

# Dead end1 is an essential partner of NANOS2 for selective binding of target RNAs in male germ cell development

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## Abstract

RNA-binding proteins (RBPs) play important roles for generating various cell types in many developmental processes, including eggs and sperms. Nanos is widely known as an evolutionarily conserved RNA-binding protein implicated in germ cell development. Mouse NANOS2 interacts directly with the CCR4-NOT (CNOT) deadenylase complex, resulting in the suppression of specific RNAs. However, the mechanisms involved in target specificity remain elusive. We show that another RBP, Dead end1 (DND1), directly interacts with NANOS2 to load unique RNAs into the CNOT complex. This interaction is mediated by the zinc finger domain of NANOS2, which is essential for its association with target RNAs. In addition, the conditional deletion of DND1 causes the disruption of male germ cell differentiation similar to that observed in *Nanos2*-KO mice. Thus, DND1 is an essential partner for NANOS2 that leads to the degradation of specific RNAs. We also present the first evidence that the zinc finger domain of Nanos acts as a protein-interacting domain for another RBP, providing a novel insight into Nanos-mediated germ cell development.

**Keywords** Dead end; germ cell; Nanos; RNA

**Subject Categories** Development & Differentiation; RNA Biology

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## Introduction

RNA-binding proteins (RBPs) control all aspects of the “life cycle” of RNA from its transcription to its degradation. RBPs form dynamic ribonucleoprotein particles, often in a cell type-specific manner, and affect various biological processes, including asymmetric cell division, cell motility, embryonic axis determination, and neuronal plasticity [1,2]. Most importantly, RBPs play vital roles in germ cell development [3].

The differentiation of mammalian germ cells into male and female gametes is a fundamental biological process. In mice, the primordial germ cells (PGCs) are segregated from the somatic cell lineage at an early gastrulation stage [4] and then begin to migrate to the future gonads. Although the migrating PGCs are potent precursors of both oogonia and spermatogonia, they start sexual differentiation depending on the environmental cues after their colonization of the embryonic gonads with somatic cells; germ cells in female gonads (female gonocytes) immediately enter meiosis, whereas the germ cells in male gonads (male gonocytes) undergo cell cycle arrest at the G0/G1 phase [5]. To date, it has been reported that at least two somatic factors are required for the masculinization of the PGCs in male embryonic gonads. CYP26B1, a retinoic acid (RA)-metabolizing enzyme, is expressed in the Sertoli cells and protects male gonocytes from exposure to RA, resulting in the suppression of meiosis [6,7]. In addition, somatically derived fibroblast growth factor 9 induces the expression of the male gonocyte-specific gene, *Nanos2*, via activation of the Nodal/Activin pathway [8]. Once the expression of NANOS2 protein begins, male-specific genetic programming is initiated.

Nanos is an evolutionarily conserved RBP implicated in germ cell development [9–12]. Three *Nanos* homologues, *Nanos1–3*, exist in mice, among which *Nanos2* is expressed only in male gonocytes and plays a key role during the sexual development of these cells by suppressing meiosis and promoting male-type differentiation [13]. We have previously shown that NANOS2 associates with the CNOT deadenylase complex via direct interaction with CNOT1, a component of the CNOT complex [14]. This complex localizes to P-bodies, the RNA–protein granules for storage of nontranslating RNAs, which likely induce the degradation of specific RNAs [15,16]. However, the mechanism of specific RNA recognition remains unclear since the zinc finger domain of Nanos, which is composed of two highly conserved, consecutive CCHC-type zinc finger motifs, has been shown to bind RNA in a sequence-unspecific manner [17,18]. In our current study, we attempted to elucidate the mechanism by which NANOS2 selects target RNAs for degradation and found that DND1

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was a partner of NANOS2. We also established a *Dnd1*-conditional knockout (*Dnd1*-cKO) mouse strain to examine the *in vivo* function of the NANOS2–DND1 complex in male gonocytes.

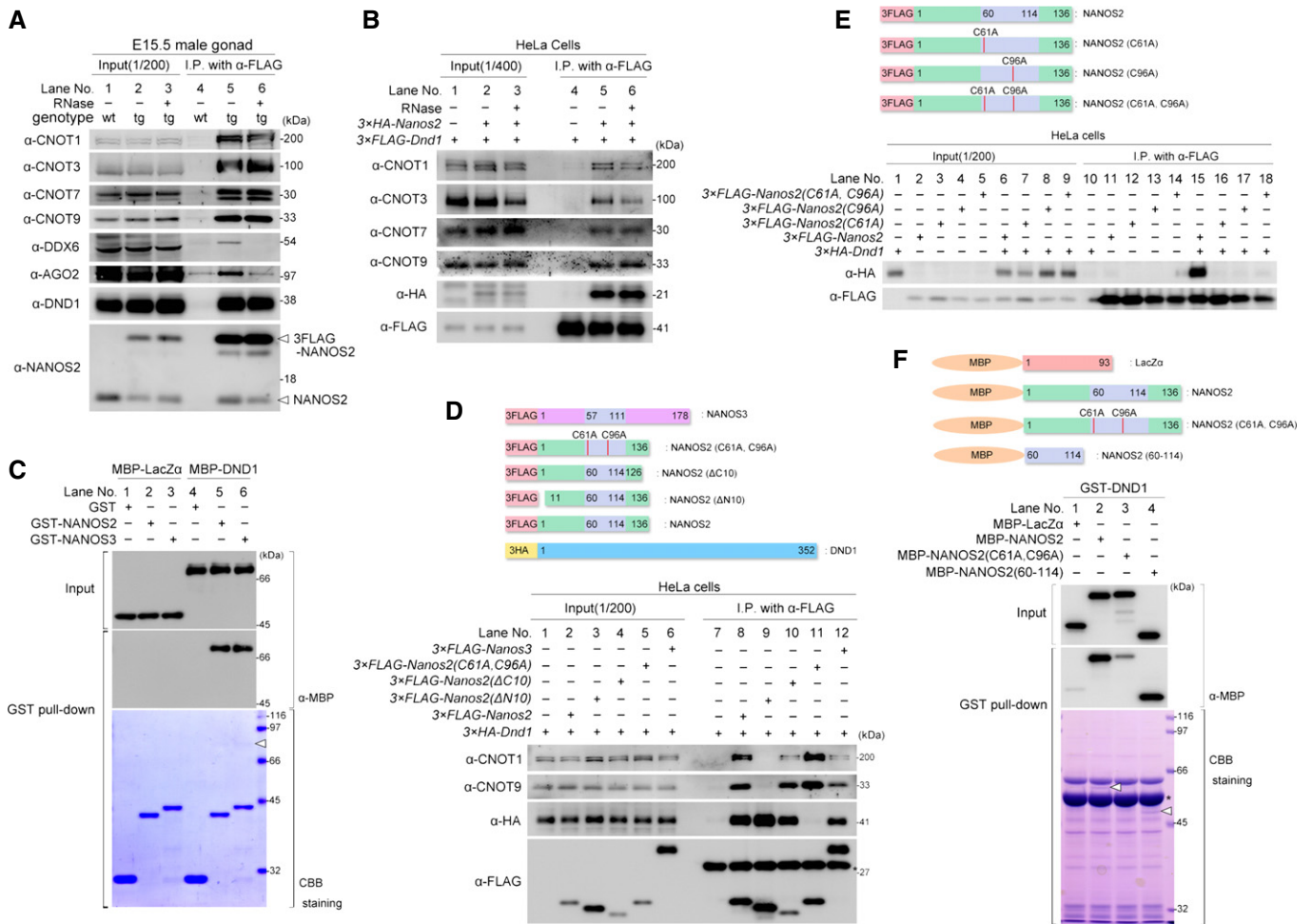
## Results and Discussion

### DND1 directly interacts with NANOS2 via the zinc finger domain

To elucidate the mechanism of NANOS2-mediated RNA regulation in the sexual differentiation of male gonocytes, we searched for NANOS2-interacting proteins by immunoprecipitation (IP) from

male gonadal extracts, followed by extensive mass-spectrometric analyses. For this purpose, we took advantage of a transgenic mouse line expressing FLAG-tagged NANOS2 under the control of the *Nanos2* enhancer, which could fully rescue the defects of *Nanos2*-knockout (*Nanos2*-KO) mice [19]. These experiments allowed the identification of several proteins, including CNOT1, Poly (A)-binding protein1 (PABP1), and DND1 (Fig EV1A–D and Table EV1). We focused on DND1 as a strong candidate.

DND1 is an RBP initially identified in zebrafish and conserved among vertebrates [20]. Mouse *Dnd1* is identified as a causable gene for the *Ter* mutation [21], by which most of the PGCs disappear at the migration stage, resulting in complete male sterility [22].



**Figure 1. DND1 interacts directly with NANOS2 via its zinc finger motif.**

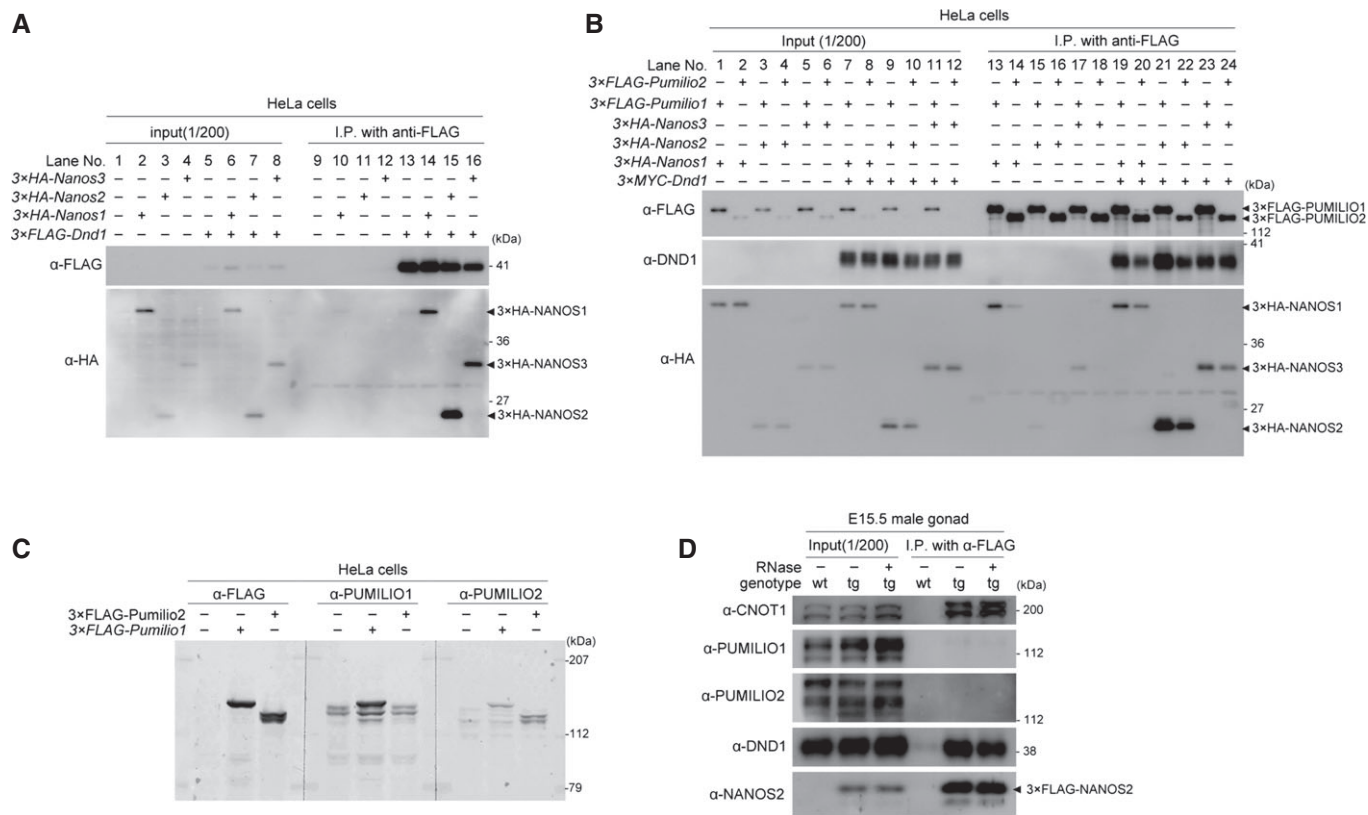
- A, B Western blotting analysis of proteins co-precipitated with anti-FLAG antibody from testis extracts of E15.5 wild-type embryos and transgenic embryos expressing FLAG-tagged NANOS2 with or without RNase (A) or from extracts of HeLa cells transfected with FLAG-tagged DND1 and with or without HA-tagged NANOS2 (B). Precipitates were analyzed with the indicated antibodies.
- C GST pull-down assay using *E. coli* extracts expressing GST, GST-fused NANOS2, or NANOS3 mixed with MBP-tagged LacZα or DND1. Precipitates were analyzed by Coomassie Brilliant Blue (CBB) staining or Western blotting with an anti-MBP antibody. An arrowhead indicates MBP-DND1 co-precipitated with GST-tagged NANOS2 or NANOS3. See also Fig EV1E.
- D, E Western blotting analyses of proteins co-precipitated with anti-FLAG antibody from extracts of HeLa cells transfected with FLAG-tagged NANOS2, NANOS2 (ΔN10), NANOS2 (ΔC10), NANOS2 (C61A, C96A) or NANOS3, and HA-tagged DND1 (D) or with FLAG-tagged NANOS2, NANOS2 (C61A), NANOS2 (C96A) or NANOS2 (C61A, C96A), and HA-tagged DND1 (E). Precipitates were analyzed with the indicated antibodies.
- F GST pull-down assay using *E. coli* extracts expressing GST-fused DND1 mixed with MBP-tagged LacZα, NANOS2, NANOS2 (C61A, C96A) or NANOS2 (60–114). Arrowheads indicate MBP-tagged NANOS2 (lane 2) or NANOS2 (60–114) (lane 4).

However, it remains unclear whether DND1 plays a role in germ cell development after the migration stage. Therefore, we first generated a specific antibody to examine the expression profile. As expected from a functional study of *Ter* mutants showing a decreased germ cell number in the early embryonic stage, DND1 was highly expressed in the migrating PGCs (Fig EV2A–L). However, its expression became restricted to male gonocytes after colonization of the embryonic gonads, while such signals became undetectable in female gonocytes (Fig EV2M–R). Western blotting analyses confirmed the expression profile; DND1 expression gradually increased in the male gonads and was absent in the female gonads by embryonic day (E) 14.5 (Fig EV2S), raising the possibility that, together with NANOS2, DND1 is involved in the sexual differentiation of male gonocytes.

To confirm the interaction between NANOS2 and DND1, we conducted IP assays from E15.5 male gonadal extracts using the transgenic mouse line expressing FLAG-tagged NANOS2, followed by Western blotting analyses (Fig 1A). This assay revealed that CNOT proteins co-precipitated with FLAG-tagged NANOS2

independently of RNA, as reported previously [19]. Factors localizing to P-bodies, DEAD box polypeptide 6 (DDX6) and Argonaute 2 (AGO2) [23,24], also co-precipitated with FLAG-tagged NANOS2 but were removed by RNase treatment, indicating that the interaction is RNA-dependent. On the contrary, DND1 co-precipitated efficiently with NANOS2 even when the extracts were treated with RNase, demonstrating that NANOS2 interacts with DND1 *in vivo*, independent of the presence of RNA.

Subsequently, we asked whether DND1 associated directly with NANOS2, since the association of DND1 with NANOS2 might be mediated by CNOT proteins. However, IP assays in HeLa cells revealed that the CNOT proteins co-precipitated with FLAG-tagged DND1 only when HA-tagged NANOS2 was co-expressed, regardless of whether RNase was added (Fig 1B). Furthermore, we found that purified recombinant maltose-binding protein (MBP)-tagged DND1 was co-precipitated with bacterially expressed glutathione-S-transferase (GST)-tagged NANOS2 or NANOS3 in a pull-down assay using antibodies against MBP (Fig 1C, lanes 5 and 6) or Coomassie Brilliant Blue (CBB) staining (Fig EV1E, lanes 5 and 6),



**Figure 2. Relationship between NANOS and PUMILIO.**

- A, B Western blotting analyses of proteins co-precipitated with anti-FLAG antibody from extracts of HeLa cells transfected with HA-tagged NANOS1, NANOS2, or NANOS3, and FLAG-tagged DND1 (A) or with FLAG-tagged PUMILIO1 or PUMILIO2, and HA-tagged NANOS1, NANOS2, or NANOS3 with or without MYC-tagged DND1 (B).
- C Characterization of antibodies against PUMILIO1 and PUMILIO2. Western blotting analyses of Flag-tagged PUMILIO1 and PUMILIO2 in HeLa cells transfected with FLAG-tagged PUMILIO1 or PUMILIO2 using antibodies against FLAG, PUMILIO1 or PUMILIO2. Note that the antibodies against PUMILIO1 specifically recognized FLAG-tagged PUMILIO1, while the anti-PUMILIO2 antibody could detect both Flag-tagged PUMILIO1 and PUMILIO2.
- D FLAG-tagged NANOS2 was immunoprecipitated with an anti-FLAG antibody from E15.5 testicular extracts from transgenic mice expressing FLAG-tagged NANOS2. Precipitates were analyzed by Western blotting with the indicated antibodies. Note that co-precipitation of both PUMILIO1 and PUMILIO2 were not detected even though both CNOT1 and DND1 were clearly co-precipitated.

indicating that DND1 interacts directly with both NANOS2 and NANOS3, independently of the CNOT complex. This result also implied that a common amino acid sequence between NANOS2 and NANOS3 might be involved in the interaction with DND1. The N-terminus of NANOS2 had already been reported to be structurally essential for the direct interaction with the CNOT1, which has been recently named as NOT1-interacting motif (NIM) [25] (Fig EV1F). We then focused on the conserved zinc finger motifs. IP assays in HeLa cells revealed that substitution of the first cysteine residues in the two CCHC motifs of NANOS2 (C61 and C96) with alanine, yielding NANOS2 (C61A, C96A), drastically reduced and almost abolished the interaction with DND1, while the interactions with the CNOT proteins were intact (Fig 1D, lane 11). In contrast, the deletion of the N- or C-terminal residues had a limited, or almost no effect, on the interaction with DND1, whereas NANOS2 ( $\Delta$ N10) could not associate with the CNOT complex as previously reported (Fig 1D, lanes 9 and 10) [14,25]. In addition, single mutations in either CCHC motif (C61A or C96A) also drastically reduced this interaction (Fig 1E, lanes 16 and 17), indicating that both of the CCHC-type zinc finger motifs are structurally essential for the interaction of NANOS2 with DND1. We next tested whether or not these two zinc finger motifs alone were sufficient for effective interaction of NANOS2 and DND1. To this end, MBP-tagged zinc finger motifs (NANOS2 (60–114)) were subjected to a pull-down assay with GST-tagged DND1. Western blotting analyses revealed that the interaction between NANOS2 (C61A, C96A) and DND1 was much weaker than that of wild-type NANOS2, and rather similar to that of negative control (Fig 1F, lanes 1–3), which could reflect the interaction observed in HeLa cells (Fig 1D, lane 11). On the other hand, NANOS2 (60–114) showed the same degree of co-precipitation as wild-type NANOS2 (Fig 1F, lanes 2 and 4). These data indicate that the zinc finger domain of NANOS2 is essential and sufficient for the direct interaction with DND1; thus, the two conserved, consecutive CCHC-type zinc finger motifs of NANOS2 constitute a protein-interacting domain for DND1.

#### Interactions between NANOS2 and PUMILIO1/PUMILIO2 are not detected in male gonocytes

Because we found that DND1 interacted with all NANOS family member (NANOS1–3) in HeLa cells (Fig 2A), DND1 is presumed to be a major partner of mouse NANOS. On the other hand, it is well known that Nanos works together with Pumilio in many species [26–28]; the interaction between human NANOS1 and PUMILIO1 was reported in cultured cells via co-transfection and IP assay [29]. However, the mouse homologues PUMILIO1 and PUMILIO2 were not detected in our mass-spec analyses of NANOS2-IP (Fig EV1A and B). To ask more directly the relationship between mouse NANOS and PUMILIO, we transfected HeLa cells with HA-tagged NANOS (NANOS1, NANOS2, or NANOS3) and Flag-tagged PUMILIO (PUMILIO1 or PUMILIO2). IP assays revealed that mouse NANOS1 seemed to interact with both PUMILIO1 and PUMILIO2 (Fig 2B, lanes 13 and 14) as was the case in human NANOS1 and PUMILIO1, whereas such interactions were not detected or very weak for NANOS2 and NANOS3 (Fig 2B, lanes 15–18). However, we found obvious co-precipitation of NANOS2 and NANOS3 with either PUMILIO1 or PUMILIO2 when MYC-tagged DND1 was co-expressed (Fig 2B, lanes 21–24), raising the possibility that NANOS2 interacts

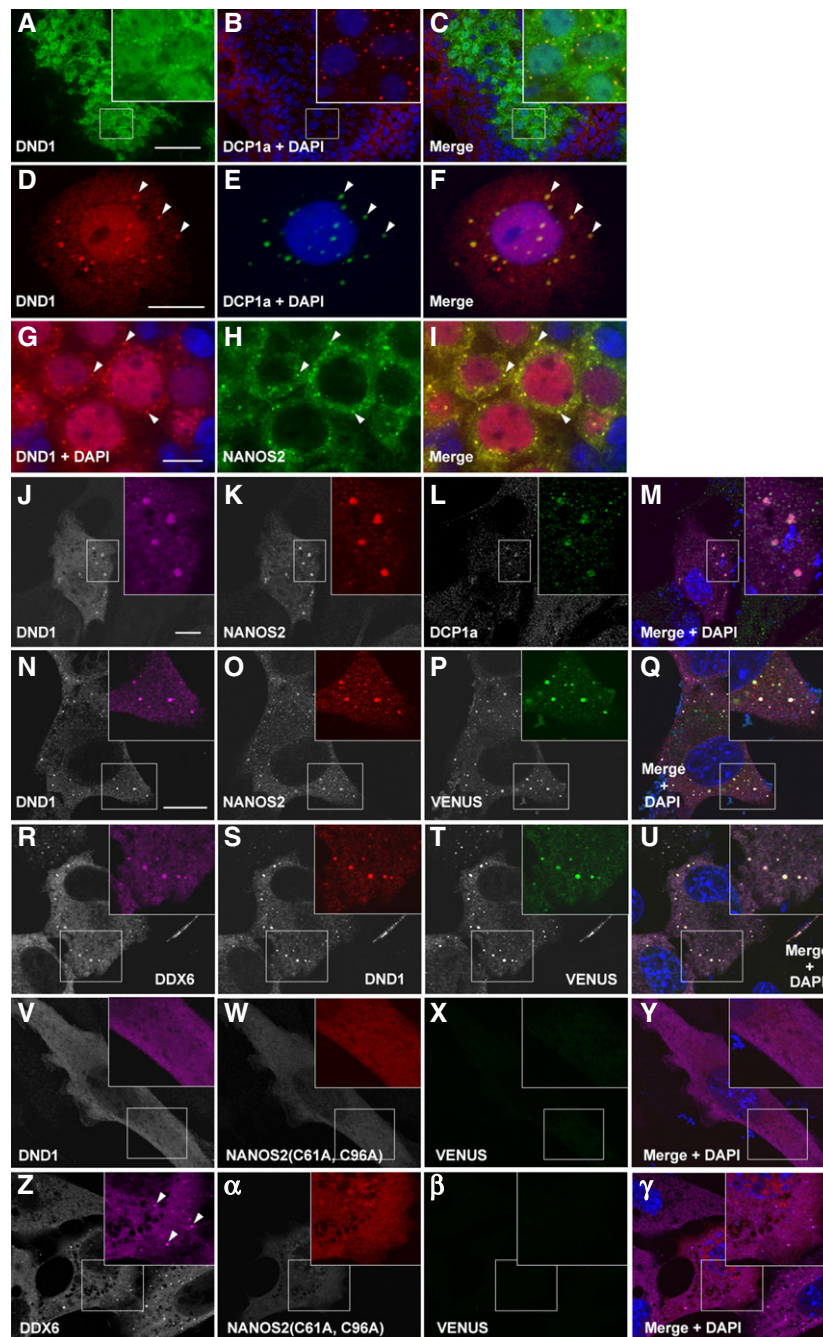
with PUMILIO1/PUMILIO2 via DND1 *in vivo*. Therefore, we conducted IP assay using gonadal extracts from E15.5 embryos expressing Flag-tagged NANOS2, but we found no interaction between NANOS2 and PUMILIO1 or PUMILIO2 (Fig 2C and D), consistent with our mass-spec analyses. These results suggest that NANOS2 does not interact with either PUMILIO1 or PUMILIO2 in male gonocytes even in the presence of DND1, although the reason is currently unknown.

#### DND1 co-localizes with NANOS2 in P-bodies

As NANOS2 localizes to P-bodies [19], we expected to find co-localization of DND1 in P-bodies. Immunostaining analyses of male gonadal sections at E15.5 revealed that DND1 was distributed in both the nucleus and cytoplasm (Fig 3A). Furthermore, cytoplasmic DND1 seemed to form discrete foci, which co-localized with DCP1a, a well-known marker for P-bodies (Fig 3B and C) [23]. To confirm this, we conducted immunostaining using squash preparations of male gonocytes at E16.5 and found clear localization of DND1 to the P-bodies (Fig 3D–F). Therefore, DND1 and NANOS2 co-localized in the cytoplasm, particularly in the P-bodies (Fig 3G–I), as expected. We next asked whether such an interaction is required for the localization to P-bodies, using a co-transfection method in cultured cells (NIH3T3 cells). Interestingly, DND1 and NANOS2 localized to P-bodies only when both proteins were concurrently expressed (Fig 3J–M), whereas expression of each protein alone was not sufficient for clear localization to P-bodies (Fig EV2T–Y). To further determine whether the direct interaction between DND1 and NANOS2 contributes to their P-body localization, we conducted a biomolecular fluorescence complementation assay using VENUS-C-fused DND1 and VENUS-N-fused wild-type NANOS2 or mutant-type NANOS2 (C61A, C96A). In the case of wild-type NANOS2, the fluorescence of VENUS protein was observed throughout the cytoplasm with some granular structures in which both NANOS2 and DND1 co-localized (Fig 3N–Q). Additionally, these granular VENUS signals were merged with granules of DDX6, a P-body marker (Fig 3R–U), indicating localization of the NANOS2–DND1 complex to P-bodies. In contrast, no VENUS signal was detected when NANOS2 (C61A, C96A) was used (Fig 3V–Y), although the assembly of P-bodies itself was observed in these cells (Fig 3Z– $\gamma$ ). These data demonstrate that the zinc finger domain of NANOS2 is required for the interaction with DND1 and that this interaction is essential for localization of the complex to the P-bodies.

#### Conditional deletion of DND1 results in similar but not identical phenotypes to those of *Nanos2*-KO male gonocytes

Given that the interaction with DND1 is physiologically significant for NANOS2 function, the loss of DND1 in male gonocytes should result in similar phenotypes to those in *Nanos2*-KO male gonocytes, including failure of mitotic arrest, entry into meiosis, apoptotic cell death, failure of male-type differentiation, and upregulation of the PGC characteristics [13]. To address this issue, we generated a conditional allele of *Dnd1* (Fig EV3A–C) and crossed the *Dnd1*-flox mouse with a transgenic mouse expressing CreER<sup>T2</sup> under the control of the Oct4 $\Delta$ PE enhancer (*Oct4* $\Delta$ PE-CreER<sup>T2</sup>) [30], followed by tamoxifen injection at E13.5 (Fig EV3D–J). As shown in Western blotting analyses, DND1 expression gradually decreased



**Figure 3. DND1 co-localizes with NANOS2 in P-bodies.**

A–C Sections of male gonads from E15.5 embryos were immunostained with antibodies against DND1 (green) and DCP1a (red).

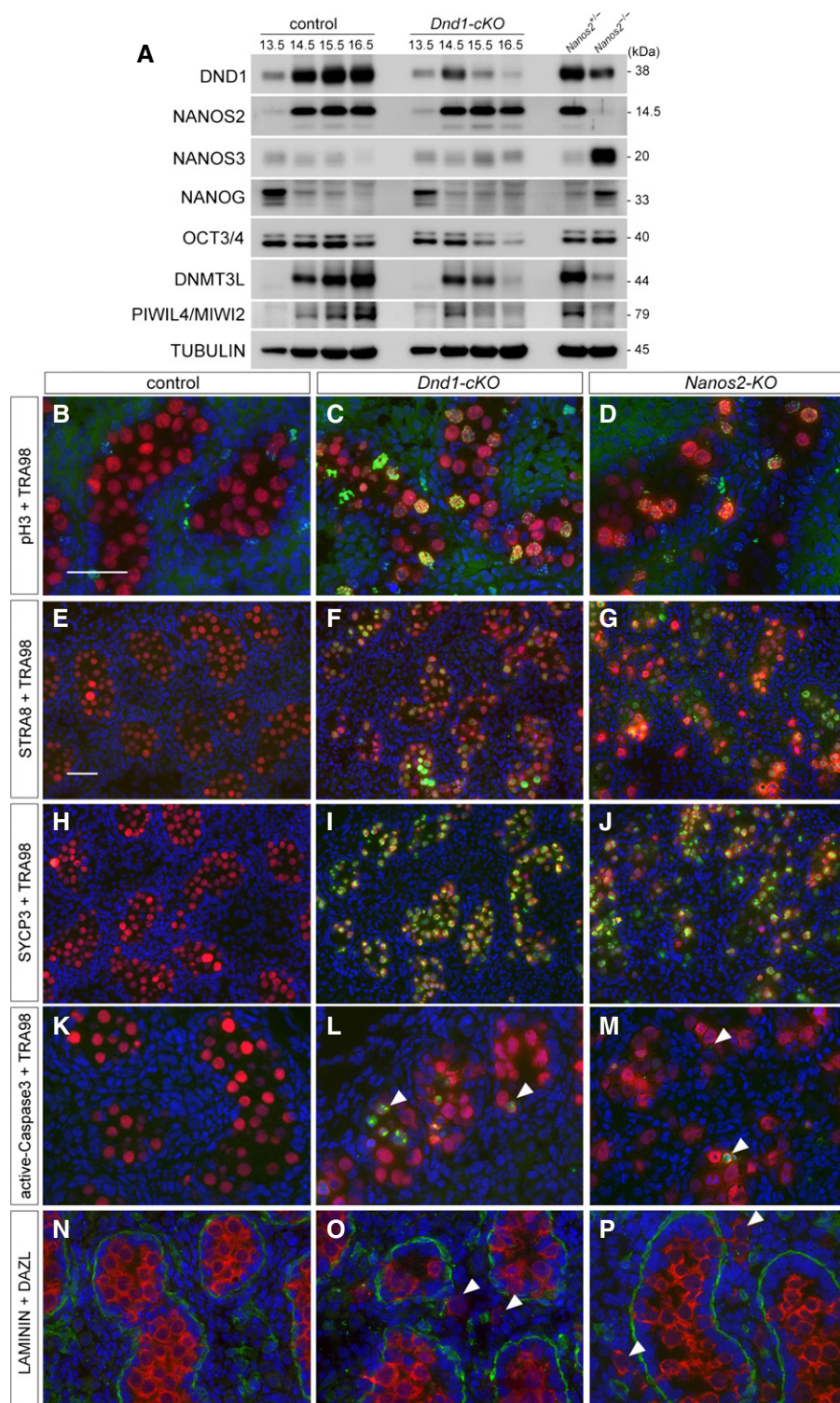
D–F Squash preparation of a male gonocyte from E16.5 embryo immunostained with antibodies against DND1 (red) and DCP1a (green). Arrowheads indicate co-localization of DND1 and DCP1a.

G–I Sections of male gonads from E15.5 embryos were immunostained with antibodies against DND1 (red) and NANOS2 (green). Arrowheads indicate co-localization of DND1 and NANOS2.

J–M NIH3T3 cells transfected with HA-tagged *Dnd1* and FLAG-tagged *Nanos2* were then immunostained with antibodies against DND1 (J) (magenta), NANOS2 (K) (red), and DCP1a (L) (green).

N– $\gamma$  Biomolecular fluorescence complementation assay. NIH3T3 cells transfected with VENU-C-fused *Dnd1* and VENU-N-fused *Nanos2* (N–U) or *Nanos2* (C61A, C96A) (V– $\gamma$ ) were immunostained with antibodies against DND1 (N, S, V), NANOS2 (O, W,  $\alpha$ ) or DDX6 (R, Z). Then, the signals of VENU fusion protein were visualized (P, T, X,  $\beta$ ). Arrowheads in (Z) indicate P-bodies.

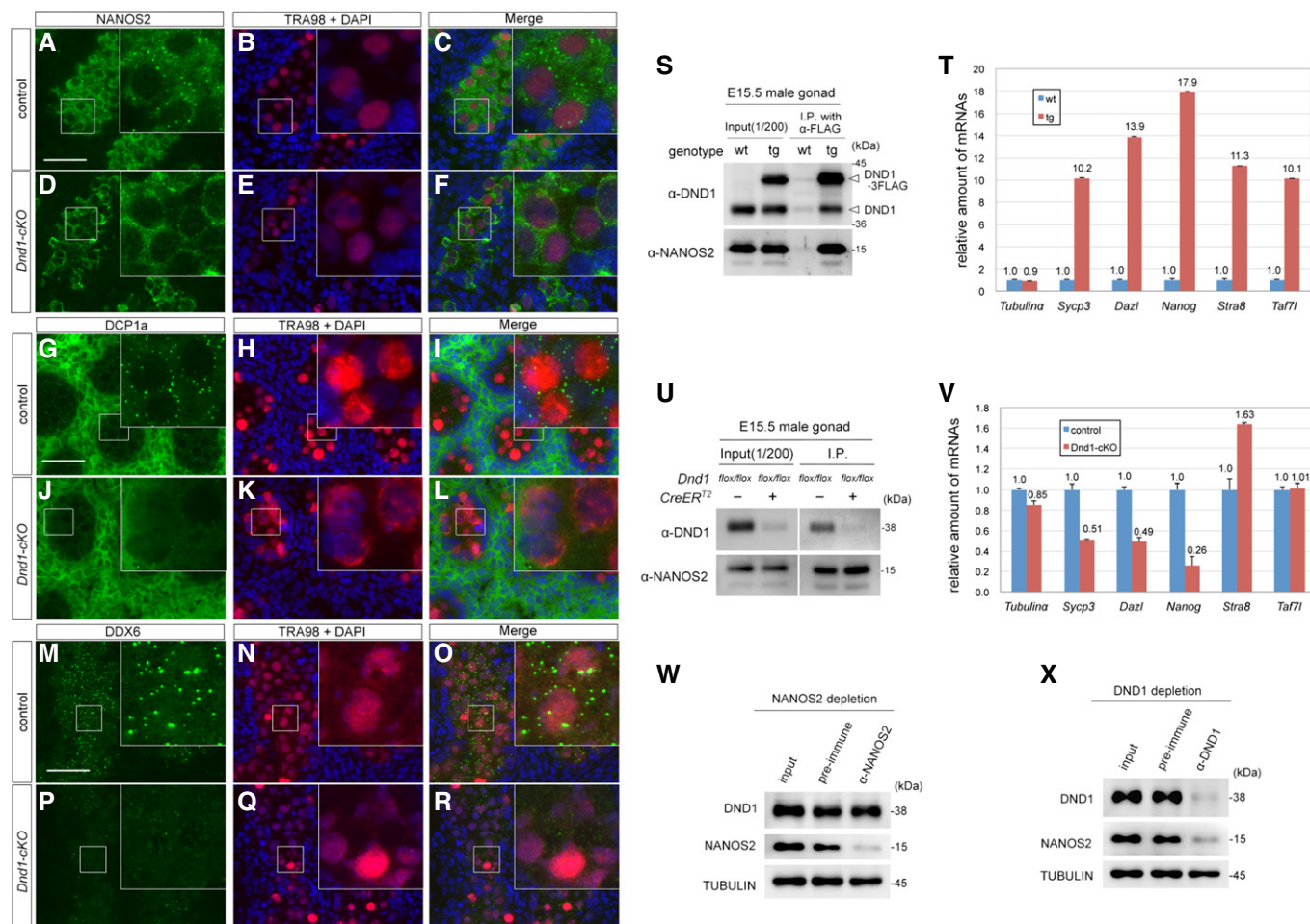
Data information: DNA was labeled with DAPI (blue). Scale bars: 50  $\mu$ m in (A) for (A–C); 10  $\mu$ m in (D, G, J, N) for (D–F), (G–I), (J–M) and (N– $\gamma$ ), respectively. Insets in (A–C) and (J– $\gamma$ ) show an enlarged version of each picture to better depict localization of each protein. See also Fig EV2T–Y.



**Figure 4. Conditional deletion of DND1 causes similar but not identical phenotypes to those of *Nanos2*-KO male gonocytes.**

**A** Western blotting analyses of proteins in testes from E13.5 to E16.5 embryos of *Dnd1<sup>fllox/fllox</sup>* or *Dnd1<sup>fllox/fllox</sup>\_Tg(Oct4.ΔPE-CreER<sup>T2</sup>)* each administered with tamoxifen at E13.5 and E15.5 embryos of *Nanos2<sup>+/+</sup>* or *Nanos2<sup>-/-</sup>* with the indicated antibodies.

**B–P** Sections of testes from *Dnd1<sup>fllox/fllox</sup>* (B, E, H, K, N), *Dnd1<sup>fllox/fllox</sup>\_Tg(Oct4.ΔPE-CreER<sup>T2</sup>)* (C, F, I, L, O) or *Nanos2<sup>-/-</sup>* (D, G, J, M, P) embryos were prepared at E16.5 and then immunostained with antibodies against pH3 (B–D), STRA8 (E–G), SYCP3 (H–J), activated caspase-3 (K–M) or LAMININ (N–P) (green). Germ cells were immunostained with TRA98 (B–D) or DAZL (N–P) (red), and DNA was labeled with DAPI (blue). Tamoxifen was administered at E13.5. Arrowheads indicate apoptotic cells (L, M) or cells outside the tubules (O, P). Scale bars: 50 μm in (B) for (B–D) and (K–P), 50 μm in (E) for (E–J). See also Fig EV3.



**Figure 5. DND1 is required for loading of the target RNAs to NANOS2-CNOT complex.**

A–R Sections of testes from *Dnd1*<sup>flax/flax</sup> (A–C, G–I, M–O) and *Dnd1*<sup>flax/flax</sup>\_Tg(*Oct4.APE-CreERT2*) (D–F, J–L, P–R) embryos were prepared at E16.5 and then immunostained with antibodies against NANOS2 (A, D) (green), DCP1a (G, J) (green), DDX6 (M, P) (green) and TRA98 (B, E, H, K, N, Q). DNA was labeled with DAPI (blue). Tamoxifen was administered at E13.5. Scale bars: 50  $\mu$ m in (A) for (A–F); 50  $\mu$ m in (G) for (G–L); 50  $\mu$ m in (M) for (M–R). Insets show an enlarged version of each picture to better depict localization of NANOS2, DCP1a and DDX6.

S–V Immunoprecipitation with an anti-FLAG antibody from E15.5 male gonadal extracts of wild-type and the transgenic mouse line expressing FLAG-tagged DND1 (S, T), or with an anti-NANOS2 antibody from E15.5 male gonadal extracts of *Dnd1*<sup>flax/flax</sup> mice with or without *Oct4.APE-CreERT2* (U, V). Precipitates were analyzed by Western blotting (S, U) or by RT-qPCR (T, V). The RT-qPCR data in (T) and (V) are shown as average relative mRNA levels  $\pm$  SE ( $n = 3$ ).

W, X Western blotting analyses of proteins in NANOS2-depleted (W) or DND1-depleted (X) testis extracts from E15.5 embryos. Proteins were analyzed with the indicated antibodies.

and almost disappeared at E16.5, whereas the expression level of NANOS2 was unaffected by the deletion of DND1 (Fig 4A). In this condition, the expression of marker proteins for PGC characteristics, NANOS3, NANOG, and OCT3/4 [30–32], was almost unaffected or downregulated in *Dnd1*-cKO male gonads regardless of upregulation of these proteins in *Nanos2*-KO male gonads at E15.5, indicating that NANOS2 suppresses PGC characteristics in a DND1-independent manner. On the other hand, the expression of DNMT3L and PIWIL4/MIWI2, marker proteins for male-type differentiation [33,34], was strongly compromised. These expressions began at approximately E14.5 and continued in the control male gonads. In the gonads of *Dnd1*-cKO mice, however, the expressions were gradually decreased in association with the reduction in DND1 to the same extent as in *Nanos2*-KO male gonads at

E15.5. These data indicate a failure of male-type differentiation, as is the case in *Nanos2*-KO male gonocytes. To examine germ cell differentiation status more precisely, we used several antibodies to examine mitotic activity, meiotic entry, and apoptosis by immunostaining [13,35]. We found strong signals of phosphorylated histone H3 (pH3) (Fig 4B and C), upregulation of the meiotic markers STRA8 and SYCP3 [36,37] (Fig 4E, F, H and I), and signals of activated caspase-3 (Fig 4K and L), traits which were all reported to be observed in *Nanos2*-KO male gonocytes [13] (Fig 4D, G, J and M). In addition, we found male gonocytes outside the tubules in *Dnd1*-cKO male gonads (Fig 4N and O), which is also a phenotype of *Nanos2*-KO mice [12] (Fig 4P). Collectively, although there is a difference in the aspect of suppression of PGC characteristics, other phenotypes are similar between

these two mutant mice, indicating the physiological significance of DND1 for NANOS2 function.

### DND1 is required for loading of the target RNAs into the NANOS2–CNOT complex

To further address the significance of the interaction between DND1 and NANOS2, we next analyzed the localization of NANOS2 in *Dnd1*-cKO male gonocytes by immunostaining and found that no cytoplasmic granular structures were observed in the absence of DND1 (Fig 5A–F). Therefore, we checked the status of P-bodies with an anti-DCP1a antibody and found that almost all of them had disappeared in *Dnd1*-cKO male gonocytes (Fig 5G–L). This dissipation of P-bodies was confirmed using an anti-DDX6 antibody (Fig 5M–R), indicating the necessity of DND1 for the assembly of P-bodies in male gonocytes. This DND1-dependent mechanism of P-body assembly may be unique to male gonocytes and different from the mechanism of assembly in cultured cells (Fig 3J–L). Nevertheless, the results suggest that target RNAs may not be loaded into the NANOS2–CNOT deadenylase complex without DND1, since loading of RNAs in the degradation pathway is a prerequisite for P-body assembly [16,38]. To test this possibility, we generated transgenic mouse lines expressing FLAG-tagged DND1 (Fig EV4A–L) and examined whether DND1-associated mRNAs included NANOS2-associated mRNAs by IP from E15.5 male gonadal extracts of the transgenic embryos. Western blotting analyses revealed co-precipitation of NANOS2 with FLAG-tagged DND1 (Fig 5S), and reverse transcription-quantitative polymerase chain reaction (RT–qPCR) results showed that the known NANOS2-associated mRNAs, *Sycp3*, *Dazl*, *Nanog*, *Stra8*, and *Taf7l* [19,35] were enriched among the RNAs precipitated with FLAG-tagged DND1 whereas  $\alpha$ -tubulin mRNA was not (Fig 5T). Finally, to test whether these target RNAs were loaded into NANOS2 in the absence of DND1, we collected *Dnd1*-cKO embryonic gonads and conducted IP using antibodies against NANOS2. We used  $\alpha$ -tubulin mRNA as a negative control. Although the amounts of NANOS2 precipitated with the antibodies were almost same between control and *Dnd1*-cKO gonadal extracts (Fig 5U), the amounts of the NANOS2-associated mRNAs, except for *Stra8* and *Taf7l* mRNAs (*Sycp3*, *Dazl* and *Nanog*), were reduced drastically in the absence of DND1 (Fig 5V). These results strongly indicate that DND1 is essential for the association of NANOS2 with certain target mRNAs. Concerning *Stra8* and *Taf7l* mRNAs, however, not all of the target mRNAs appear to interact with NANOS2 in a DND1-dependent manner. We, therefore, validated what quantity of NANOS2 associated with DND1 in the male gonocytes and vice versa. Immunodepletion analyses using E15.5 male gonadal extracts revealed that the amount of DND1 was slightly affected by NANOS2 depletion (Fig 5W), whereas a large fraction of NANOS2 was eliminated by DND1 depletion (Fig 5X), suggesting that DND1 is a major partner of NANOS2 in male gonocytes. However, a small fraction of NANOS2 seemed to remain in the extracts even after DND1 depletion (Fig 5X), indicating that some NANOS2 is found outside of the DND1–NANOS2 complex. Therefore, certain RNAs, such as *Stra8* and *Taf7l* mRNAs, might associate with a small fraction of NANOS2 that is not associated with DND1; or an alternate RBP regulates the association of these RNAs with the NANOS2–DND1 complex independently of DND1.

On the other hand, NANOG expression was upregulated in *Nanos2*-KO male gonads (Fig 4A) and its mRNA associated with NANOS2 in a DND1-dependent manner (Fig 5V). However, NANOG expression in *Dnd1*-cKO male gonads was almost unaffected (it was similar to that in the wild-type) (Fig 4A). These data suggest that *Nanog* is suppressed mainly by an unknown mechanism under the control of NANOS2 but not DND1, and the DND1–NANOS2 complex contributes to only a part of the suppression mechanism for *Nanog* mRNA. Since DND1 is also implicated in mRNA protection from miRNA-mediated RNA degradation in other context [39], identification of RNAs associated with the DND1–NANOS2 complex and DND1-partner proteins except for NANOS2 is required for understanding the *Nanos2*-KO and *Dnd1*-cKO phenotypes.

## Materials and Methods

The detailed experimental procedures are available in the Appendix.

### Ethics statement

Experiments were carried out with the permission of the animal experimental committee at the Yokohama National University and the National Institute of Genetics.

### Mice

The *Nanos2*-KO mouse line, the transgenic mouse line expressing FLAG-tagged NANOS2 and the *Ter*-mutant mouse line were previously described [12,19,21].

### Immunoprecipitation – mass spectrometry

For the mass-spectrometric analyses, 100 testes from either wild-type or transgenic E15.5 embryos expressing FLAG-tagged NANOS2 were used for the generation of extracts as previously described [19].

### Biomolecular fluorescence complementation assay

Both VENUS-C-fused *Dnd1* and VENUS-N-fused *Nanos2* were cloned into pcDNA3.1 as described previously [40] and transfected into NIH3T3 cells.

**Expanded View** for this article is available online.

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## Author contributions

AS and YS developed the concept of this paper. KS carried out the mass-spectrometric analyses and ZZ conducted the biomolecular fluorescence complementation assay, and MK generated the *Dnd1* conditional knockout mouse line. AS and YN carried out all other experiments and data analyses. AS and YS prepared the manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

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