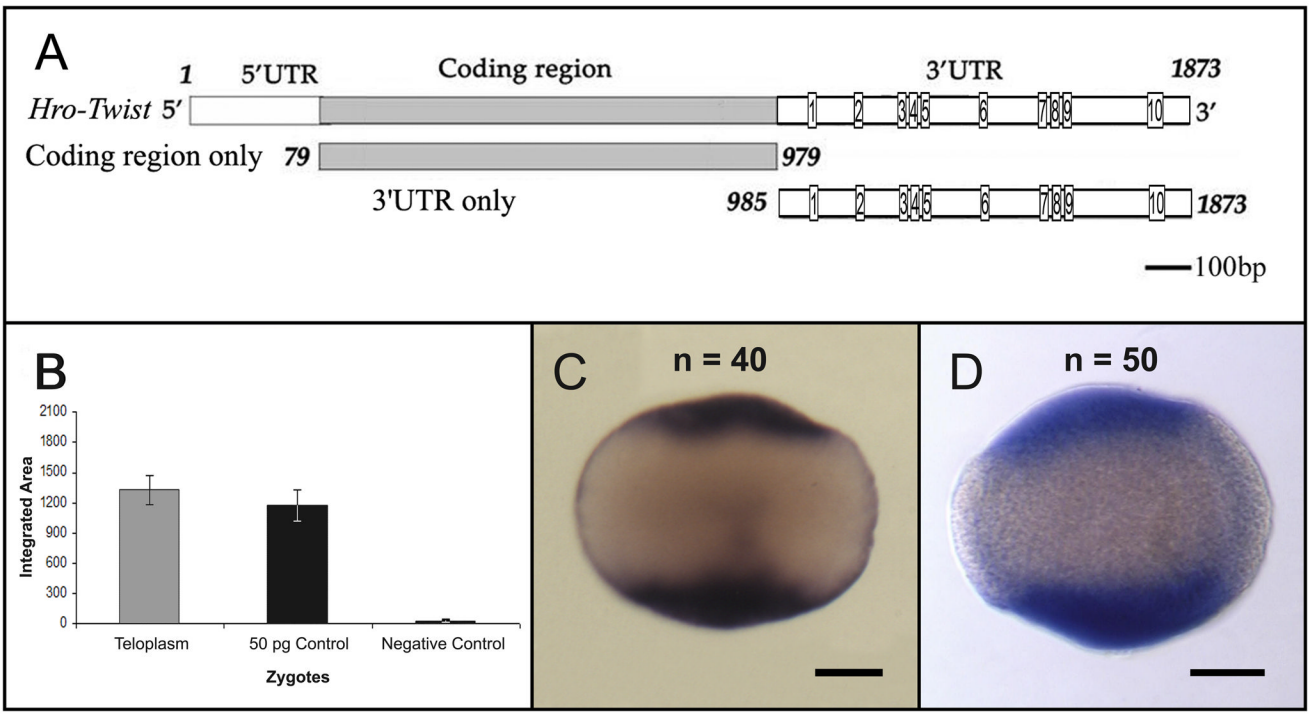

Fig. 1.

(A) Schematic diagram of predicted ARE2 elements in *Hro-twist* mRNA. The ten AU-rich elements ARE2 (AAUAAUA) are indicated by boxes at the 3′UTR. Diagrams of the coding region (ORF) and 3′UTR-only transcript templates used for experiments shown in Figure 2. Scale bar 100 bp. (B) Endogenous *Hro-twist* mRNA at 4h AZD. The slot blot experiment was repeated three times. Endogenous *Hro-twist* mRNA and exogenous *Hro-twist* 3′UTR localize to the teloplasm. (C) Endogenous *Hro-twist* mRNA was detected using *in situ* hybridization. (D) DIG-labeled exogenous *Hro-twist* 3′UTR was microinjected 20 min AZD and the embryos fixed 4 h later before the first cleavage. Equatorial views are shown on both C and D. Animal is up. At 4 h AZD, *Hro-twist* mRNA is detected at both poles. Scale bar 100 μm.

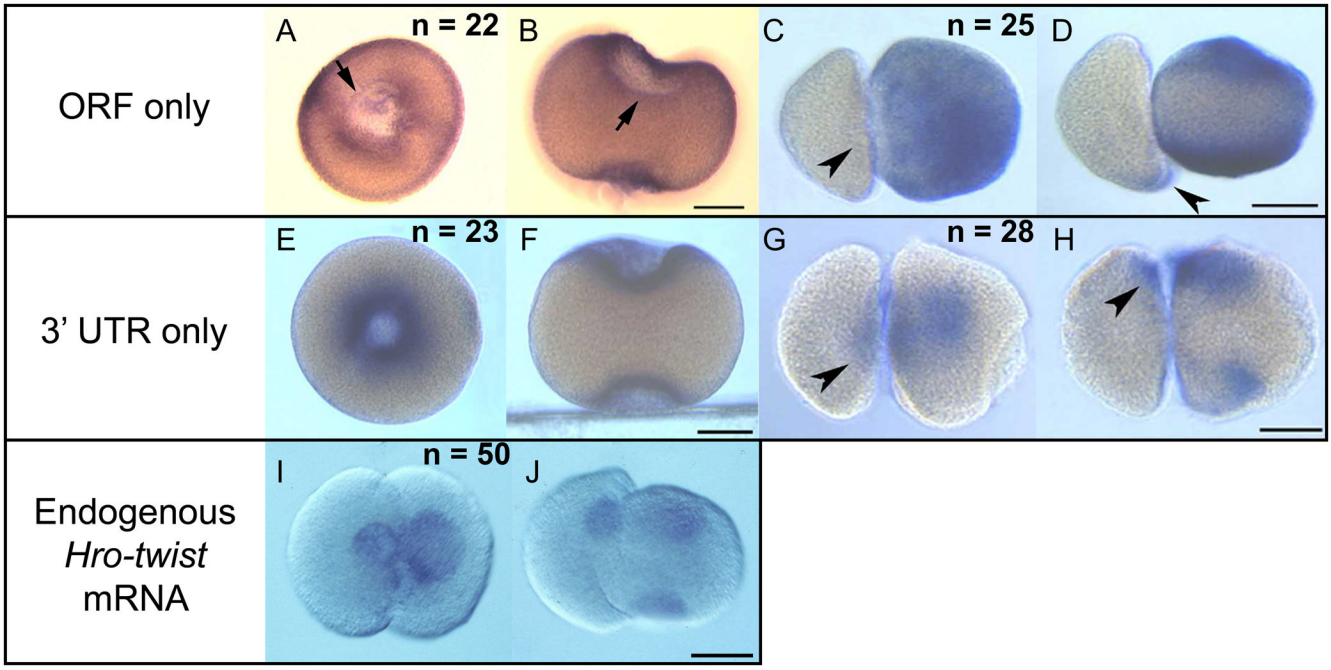

Fig. 2.

Animal (A) and equatorial (B) views of localized exogenous *Hro-twist* coding region of transcripts during the pre-cleavage stage. Black arrows indicate the region devoid of exogenous *Hro-twist* transcripts in the animal pole. Animal (C) and equatorial (D) views of localized exogenous *Hro-twist* coding region transcripts at the two-celled embryo. Animal (E) and equatorial (F) views of localized exogenous *Hro-twist* 3′ UTR (only) transcripts during the pre-cleavage stage. Animal (G) and equatorial (H) views of localized exogenous *Hro-twist* 3′ UTR transcripts at the two-cell stage. Black arrowheads point to localized exogenous transcripts in the AB cell. Animal (I) and equatorial (J) views of endogenous *Hro-twist* mRNA detected using *in situ* hybridization. Scale bars 100 μm.

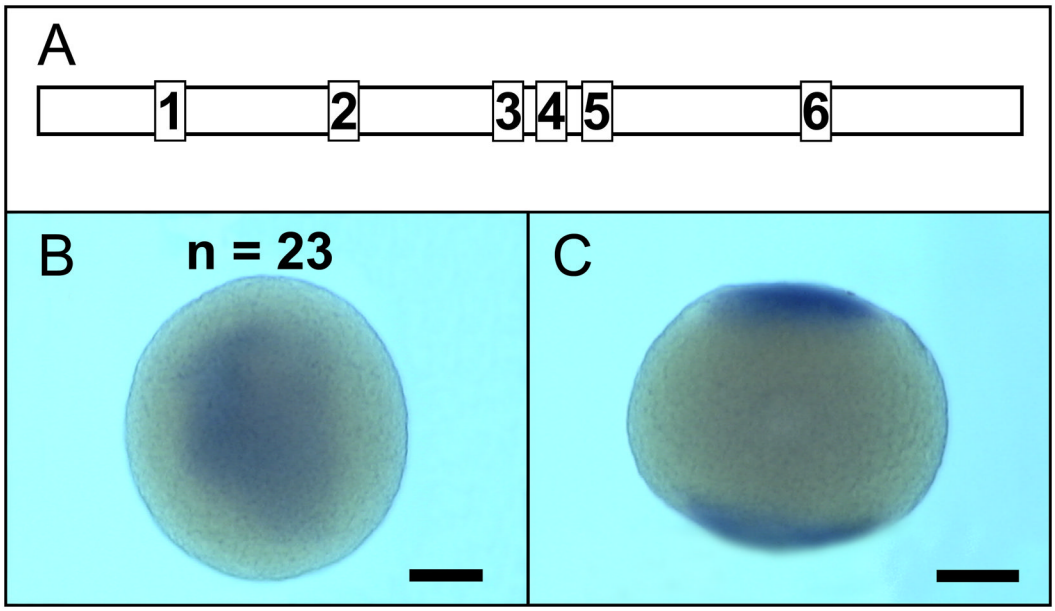

Fig. 3.

The 279 nucleotides at the 3′ end of the transcript are not necessary for Hro-twist mRNA localization. (A) Diagram of 3′UTR indicating relative position of ARE2 elements (not drawn to scale). Animal (B) and equatorial (C) views of localized exogenous 3′UTR transcripts containing a 279-nucleotide deletion at the 3′ end. Transcripts were injected within 25 min AZD and fixed once teloplasm was observed (4h AZD). Scale bar 100 μm.

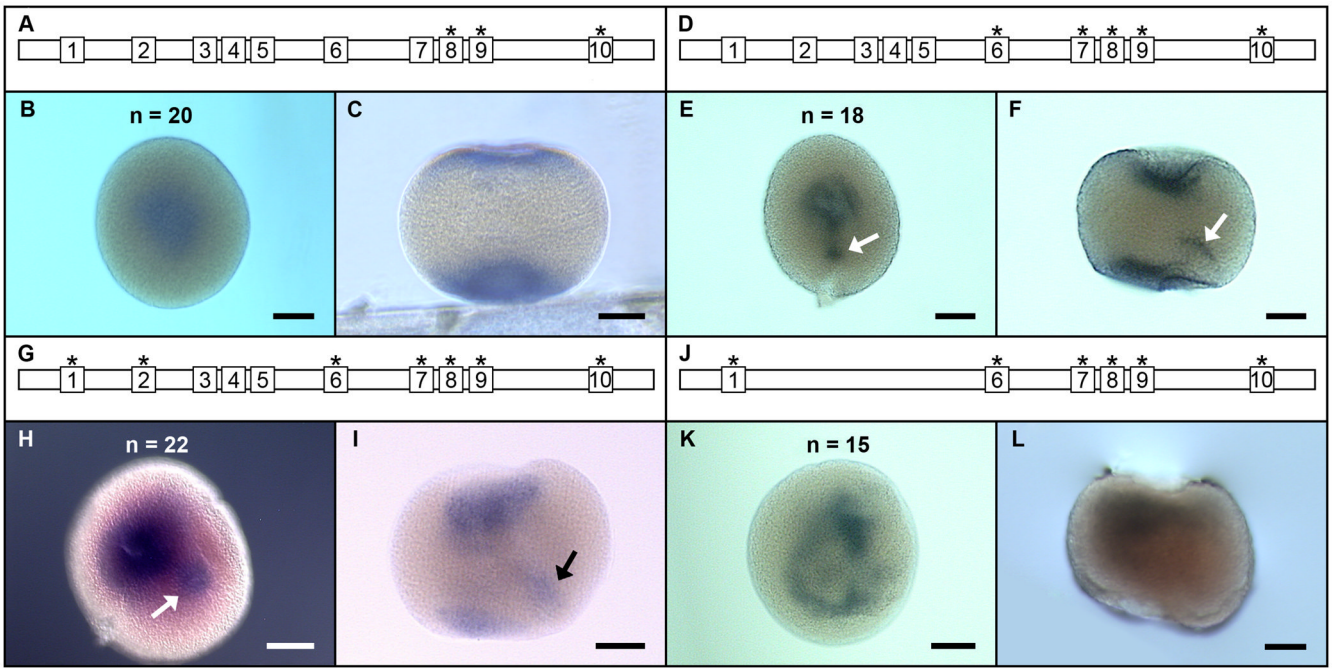

Fig. 4.

Localization patterns of exogenous *Hro-twist* 3′UTR transcripts with ARE2 mutations. (A, D, G, J) Diagrams of 3′UTR indicating relative position of ARE2 elements (not drawn to scale). Asterisks (*) indicate mutated ARE2 elements. Animal (B) and equatorial (C) views of localized exogenous 3′UTR transcripts containing mutations in ARE2 elements 8–10. Animal (E) and equatorial (F) views of localized exogenous 3′UTR transcripts containing mutations in ARE2 elements 6–10. Animal (H) and equatorial (I) views of localized exogenous 3′UTR transcripts containing mutations in ARE2 elements 1–2 and 6–10. Animal (K) and equatorial (L) views of localized exogenous 3′UTR transcripts containing mutations in both ARE2 elements 1–2 and 6–10, as well as a deletion of ARE2 elements 2–5. Transcripts were injected within 25 min AZD and fixed once teloplasm was observed (4h AZD). White arrows indicate mislocalization of exogenous transcripts near the vegetal pole. Scale bar 100 μm.

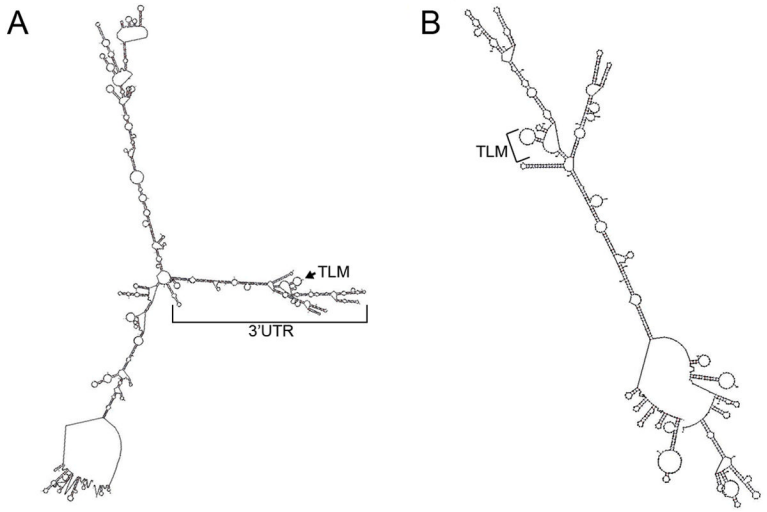

Fig. 5.
Mfold predictions of the *Hro-twist* mRNA (A) and 3′UTR (B). The teloplasm localization motif (TLM) is indicated by a bracket (mRNA) or an arrow (3′UTR).

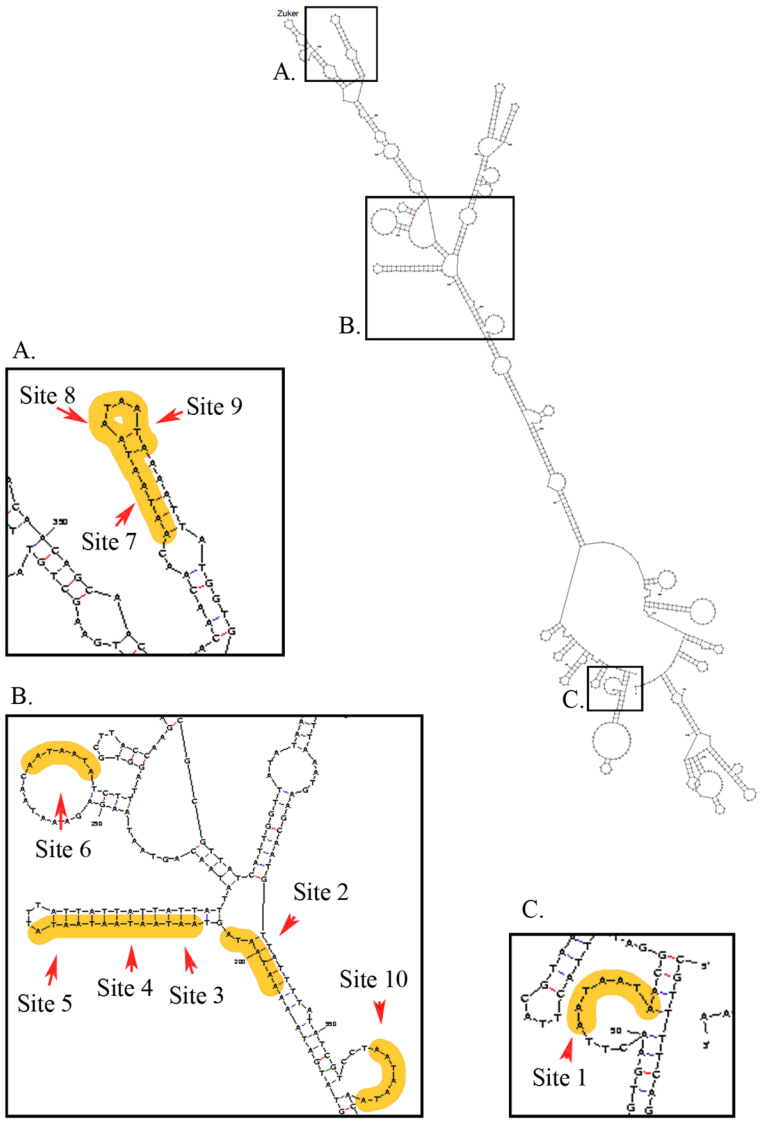

Fig. 6.

Hro-twist 3′ UTR transcript secondary structure folding and location of *ARE2* elements as determined by MFold. (A) ARE2 elements 7–9. (B) ARE2 elements 2–6, and 10. (C) ARE2 element 1. Nucleotide sequences are highlighted in yellow.

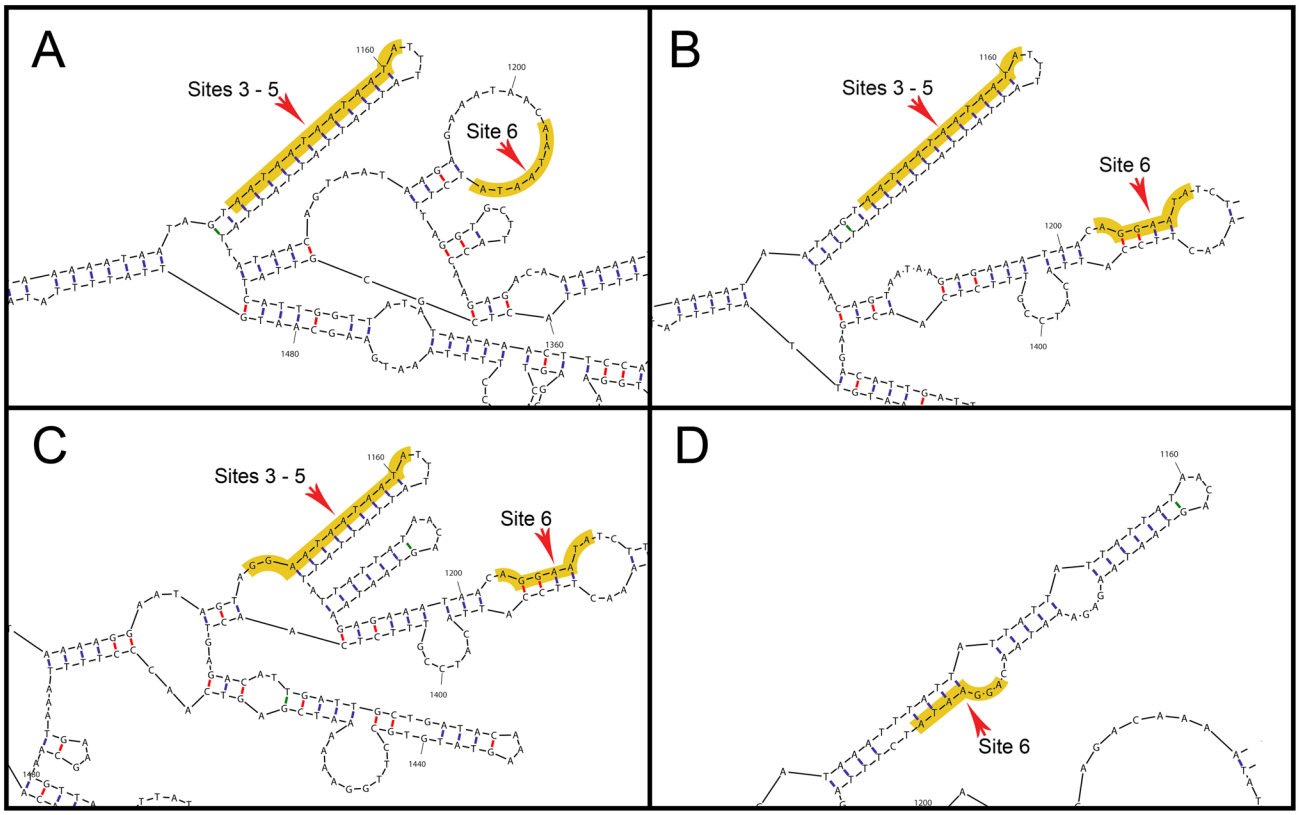

Fig. 7.

Predicted changes of the hairpin loop (ARE2 elements 3–5) and TLM structures of transcripts containing ARE2 mutations. For simplicity only the hairpin loop and the TLM are shown. (A) 3′UTR transcripts containing mutated ARE2 elements 8–10. (B) 3′UTR transcripts containing mutations in ARE2 elements 6–10. (C) 3′UTR transcripts containing mutations in ARE2 elements 1–2 and 6–10. (D) 3′UTR transcripts containing mutations in ARE2 elements 1 and 6–10, and a deletion of ARE2 elements 2–5.

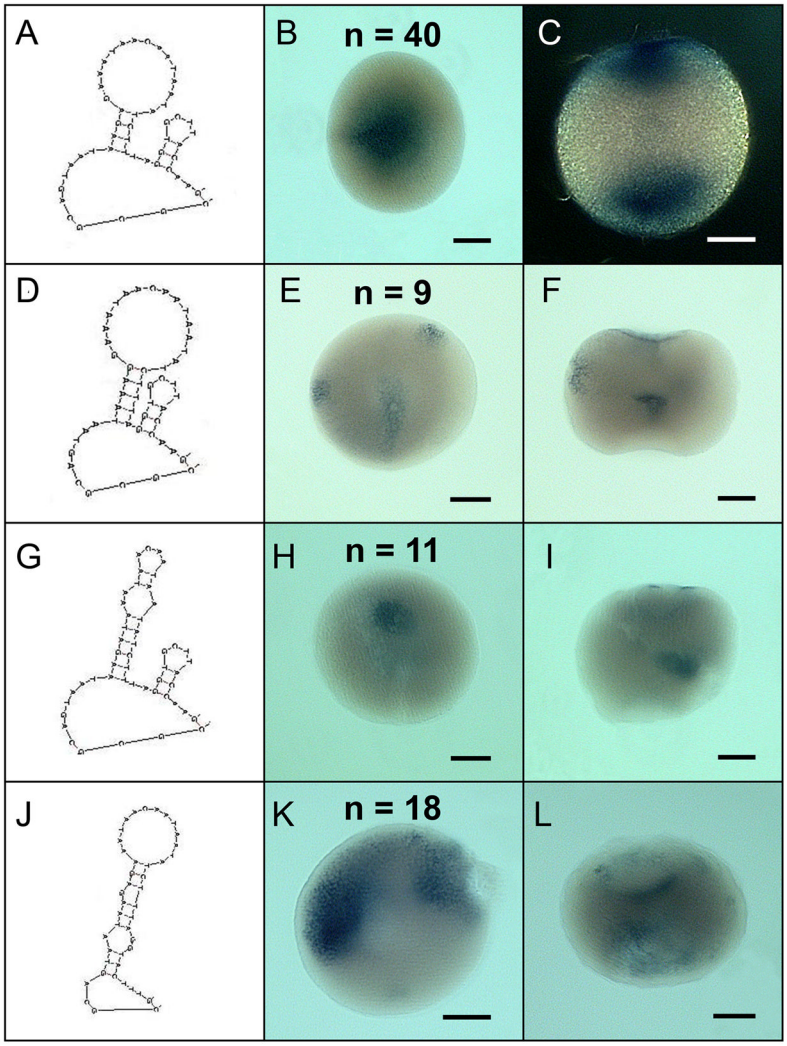

Fig. 8.

(A) Predicted secondary structure of TLM with no mutations. (B) Animal pole view of exogenous non-mutated *Hro-twist* 3′UTR localization pattern. (C) Equatorial view of exogenous non-mutated *Hro-twist* 3′UTR localization pattern. (D) Predicted secondary structure of TLM with mutation D. (E) Animal pole view of 3′UTR localization pattern of exogenous *Hro-twist* transcript containing TLM mutation D. (F) Equatorial view of 3′UTR localization pattern of exogenous *Hro-twist* transcript containing TLM mutation D. (G) Predicted secondary structure of the TLM with mutation A. (H) Animal pole view of the 3′UTR localization pattern of exogenous *Hro-twist* transcript containing TLM mutation A. (I) Equatorial view of the 3′UTR localization pattern of exogenous *Hro-twist* transcript containing TLM mutation A. (J) Predicted secondary structure of the TLM with mutation F. (K) Animal pole view of the 3′UTR localization pattern of exogenous *Hro-twist* transcript containing TLM mutation F. (L) Equatorial view of the 3′UTR localization pattern of exogenous *Hro-twist* transcript containing TLM mutation F. Scale bar 100 μm.

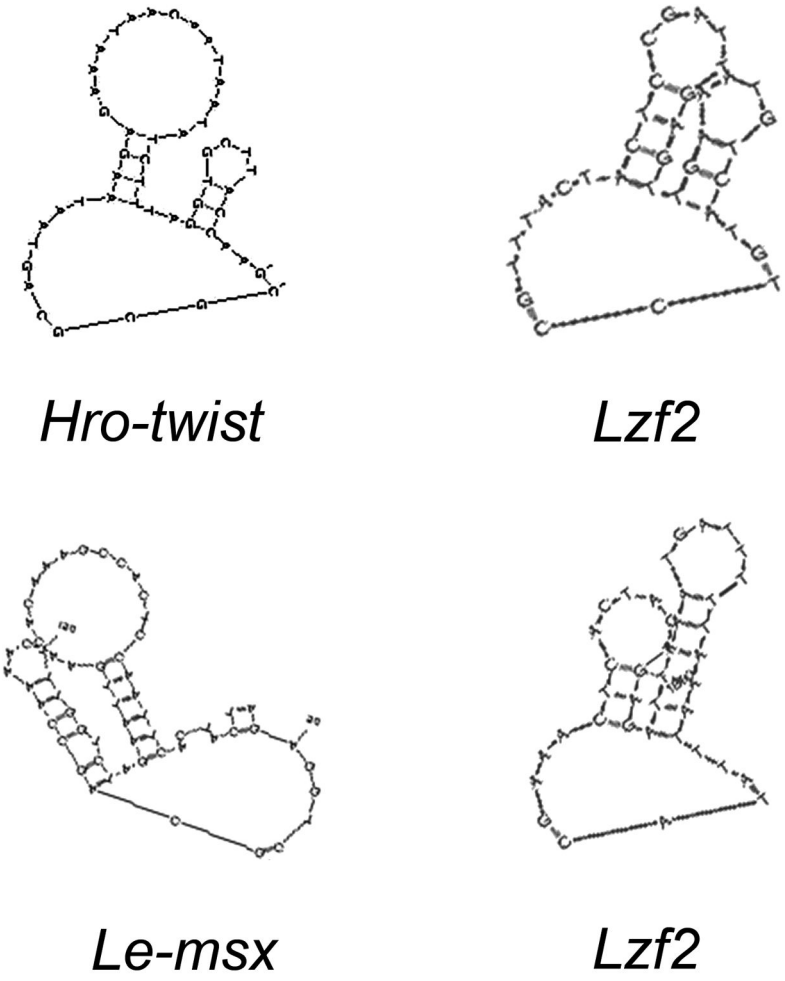

Fig. 9.
Predicted structure of the TLM in *Hro-twist* mRNA and similar motifs found in the 3′UTR of *LzF2* and *Le-msx* transcripts.

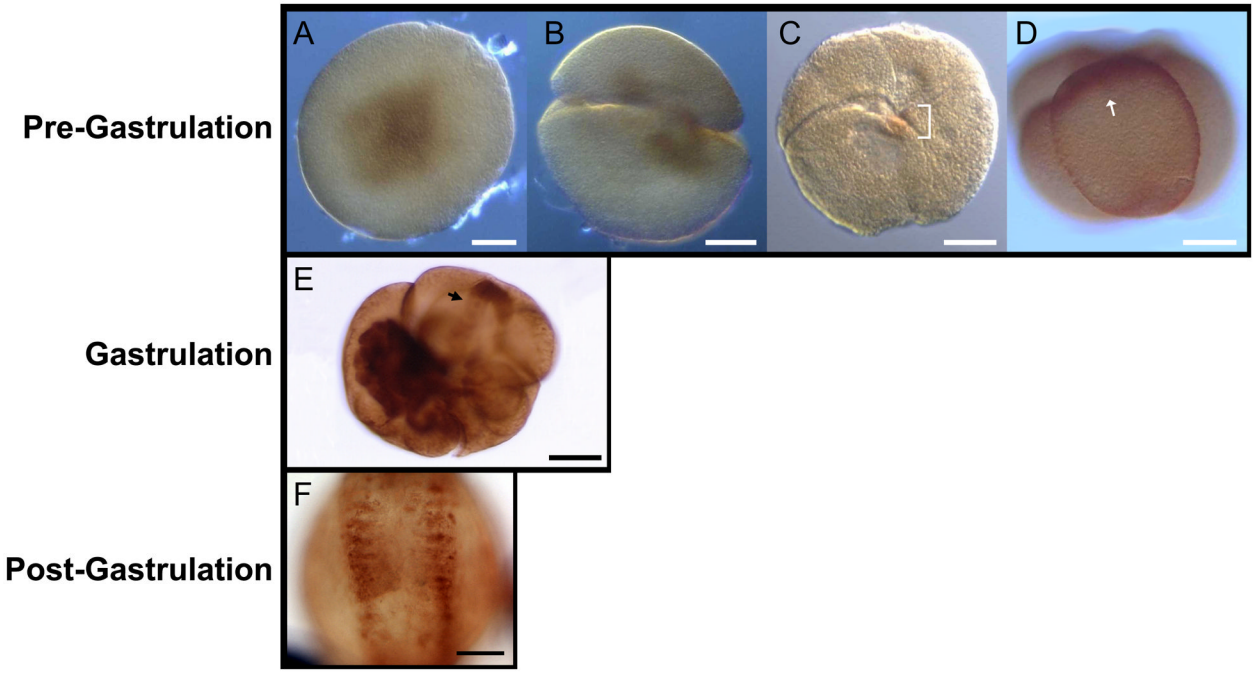

Fig. 10.

Hro-Twist protein expression during early leech development. Representative embryos at cleavage stages are shown on the top row. (A) Animal view of a one-celled embryo showing that Hro-Twist protein is present in the teloplasm. (B) Hro-Twist is mostly distributed to the CD cell. (C) At stage 3, Hro-Twist is present at low levels in the region that will form the quartet micromere. (D) At stage 4b, Hro-Twist levels have increased. Side view of embryo at stage 4b. The white arrow indicates animal teloplasm in cell DNOPQ. Embryos are viewed from the animal side with the exception of (D). (E) Animal view of an early stage 7 embryo. Hro-Twist is expressed in many of the cells in the micromere cap and in some of the ectodermal and both mesodermal teloblasts (black arrow indicates the right M bandlet). (F) Hro-Twist is expressed in an iterated pattern along the rostro-caudal axis during late 8-early 9 stages. Scale bars 100 μm.

Table 1

Presence of ARE2 sites and TLM structures in leech transcripts that localize to the teloplasm.

Transcript	ARE2 elements	TLM structures
Hro-twist	10	1
Hro-sna1[*]	none	none
Hro-dl[*]	none	none
Hro-nos	1	none
Hro-hes	2	none
Lzf2	none	2
Le-msx	4	1
Hau-pax 3/7a	none	none
Hau-pax beta1	none	none

[*] mRNA localization pattern not reported, but protein expressed at the zygotic teloplasm (Goldstein *et al.*, 2001).