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T-cell internal antigen 1 counteracts somatic RNA degradation during early *Xenopus* embryogenesis

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ABSTRACT In *Xenopus laevis*, maternal transcripts that localize to the vegetal cortex of the oocyte are specifically inherited by prospective germ cells during cleavage stages. While a large fraction of maternal transcripts is degraded during the maternal to zygotic transition (MZT), transcripts associated with the germ-line are stable. A sequence in the *dead end 1* 3'UTR mediates vegetal localization in the oocyte as well as miR mediated clearance in somatic cells and germ cell specific stabilization during the MZT in embryos. We could identify Tia1 to co-precipitate with known components of vegetal localization RNPs in *X. laevis* oocytes. Tia1 interacts and co-localizes with various localization elements from vegetally localizing RNAs. In *X. laevis* embryos, ectopic expression of Tia1 counteracts somatic degradation of *dnd1* localization element reporter RNAs and it can synergize with Dnd1 protein in reporter RNA stabilization. Ectopic Tia1 also protects several endogenous localizing and germ cell specific mRNAs from somatic degradation. Thus, proteins that protect germ-line transcripts from miR mediated decay during the MZT in embryos might bind these RNAs already in the oocyte.

KEY WORDS: *primordial germ cell, microRNA, RNA localization, vegetal, germline*

Introduction

The earliest stages of animal embryogenesis are under the control of maternal determinants. During the maternal to zygotic transition (MZT), the control of embryonic development shifts from maternally provided materials to RNA and proteins produced upon zygotic activation of transcription. This transition also involves the degradation of a large fraction of maternal mRNAs (reviewed in Tadros and Lipshitz, 2009; Langley *et al*., 2014). In zebrafish, *Drosophila* and *Xenopus*, this degradation of maternal mRNAs is mediated by microRNAs (miR) (Giraldez *et al*., 2006; Bushati *et al*., 2008; Lund *et al*., 2009).

Several germline specific mRNAs have been demonstrated to localize to the vegetal cortex during early phases of *X. laevis* oogenesis, some of these becoming part of the germ plasm (reviewed in King *et al*., 2005). During early cleavage stages, the germ cell precursor cell lineage segregates from somatic cell lineages by selective inheritance of the germ plasm (reviewed in Zhou and King, 2004). Maternally provided *dnd1* is an important regulator of germ cell development. It is however not restricted to the germ plasm. During mid-oogenesis, *X. laevis dnd1* mRNA localizes in a broad region of the vegetal cortex, nevertheless overlapping with the germ plasm (Horvay *et al*., 2006). Several proteins have been identified to mediate vegetal RNA localization during mid-oogenesis; they include Igf2bp3 (Havin *et al*., 1998), Ptbp1 (Cote *et al*., 1999), Stau1 (Yoon and Mowry, 2004), Hnrnpab (Czaplinski *et al*., 2005), Elavl1/2 (Arthur *et al*., 2009) and Celf1 (Bauermeister *et al*., 2015). During embryogenesis, *dnd1* then becomes specifically restricted to the germ cells, where the Dnd1 protein is required for germ cell migration and survival (Horvay *et al*., 2006). The restriction of *dnd1* transcripts to the germ cells is at least in part a consequence of their miR-18 mediated degradation in the soma during MZT. The

Abbreviations used in this paper: dnd1, dead end 1; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; K_D, dissociation constant; mut, mutant; LE, localization element; miR, microRNA; MZT, maternal to zygotic transition; RBP, RNA binding protein; RNP, ribonucleoprotein; RRM, RNA recognition motif; Tia1, T-cell internal antigen 1; UTR, untranslated region; wt, wild-type.

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sequence critical for somatic degradation of *dnd1* is located in its 3'UTR and it overlaps with the one that mediates its vegetal localization in the oocyte, hence referred to as localization element (LE) (Horvay *et al.*, 2006; Koebernick *et al.*, 2010).

Protein binding of Elavl2 to the *dnd1*-LE can promote vegetal localization in the oocyte as well as stability of *dnd1* mRNA in the early embryo (Arthur *et al.*, 2009; Koebernick *et al.*, 2010). The Dnd1 protein itself also appears to function in the stabilization of germ cell specific RNAs including its own mRNA. In the zebrafish, Dnd1 was previously demonstrated to serve a critical function in stabilizing the germ cell specific *nanos* and *tdrd7* mRNAs by preventing its miRNA mediated degradation (Kedde *et al.*, 2007). Similarly, in the frog, Dnd1 was demonstrated to cooperate with Elavl2 in protecting its own mRNA from miR mediated degradation in the early embryo (Koebernick *et al.*, 2010).

Here we report on the identification of Tia1 (T cell internal antigen-1) as an additional protein that is likely be part of the vegetal localization machinery on the one hand and involved in RNA protection against miR mediated degradation at MZT on the other. Tia1 was identified in the context of an attempt aiming at the identification of novel *dnd1*-LE binding proteins in *X. laevis* oocytes, making use of an RNA affinity purification approach (Bauermeister *et al.*, 2015). Our results reveal that, in *X. laevis* oocytes, Tia1 is part of one and the same RNP complex together with other proteins known to function in vegetal RNA localization. Tia1 co-localizes with granules containing vegetally localizing RNA. During MZT, overexpression of Tia1 results in the stabilization of a specific subset of vegetally localizing mRNAs, also including the *dnd1* mRNA. Moreover, ectopic Tia1 can synergize with ectopic Dnd1 protein in RNA stabilization. These findings suggest that miRNA mediated RNA turnover during MZT is at least in part regulated by proteins that bind their target RNAs already during vegetal RNA localization in *X. laevis* oocytes.

Results

Tia1 is a novel candidate component of vegetal RNA localization complexes in Xenopus *oocytes*

Using an RNP reconstitution/purification approach combined with tandem mass spectrometry (Bauermeister *et al.*, 2015), we identified *X. laevis* Tia1 as one of the proteins that specifically interact with the LE of the vegetally localizing *dnd1* mRNA using extracts from stage III/IV oocytes. Tia1 and the highly similar Tiar (Tia-related) represent the TIA-1 family of ARE (AU-rich element) binding proteins (Dember *et al.*, 1996). Both proteins possess three RNA recognition motifs (RRM) in their amino terminus as well as a glutamine-rich domain in their carboxy-terminus. In mice and humans, two isoforms of Tia1 are generated by alternative splicing, referred to as Tia1a (43 kDa) and Tia1b (40 kDa). Tia1a carries an insertion of 11 amino acids within its RRM2 (Beck *et al.*, 1996). We cloned the *X. laevis* ortholog of Tia1a, and this isoform was utilized in all subsequent experiments; it is referred to as Tia1 in the context of this study.

In order to test for a potential incorporation of Tia1 in vegetally localizing RNPs in *X. laevis* oocytes, a Flag-tagged version of Tia1 was overexpressed in localization competent stage III/IV oocytes and immunoprecipitated (Fig. 1). Celf1 and Ptbp1 were previously shown to be components of localization RNPs in *X. laevis* oocytes and served as controls (Bauermeister *et al.*, 2015; Cote *et al.*, 1999). Tia1 co-precipitates other known localization components such as Igf2bp3, Stau1, Hnrnpab and Elav-like proteins, indicating that Tia1 could indeed be a component of vegetal localization RNP complexes in *X. laevis* oocytes. The co-precipitation of these proteins is sensitive to RNAse treatment, suggesting that their interactions depend on an intact RNA scaffold.

Tia1 is a predominantly cytoplasmic protein that co-localizes with the dnd1*-LE at the vegetal cortex of* Xenopus *oocytes*

Endogenous Tia1 was detected in oocyte and embryonic extracts using a polyclonal α-Tia1 antibody, which was previously shown to detect both murine isoforms of TIA1 but not TIAR (Dixon *et al.*, 2003). We detect both isoforms of Tia1 throughout *X. laevis* oogenesis and embryonic development with highest protein levels in later stages of oogenesis and embryonic development (Fig. 2A). Tia1 was shown to shuttle between nucleus and cytoplasm in mammalian tissue culture cells (Zhang *et al.*, 2005). In *X. laevis* stage VI oocytes, Tia1 was only detected in the cytoplasm (Colegrove-Otero *et al.*, 2005). We detect Tia1 mainly in the cytoplasm throughout oogenesis, with traces of Tia1 in the nucleus of later stage oocytes (Fig. 2B). For the two proteins employed as controls, Igf2bp3 had been demonstrated to be predominantly cytoplasmic (Loeber *et al.*, 2010) and Hnrnpab to be present in both nuclear and cytoplasmic fractions (Czaplinski *et al.*, 2005).

In order to further analyze the subcellular distribution of Tia1,

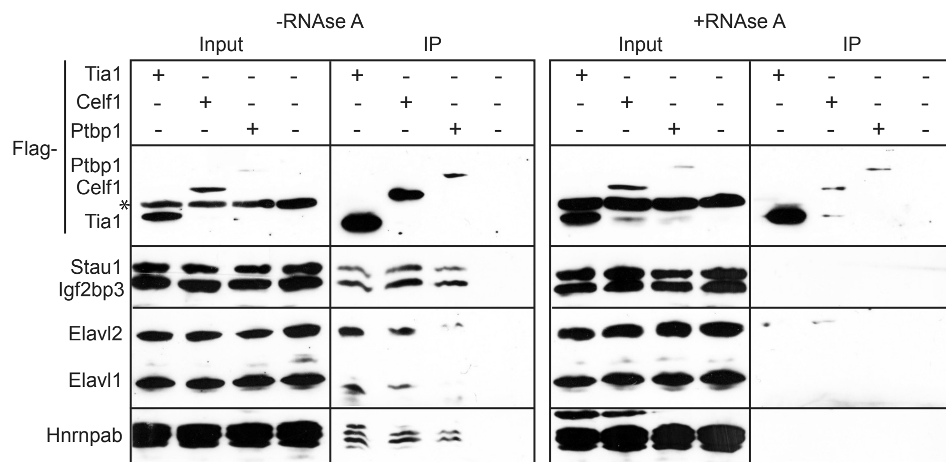

Fig 1. Tia1 co-precipitates known localization RNP complex components in *Xenopus* oocytes. *Flag-tagged versions of different localization proteins and Tia1 were expressed in* Xenopus *oocytes by means of RNA microinjection and analyzed by Western blot for co-precipitation of known localization factors in the absence or presence of RNase A. Celf1 and Ptbp1 served as controls for known localization complex components. Immunoprecipitation with extract from uninjected oocytes served as a negative control. The asterisk indicates an unspecific signal caused by cross-reaction of the anti-Flag antibody.*

immunostaining of albino stage III oocytes injected with Cy3-*dnd1*-LE RNA was performed. As reported before (Bauermeister *et al.*, 2015), Cy3-*dnd1*-LE becomes enriched at the vegetal cortex and in granular structures in the cytoplasm of the vegetal hemisphere (Fig. 2C). Endogenous Tia1 co-localizes with Cy3-*dnd1*-LE at the vegetal cortex and in the vegetally located granular structures (Fig. 2C). In summary, Tia1 is predominantly cytoplasmic throughout oogenesis and it is enriched at the vegetal cortex, co-localizing with the *dnd1*-LE.

Tia1 directly interacts with vegetal LEs and binding to the dnd1*-LE requires an AU-rich region*

Tia1 was identified by an RNA affinity approach using the *dnd1*-LE (Bauermeister *et al.*, 2015). In order to analyze if Tia1 directly binds to the *dnd1*-LE and/or to other vegetal LEs, an *in vitro* interaction assay was performed. *In vitro* translated Flag-Tia1 (Fig. S1A) was incubated with different Cy3-labeled LE RNAs followed by an anti-Flag-co-immunoprecipitation. Tia1 interacts with the *dnd1*-LE and not with the *velo1*-LE nor with the *β-globin*-3'UTR; weak interaction was detected with the *gdf1*-LE and the *grip2*-LE (Fig. 3A). Celf1 and Ptbp1 served as controls, as they are known to interact with the *dnd1*-LE as well as with the *grip2*-LE, and the latter protein also with the *gdf1*-LE (Fig. 3A; Bauermeister *et al.*, 2015).

In order to determine the region critical for Tia1 interaction within the *dnd1*-LE, we used *in vitro* translated Flag-Tia1 (Fig. S1 B,C) and Cy3-labeled full length or 5'- and 3'- truncated fragments derived from the *dnd1*-LE (Fig. 3B). A loss of Tia1 binding is observed with deletions beyond nucleotide 40 (5'del2) of the *dnd1*-LE, while 3' deletions up to nucleotide 106 (3'del5) maintain binding to Tia1 (Fig. 3B). Thus, the region critical for Tia1 binding is located between nucleotides 40-106 (Fig. 3B).

Tia1 was previously shown to bind to AU-rich elements (ARE) (Dember *et al.*, 1996; Piecyk *et al.*, 2000; López de Silanes *et al.*, 2005). The Tia1 binding region in the *dnd1*-LE exhibits AREs composed of several adenines embedded in uracil-rich stretches (Fig. 4A). In order to create a mutant version of the *dnd1*-LE that is deficient in Tia1 binding, several uracil bases (U) were substituted for adenine bases (A), while the miR-18 binding site is maintained (Fig. 4A). Since the 5'del1 truncated *dnd1*-LE fragment shows full vegetal localization activity in *X. laevis* oocytes (data not shown), the effect of A to U point mutations on Tia1 binding was analyzed using this 232 nucleotide long *dnd1*-LE fragment (Fig. 3B, 4B). Binding of Tia1 to wild-type and mutated *dnd1*-LEs was analyzed by electrophoretic mobility shift assays (EMSA) using bacterially expressed Tia1 and Cy3-labeled RNAs (Fig. S2A, B, 4B). Binding of Tia1 to the mutant *dnd1*-LE is reduced in comparison to the wild-type *dnd1*-LE, with a dissociation constant (K_D) that is approximately 5 fold increased (Fig. 4B), while high affinity binding of Elavl2 was maintained; however, although mutant *dnd1*-LE is reduced considerably in binding to Tia1, it shows full localization capacity when injected into oocytes (data not shown). Thus, a possible function of Tia1 in the context of vegetal RNA localization remains to be demonstrated.

Ectopic expression of Tia1 leads to stabilization of Tia1 binding RNAs during early embryogenesis

In previous studies, we have reported that Elavl proteins, which are components of vegetal localization RNPs in oocytes, counteract miR mediated

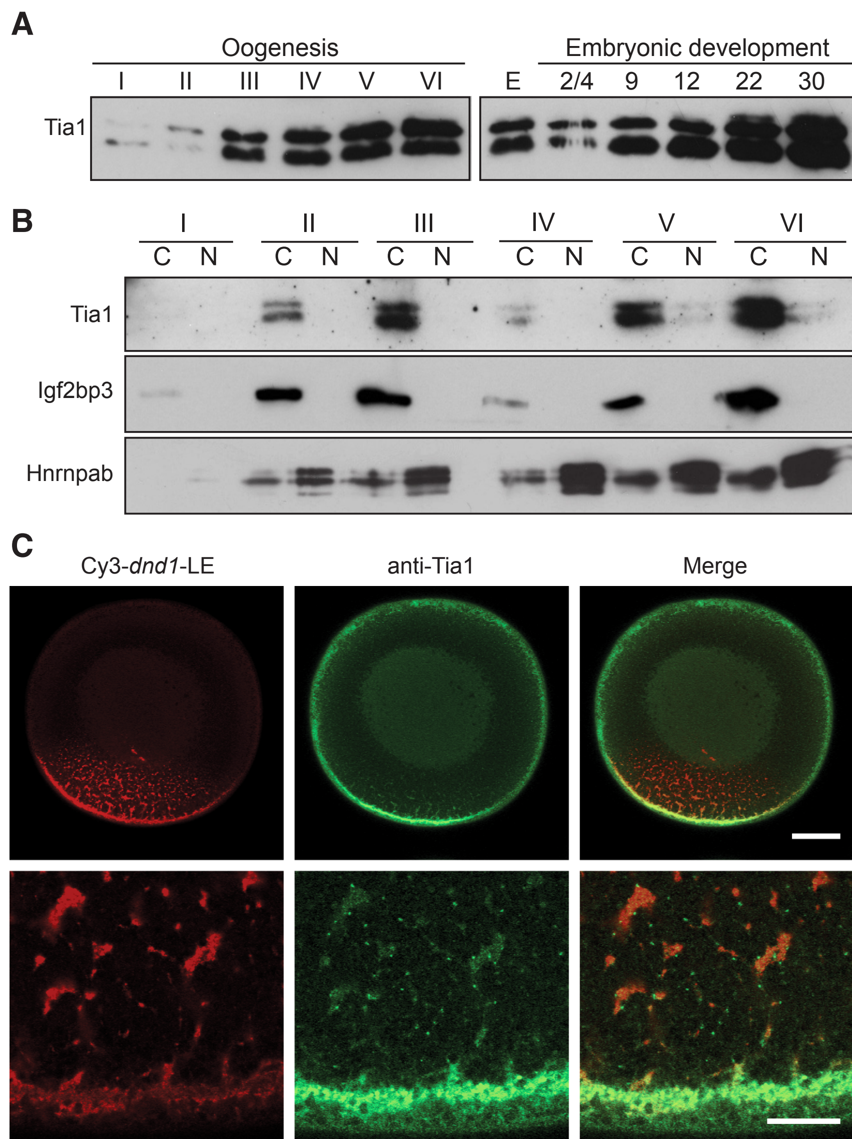

Fig 2. Tia1 is predominantly cytoplasmic and co-localizes with *dnd1*-LE-RNA at the vegetal cortex in *Xenopus* oocytes. (A) *Temporal analysis of Tia1 expression during* Xenopus *oogenesis and embryogenesis. Western blot analysis of Tia1 with equivalent amounts of oocyte and embryonic extracts; stages of oogenesis and embryogenesis were as indicated.* **(B)** *Western blot analysis of Tia1 with cytoplasmic (C) and nuclear (N) fractions from staged oocytes. Igf2bp3 served as control for a cytoplasmic protein and Hnrnpab was utilized as control for a predominantly nuclear protein.* **(C)** *Co-localization analysis of endogenous Tia1 protein and vegetally localizing, microinjected Cy3-*dnd1*-LE in* Xenopus *oocytes. Tia immunostaining was performed on Cy3-*dnd1*-LE RNA injected stage III oocytes. Scale bars indicate 100 μm (whole oocyte) and 20 μm (magnification).*

degradation of the *dnd1*-LE during MZT in *X. laevis* embryos (Arthur *et al*., 2009; Koebernick *et al*., 2010). In order to analyze if Tia1 carries a similar function in *dnd1* mRNA protection, wild-type and mutant *dnd1*-LE reporter RNAs were injected into 2-cell stage embryos and RNA levels were detected by whole mount *in situ* hybridization in stage 32-34 (Fig. 5A). Both wild-type and mutant *dnd1*-LE-reporter RNAs are degraded in somatic cells during MZT but are stable in PGCs (Fig. 5B, C). However, ectopic expression of Tia1 leads to somatic stabilization of wild-type *dnd1*-LE, but not of the mutant *dnd1*-LE-reporter RNA that is reduced in Tia1 binding (Fig. 5B, 5C), suggesting that somatic stabilization of wild-type *dnd1*-LE-reporter RNA during MZT involves Tia1 binding. Although mutations in the *dnd1*-LE reporter RNA that result in moderately or highly impaired Elavl2 binding lead to its global destabilization (Köbernick *et al*., 2010), the mutant *dnd1*-LE utilized in this study, which is defective in Tia1-binding but maintains high affinity Elavl2-binding, is stable in PGCs (Fig. 5). This result indicates that, while Tia1-binding is not essential, Elavl2-binding could be sufficient for the stabilization of *dnd1*-LE reporter RNA in PGCs.

Next, we analyzed if endogenous *dnd1* mRNA and/or other localizing mRNAs are stabilized by ectopic expression of Tia1. Two different concentrations of *tia1* mRNA were injected into 2-cell stage embryos and total RNA was extracted just before MZT (stage 8), during MZT (stage 11) and after MZT (stage 14). Different localizing mRNAs (*dnd1*, *grip2*, *germes* and *gdf1*) show increased RNA levels during or after MZT, but not before MZT, in *tia1* injected embryos in a dose dependent manner (Fig. 5D). Other localizing mRNAs (*velo1*, *pgat*, *trim36*), the non-localizing miR target RNA *ccnb2* (Lund *et al*., 2009) as well as a housekeeping gene (*g6dph*) are not affected by Tia1 overexpression (Fig. 5D). With the exception of *gdf1* mRNA, stabilized mRNAs are germ cell specific (*germes*, *dnd1* and *grip2*). Thus, Tia1 seems to be involved in stabilization of several, but not all vegetally localizing mRNAs during MZT.

Tia1 and Dnd1 proteins synergize in the somatic stabilization of dnd1*-LE RNA*

As described previously (Rothé *et al*., 2006), we detect *tia1* expression mainly in neural tissues of *X. laevis* embryos, but not enriched in PGCs. However, RNA sequencing analyses had revealed that *tia1* transcripts are present in *X. laevis* PGCs (our own unpublished results). This raises the question as to how Tia1 mediates protection of germline-specific mRNAs. We could show earlier that Elavl2 and Dnd1 cooperate in stabilizing *dnd1* mRNA (Koebernick *et al*., 2010). To test if Tia1 and Dnd1 might also act synergistically, we co-injected suboptimal doses of RNA encoding for either protein into embryos and analyzed *dnd1*-LE reporter RNA stability by WMISH (Fig. 6A, B). Ectopic expression of Tia1 or Dnd1 proteins in these low amounts leads to weak or no stabilization of *dnd1*-LE reporter RNA, while co-expression of both proteins leads to robustly increased *dnd1*-LE reporter RNA levels (Fig. 6A, B). Thus, Tia1 and Dnd1 proteins might synergize in protecting germline RNAs from miR mediated decay.

In summary, our results suggest that

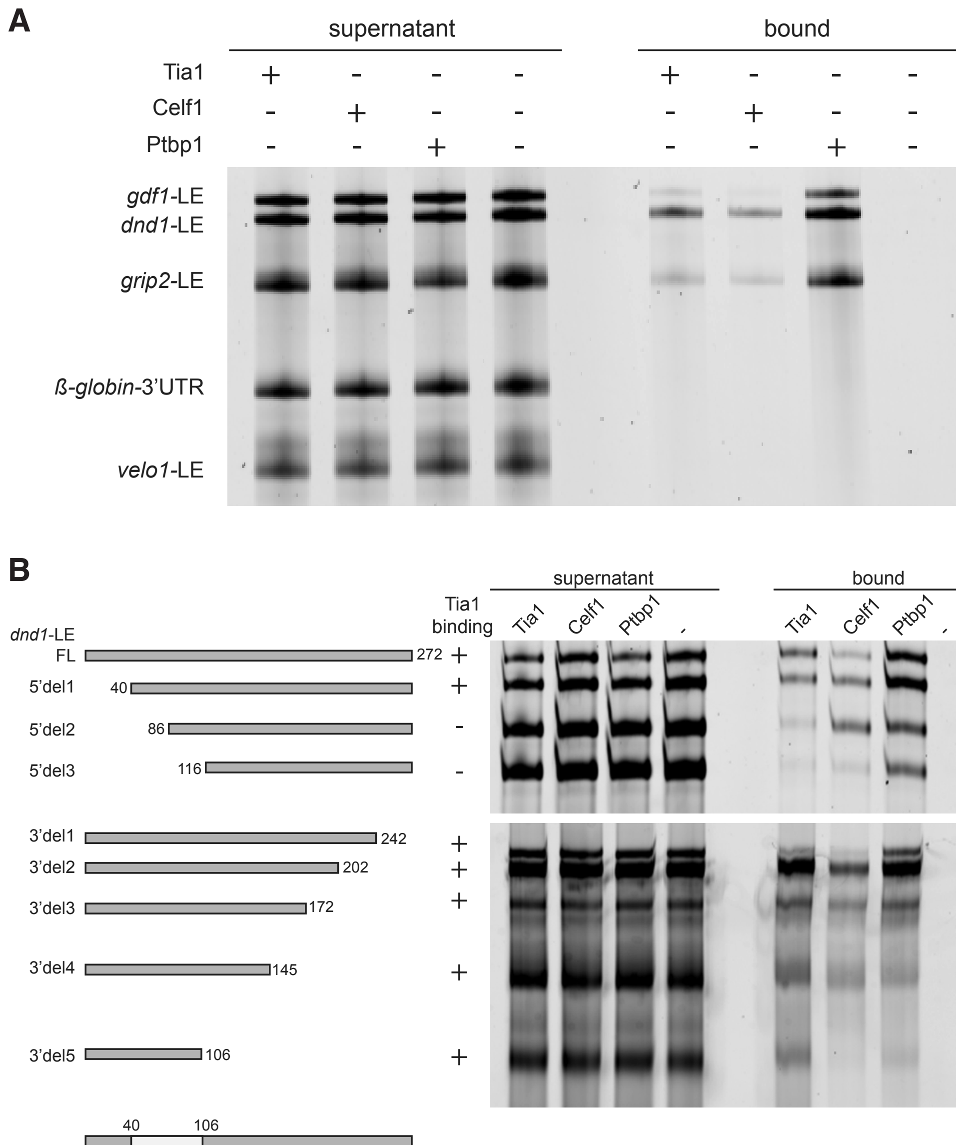

Fig 3. Tia1 binds to the 5′ region of the *dnd1*-LE. (A) In vitro *interaction of Flag-tagged Tia1 with different LE RNAs. Cy3-labeled LE RNAs and* β-globin-*3'UTR control RNA were co-immunoprecipitated with Flag-tagged,* in vitro *translated Tia1, Celf1 or Ptbp1. Non-programmed reticulocyte lysate served as a negative control (-). Supernatant and bound RNAs were separated by UREA-PAGE and detected by fluorescence imaging.* **(B)** In vitro *interactions of Flag-tagged Tia1 with Cy3-labeled full length (FL) and 5´- or 3´-deleted fragments of the* dnd1*-LE RNA. The region critical for Tia1 binding is marked in light grey (nt 40-106).*

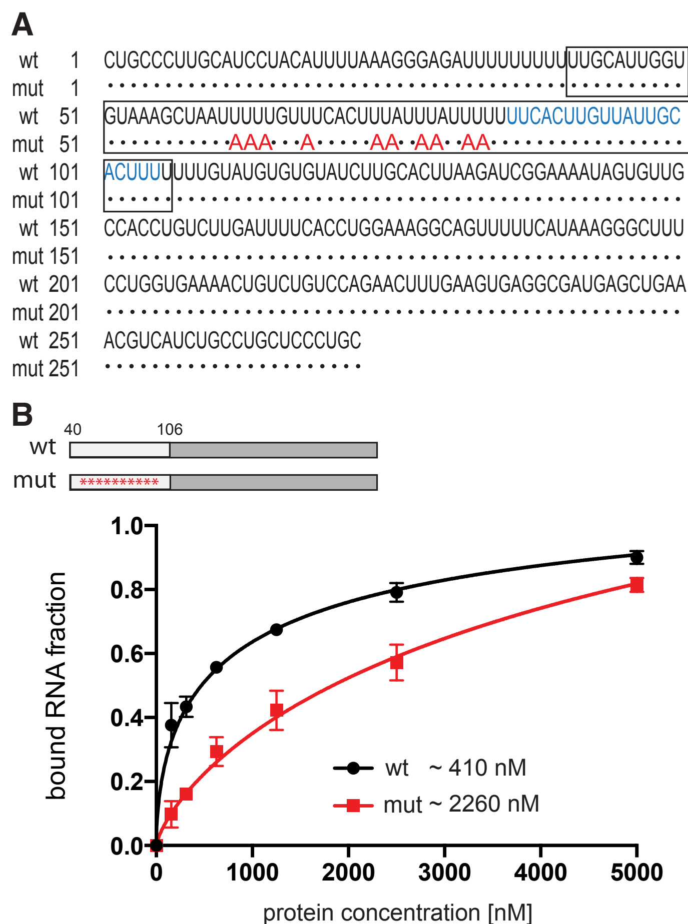

Fig 4. Mutation of the uracil-rich 5′region in the *dnd1*-LE reduces Tia1 binding. (A) *Sequence of full length wild-type (wt) and mutant (mut)* dnd1-*LE. Multiple uracil bases in the Tia1 binding site (boxed) were replaced by adenines by site-directed mutagenesis (red letters). The miR-18 target is shown in blue.* **(B)** *Binding of bacterially expressed Tia1 to wild-type (wt) and mutant (mut)* dnd1*-LE was analyzed by electrophoretic mobility shift assays (see Figure S2B). The relative amount of complexed RNA is plotted against the individual protein concentrations using non-linear curve fitting; approximate K_D values for wild-type and mutant* dnd1*-LE RNAs are indicated.*

Tia1 is a potential novel component of vegetal localization RNPs in *X. laevis* oocytes and, although its role during vegetal RNA localization is unclear, we found that it may function in the context of stabilizing vegetally localizing mRNAs during MZT.

Discussion

We identified the RNA binding protein Tia1 as a potential novel component of vegetal localization complexes in *X. laevis* oocytes. Tia1 directly interacts and co-localizes with vegetal LE RNAs. Ectopic expression of Tia1 in *X. laevis* embryos leads to somatic stabilization of a *dnd1*-LE reporter RNA and several other endogenous vegetally localizing germ cell RNAs. Co-expression of Tia1 with Dnd1 increases *dnd1*-LE reporter RNA stabilization.

Tia1 is an RRM-type protein that has been reported to act in the regulation of alternative splicing (Del Gatto *et al.*, 1997; Förch *et al.*, 2000; Zhu *et al.*, 2003) and translation of diverse transcripts by binding to AU-rich sequence elements (Piecyk *et al.*, 2000; Dixon *et al.*, 2003; Kandasamy *et al.*, 2005). Tia1 can also promote a general translational arrest in response to environmental stress by formation of stress granules (Kedersha *et al.*, 1999; Gilks *et al.*, 2004). Tia1 was shown to shuttle between nucleus and cytoplasm with a predominantly nuclear localization in somatic cells (Kedersha *et al.*, 1999; Zhang *et al.*, 2005). In *X. laevis* oocytes, Tia1 has been detected predominantly in the cytoplasm (Colegrove-Otero *et al.*, 2005) and *tia1* transcripts are expressed throughout embryogenesis with highest RNA levels in neural tissues (Rothé *et al.*, 2006).

Tia1, a translational regulator in oocytes?

Although Tia1 might be a component of vegetally localizing RNPs in oocytes, we were unable to provide direct experimental evidence for an essential function in vegetal RNA transport. However, Tia1 might exert other functions on vegetal RNA localization. Tia1 has been shown to act in the translational repression of diverse mRNAs in mice (Piecyk *et al.*, 2000; Dixon *et al.*, 2003; Yu *et al.*, 2003). A number of other localization complex components have been reported to function in translational repression in different biological systems (Kim-Ha *et al.*, 1995; Colegrove-Otero *et al.*, 2005; Hüttelmaier *et al.*, 2005). However, tethering of Tia1 to a reporter RNA did not mediate translational repression in oocytes (data not shown), indicating that Tia1 is not sufficient for translational repression of transcripts.

The translational activity of a mutant *dnd1*-LE reporter RNA with reduced affinity for Tia1 as compared to a wild-type *dnd1*-LE reporter RNA was also not affected (data not shown). Similarly, Tia1 was found to bind to the *gdf1* translational element, but depletion of Tia1 from oocyte extracts did not affect translational activity of a *gdf1* reporter RNA (Colegrove-Otero *et al.*, 2005). Thus, Tia1 might not regulate the translation of dnd1 or gdf1 transcripts, or Tia1 mediated translational repression is not detectable by the reporter assays described above, as it might require other regions in the mRNA.

Self aggregation of Tia1 - a role in the multimerization of localization RNPs and formation of stress granules?

Stress granules are transient cytoplasmic aggregates of mRNA that contain 48S translation initiation factors, poly(A)-binding protein 1, and small ribosomal subunits (reviewed in Anderson and Kedersha, 2008). Tia1 is a known component of stress granules in somatic cells, promoting their formation by self-aggregation via its prion-like C-terminal domain (Tian *et al.*, 1991; Gilks *et al.*, 2004). Strikingly, other known localization RNP components like Staufen1 and Elavl proteins have been reported to be components of stress granules in other systems (Gallouzi *et al.*, 2000; Thomas *et al.*, 2005). Thus, localization RNPs and stress granules appear to contain overlapping components. *C. elegans* oocytes form large RNP granules in response to stress that contain Tia1 (Jud *et al.*, 2008; Patterson *et al.*, 2011), whereas, to our best knowledge, the presence of stress granules in *Xenopus* oocytes has not been reported to date. Nevertheless, Tia1 might promote multimerization of localization RNPs. Indeed, localization RNPs are thought to oligomerize into larger complexes that are co-transported to the vegetal cortex (reviewed in Pratt and Mowry, 2012; Xing and Bassell, 2013). *Oskar* mRNA, for example, localizes to large granules that are translationally silent during transport to the posterior pole of *Drosophila* oocytes (Chekulaeva *et al.*, 2006). Further evidence for the transport in granules containing multiple different RNA mol-

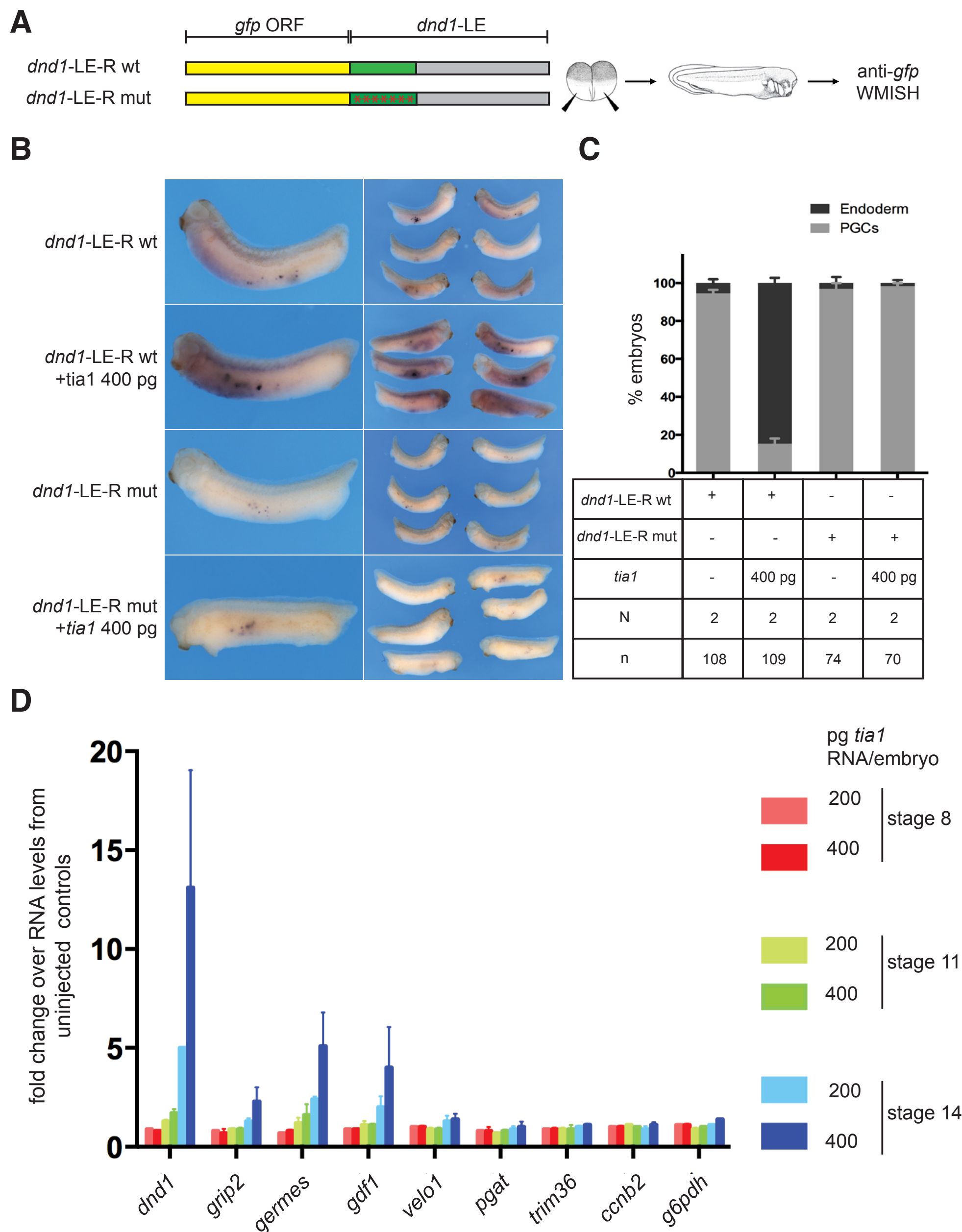

Fig 5. Somatic expression of Tia1 stabilizes injected *dnd1*-LE reporter RNA and endogenous localizing mRNAs. (A) *Wild-type (wt) or mutant (mut)* dnd1*-LE-reporter (*dnd1*-LE-R) RNAs containing the gfp open reading frame were injected into both vegetal blastomeres of 2-cell stage* Xenopus *embryos. At stage 32, whole mount* in situ *hybridization was performed against* gfp. **(B)** *Embryos injected with wildtype or mutant* dnd1*-LE-reporter RNAs alone or co-injected with 400 pg RNA encoding Tia1.* **(C)** *Quantification of* dnd1*-LE-reporter RNA levels as scored in the injected embryos. Mean values of 2 independent experiments are shown. Error bars indicate the standard error of the mean.* **(D)** *Fold change in RNA levels of different endogenous localizing and control mRNAs at the developmental stages indicated after overexpression of Tia1.* Xenopus *2-cell stage embryos were injected with 200 or 400 pg* tia1 *RNA. Embryos were grown until stages 8, 11 or 14 and subjected to total RNA extraction. RNA samples were analyzed using the nCounter® Gene Expression assay (NanoString Technologies). The averaged fold change of selected RNAs over uninjected control embryos of two independent experiments are shown, error bars indicate the standard error of the mean.*

ecules comes from studies on *ASH1* and *IST2* mRNAs in yeast (Lange *et al.,* 2008). However, yet different experiments support the idea of independent localization of singular RNPs; Amrute-Nayak and Bullock (2012) found that individual mRNA molecules are present in distinct reconstituted *Drosophila* RNPs. Similarly, different dendritic mRNAs were shown to localize to distinct RNPs, and might thus contribute to a higher efficiency of temporal and spatial control of protein expression in neurons (Mikl *et al.*, 2011). We observed that a mutant *dnd1*-LE RNA with reduced affinity for Tia1 shows full localization capacity in *Xenopus* oocytes and is found to be present in similar granular structures as observed for wild-type *dnd1*-LE RNA (data not shown). Different localization RNP components might thus act redundantly in RNP multimerization, or the same process might be dispensable for vegetal RNA transport.

Tia1 – a function in RNA stabilization during embryogenesis?

Over-expression of Tia1 counteracts the somatic degradation of several maternal, vegetally localizing mRNAs in *X. laevis* embryos at MZT. Tia1 also co-localizes with these RNAs in the oocyte, suggesting that it remains bound to its RNA targets upon fertilization. Similarly, Elavl2 was also shown to bind localizing RNAs in the oocyte and to stabilize the same transcripts in the embryo (Koebernick *et al.*, 2010). Most of the RNAs stabilized by Tia1, such as *dnd1, germes* and *grip2,* are germ cell specific, indicating that Tia1 might exert a specific function in the protection of germline RNAs from miR mediated decay. However, although other germ cell specific mRNAs are largely degraded during MZT *(pgat, trim36*), they are not affected by Tia1 overexpression, suggesting that Tia1 is involved in the stabilization of only a subset of maternally expressed germline RNAs. Tia1 seems not to be essential for the stabilization of germ cell specific transcripts, as a mutant *dnd1*-LE reporter RNA with vastly reduced affinity for Tia1 is stable in PGCs. Thus, there must be additional proteins involved in the protection of germ cell specific transcripts, such as Elavl2 (Koebernick *et al.*, 2010). Tia1 has been reported to be mainly expressed in neural tissue during *X. laevis* development (Rothé *et al.*, 2006), but *tia1* transcripts are detectable in *X. laevis* germ cells as well, as revealed by RNA sequencing (our own unpublished results). Thus, germ

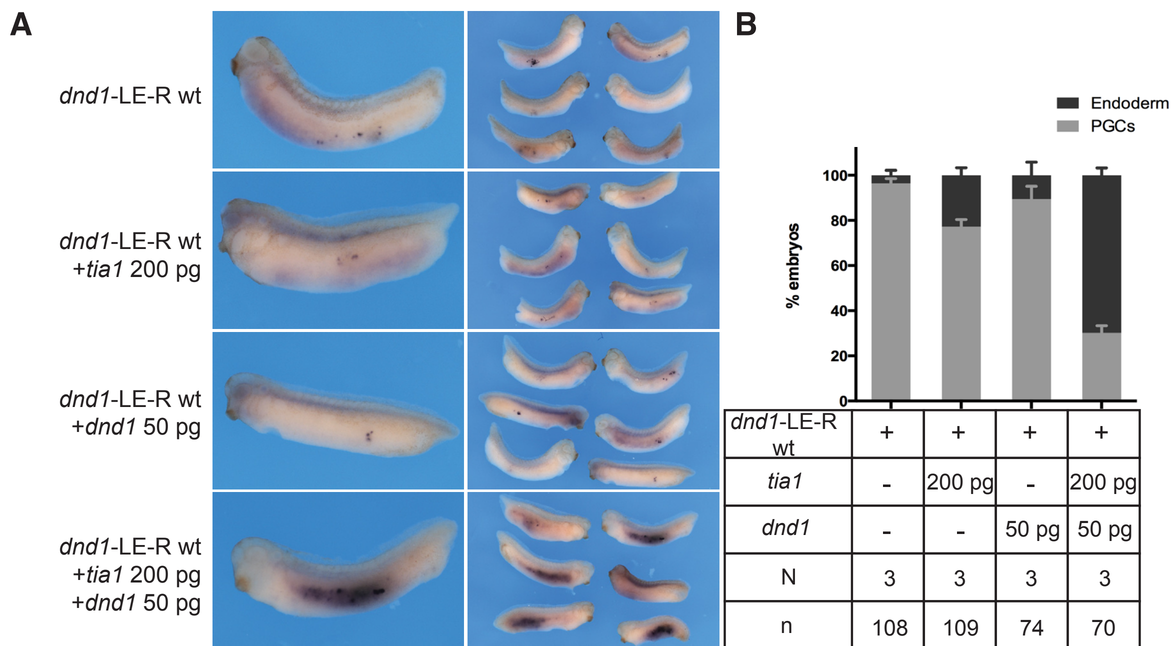

Fig 6. Tia1 synergizes with Dnd1 in the stabilization of *dnd1*-LE-reporter RNA in *Xenopus* embryos. (A) *Embryos injected with* gfp-dnd1*-LE reporter RNA (*dnd1*-LE-R wt) alone or co-injected with low doses of RNA encoding either Tia1 or Dnd1 or both. Reporter RNA levels were detected by WMISH at stage 32. Injected wild-type* dnd1*-LE reporter RNA alone is degraded in the soma and stable in PGCs. Over-expression of low doses of Tia1 or Dnd1 does not stabilize* dnd1*-LE reporter RNA in the soma. Co-expression of same doses of Tia1 and Dnd1 leads to somatic stabilization of* dnd1*-LE reporter RNA.* **(B)** *Quantification of* dnd1*-LE reporter RNA levels scored in the injected embryos. Mean values of three independent experiments are shown, error bars indicate the standard error of the mean.*

cell specific RNA protection might involve interaction of Tia1 with additional germ cell specific proteins, similar to what was shown for Elavl2 and Dnd1; even though Elavl2 is not germ cell specific, it synergizes with the germ cell specific Dnd1 in RNA stabilization.

Although TIA1 and the highly similar TIAR are suggested to act partially redundant, TIAR is essential for germ cell development in mice (Beck *et al.*, 1998), while TIA1 knock-out mice are fully fertile (Piecyk *et al.*, 2000). However, the differences of both proteins that enable a unique function of TIAR in germ cell development are not defined so far.

Ectopic expression of Tia1 also leads to stabilization of localizing mRNAs that are not germ cell specific, including *gdf1*. Although the LE of *gdf1* mRNA does not mediate somatic degradation (Koebernick *et al.*, 2010), *gdf1* transcript levels decrease during MZT (Birsoy *et al.*, 2006; Table S1-S4). Thus, *gdf1* mRNA might contain miR target sites outside of the LE, or *gdf1* mRNA is degraded by other mechanism than miR-mediated decay, which endogenous Tia1 seems to counteract. Maternal *gdf1* transcripts seem to be essential for early development, as the antisense depletion of *gdf1* mRNA in *Xenopus* embryos leads to gastrulation defects (Birsoy *et al.*, 2006). Thus, Tia1 might also stabilize other maternal RNAs that are involved in specific aspects of early embryonic development other than germ cell development.

In *X. laevis*, the degradation of *dnd1* mRNA during MZT appears to be mediated by miR-18 (Koebernick *et al.*, 2010). Thus, *X. laevis* Tia1 might protect target RNAs by antagonizing miR-18 binding. Similarly, Dnd1 protein is suggested to prevent binding of miR-430 to the germline mRNAs *nanos* and *tdrd7* in zebrafish (Kedde *et al.*, 2007). Indeed, Tia1 binds to the 5'-part of the *dnd1*-LE, overlapping with the miR-18 target site, and it might thus prevent miR-mediated RNA degradation by physically blocking the target site of miR-18. On the other hand, Tia1 could also alter the RNA structure and thereby inhibit miR binding. We also can not exclude other mechanisms to counteract miR mediated decay for example by controlling poly A tail length as suggested for Dazl (Takeda *et al.*, 2009). Additional efforts will be required in order to clarify the mechanism of Tia1 mediated RNA stabilization.

Materials and Methods

DNA cloning and protein expression

The open reading frame of Tia1 was amplified by PCR from *X. laevis* oocyte cDNA and subcloned into EcoRI and XhoI of pCS2+Flag vector using the following primers GGAATTCTATGGAGGAAGATCTACCCAAAACG (forward), CCGCTCGAGTCATTGTGTTTGGTATCCAGC (reverse). The sequence of the Tia1 ORF cloned here is identical to the Tia1 nucleotide sequence published by Rothé *et al.,* (2006) (NCBI accession number AJ416631), with the exception of a 9 nucleotide deletion. Celf1 and Ptbp1 expression constructs were cloned as described in Bauermeister *et al.,* (2015). The Dnd1 expression construct is described in Koebernick *et al.,* (2010).

For expression of recombinant proteins, the Tia1 coding region was amplified using primers containing Nhe1 and Xho1 restriction sites, and the PCR product was cloned into the expression vector pET21a (Novagen). His-tagged, recombinant protein was purified as described previously (Bauermeister *et al.*, 2015; Git and Standart, 2002). The generation of dnd1-LE deletion fragments was as we have described earlier (Bauermeister *et al.*, 2015). The mutant version of 5'del1 *dnd1*-LE was generated by site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis Kit (Agilent technologies) following the manufacturer's instructions. For embryo injection, the mutant and wt 5'del1 *dnd1*-LE was amplified with primers containing XhoI and Not1 restriction sites and cloned into the GFP

construct that was previously described (Koebernick *et al*., 2010).

In vitro *transcription*

In vitro transcription of capped and labeled RNAs was done as described before (Bauermeister *et al*., 2015).

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as previously described (Bauermeister *et al*., 2015).

Oocyte and embryo treatment

Oocytes were isolated and extracts prepared as described before (Bauermeister *et al*., 2015). For Western blot analysis 2 oocytes or nuclei/ cytoplasm were used per lane. Oocyte injection was done essentially as described by Claussen and Pieler, 2004. For injection of RNA encoding Flag-tagged proteins, 1 to 1.5 ng capped RNA was injected into the cytoplasm of stage III oocytes in 3 nl volume. Oocytes were incubated for 24 h at 18 °C in MBSH and homogenized. Oocytes for immunofluorescence analysis were injected and cultured as described (Bauermeister *et al*., 2015). Embryos were obtained according to standard procedures (Sive *et al*., 2000). 2-cell stage embryos were injected vegetally with varying amounts of capped RNA and 500 pg *dnd1*-LE reporter RNA in a volume of 4 nl into both blastomeres, cultivated at 12.5 °C for 6 days and fixed at stage 32-34. Embryos were processed for *in situ* hybridization as described previously (Harland, 1991) using digoxigenin-labeled RNA probes directed against the GFP encoding portion.

Immunofluorescent staining of oocytes

Immunostaining of oocytes was done as described (Gagnon and Mowry, 2011). The primary anti-Tia1 antibody (sc-1751, Santa Cruz) was diluted 1:250 and the secondary anti-goat-Alexa633 antibody (A21082, Invitrogen) was diluted 1:500 in PBT + 2 % horse serum and 2 % BSA. Fluorescence was visualized by confocal imaging (LSM780, Zeiss).

Co-immunoprecipitations

Co-immunoprecipitation of proteins from oocyte lysate was done as described (Bauermeister *et al*., 2015). Co-immunoprecipitation of Cy3-labeled RNAs was performed essentially as described by Claussen *et al*., 2011 using anti-Flag M2 affinity gel (Sigma).

Western blotting

Protein samples were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Anti-Tia1 (sc-1751, Santa Cruz) was diluted in 5 % non-fat milk in TBST at 1/1000 and incubated overnight at 4 °C. The primary antibodies anti-Elavl (anti-HuR; sc-5261; Santa Cruz Biotechnology, Inc.), anti-Igf2bp3 (Vg1RBP) (Zhang *et al*., 1999), anti-Stau1 (Allison *et al*., 2004), anti-Hnrnpab (40LoVe) (Czaplinski *et al*., 2005) as well as secondary antibodies were employed and detection performed as described (Bauermeister *et al*., 2015).

Quantitative multiplex RNA analysis

Injected *X. laevis* embryos were grown until stages 8, 11 or 14 and total RNA was extracted from a pool of five embryos using peqGOLD TriFast (PEQLAB) after manufacturer's instructions followed by DNase I treatment (Thermo Scientific). RNA samples were analyzed using the nCounter® Gene Expression assay (NanoString Technologies) following the manufacturer's instructions. 500 ng of each RNA sample were incubated for 16 h at 65 °C in hybridization buffer containing the CodeSet. Data acquisition was done by imaging on a digital analyzer. Raw counts were normalized using internal positive controls, and target genes were normalized to the internal housekeeping genes *odc, gapdh, laminB1* and *h4*. For background correction, the mean and two times the standard deviation of the 8 internal negative control counts (mean + 2 x SD) was subtracted from each target gene count. For data processing, the nSolver software v1.1 (NanoString Technologies) was used.

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