Combined functions of two RRMs in Dead-end1 mimic helicase activity to promote nanos1 translation in the germline

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1 | INTRODUCTION

A challenge for all metazoans is how to preserve the full potential of their germline while surrounding somatic cells become increasingly more restricted as to their cell fates. Although the genes that play critical roles in this process are frequently conserved across phyla, the timing and means of their expression varies. In organisms that employ germ plasm to contain and move germline determinants into the presumptive primordial germ cells (PGCs) after fertilization, gene expression begins early during oogenesis when the germ plasm forms. At that time, specific maternal RNAs and proteins accumulate in the germ plasm, many of which are RNA-binding proteins that function posttranscriptionally to either block somatic gene expression or to promote germline specific expression (Aguero et al., 2016; Aguero, Kassmer, Alberio, Johnson, & King, 2017).
In germlines, including Drosophila, Xenopus, zebrafish, mice, and humans, Nanos1 works with Pumilio to translationally repress specific messenger RNAs (mRNAs) encoding proteins that promote somatic fates. Loss of Nanos1 activity in Xenopus results in misexpression of the endoderm determinant VegT, and ultimately the loss of the germline resulting in infertility (Lai, Singh, & King, 2012). Premature translation of nanos1 in the oocyte results in abnormal embryonic development consistent with a loss of VegT (Luo et al., 2011). This region is highly conserved from amino acids (AA) 76 to 153 (NCBI, #Q6DCB7). Therefore, to test whether the RRM1 was required to bind nanos1 mRNA, we generated Xenopus tropicalis Dnd1 recombinant protein containing two point mutations within this conserved region: F110M/S117D, which corresponds to F106M/S113D in Xenopus laevis. Glutathione S-transferase (GST)-tagged recombinant proteins Dnd1 and Dnd1-F110M/S117D were tested in RNA immunoprecipitation (RIP) assays and the amount of nanos1 RNA bound determined by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). GST alone served as a negative control. While wild-type Dnd1 bound nanos1 mRNA (40% of input), the double point mutation within the RRM1 was sufficient to prevent binding to nanos1 mRNA, confirming a role for RRM1 in nanos1 mRNA binding (Figure 1a). As Dnd1 has two RRMs, these results also suggested that either RRM2 (also called Dnd1 motif) does not bind nanos1 mRNA or all RRM2 alone is not sufficient for mRNA binding.

2.2 | Dnd1 RRM2 is required for nanos1 translation

To determine what region(s) of Dnd1 protein was required to support nanos1 translation, we created a series of deletions in Dnd1 and subcloned them into the pCS2+ expression vector (Figure 1b). All deletions were examined for Dnd1 expression by western blot analysis with anti-Dnd1 antibody and found to be expressed at comparable levels (Figure 4b). We used two different assays, Xenopus oocytes and wheat germ (WG) extracts (Aguero, Jin, et al., 2017; Luo et al., 2011), to test each of the mutant Dnd1 proteins for their ability to promote nanos1 mRNA translation. Oocyte injections or in vitro translation (WG assay) yielded the same results (Supporting Information Figure 1) and these results are summarized in Figure 1b. Zebrafish Dnd1 contains an ATPase domain, and a C-terminal region that is important for its function (Liu & Collodi, 2010). However, xDnd1 missing the C-terminal 100 AA (Δ273–371) still promoted nanos1 translation. Interestingly, deletion of the RRM2 (ΔRRM2) or just its C-proximal half (ΔRRM2-R; missing 198–237 AA) resulted in the failure of Dnd1 to promote nanos1 translation (Figure 1b, Supporting Information Figure 1).

Interestingly, the Dnd1<sup>1er</sup> point mutation that results in tumors and infertility in mice and humans (Youngren et al., 2005; Zech et al., 2013), generates a stop codon within the RRM2 domain. We created the identical mutant in xDnd1 that results in a truncated protein by replacing the codon for arginine at position 209 with a stop codon (Figure 1c). The xDnd1<sup>1er</sup> mutant did not promote nanos1 translation as tested in either after fertilization (Aguero, Jin, et al., 2017). In that work, we showed that Dnd1 is capable of binding directly to nanos1 mRNA with a dissociation constant of ~140 nM (Aguero, Jin, et al., 2017). Dnd1 contains two motifs, RRM1 and RRM2, in its N-terminus (Marchler-Bauer et al., 2015; Weidinger et al., 2003). Point mutations within the RRM1 prevented RNA binding in zebrafish Dnd1 (Slanchev et al., 2009). This region is highly conserved from amino acids (AA) 76 to 153 (NCBI, #Q6DCB7). Therefore, to test whether the RRM1 was required to bind nanos1 mRNA, we generated Xenopus tropicalis Dnd1 recombinant protein containing two point mutations within this conserved region: F110M/S117D, which corresponds to F106M/S113D in Xenopus laevis. Glutathione S-transferase (GST)-tagged recombinant proteins Dnd1 and Dnd1-F110M/S117D were tested in RNA immunoprecipitation (RIP) assays and the amount of nanos1 RNA bound determined by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). GST alone served as a negative control. While wild-type Dnd1 bound nanos1 mRNA (40% of input), the double point mutation within the RRM1 was sufficient to prevent binding to nanos1 mRNA, confirming a role for RRM1 in nanos1 mRNA binding (Figure 1a). As Dnd1 has two RRMs, these results also suggested that either RRM2 (also called Dnd1 motif) does not bind nanos1 mRNA or all RRM2 alone is not sufficient for mRNA binding.

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assay, further supporting a role for the RRM2 in translation (Figure 1b, Supporting Information Figure 1). These results suggested that the two Dnd1 RRM domains evolved to have different functions: One in RNA binding and the other in promoting translation.

2.3 Overexpression of xDnd1<sup>ter</sup> or Dnd1<sup>ΔRRM2-R</sup> results in loss of PGCs

The failure of xDnd1<sup>ter</sup> and Dnd1<sup>ΔRRM2-R</sup> to support <i>nanos1</i> translation, prompted us to ask what the effects of these mutations
might have on PGCs when overexpressed in the embryo. To assess the effect on PGCs, we injected one-cell stage embryos at the vegetal pole with either Dnd1 ΔRRM2-R or xDnd1ΔR RNA. Green fluorescent protein (GFP) served as a negative control (Figure 2a–c). The embryos were allowed to develop until Stage 33 (late tailbud stage) when PGCs were assessed by whole mount in situ hybridization with the PGC marker Xpat (Hudson & Woodland, 1998). Although PGC migration was not affected by either mutant when overexpressed, the number of PGCs were significantly reduced by both mutants within the context of otherwise normal looking embryos (40% of embryos had less than 20 PGCs, and 50–60% less than 10; n = 32; Figure 2d). These results show that the absence of 198–237 AA within the RRM2 (ΔRRM2-R) is sufficient to phenocopy the nanos1 loss-of-function mutant as well as the mDnd1ΔR mutation (Lai et al., 2012; Zechel et al., 2013). Taken together, our findings have narrowed the region required for nanos1 translation to 40 residues within the RRM2. We next asked what function might this region have.

2.4 | Dnd1 possesses ATPase activity

Zebrafish Dnd1 (zDnd1) protein is known to possess ATPase activity. Furthermore, this activity is required for PGC development in zebrafish, although it remains unclear why this is the case (Liu & Collodi, 2010). We have previously shown that nanos1 translation is repressed by its secondary structure, TCE, and the loss of this structure is sufficient to allow translation (Luo et al., 2011). Since xDnd1 promotes nanos1 translation (Aguero, Jin, et al., 2017), we speculated that xDnd1 may also have ATPase activity, and that the ATPase activity may be required to melt the TCE to allow nanos1 translation. If this were true, it would explain the requirement of Dnd1 ATPase activity for normal PGC development as Nanos1 is required for PGCs survival (Lai et al., 2012).

As a first step, we used purified recombinant GST-Dnd1 protein in a standard assay for ATPase activity (Figure 3a,b). xDnd1 had the equivalent ATPase activity as that of recombinant zDnd1, used as a positive control. The amount of released phosphate from ATP by GST-Dnd1 increased in a time-dependent manner and required Mg2+ (Supporting Information Figure 2). The ATPase activity of the well-studied DEAD-box helicase family is also stimulated by its RNA substrate (Cordin, Banroques, Tanner, & Linder, 2006). To determine if Dnd1 shares similar properties to this family of helicases, we included nanos1 mRNA in the reaction and asked if it can synergistically stimulate the Dnd1 ATPase activity in the presence of Mg2+. As shown, ATPase activity was not further enhanced by the presence of nanos1 mRNA (Figure 3c). Thus, although xDnd1 has ATPase activity and binds RNA, it does not function as a typical helicase, at least by this criterion.

2.5 | xDnd1-mediated activation of nanos1 translation requires the putative ATPase site

In a tour-de-force, Liu and Collodi (2010) mapped the precise location of the ATPase activity in zDnd1 to five residues, RAAAE (392–396), near the end of the nonconserved C-terminus (Figure 4a). However, unexpectedly, deletion of 300 nts (100 AA residues) from the xDnd1 C-terminus retained full ATPase activity (data not shown). To determine if the ATPase site was conserved, but found elsewhere in xDnd1, we blasted the xDnd1 sequence for RAAAE. Only one related polypeptide was identified, RAAAM. RAAAM resides within the RRM2-R, a region required for nanos1 translation and conserved in mammals (Figure 1b). To determine if this putative ATPase (P-ATPase) site was required for nanos1 translation, synthetic transcripts encoding wild-type Dnd1 and the mutant missing the RAAAM site (Dnd1-ΔP-ATPase), were tested in WG extracts in the presence of nanos1 mRNA and its translational inhibitor, eukaryotic translation initiation factor 3 subunit F (eIF3f; Aguero, Jin, et al., 2017). Nanos1 protein was detected by western blot analysis with anti-Nanos1 antibody. Dnd1 mutant proteins were translated at comparable levels to Dnd1-FL (Figure 4b). In all cases, Dnd1 mutants, abrogating the putative ATPase site, failed to derepress nanos1 translation (Figure 4b,c). These results strongly suggested that the putative ATPase site in Dnd1 was required for translational activation of nanos1 mRNA, and likely other germline RNAs as well (Aguero, Jin, et al., 2017).

2.6 | Dnd1 forms homodimers in vivo

Many RNA-binding proteins function as a dimer to bind RNA (Marchione, Leibovitch, & Lenormand, 2013; Wang et al., 1999). To
determine if Dnd1 could form a homodimer, we generated plasmids containing Dnd1 tagged with either Flag or Myc and transfected corresponding plasmids into HEK293T cells. In coimmunoprecipitation experiments Myc‐Dnd1 could bring down Flag‐Dnd1, and vice versa, from cell lysates (Figure 5a). Addition of RNase A did not affect homodimerization of Dnd1 (data not shown). We extended the above studies by creating a series of Dnd1 deletions and mapping what regions in Dnd1 were required for its homodimerization. The smallest region able to form homodimers resided in the C‐terminal region (Figure 5b, summarized in c; X‐257–327 AA). To determine if Dnd1 forms homodimers in embryos at the time of nanos1 translation, GST‐Dnd1 purified protein was added to embryo extracts made from 1,2,8, and 32‐cell stage embryos. After an incubation period (1–2 hr), GST‐Dnd1 was immunoprecipitated with anti‐GST antibody and analyzed by western blot analysis for interaction with endogenous Dnd1 using an anti‐Dnd1 antibody. The control lane without GST‐Dnd1 added (−) revealed a nonspecific protein that migrated similarly to Dnd1 (+). However, the nonspecific protein was present at a much lower level, allowing us to conclude that GST‐Dnd1 could bring down endogenous Dnd1 at all stages tested (Figure 5d). Taken together, these results show that Dnd1 is capable of forming homodimers in embryos at the correct time to support nanos1 translation.

### 2.7 RRM1 and RRM2 have different structures

We created homology models of the two RRM domains to see if the basis for their functional differences was consistent with their predicted structure. Both domains adopt the classical RRM structure with a flat concave surface composed of four antiparallel β strands supported by two helices on the back (Lunde, Moore, & Varani, 2007). RRM domains typically bind single‐strand RNA along their concave β sheet surface, stabilized by conserved aromatic residues that stack with the RNA bases. RRM1 (green) domain of Dnd1 has three aromatic residues (Figure 6a; shown as red sticks) poised for stacking with RNA, while RRM2 (orange) has no aromatic side chains facing out from its β sheet. The location of putative ATP binding on RRM2 is highlighted by a red circle. This model also shows the molecular surface and was colored to indicate the electrostatic potential (Figure 5b; red is acidic and blue is basic). RRM1 presents a basic surface along its beta sheet which is conducive for RNA binding, while RRM2’s β sheet has a very acidic patch that is unlikely to bind.
However, RRM domains are unusually versatile in their RNA-binding modes (Maris, Dominguez, & Allain, 2005), and it is possible that the RRM2 binds RNA in a noncanonical manner via the basic patch near the helical regions. Taken together, the model suggests that only the N-terminal RRM1 domain is likely to bind RNA, consistent with our functional studies. RRM2 also has a basic nonstandard P-loop/helix that is consistent with ATP binding sites, suggesting that an ATPase site resides in this domain.

2.8 | Dnd1 requires a region adjacent to the nanos1 TCE to promote nanos1 translation

We next determined what region of nanos1 mRNA was required for Dnd1 to promote translation. Based on our previous findings, the simplest working model suggested that Dnd1 binds to the TCE, altering its structure and thus promoting translation (Luo et al., 2011). A series of deletions or substitution mutants were created within nanos1 mRNA, and these mutant mRNAs were tested, in WG extracts, to determine if Dnd1 protein could promote their translation. In these studies, we also sought to determine if the nanos1 TCE was required for Dnd1-mediated nanos1 translation. However, we could not simply investigate the ability of Dnd1 protein to promote translation of a nanos1Δ-TCE mutant because, unlike any other region, such a deletion dramatically promotes the translation of nanos1 (Luo et al., 2011). Furthermore, levels of nanos1 translation in recent commercial WG extracts were found to be variable (Figure 7a). To circumvent these problems, we blocked translation of nanos1 by including in the reaction the translational repressor, eIF3f, part of the eIF3 complex (Aguero, Jin, et al., 2017; Daubner, Clery, & Allain, 2013). As expected, in the presence of eIF3f, nanos1 translation was repressed, allowing us to test if Dnd1 could promote translation in the absence of the TCE (Figure 7a). Addition of Dnd1 protein was able to enhance translation of the Δ-TCE mutant (Figure 7a). Therefore, Dnd1 must interact with a region other than the TCE to promote translation.

**FIGURE 5** Dnd1 forms homodimers in vitro and in vivo. (a) Coimmunoprecipitation shows the interaction between myc-Dnd1 and FLAG-Dnd1 in transfected HEK293T cells. Anti-FLAG (top panel) or anti-Myc (bottom panel) antibodies were used to pulldown xDnd1 from cell lysates. (b) Different Myc-tagged Dnd1 deletion constructs were transfected into HEK293T cells, lysed and incubated with GST-xDnd1. Copurified Dnd1 was detected by western blot analysis using anti-Myc antibodies. (c) Schematic summarizing experiments shown in (b). “+” for Dnd1 homodimerization; “-” lack of Dnd1 dimerization. Red rectangle highlights the smallest xDnd1 region required for homodimerization (Figure 4a). Analysis based on at least two-independent experiments. (d) Dnd1 dimerizes in vivo. Extract from 50 1-, 2-, 8-, and 32-cell stage Xenopus embryos were made and incubated with GST-xDnd1 purified protein. Anti-GST antibody was used to pulldown GST-xDnd1 and xDnd1 proteins were detected by western blot analysis using anti-Myc antibodies. Endogenous xDnd1 bands are indicated by asterisks, exogenous pulled-down GST-xDnd1 bands are indicated with black dots. Negative controls have a weak nonspecific protein that migrates similarly to GST-xDnd1 protein. This experiment was repeated twice using embryos from four different females. Dnd1: dead-end1; GST: glutathione S-transferase; xDnd1: Xenopus Dnd1 [Color figure can be viewed at wileyonlinelibrary.com]
Previously, we have shown that the nanos1 3′-untranslated region (3′-UTR) is not required to promote translation (Aguero, Jin, et al., 2017). To rule out any possibility that the 3′-UTR facilitates Dnd1 binding to nanos1 mRNA, we removed the 3′-UTR and replaced it with the SV40polyA to stabilize the transcript. Consistent with our previous findings, Dnd1 could promote nanos1 translation in the absence of the nanos1 3′-UTR (Figure 7a). To rule out any involvement of the 5′-UTR, we replaced that region with six myc-tags. Dnd1 also greatly enhanced translation without the native 5′-UTR. Therefore, Dnd1 required a region within the open reading frame (ORF) downstream of the TCE to promote translation of nanos1.

Next, we did a more complete series of nanos1 deletions downstream of the TCE and within the ORF, dividing it into four regions (Figure 7b, I–IV). A progressive series of deletions from the 3′ end of the nanos1 ORF was tested in WG extracts for the ability of Dnd1 to significantly increase nanos1 translation (Figure 7b; Supporting Information Figure 3). Although all progressive deletions of the ORF were less efficient in translating nanos1 mRNA, translation of each region was improved by the addition of Dnd1 protein (Fig. 7b). Thus, Dnd1 was able to promote nanos1 translation when only region I was present and associated with the TCE, suggesting that Dnd1 binds region I.

Therefore, to test region I alone as a putative site of Dnd1 interaction, we deleted it (Figure 8a) while retaining all other regions (ΔI; Figure 8a). We asked if region I was required for Dnd1 to promote nanos1 translation in the presence of eIF3f as detailed for Figure 7. We found that Dnd1 protein could not rescue nanos1 translation when region I was deleted, and nanos1 translation remained at the fully repressed level (Figure 8b). To determine even more precisely the site of interaction, we introduced two 24-nt deletions within region I, closest to the TCE (Figure 8a; labeled a,b). Subregion B was of special interest as it contains a poly(U)-rich region, a putative Dnd1 binding site (Kedde et al., 2007; Ketting, 2007; Yamaji et al., 2017). To determine if the poly(U)-rich region was necessary for Dnd1 function, we also substituted uridines for adenines within subregion B (IB-Mut in Figure 8a,c). Addition of Dnd1 protein to WG extracts had no effect on the translation of nanos1 mutants, as expected if region I was required for Dnd1 function promoting nanos1 translation (Figure 8c).

Lastly, to test region I and the poly(U)-rich site for Dnd1 protein binding, we carried out RIP analysis. We incubated purified recombinant GST-Dnd1 protein with nanos1 3′ΔI RNA. Dnd1 was precipitated and the levels of bound nanos1 mRNA was detected by RT-qPCR (Figure 8d). RIP analysis showed that Dnd1 binding to nanos1 mRNA decreased only upon the deletion of region I (reduced to 25% of control). Deletion of IB and, importantly, the IB-Mut, reduced Dnd1 binding to similar levels as detected when the entire region I was deleted. Interestingly, deletion of IA caused a mild reduction in Dnd1 binding. We speculate that deletion of IA may influence the Dnd1 binding by altering the secondary structure of RNA around the poly(U)-rich site. Taken together, these results show that a small region downstream of the TCE is required for Dnd1 binding and promotion of nanos1 translation. Our results also suggest that Dnd1 binds the poly(U)-rich region within subregion B.

3 DISCUSSION

In this study, we provide evidence as to how nanos1 mRNA and Dnd1 interact to achieve proper translation of nanos1, an early event essential to the survival of the germline. We show that, similar to zebrafish, xDnd1 possesses ATPase activity. However, surprisingly, the homologous ATPase site was mapped within the RRM2 or Dnd1 domain, and is very different from its C-terminus location in zebrafish (Liu & Collodi, 2010), but conserved in mouse. More importantly, we show that this site is required for nanos1 translation, but not RNA binding, whereas the canonical RRM1 is required for binding nanos1 mRNA. Overexpression of xDnd1 mutants lacking the putative RRM2-ATPase site caused a dominant negative effect, which resulted in the loss of the germline, further confirming its functional
Dnd1 requires a region adjacent to the nanos1 TCE to promote nanos1 translation. (a) Schematic of nanos1 deletions used in in vitro translation (WG) experiments shown below. WG extract was supplemented with 500 ng of nanos1 mRNA with or without purified Dnd1 protein and 1 µg of mRNA encoding the translational repressor eIF3f. Samples were analyzed for Nanos1, eIF3f, and Dnd1 protein expression by western blot analysis. Experiments were repeated twice. Dnd1 protein promoted translation of each nanos1 mutant tested. (b) Schematic of deletions within the nanos1 ORF downstream of the TCE (red box). Each transcript was tested for translation in the presence or absence of xDnd1 protein and eIF3f mRNA as shown in (a). Results from blots are presented in the histogram. xDnd1 failed to promote nanos1 translation when region I (96–nt downstream of TCE) was deleted, suggesting that this region of nanos1 is required for Dnd1 function. Experiments were repeated twice using different WG extracts. Dnd1: dead-end1; eIF3f: eukaryotic translation initiation factor 3 subunit F; ORF: open reading frame; TCE: translational control element; WG: wheat germ; xDnd1: Xenopus Dnd1 [Color figure can be viewed at wileyonlinelibrary.com]

3.1 Different roles for RRM1 in Dnd1?

In vertebrates, the RRM is the most abundant RNA-binding domain and its unusual versatility is well documented (Clery, Blatter, & Allain, 2008). As expected, the canonical RRM was found to bind nanos1 mRNA. Two conserved point mutations within the RRM1 rendered Dnd1 unable to bind nanos1 mRNA (Figure 1a). Two additional functions suggested for the Dnd1 RRM1 appear to involve protein association. RRM1, a region required for RNA binding, is also required for eIF3f protein binding (Xt: 96–127 AA; Aguero, Jin, et al., 2017). Furthermore, point mutations within the zebrafish RRM1 prevented Dnd1 in PGCs from shuttling between the nucleus and cytoplasm (Slanchev et al., 2009). As Dnd1 does not contain a nuclear localization signal, these results imply that the RRM1 may also interact with a cofactor for nuclear export after RNA binding.

3.2 xDnd1 has ATPase activity

xDnd1 has been shown to possess ATPase activity, although it does not share similarity to any known ATPase families. We showed that xDnd1 also exhibits ATPase activity that is commensurate with that of zebrafish. Consistent with other characterized ATPases, Mg²⁺ is required for the ATPase activity in both zebrafish (Liu & Collodi, 2010) and Xenopus (Figure 3). Typically, RNA substrates can stimulate the ATPase activity of DEAD-box helicase proteins such as Vasa (Cordin et al., 2006; Liang, Diehl-Jones, & Lasko, 1994). Although Dnd1 interacted with nanos1 directly, the Dnd1 ATPase activity was not stimulated by nanos1 mRNA. Dnd1 may need additional factors in vivo to stimulate its activity in a manner similar to that of other RNA helicases. For example, the eIF4A ATPase activity increases three-fold in the presence of accessory proteins eIF4B and eIF4H (Rogers, Richter, Lima, & Merrick, 2001). It will be interesting to determine if accessory factors, like DeadSouth, interact with Dnd1 in PGCs and enhance ATPase activity.

xDnd1 is a much larger protein than either mDnd1 or xDnd1 and contains large stretches in the C-terminus that are not well conserved (Liu & Collodi, 2010; Figure 3a). It is in the nonconserved C-terminus where the zebrafish RAAAE ATPase site maps. Deletion of the C-terminal region from xDnd1 retained both ATPase activity (data not shown) and the ability to promote nanos1 translation (Figure 1c). Interestingly, the homologous ATPase site to that found in zebrafish mapped to a second recently identified RRM2 or Dnd1 domain (Slanchev et al., 2009). RRM2 contains RAAAM, a site which is precisely conserved in chicken, mouse, and human as to sequence and location with xDnd1 (Figure 1a; Aramaki et al., 2007; Aramaki, Kubota, Soh, Yamauchi, & Hattori, 2009; Kedde et al., 2007; Kito et al., 2010). The basic patch near the helical regions in RRM2 corresponds to the putative ATPase site, and potentially promotes interactions required for translation of nanos1. Consistent with that interpretation, deletions introduced into the RRM2 covering that site prevented Dnd1 from promoting nanos1 translation. Thus, our data support different functions for each of the two RRMs in Dnd1.

3.3 Dnd1 ATPase activity is required for PGC survival

Dnd1 ATPase activity is required for zebrafish PGC survival (Liu & Collodi, 2010), however, the mechanism for the involvement of such
activity in germline development is not clear. We show that deletion of the homologous ATPase site in xDnd1 resulted in the loss of the ability of Dnd1 to activate nanos1 translation and converted Dnd1 into a dominant negative mutant which interfered with PGC development (Figure 1d). Our findings suggest that PGCs are lost after overexpression of Dnd1ΔRRM2 because ATPase activity is required to promote germline RNA translation essential for normal PGC development. We previously found that Dnd1 directly interacts with eIF3f, and through it, the whole eIF3 complex, which is required to initiate translation (Aguero, Jin, et al., 2017). Dnd1 may be recruited to nanos1 RNA and function as a chaperone that facilitates RNA structural remodeling at the cost of ATP (Jarmoskaite & Russell, 2011). In zebrafish PGCs, zDnd1 protects germ plasm-associated nanos1 mRNA from miRNA-mediated degradation that clears nanos1 from the soma (Kedde et al., 2007). However, a direct interaction between the zDnd1 and nanos1 mRNA has not been reported. Furthermore, whether the zDnd1 ATPase activity is involved in nanos1 protection is not clear. A recent study tethering zDnd1 to a reporter shows Dnd1 can act as a repressor of translation and this activity persists even if the ATPase site is deleted (Kobayashi, Tani-Matsuhana, Ohkawa, Sakamoto, & Inoue, 2017). xDnd1 interacts with eIF3f, a repressor of translation within the eIF3 complex at
the earliest stages in development, and blocks its repressive activity, promoting nanos1 translation (Aguero, Jin, et al., 2017). Since the teleost genome is highly rearranged in comparison with other vertebrates such as mouse, chick, and Xenopus, it seems likely that the ATPase site moved to the end of the C-terminal region during teleost evolution and genome duplication. We would argue that the ATPase site within the RRM2 is the conserved site and functions to facilitate the role of Dnd1 in promoting translation.

3.4 | xDnd1 forms homodimers

It is not uncommon for RRM-containing proteins to form homodimers (Marchione et al., 2013; Wang et al., 1999), and in some cases, it is shown to be a requirement for RNA binding (van Gelder et al., 1993; Varani et al., 2000; Wang et al., 1999). We found that Dnd1 is capable of forming homodimers within the context of HEK293T cells as well as in embryos at the correct time for nanos1 translation. GST-Dnd1 coimmunoprecipitated with endogenous Dnd1 in embryos. This event did not require RNA, suggesting that dimerization occurs first. To complicate matters, Dnd1 is also capable of forming heterodimers with specific subunits of the eIF3 complex (Aguero, Jin, et al., 2017). xDnd1 directly binds eIF3f and in IP experiments, brings down eIF3m with specific subunits of the eIF3 complex (Aguero, Jin, et al., 2017).

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Although the RRM is required to form homodimers in some proteins (Crichlow et al., 2008; van Gelder et al., 1993), xDnd1 requires the C-terminus to form homodimers (Figure 5) and to associate with eIF3f (Aguero, Jin, et al., 2017). In general, the C-terminus is less well conserved among different species, however the region that correlates with the ability to dimerize (304–350 AA) contains two identical stretches of 10 residues in Xenopus, mouse, and humans that is not present in zebrafish. Finner mapping will be required to determine if these conserved regions are critical for dimerization. Deletion of the C-terminus in zDnd1 results in an 85% reduction in the number of PGCs, however, that phenotype is attributed to the loss of the ATPase site located in the last 91 AA. It will be important to determine if Dnd1 from other species, including zebrafish, functions as homodimers.

3.5 | Model: nanos1 activation by xDnd1

We had anticipated that xDnd1 would bind the TCE in nanos1 to promote the melting of this repressive structure. However, our data identified a U-rich region immediately juxtaposed to the TCE required for Dnd1 binding. RIP analysis showed that substitution of these U’s greatly reduced the ability of xDnd1 to bind nanos1 mRNA (Figure 8d). Previous studies proposed that Dnd1 binds to a stretch of U-rich sequences around the miR-430-binding site in the 3′-UTR and prevents access of miRNA machinery (Kedde et al., 2007). Similarly, in Xenopus, miR-18 is blocked by xDnd1 binding to U-rich sites within the 3′-UTR that are embedded in mRNA localization signals (Koebernick, Loeber, Arthur, Tarbashevich, & Pieler, 2010). Here we propose another U-rich region within the ORF that binds xDnd1 and is required for nanos1 translation. Taken together, our results suggest a model whereby xDnd1 binds subunits of the eIF3 complex as well as RNA, promoting structural remodeling of the TCE at the cost of ATP. Disruption of the TCE allows the preinitiation complex to scan and translation to proceed. The temporal order in which these events occur and whether binding requires the dimerization of xDnd1 remains to be determined in future work.

3.6 | Dnd1 as master regulator-gatekeeper of the germline

In germ cells, Dnd1 appears to function at each one of three critical transition points for gene expression, in oocytes, just after fertilization (Aguero, Jin, et al., 2017), and during gastrulation as the maternal to zygotic transition peaks (Crichlow et al., 2008; Kedde et al., 2007; Mickoleit, Banisch, & Raz, 2011). In the fully-grown Xenopus oocyte, Dnd1 binds to trim36 RNA anchoring it within the vegetal cortex, a requirement for Trim36 E ligase protein to accumulate, microtubule arrays to form, and the dorsal/ventral axis to be established (Mei et al., 2013). Postfertilization, Dnd1 is required to activate the translation of nanos1 mRNA through interaction with the eIF3 complex (this report and Aguero, Jin, et al., 2017). Nanos1 is required early on to translationally block maternally supplied somatic determinants and to preserve the germline (Lai et al., 2012). At the time of transition from maternal to zygotic gene expression, germline specific Dnd1 plays yet another role; protection of germ plasm-associated nanos1 mRNA from miRNA-mediated degradation (Crichlow et al., 2008; Kedde et al., 2007).

Early development depends on the correct activation and regulation of maternal RNAs. Although we have focused on nanos1 mRNA, there is abundant evidence that Dnd1 binds many germline mRNAs (Aguero, Jin, et al., 2017; Yamaji et al., 2017; and our unpublished data). Several germline mRNAs, including nanos1 mRNA, could be coimmunoprecipitated (RIP) with Dnd1 protein in early staged embryos. The storage of these messages involves formation of stable and detergent-resistant mRNP complexes. RNA helicases may not only play a role in RNA regulation, but also act as a remodeler to regulate RNA-protein complexes at the cost of ATP and promote translation. We propose that Dnd1, at the top of the regulatory pathway for germline mRNAs, may be the master regulator of gene expression in the germline.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plasmids

The ORF of xnd1 was cloned by RT-PCR (Invitrogen, Fisher Scientific, Hanover Park, IL) from RNAs extracted from Stage VI oocytes and sequenced. We identified an AUG start site upstream (MATS) of the start site originally reported by Horvay et al., 2006 (MELS). Our sequence adds 17 AA for a total number of 371 AA. Synthetic xnd1 transcripts for oocyte injection or in vitro translation were generated by PCR and subcloned into a pCS2-MT vector at EcoRI/XhoI sites. xnd1 transcripts with Myc tags at the 5′ end were
produced by SP6 transcription (mMESSAGE mMACHINE, Ambion/Fisher Scientific, Hanover Park, IL). To obtain recombinant GST-xDnd1 protein, xDnd1 was subcloned into pGEX vector (GE Health, Princeton, NJ) after the GST tag, between EcoRI/Xhol sites. Nanos1 full length, ΔTCE, and ORF deletions (I–IV) were generated by PCR and subcloned in pCS2+ vector. Nanos1 region I deletions (Δ1, ΔIA, ΔIB, and B mut) were artificially synthesized and cloned into pUC57-Amp vector (Genewiz, South Plainfield, NJ). Synthetic transcripts of ΔI, ΔII, ΔIII, and ΔIV were generated in vitro by SP6 RNA polymerase (mMES- SAGE mMACHINE, Ambion) using NotI-linearized plasmids as templates.

4.2 | Protein expression and purification
Recombinant GST-xDnd1 protein was expressed in Escherichia coli (BL21 stain; Invitrogen) at 37°C for 3 hr by IPTG induction (1 mM). The bacteria were harvested by centrifugation and subjected to cell disruption using a French Press. The protein extracts were loaded onto the GSTrap column (GE Health, Princeton, NJ). The column was disruption using a French Press. The protein extracts were loaded onto the GSTrap column (GE Health, Princeton, NJ). The column was washed with phosphate buffered saline before elution by addition of 10 mM reduced glutathione.

4.3 | In vitro RNA pull-down assay (RIP)
GST-xDnd1-bound glutathione beads were incubated with 10 µg yeast transfer RNA in 1 ml RIP buffer (50 mM Tris, pH 7.6, 125 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.25% NP-40, 0.2% glycerol, 0.1 mM dithiothreitol [DTT], and 100 U/ml RNasin) at 4°C for 1 hr for preabsorption. A quantity of 100 ng synthesized mRNAs was added to 1 ml RIP buffer containing preabsorbed beads and incubated for an additional 4 hr at 4°C. The mixture was then centrifuged and 50 µl supernatants were set aside as "5% of mRNA input." Beads were washed five times with RIP buffer and once with RIP buffer without NP-40 and DTT (50 mM Tris, pH 7.6, 125 mM NaCl, 1 mM EDTA, 0.2% glycerol, and 100 U/ml RNasin). mRNAs were extracted from beads using TRIzol reagent, and subjected to complementary DNA synthesis. Subsequently, qPCR was performed using Power SYBR Green Master Mix (Life Technologies, Grand Island, NY) on an Applied Biosystems 7500 real-time PCR system. The ratio between pulled down mRNA and 5% of mRNA input was used to determine binding of mRNAs by GST-Dnd1. GST alone served as the negative control. Primers for nanos1 were 5′-GGAGGGCGCTGTCTCTATAC and 5′-GGGAGGCGCTGTCTCTATAC

4.4 | Oocytes, embryos, and microinjection
Xenopus oocytes and embryos were obtained and microinjected as described (Aguero, Newman, & King, 2018; Newman, Aguero, & King, 2018; Sive, Grainger, & Harland, 2000). For injection, mRNAs were synthesized using mMESSAGE mMACHINE Kit (Ambion) and the plasmid templates described above. The protocol for Xenopus studies has been approved by the University of Illinois Institutional Animal Care and Use Committee (#14249) and by the University of Miami IACC (#12–276).

4.5 | In vitro translation
In vitro translation was carried out using WG extracts according to the manufacturer’s instructions (Promega, Madison, WI). Either 0.5 µg (nanos1) or 1.0 µg (elf3f and xDnd1) capped-mRNA and GST-Dnd1 protein were used per reaction.

4.6 | Phosphate release assay
The assay was performed as outlined in the manufacturer’s instructions using BioMol Green Reagent (Biomol, Enzo Life Sciences, Farmingdale, NY). Recombinant Dnd1 proteins (1 µM) and ATP (0.8 mM) were added to reaction buffer (10 mM Tris-HCl, pH 7.5, 75 mM KCl, 0.2 mM EDTA with indicated cations) and incubated at room temperature for 20 min. A volume of 200 µl BioMol Green Reagent was subsequently added to allow the color reaction. After 10 min, the absorbance was measured at 620 nm.

4.7 | Homology modeling
Homology models of Dnd1 (Uniprot entry Q6DCB7, residues 76–235) were constructed using the SWISS-MODEL web server (Biasini et al., 2014). Figure was generated using Pymol (http://www.pymol.org).

4.8 | Homodimer analysis in vitro and in vivo
Homodimer in vitro analysis was performed by GST-pulldown as described previously (Jin et al., 2015). For in vivo analysis, total embryo extracts from 1-, 2-, 8- and 32-cell stage embryos were incubated with purified GST-Dnd1 protein on ice for 1 hr (Aguero, Jin, et al., 2017). For IP, embryo extracts were incubated with anti-GST antibody for 3 hr, followed by an overnight incubation at 4°C with protein G-magnetic nanobeads (NVIGEN Inc., Sunnyvale, CA). Coprecipitated endogenous Dnd1 proteins were detected by western blot using anti-Dnd1 antibody 1:1,000 (Aguero, Jin, et al., 2017).

4.9 | Cell culture, transfection, antibodies, coimmunoprecipitation, and western blot analysis
HEK293T cells, which were authenticated and tested for contamination, were cultured and transfected as previously described (Jin et al., 2009). Protocols for coimmunoprecipitation and western blot analysis were as previously described (Jin et al., 2009). Antibodies were: anti-myc (#5546, Sigma-Aldrich, St Louis, MO, 1:1,000), anti-FLAG (#F1804, Sigma-Aldrich, 1:1,000), anti-α-tubulin (#T5293, Sigma-Aldrich, 1:2,500), anti-xDnd1 (Mei et al., 2013, 1:1,000), anti-Nanos1 (Luo et al., 2011, 1:1,000), anti-mouse elf3f (#390413; Santa Cruz Biotechnology, Dallas, TX; 1:500), anti-GST (ab19256, Abcam,
Cambridge, MA). All blots consistently showed a high molecular weight nonspecific band, which was used as a loading control.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

T. A., Z. J., M. L. K., and J. Y. were responsible for conceptualization; T. A., Z. J., K. N., D. O., M. L. K., and J. Y. for methodology; T. A., Z. J., and D. O. for formal analysis; T. A., Z. J., and J. Y. were involved in investigation; M. L. K. and J. Y. were involved in writing—original draft; A. M., M. L. K., and J. Y. were involved in writing—review and editing; M. L. K. and J. Y. carried out supervision; M. L. K. and J. Y. were involved in project administration; M. L. K. and J. Y. were responsible for funding acquisition.

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