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MECHANISMS OF DEVELOPMENT

Control of dead end localization and activity – implications for the function of the protein in antagonizing miRNA function

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ABSTRACT

Dead end (dnd) is a vertebrate-specific component of the germ plasm and germ-cell granules that is crucial for germ cell development in zebrafish and mouse. Dnd counteracts the inhibitory function of miRNAs, thereby facilitating the expression of proteins such as Nanos and Tdrd7 in the germ cells. Here we show that *cis*-acting elements within *dnd* mRNA and the RNA recognition motive (RRM) of the protein are essential for targeting protein expression to the germ cells and to the perinuclear granules, respectively. We demonstrate that as it executes its function, Dnd translocates between the germ-cell nucleus and germ-cell granules. This phenomenon is not observed in proteins mutated in the RRM motif, correlating with loss of function of Dnd. Based on molecular modeling, we identify the putative RNA binding domain of Dnd as a canonical RRM and propose that this domain is important for protein subcellular localization and function.

1. Introduction

The germline is responsible for the transmission of genetic information between the generations in sexually reproducing organisms. In many organisms (e.g. *Drosophila*, *Caenorhabditis elegans*, *Xenopus* and zebrafish), specification of germline cells relies upon the inheritance of a specific set of molecules collectively termed germ plasm. The germ plasm is composed of proteins as well as coding and non-coding RNA that assume perinuclear localization as the germ cells are formed (Eddy and Ito, 1971; Hay et al., 1988; Mahowald, 1968; Mahowald and Hennen, 1971; Strome and Wood, 1982; Strome and Wood, 1983). Even in organisms in which germ cells are specified by induction (e.g. mammals and Urodeles), structures of similar molecular composition are found in the cells following their specification (Kotaja et al., 2006; Tanaka et al., 2000). Indeed, specific germ-plasm components that are important for germ cell specification and fate maintenance in *Drosophila*, *C. elegans*, *Xenopus* and zebrafish were found to be crucial for proper germline development in mouse (Tsuda et al., 2003; Raz, 2000 #1249, Youngren et al., 2005 #1961).

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Dead end (*dnd*) is a recently discovered zebrafish germ plasm component that is vertebrate-specific (Weidinger et al., 2003). In Dnd depleted zebrafish embryos the PGCs fail to acquire motility, loose specific marker gene expression and die by the end of the first day of development (Weidinger et al., 2003). The mouse homologue of Dead end (*dnd1*) known as Ter (Asada et al., 1994) is also expressed in PGCs. Mutations in the mouse Dnd1 locus result in loss of primordial germ cells (PGCs) and increased susceptibility to spontaneous testicular germ cell tumors in a certain genetic background (Youngren et al., 2005). The molecular function of Dnd was recently revealed by the demonstration that the protein acts to counteract the inhibitory function of several miRNAs, thereby allowing the expression of PGC specific proteins such as Nanos and Tdrd7 (Kedde et al., 2007).

In this work we investigate the molecular mechanisms controlling Dnd localization to the germ cell, to germ-cell granules, and determine the protein domains required for its function. We show that the combination of a *cis*-acting *element of the dnd* mRNA and the RNA recognition motive (RRM) of the protein are essential for targeting Dnd expression to the germ cells and for subcellular localization of the protein to the perinuclear granules (germ cell granules, hereafter termed GCGs). We demonstrate that Dnd translocates between the germ cell nucleus and the GCGs, a feature that appears to be essential for its function. In addition, based on molecular modeling, we identify the putative RNA binding domain as a canonical RRM and propose that RNA binding might be important for nucleocytoplasmic shuttling.

2. Results

As a first step in determining the mechanisms controlling Dnd function, we investigated the mechanisms directing the expression of the protein to the germ cells. To this end, we fused the open reading frame of the green fluorescence protein (GFP) to the 3′ untranslated region (UTR) of *dnd*. *in vitro* transcribed RNA synthesized from this construct was injected into 1-cell stage zebrafish embryos and its distribution compared with that of a control *gfp-globin* 3′UTR RNA. In 6 h post fertilization (hpf) embryos injected embryos, *gfp-dnd* 3′UTR mRNA was strongly degraded in somatic cells, but was protected from degradation in germ cells (Fig. 1A). In contrast, *gfp-globin* 3′UTR injected embryos showed a strong uniform distribution of the mRNA at this stage (Fig. 1B). At 28hpf, no *gfp-dnd* 3′UTR mRNA could be detected by *in situ* hybridization (data not shown), but the germ cells specifically expressed GFP (Fig. 1C). We conclude that, similar to findings concerning *nanos1 (nos1)* and *vasa* RNA (Köprunner et al., 2001; Wolke et al., 2002), the 3′UTR of *dnd* could play a role in directing germ cell specific expression of the protein.

To determine which regions of the Dnd protein were required to direct correct subcellular distribution we studied the localization of various truncated versions of the protein (Fig. 2A). *In-vitro* transcribed mRNAs encoding for these modified Hemagglutinin (HA)-tagged Dnd variants were expressed in the PGCs using the *nanos*1-3′UTR (Köprunner et al., 2001) and localization of the proteins detected using anti-HA antibodies.

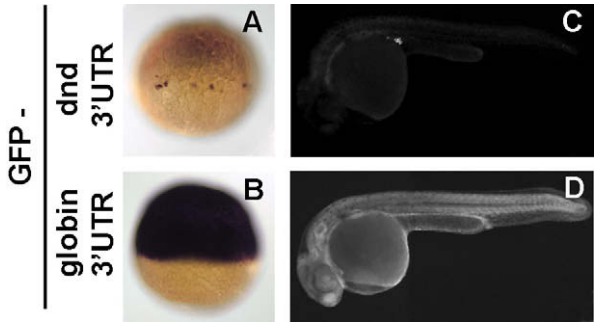

Fig. 1 – Dead end 3′UTR is responsible for targeting of *dnd* mRNA to the PGCs. (A, B) 1-cell stage embryos injected with GFP mRNA fused to *Dnd* 3′UTR were subjected to *in situ* hybridization with GFP anti-sense probe. GFP mRNA is restricted to the PGCs only by the *dnd* 3′UTR (A), in contrast to the *globin* driven ubiquitous mRNA expression (B). Accordingly, the GFP protein produced by the fusion mRNA follows the same pattern and is detected by fluorescent microscopy at 24hpf only in the PGCs in the case of *dnd* UTR (C) or ubiquitously as for *globin* UTR (D).

The full length Dnd fusion protein showed strong localization to the perinuclear granules in germ cells ((Weidinger et al., 2003) and Fig. 2B), but not in somatic cells where it exhibited uniform distribution (Suppl. Fig. 1B). Significantly, extensive C-terminal truncations of Dnd did not abrogate the localization of the protein to these structures, suggesting that the RNA recognition motif could be involved in targeting the protein to its normal location in the cell (Fig. 2B). Strikingly, HA tagged mouse Dnd1 (encoded by open reading frame AY321066) exhibited strong localization to zebrafish germ-cell granules (Fig. 2B). Despite the normal localization of some of the C-terminally truncated Dnd proteins, introduction of these versions into embryos in which Dnd was knocked down using morpholino antisense oligonucleotides revealed no, or little activity of these proteins (less than 20%) as measured by the number of PGCs in those embryos (Fig. 2C). These findings suggest that while not critical for proper localization, protein sequences within the C-terminal part of Dnd play an important role for in protein function, for example by interacting with other proteins to facilitate Dnd task. Interestingly, consistent with the finding that the zebrafish protein can function in mouse cells (Kedde et al., 2007), the mouse Dnd protein is partially functional in zebrafish germ cells (Fig. 2C).

To examine the role of specific domains of Dnd at a higher resolution, we generated point mutations altering 31 single amino acids. These amino acids that are identical among the zebrafish, human and the mouse proteins, were replaced by non-conserved residues (Fig. 3A). As for the deletion constructs, RNA-expression vectors were generated where mutated *dnd* variants were placed upstream of the *nanos1* 3′UTR, transcribed *in vitro* and injected in 1-cell stage embryos together with *dnd* morpholino antisense oligonucleotides. Injected embryos were fixed at 24hpf, subjected to *in situ* hybridization using a germ cell specific probe (*nanos*1), and their PGCs were counted. The vast majority of the mutations lying outside of the RRM domain did not result in a complete

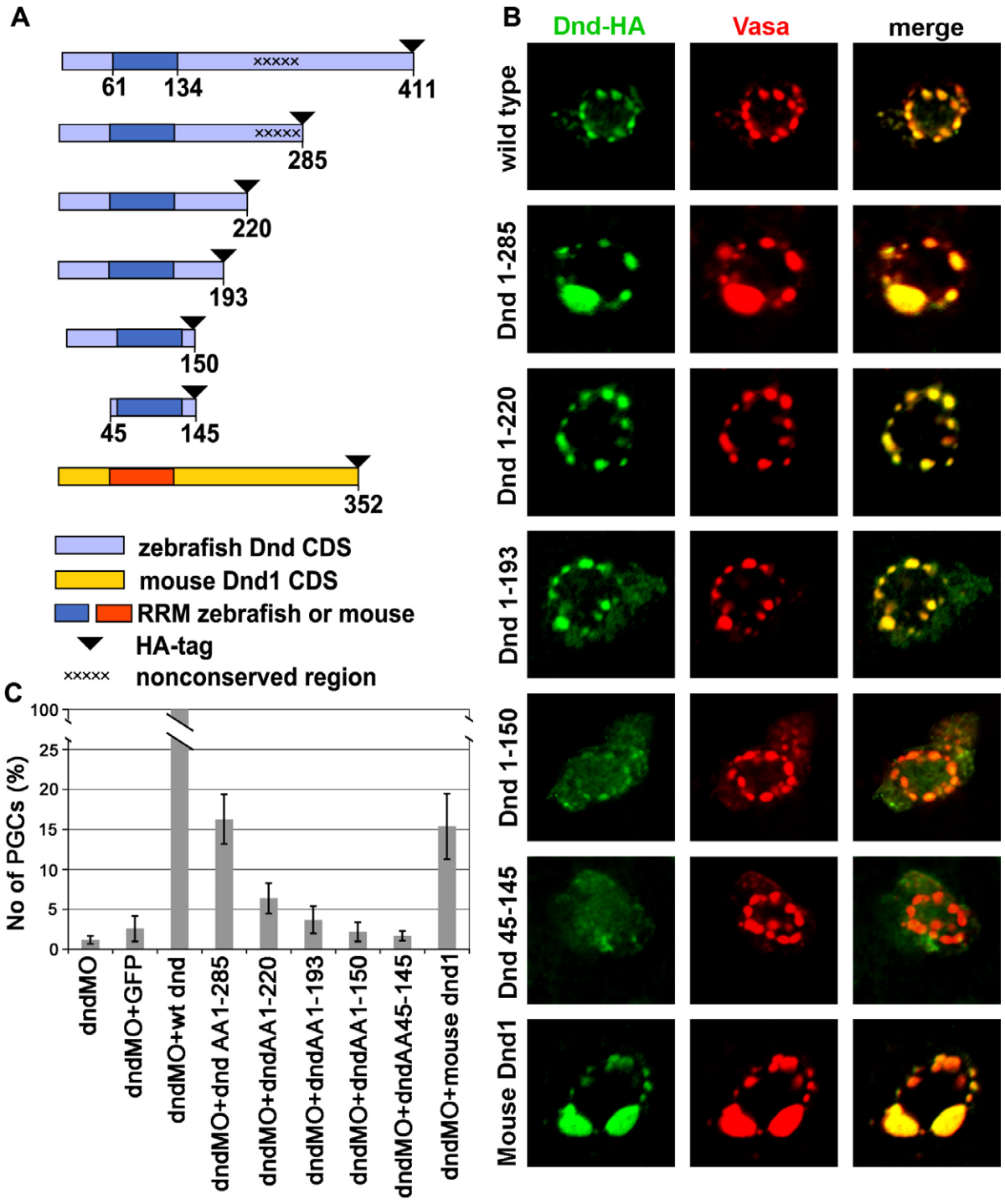

Fig. 2 – Deletions of parts of Dead end protein reveal domains important for protein localization and function. (A) Schematic drawing of Dead end deletion constructs. (B) Intracellular localization of the various Dnd-deletion fusion proteins detected by immunohistochemistry with anti HA antibody (Green) in comparison with the position of the GCGs reported by anti Vasa antibody staining (Red). (C) A chart depicting the ability of the various deletions constructs to supplement for wild type Dead end. The values represent the number of rescued PGCs in embryos co-injected with a specific Dnd truncated protein and *dnd* MO, normalized to the PGCs rescued by a wild type *dnd* mRNA injection (see the text for details).

inactivation of Dnd function (20/21 mutations) and, consequently, these mutant protein variants rescued a significant number of PGCs (Fig. 3B). Coupled with the finding that deletion of almost half of the protein does not completely abrogate its function (Fig. 2C, construct Dnd 1–285) it could be postulated that while this domain is essential for Dnd function, it plays a less crucial role as compared with the function of the RRM region where a large proportion of the mutations

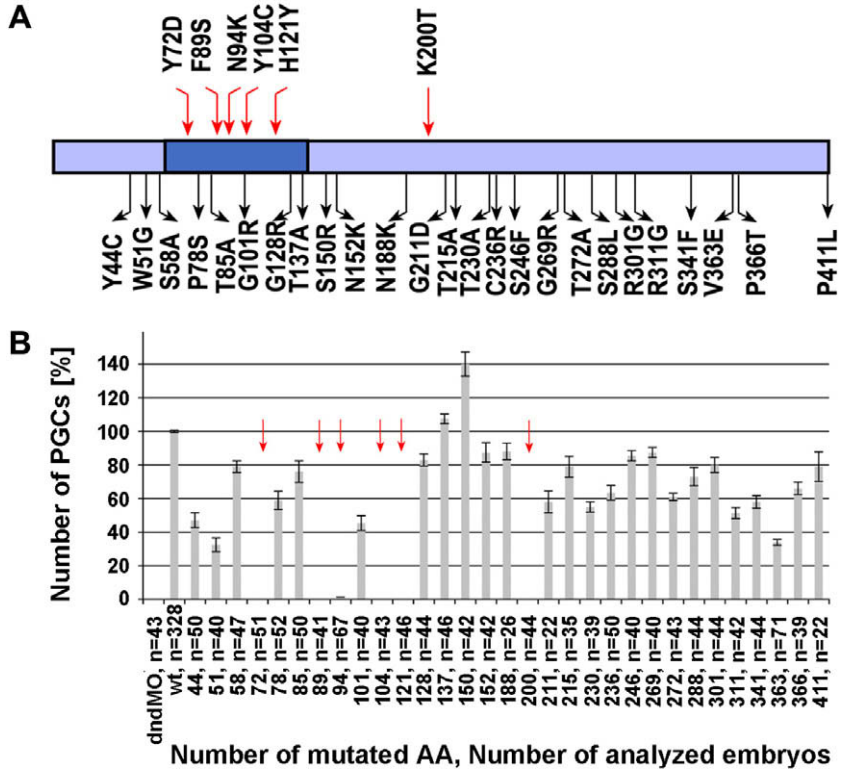

Fig. 3 – Screen for amino acids crucial for Dead end localization and function. (A) Schematic drawing of the relative positions of each altered amino acid along the Dead end axes. The mutations that caused loss of function of the protein are depicted with red arrows; black arrows mark substitutions that did not display any effect on the protein function. (B) Chart quantifying the effect of each individual amino acid on the Dead end protein functionality, as reported by the relative number of rescued PGCs upon an endogenous *dnd* knock down.

completely inactivated the protein (5/10 mutations, Fig. 3A and 3B, red arrows). To confirm that the results obtained in this section do not depend on the 3′UTR used for directing Dnd expression to the PGCs, we have repeated the experiment for a few key mutations while utilizing the *vasa* 3′UTR with similar results (Suppl. Fig. 2)

To determine whether the effect on cell survival correlates with the role of dead end in counteracting miRNA function (Kedde et al., 2007), we have assayed potency of different mutated Dead end versions in promoting GFP translation in PGCs of RNA containing the *nanos* 3′UTR. Indeed, similar to their loss of function in supporting PGC survival, several representative Dead end point mutations and the deletion AA1-220 exhibited reduced activity as measured by GFP translation from *nanos* 3′UTR-containing RNA at an earlier developmental stage (Suppl. Fig. 3).

Our attempts to obtain structural data to improve our understanding of Dnd function failed as none of the several expression systems used overcame protein insolubility problems. We have therefore resorted to computer homology modeling and investigated the effect of the loss of function mutations we identified on the structure of the RRM domain. We built a model for the RRM of zebrafish Dnd based on the available structures of highly homologues RRMs (Fig. 4A, for details see Section 4). The calculated model showed a typical RRM fold (babbab, where *b* is beta sheet and *a* is alpha helix) with a core backbone rmsd (the root mean square deviation between two different structures) of 1.99, 1.96, 2.95 and 1.14 relative to the three templates. Fig. 4B shows the positions of the essential residues Y72, N94, F89, Y104 and H121 in the 3 dimentional (3D) model of Dnd RRM. All of these, except Y72, are located close to or on the β sheet of the RRM, a region that was shown to be the RNA binding interface in other RRMs (Maris et al., 2005). To investigate how the point mutations might interfere with the RNA binding properties of the Dnd RRM, we obtained models for the Dnd–RNA interaction by overlaying the calculated 3D model of the RRM on three different structures of RRM–RNA complexes (see Section 4). We found that Y104 shows prominent stacking ability with RNA bases (Fig. 4C). With no exception, the overlaid structures (including ones that were not used for calculating the RRM model) revealed RRM–RNA stacking interactions involving aromatic residues equivalent to Y104 and F63 in the Dnd RRM. Furthermore, only a small change in the side-chain orientation of Y72, F89, and N94 is required for a direct contact between these amino acids and the RNA backbone.

To determine how mutants in the RRM domain affect the subcellular localization of Dnd, we tagged the Y72D, F89S, N94K and Y104C mutated proteins and followed their distribution in the cells. Surprisingly, we found that instead of the expected localization to the Vasa-containing perinuclear granules, these mutated proteins were found exclusively in germ cells nuclei despite the lack of a canonical nuclear localization sequence (Fig. 5A and Supplememtary Fig. 1A, unlike

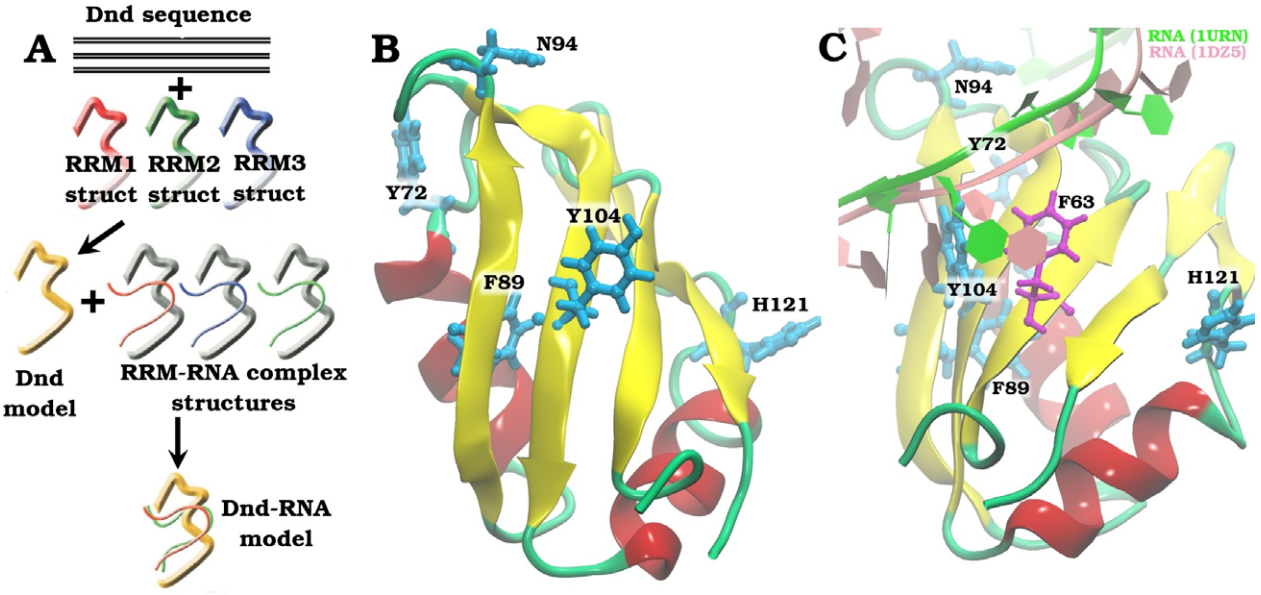

Fig. 4 – Computer homology modeling of Dnd RRM domain. (A) Procedure for obtaining a 3-D model for the Dnd RRM (see Section 4 for details). (B) The position of the essential residues (shown in blue Licorice representation) found by single-point mutations on the 3D model structure. (C) The model of the interaction between the Dnd RRM and RNA. The RNAs from the 1URN and 1DZ5 are shown in green and pink ribbons, respectively. For clarity, the RRMs in the RRM–RNA structures used for the alignment, as well as the RNA in the 1AUD structure are not shown. F63 is shown in a violet Licorice representation. In (B) and (C) The protein is shown in a Cartoon representation, helices colored red and the β-sheet yellow.

the K200T mutant protein, Suppl. Fig. 1A). It is important to note, however, that localization to the granules *per se* is not sufficient for providing wild-type Dnd activity as properly localized C-terminally truncated Dnd versions exhibited only low activity as judged by small number of rescued germ cells in embryos co-injected with *dnd* morpholino antisense oligonucleotides (Fig. 2B and C Dnd 1–220).

Together, these findings are consistent with the idea that Dnd binds RNA in the nucleus and then shuttles to the perinuclear granules where it protects the RNA from miRNA-mediated inhibition. To test this hypothesis more directly, we inhibited the nucleocytoplasmic export using leptomycin B (LMB), a specific inhibitor of nuclear export (Fornerod et al., 1997; Fukuda et al., 1997; Steinbach et al., 1997) and followed the distribution of tagged Dnd in the cell. Indeed, unlike in control embryos, tagged Dnd protein could be detected in the nucleus of LMB treated embryos (Fig. 5B). These findings show that in contrast to other components of the GCGs (e.g. Vasa, whose distribution is unaffected by LMB treatment, Fig. 5B), Dnd translocates from the nucleus to the perinuclear granules.

3. Discussion

In this work we show that the 3′UTR of dead end contains sequences that promote specific expression of the mRNA in the PGCs. Interestingly, similar results were reported for three other components of the germ plasm in zebrafish, *vasa, nanos1* and TDRD7 (Köprunner et al., 2001; Mishima et al., 2006; Wolke et al., 2002). Nevertheless, while the basis for *nanos1* and TDRD7 UTRs function is differential susceptibility to miRNA inhibition in the soma and in the germ cells, the 3′UTR of *vasa* appears to mediate PGC specific expression using a different mechanism (Mishima et al., 2006). As the 3′UTR of *dnd* lacks a perfect canonical miR-430 binding consensus sequence (GCACUU), the mechanisms responsible for *dnd* mRNA degradation in somatic cells and protection in germ cells remain to be determined.

We found that even minor deletions from the less conserved C-terminus of Dnd resulted in a dramatic reduction in protein function. Thus, despite the fact that single point mutations in this part of the protein did not abrogate the function of the protein, we have shown that it is essential for Dnd function, presumably by mediating interaction with other proteins. Interestingly, the deletion analysis revealed a correlation between the proper localization of Dnd protein to perinuclear granules and its function. Thus, consistent with the strong enrichment of Dnd in perinuclear granules, our results suggest that the function of the protein is probably exerted within this subcellular structure.

The apparent clustering of amino acids that are crucial for Dead end function within the RRM domain is a clear demonstration of the critical role it plays in Dnd function. Interestingly, mutations in this domain affected not only RNA binding ((Kedde et al., 2007) and the computer modeling results described above), but also the subcellular localization of the protein, such that the mutated Dnd protein was predominantly localized to the nucleus. This important finding, coupled with the analysis of Dnd localization in LMB treated cells, is consistent with the idea that Dnd translocates between the nucleus and the perinuclear granules following RNA binding. We therefore suggest that Dnd binding of

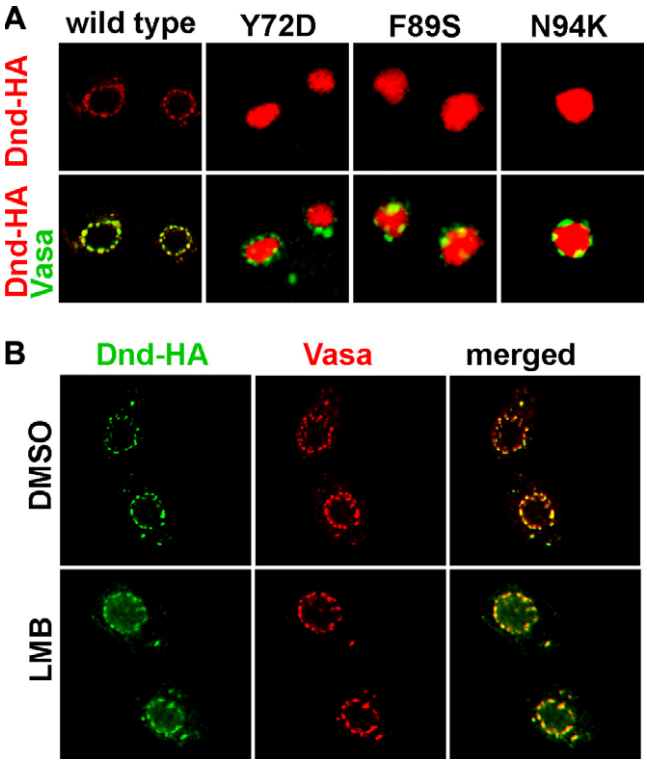

Fig. 5 – Dead end shuffles between the nucleus and the cytoplasm. (A) Dnd subcellular localization was changed from cytoplasmic to nuclear when amino acids Y72, F89 and N94 were altered. (B) Dead end protein was trapped in the nucleus when the Exportin 1 mediated nucleocytoplasmic export was blocked by leptomycin B (LMB). The localization of Dead end (green) was compared to that of Vasa protein (red), which labels the perinuclear granules in the control embryos treated with DMSO only (upper panel) and LMB treated embryos (lower panel).

specific RNAs in the nucleus facilitates their utilization in translation or protects them from miRNA-mediated inhibition in the perinuclear granules.

Our computer model of Dnd RRM structure together with biochemical data from Kedde et al. (2007) suggests that inactivation of Dnd by some mutations is due to disruption of the protein–RNA binding as illustrated by Y104C mutation. Due to the approximations used to calculate the 3D model, alternative orientations of the side-chains of Y72, F89, or N94 cannot be excluded. Such motions that could lead to direct Dnd RRM–RNA contacts involving these amino acids could be investigated via more advanced simulation techniques such as molecular dynamics.

Last, while this work focused on zebrafish PGCs, our results are likely to be relevant for the mouse gene as well (Youngren et al., 2005). Specifically, we could show that mouse Dead end localizes to the germ plasm in zebrafish and is capable, at least to some extent, of complementing loss of function of the zebrafish orthologue. Furthermore, the chromatoid body, a germ-cell-specific cytoplasmic structure found in 4.5 days old mouse embryos and male germ cells of various mammals (Fawcett, 1970; Kotaja et al., 2006; Reunov, 2006), strongly resemble germ plasm granules in its ultrastructural and molecular characteristics. It can thus be speculated that a further understanding of the function of components of zebrafish germ plasm would shed light on the function of their mouse counterpart.

4. Experimental procedures

4.1. *Cloning and DNA constructs*

To obtain the zebrafish dead end mutant variants, full-length coding sequence (Weidinger et al., 2003) was modified by introducing single nucleotide exchanges, using four primers and three PCR reactions as described in Site-specific Mutagenesis by Overlap Extension (Sambrook and Russel, 2001). Obtained PCR fragments were cloned according to the standard protocol of TOPO kit (Invitrogen). Two variants of the TOPO vector, TOPO PCRII and TOPO 2.1 were used. Dnd fragments were then sub-cloned upstream from *nos1* 3′UTR*globin* 3′UTR *or vasa* 3′UTR (Köprunner et al., 2001; Wolke et al., 2002) and verified by sequencing. GFP-Nos1-3′UTR was described in (Weidinger et al., 2003).

4.2. *Immunohistochemistry*

For the antibody staining anti Vasa rabbit polyclonal (Knaut et al., 2000) and mouse monoclonal anti HA antibodies were used. Embryos were fixed for 24 h in 4% PFA at 4 °C, dechorionated and incubated in 100% MeOH for 1 h at −20 °C. After rehydration embryos were blocked in PBS with 0.2% Triton X-100 and 1% BSA (PBTB) for 1 h at room temperature, incubated with the primary antibody at appropriate dilution (1:2000 for anti Vasa and MAb414, 1:1500 for anti HA) in PBTB at 4 °C overnight, washed with PBTB four times for 20 min, incubated with the secondary antibody (Alexa 488 goat anti-rabbit IgG and Alexa 546 goat anti mouse (Molecular probes)) for 2 h at room temperature, and washed with PBT four times for 20 min.

4.3. *Whole-mount in situ hybridization*

In situ hybridization was performed as described in (Jowett and Lettice, 1994), with modification according to Hauptmann and Gerster (1994).

4.4. *Fish stocks and maintenance*

Zebrafish embryos were raised, maintained and staged as described by Kimmel et al. (1995). Embryos were obtained from natural spawning and incubated at 28 °C until the desired stage in Daniou's solution.

4.5. *mRNA and morpholino microinjection*

Caped sense mRNA was synthesized *in vitro* by using the Message Machine kit (Ambion, Austin, TX) and injected in the quantities indicated in the text into embryos of the AB genetic background. Every egg was injected in the yolk below the first developing cell with about 0.5 nl solution.

4.6. *Leptomycin treatment*

Leptomycin stock solution was prepared in DMSO and diluted to final concentrations of 15 ng/ml in Danieaus solution. 3–6 somites old embryos were incubated for 3 h in the solution, fixed in 4% PFA and subjected to Immunohistochemistry.

4.7. *Homology modeling*

The Robetta web-based server (Kim et al., 2004) was used to find high-homology templates in the Protein Data Bank for the RNA recognition motif of the Zebrafish Dead-end protein. Based on sequence and structure alignments, a 3D model structure was obtained with the Swiss-Model program (Guex and Peitsch, 1997) using the identified templates: (i) two of the five models of the NMR structure of the N-terminal RRM domain of cleavage simulation factor 64 Kda subunit (pdbid 1P1T; (Perez Canadillas and Varani, 2003)), (ii) the X-ray structure of the RRM of the heterogeneous nuclear ribonucleoprotein A1 (pdbid 1L3K; (Vitali et al., 2002)), and (iii) the NMR structure of the RRM of human U2 small nuclear ribonucleoprotein auxiliary factor U2Af65 (pdbid 2U2F; (Ito et al., 1999)). The 1P1T structure was found by the Robetta server to be the highest-homology template, while the 1P1T, 1L3K and 2U2F were found to have the highest sequence homologies with the RRM to be modeled. We then overlaid the model on three different structures of RRM–RNA complexes (Fig. 4A): (i) U1A-Pie RNA complex (pdbid 1DZ5; (Varani et al., 2000)); (ii) human U1A protein bound to the 5′UTR of (pdbid 1AUD; (Allain et al., 1997), (iii) spliceosomal protein U1A complexed with a hairpin RNA (pdbid 1URN; (Oubridge et al., 1994)). Thus, we obtained three models for the RNA-binding interface of the Dnd RRM. The structure visualization and rendering was perfomed with VMD (Humphrey et al., 1996).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2008.10.006.

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