Interaction between NANOS2 and the CCR4-NOT Deadenylation Complex Is Essential for Male Germ Cell Development in Mouse

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Abstract

Nanos is one of the evolutionarily conserved proteins implicated in germ cell development and we have previously shown that it interacts with the CCR4-NOT deadenylation complex leading to the suppression of specific RNAs. However, the molecular mechanism and physiological significance of this interaction have remained elusive. In our present study, we identify CNOT1, a component of the CCR4-NOT deadenylation complex, as a direct factor mediating the interaction with NANOS2. We find that the first 10 amino acids (AAs) of NANOS2 are required for this binding. We further observe that a NANOS2 mutant lacking these first 10 AAs (NANOS2-AN10) fails to rescue defects in the Nanos2-null mouse. Our current data thus indicate that the interaction with the CCR4-NOT deadenylation complex is essential for NANOS2 function. In addition, we further demonstrate that NANOS2-AN10 can associate with specific mRNAs as well as wild-type NANOS2, suggesting the existence of other NANOS2-associated factor(s) that determine the specificity of RNA-binding independently of the CCR4-NOT deadenylation complex.

Introduction

The sexual development of mammalian germ cells leading to the generation of eggs and sperm is a critically important biological process. In the mouse, the primordial germ cells (PGCs) are segregated from the somatic cell lineage at an early gastrulation stage [1]. Although the PGCs are potent precursors for both oogonia and spermatogonia, sexual differentiation is induced after the colonization of the embryonic gonads with somatic cells. Retinoic acid (RA) signaling is implicated as the molecular mechanism and physiological significance of this interaction have remained elusive. In our present study, we identify CNOT1, a component of the CCR4-NOT deadenylation complex, as a direct factor mediating the interaction with NANOS2. We find that the first 10 amino acids (AAs) of NANOS2 are required for this binding. We further observe that a NANOS2 mutant lacking these first 10 AAs (NANOS2-AN10) fails to rescue defects in the Nanos2-null mouse. Our current data thus indicate that the interaction with the CCR4-NOT deadenylation complex is essential for NANOS2 function. In addition, we further demonstrate that NANOS2-AN10 can associate with specific mRNAs as well as wild-type NANOS2, suggesting the existence of other NANOS2-associated factor(s) that determine the specificity of RNA-binding independently of the CCR4-NOT deadenylation complex.

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each of the functions of NANOS2 relies on its association with the CCR4-NOT deadenylation complex.

In our current study, we have explored the molecular basis of the interaction between NANOS2 and the CCR4-NOT deadenylation complex in vitro and identified CNOT1 as a direct interacting protein. We further examined the biological significance of this interaction by generating a transgenic mouse that expresses a NANOS2 variant lacking the domain required for its interaction with the CCR4-NOT deadenylation complex.

Results

NANOS2 associates with the CCR4-NOT deadenylation complex via a direct interaction with CNOT1

In a previous study, we showed that NANOS2 associates with the CCR4-NOT deadenylation complex in male gonocytes, and that this interaction is responsible for the deadenylation activity of NANOS2 [8]. However, the mechanism underlying this association had remained unknown. To address this issue, we first compared the amino acid sequences of the Nanos proteins among several species from fish to human to screen for possible consensus sequences. Conserved sequences at both the N and C-terminus were identified in addition to two highly conserved CCHC-type zinc finger motifs (Figure S1). The N-terminal sequence was found to be common to all of the species compared whilst the C-terminal sequence was specific to mammals. We thus analyzed the function of the N-terminal conserved sequence given that the CCR4-NOT deadenylation complex is evolutionarily conserved in all of the species compared here.

We generated several N-terminal deletion mutants of Nanos2 (Figure 1A) and co-transfected them into HeLa cells with HA-tagged CNOT6, a component of the CCR4-NOT deadenylation complex (since no antibody is currently available). Immunoprecipitation assays revealed that full length NANOS2 efficiently co-precipitated endogenous components of CCR4-NOT deadenylation complex (CNOT1, 3, 7, 9) and also HA-tagged CNOT6 (Figure 1B, lane7), indicating that the interaction between NANOS2 and the CCR4-NOT deadenylation complex can be reproduced in HeLa cells. However, a deletion of the first 10 N-terminal residues of NANOS2 (yielding NANOS2-DN10) completely abolished this interaction (Figure 1B, lane 9) whereas there was no such affect if the first 5 amino acids (AAs) were deleted (Figure 1B, lane 8). This indicated the importance of residues 6–10 for this interaction and we generated the corresponding deletion

Figure 1. NANOS2 associates with the CCR4-NOT deadenylation complex through a direct interaction with CNOT1. (A) Schematic representation of NANOS2 deletion mutants. (B) Flag-tagged NANOS2 or its deletion mutants were precipitated with anti-FLAG antibodies from HeLa cell extracts co-transfected with 3×HA-Cnot6. Precipitates were analyzed by western blotting with the indicated antibodies. (C) Immunoprecipitated Flag-tagged NANOS2 or NANOS2-DN10 were incubated with 5′-fluorescein isothiocyanate-labeled poly(A) RNA substrate for 0, 30, 60 and 120 minutes. Samples were then analyzed on a denaturing sequencing gel. (D) Schematic representation of GST-fused CNOT1 protein. CNOT1 was divided into three parts due to its length: the N-terminal region (CNOT1-1), middle region (CNOT1-2) and C-terminal region (CNOT1-3). (E) E. coli extracts expressing GST-fused CNOT1-1, CNOT1-2 or CNOT1-3 were mixed with MBP-NANOS2 and subjected to a GST pull-down assay. (D) E. coli extracts expressing CNOT1-3 were mixed with MBP-lacZ, MBP-NANOS2-DN10 or MBP-NANOS2 respectively, and subjected to a GST pull-down assay.

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mutant of NANOS2 and found that only small amounts of CNOT protein were precipitated with this product compared with the full length and 1–5 AA-deleted variants (Figure 1B, lane 10). From these data, we concluded that the first 10 AAs of NANOS2 are required for a full interaction with the CCR4-NOT deadenylation complex. We further assayed the deadenylase activity levels of both NANOS2 and NANOS2-AN10 using synthetic poly(A) RNA. The resulting data showed that cleavage of poly(A) RNA occurred only with wild-type NANOS2 whereas this activity was not observed in NANOS2-AN10 precipitates (Figure, 1C).

We next searched for a direct binding partner of NANOS2 that could mediate the recruitment of the CCR4-NOT deadenylation complex. Given that this interaction can be reproduced even in HeLa cells, we surmised that germ cell specific factors would be unnecessary, which in turn raised the possibility that the direct partner may be one of the components of the CCR4-NOT deadenylation complex. Of note in this regard, Drosophila Nanos has been reported to directly bind to CNOT4 in yeast two-hybrid experiments [15]. Hence, we cloned all of the known components of the CCR4-NOT deadenylation complex [16], which include CNOT1–4, 6, 6L, and 7–10, and D1Bwg2012e (a human C2orf29 homologue), into a GST-fusion bacterial expression vector (File S1). CNOT1 was divided into three segments as indicated in Figure 1D because of its long peptide sequence. Following the expression of these components in bacteria, pull-down assays were performed with purified recombinant MBP-NANOS2 (File S1) and revealed that NANOS2 associates only with the C-terminal region of CNOT1 (Figure S2, Figure 1E). We further found that deletion of the 10 N-terminal AAs of MBP-NANOS2 abolishes this interaction (Figure 1F). These data thus revealed that NANOS2 associates with the CCR4-NOT deadenylation complex via a direct interaction with CNOT1.

The NANOS2-AN10 mutant fails to rescue the Nanos2-null phenotype

NANOS2 regulates several aspects of male gonocyte development such as the suppression of meiosis, promotion of male characteristics and suppression of apoptosis [6]. It is not known however whether all of the functions of NANOS2 are mediated by its interaction with the CCR4-NOT deadenylation complex. We thus tried to express NANOS2-AN10 in male gonocytes instead of wild-type NANOS2 to further analyze the physiological significance of this association. We generated a transgenic mouse line that expressed Flag-tagged NANOS2-AN10 under the direct control of the Nanos2 enhancer (Figure 2A, AN10). We confirmed the expression of this transgene in the embryonic gonads of two lines. Western blotting revealed that the corresponding transgenic mice produced an appreciable quantity of Flag-tagged NANOS2-AN10, and that line #1 expressed this truncated protein at levels that were comparable to the full-length Flag-tagged Nanos2 (Figure 2B, lane AN10/#1) and the total RNA amount was elevated in comparison with the protein levels (Figure S3D). These results suggest that in the presence of Flag-tagged NANOS2-AN10, endogenous Nanos2 mRNA produces more protein than expected, whereas mRNA from the transgene produces less, indicating that there is an unknown mechanism underlying the regulation of the NANOS2 protein levels independently of transcription. Further analysis was conducted using line #1, which showed higher expression of the Nanos2-AN10 transgene.

We crossed the Nanos2-AN10 transgenic mice with Nanos2<sup>−/−</sup> mice to assess the function of NANOS2-AN10 in the absence of wild-type NANOS2. Since the Nanos2-AN10 transgene was successfully transmitted via males, we therefore introduced the Flag-tagged Nanos2-AN10 transgene into Nanos2-null testes and compared the phenotype with those of Nanos2-null mice to further examine the function of NANOS2-AN10 in vivo. As shown previously, Nanos2-null males have significantly smaller testes than their wild-type counterparts, in which no germ cells exist from about 4 weeks [7]. In our current experiments, we similarly observed smaller testes in the transgenic mice with a Nanos2-null background (Figure 2C). A subsequent histological study of these transgenic tissues revealed a complete loss of germ cells from the seminiferous tubules (Figure 2D, E, F). We next assessed whether this was due to a failed rescue event during embryogenesis. We performed immunostaining for activated cleaved caspase 3 at E16.5 and found cells undergoing apoptosis, as predicted from the lack of germ cells in the adult testes (Figure 2G, H, I). We further found an upregulated meiotic marker, SCP3 (Figure 2J, K, L) [17], and downregulated male-specific marker, DNMT3L (Figure 2M, N, O) [18]. These phenotypes were almost identical to those observed in Nanos2-null mice [6] and we thus concluded that the first 10 residues of NANOS2 are essential for almost all of its functions. These results also suggest that the interaction of NANOS2 with the CCR4-NOT deadenylation complex is essential for its developmental functions, although we cannot exclude the possibility that the association of other factors with the 10 N-terminal AAs of NANOS2 is also critical.

To further examine the rescue events that cannot be initiated by Nanos2-AN10, we compared the gene expression profiles among E14.5 male gonads of Nanos2<sup>+/−</sup>, Nanos2<sup>−/−</sup> and Nanos2<sup>−/−</sup> mice expressing Nanos2-AN10 (Figure 3A, B) by microarray. Although a small set of genes showed significant expression changes between Nanos2<sup>−/−</sup> and Nanos2<sup>−/−;Tg</sup> (Table 1), box plot analyses of these genotypes showed that the gene expression profiles were mainly similar, as predicted (Figure 3A–B). Groups of both meiotic genes (Strahl, Syce1, Taf7l; Figure 3C, D, E) [4,19,20] and PGC genes (Eger1/Dppa3, Stella/Dppa3, Sox2; Figure 3F, G, H) [21,22] were found to be regulated whilst male-type genes (Dnmt3l, Macf2/Pcfl1, Tfb1d1; Figure 3I, J, K) [18,23,24] were down regulated in E14.5 male gonads as compared with wild-type male gonads, even in the presence of the transgene. These data support our contention that the interaction of NANOS2 with CCR4-NOT deadenylation complex is essential for it to exert its biological roles, and we predict that there are few, if any, CCR4-NOT deadenylation complex-independent NANOS2 functions.

NANOS2-AN10 interacts with specific RNAs

To further examine the properties of NANOS2-AN10, we next analyzed the cellular localization of this mutant in Nanos2 knockout mice by immunostaining with the antibody against NANOS2. As previously mentioned, NANOS2 is dispersed throughout the
cytoplasm with some localization in P-bodies in male gonocytes (Figure 4A, B, C) [8]. In contrast to this, however, NANOS2-ΔN10 was mainly found within the nucleus, although was partly still detectable in the cytoplasm and localized at the P-bodies, as seen for wild-type NANOS2 (Figure 4D, E, F). This indicated that the interaction with the CCR4-NOT deadenylation complex is required for the proper localization of NANOS2. These data raise the question of whether or not its interaction with CCR4-NOT deadenylation complex is also essential for the association of NANOS2 with its target RNAs. To address this issue, we purified FLAG-tagged NANOS2-ΔN10 from E15.5 male gonad extracts to analyze co-precipitated RNA molecules as described previously [8]. Subsequent western blotting analyses revealed that there was no detectable association between NANOS2-ΔN10 and the CCR4-NOT deadenylation complex as the components of which could be efficiently co-precipitated with wild-type NANOS2 (Figure 4G, lane 5) but were undetectable in NANOS2-ΔN10 precipitates (Figure 4G, lane 6). This confirmed that a deletion of the first 10 AAs of NANOS2 abolishes its interaction with CCR4-NOT deadenylation complex in vivo.

We also examined the co-precipitated RNAs by real-time RT-PCR, and found that FLAG-tagged NANOS2-ΔN10 efficiently co-precipitated meiotic gene transcripts (Stra8, Sycp3, Taf7l, Dazl, Meisetz) (Figure 4H–I) that are also associated with wild-type NANOS2 as previously shown [8]. These data indicate that NANOS2 binds specific RNAs independently of its interaction with the CCR4-NOT deadenylation complex.

Discussion

In our current study, we have identified CNOT1 as a direct NANOS2-associated protein, and shown that the first 10 AAs of...
NANOS2 is required for this interaction. In addition, we have further shown that the interaction of NANOS2 with the CCR4-NOT deadenylation complex is essential for it to exert its biological roles in vivo by using transgenic mouse that expresses a NANOS2 variant lacking these first 10 AAs (NANOS2-DN10). As this NANOS2 variant still retains both the CCHC-type zinc finger motif and a C-terminal region highly conserved among mammal (Figure S1B), it is assumed that the Nanos2-DN10 transgenic mouse would have some defects in germ cell development due to presumptive dominant effects. However, we observed normal spermatogenesis and successful transmission of this transgene to next generation, which led us to speculate that endogenous NANOS2 may be sufficient to suppress dominant-negative effects of NANOS2-DN10.

On the other hand, NANOS2-DN10 did not rescue any major defect observed in Nanos2-null mouse, indicating that the NANOS2 function is mediated via interaction with the CCR4-NOT complex. However, it is shown that the complex has various

**Table 1. Results of Microarray analyses.**

| Subtraction procedures          | Nanos2+/− | Nanos2+/− | Nanos2+/− | Total |
|---------------------------------|-----------|-----------|-----------|-------|
| All probe sets                  | 41,326    | 41,326    | 41,326    | 41,326|
| Present *                       | 25,759    | 26,036    | 25,695    | 27,033*|
| 2 fold change vs Nanos2+/− of 27,033* b | -        | 804       | 1,014     | -     |
| T-Test, p<0.05 vs Nanos2+/− of b * | -        | 144       | 219       | -     |
| (Ratio of c/*)                  | -         | 0.98%     | 0.81%     | -     |
| 2 fold change in c, <Nanos2+/− | -         | 62        | -         | -     |
| 2 fold change in c, >Nanos2+/− | -         | 82        | -         | -     |
| 2 fold change vs Nanos2+/− of 27,033* a | -        | -         | 310       | -     |
| T-Test, p<0.05 vs Nanos2+/− of b d | -        | -         | 37        | -     |
| (Ratio of d/*)                  | -         | -         | 0.14%     | -     |

**Figure 3. Comparative expression analysis of various genes in Nanos2+/− and Nanos2+/−_Tg male gonads.** (A, B) Box plots showing the expression profiles of 144 genes that are significantly altered in the male gonads of E14.5 Nanos2+/− embryos compared with Nanos2+/− embryos. Note that the averages of the plots for Nanos2+/−_Tg are very similar to those of Nanos2+/− in terms of both the increased (A) and decreased (B) genes in Nanos2+/−. (C–K) Expression levels of genes relevant to the sexual differentiation of germ cells in the male gonads of Nanos2+/−, Nanos2+/− and Nanos2+/−_Tg embryos at E14.5. These data were obtained using the Agilent GeneChip System and analyzed with Genespring GX software.

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functions other than deadenylation, including transcriptional, post-transcriptional RNA regulation and protein ubiquitylation [9,14]. For instance, CNOT1 interacts with nuclear receptors [25], and CNOT3 is involved in chromatin remodeling [26], thereby playing a role in transcriptional mechanism. In addition, CNOT4 harbors E3 ligase activity, placing the CCR4-NOT complex in the protein ubiquitylation/degradation pathways [27,28]. Nevertheless, considered that NANOS2 is cytoplasmic RNA-binding protein localizing in P-bodies and that NANOS2-associated CCR4-NOT complex has deadenylase activity in vitro [8], it would be reasonable to assume that a major function of NANOS2-associated CCR4-NOT complex is deadenylation for RNA degradation. However, at this point, we cannot rule out the possibility that some other function(s), such as ubiquitylation or post-transcriptional regulation, is also responsible for a part of NANOS2 function.

We have also shown that a mutant NANOS2 lacking association with CNOT1 still retains an ability to interact with specific mRNAs, indicating that the RNA-binding specificity is determined independently of the interaction with the CCR4-NOT deadenylase complex. However, it is known that the CCHC-type zinc finger motif in NANOS protein binds RNAs non-specifically in vitro [29], indicating the other protein(s) is required to confer the specificity. Consistently, our preliminary MAS analyses revealed that several other proteins other than the CCR4-NOT complex are co-precipitated with NANOS2, including several RNA-binding proteins. These factors are currently under investigation.

Materials and Methods

Ethics statement

Experiments were carried out with the permission of the animal experimental committee at the Yokohama National University (project number; 1), which is approved March 3, 2009.

Mice

The Nanos2-knockout mouse lines and PCR methods used for the verification of mutant alleles have been previously described.
[7]. A 3×FLAG-tagged Nanos2-ΔN10 vector with a 3′-UTR under the control of the Nanos2 enhancer (9.2 kb upstream sequence) was used for the production of the transgenic mouse line. The primer pairs used for the genotyping of these lines were as follows: 5′-CTACAAGAAGCCATGACCGGTG-3′ and N2-3′U-R2; 5′-CCCCAGAAAGTCATCCACACAG-3′.

Immunoprecipitation and western blotting

The 3×Flag expression vectors for Nanos2 and Nanos2-ΔN10, and 3×HA-Cnot6 were constructed using pcDNA3.1 (Invitrogen). HeLa cells were then transfected with 12 μg of these constructs per 10 cm dish using polyethyleneimine [30]. After 48 hours, cellular proteins were extracted with 1 ml of lysis buffer (50 mM Tris-HCl [pH7.4], 150 mM NaCl, 0.5% NP-40, 7.5 mM β-glycerophosphate, 0.1 mM Na3VO4, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 mM leupeptin, 1 mM aprotinin, 1 mM pepstatin), and spun at 20,000 g for 15 min at 4°C. The supernatants were then incubated with 10 μl of anti-FLAG M2 affinity gel (Sigma) on a rotator for 3 h at 4°C. After several washes, precipitates were boiled with 10 μl of 2×Sample buffer, separated by SDS-PAGE, and then subjected to western blotting analysis as described previously. The membranes were incubated with primary antibodies against Flag (1:3,000; Sigma-F3165), HA (1:10,000; 12CA5), CNOT1 (1:500, a gift from H. T. Timmers), CNOT3 (1:500, a gift from T. Tamura), CNOT7/Caf1 (1:500, a gift from A. B. Shyu), and CNOT9/Red1 (1:500, a gift from H. Okayama). Positive signals were visualized by incubation with an appropriate secondary antibody conjugated with horseradish peroxidase followed by detection using an ECL Advance TM Western Blotting Analysis System (GE Healthcare). All antibodies were diluted using Can Get Signal Immunoreaction Enhancer Solution (Toyobo).

In vitro deadenylylase assay

After immunoprecipitation as mentioned above, precipitates were subjected to a deadenylylase assay as previously described [3,31].

GST pull-down assay

MBP-LacZ, MBP-NANOS2 or MBP-NANOS2-ΔN10 fusion proteins were expressed in the E. coli BL21 (DE3) strain and purified with Amylose Resin (New England Biolabs). All CCR4-NOT deadenylylase complex components were cloned from a single stranded E15.5 mouse male gonad cdNA library into pGEX-5X vectors (GE Healthcare), and then expressed in E. coli, BL21 Star (DE3) (Invitrogen) cells. Bacterial pellets were sonicated in a binding buffer (25 mM HEPES-KOH [pH 7.4], 150 mM NaCl, 0.1% NP-40, 1 mM DTT, 1 mM EDTA, 1 mM PMSF), and then spun at 15,000 rpm at 4°C. The supernatants were mixed with 1–5 μg of MBP-NANOS2, NANOS2-ΔN10 or LacZz incubated for 2 h at 4°C, and then mixed with 30 μl of glutathione-sequaphorose 4FF (GE Healthcare) followed by a further incubation for 2 h. After extensive washing with the above binding buffer supplemented with 350 mM NaCl, precipitates were separated by SDS-PAGE and analyzed by western blotting with anti-MBP antibody (1:2000; New England Biolabs) or by CBB staining.

Histological methods

For immunostaining, mouse gonads were directly embedded in O.C.T. compound (Sakura) and frozen in liquid nitrogen. After sectioning (8 μm), samples were stained according standard procedures. Details of these methods have been previously described [6].

Microarray

For one hybridization assay, 200 ng of total RNA was labeled with Cy3 and then hybridized to a Whole Mouse Genome Oligo Microarray (G4122F, Agilent) in accordance with the manufacturer’s protocols (Agilent) for the Low RNA Input Linear Amplification Kit, and the One Color Gene Expression Hybridization Kit, respectively. Arrays were analyzed using a Microarray Scanner System (G2565BA, Agilent) and the images were processed with Feature Extraction, version 9.1 (Agilent) to generate signal values and present/absent calls for each probe set. Two independent datasets were obtained for each collagen. Processed data were analyzed with Genespring GX software. The following normalization steps were applied to each dataset: measurements were set from less than 5 to equal to 5 for data transformation, per chip normalization was set to the 50th percentile, and per gene normalization was set to median. All data is MIAME compliant and the raw data has been deposited in a MIAME compliant database (GEO, accession number: GSE33136).

Immunoprecipitation and real-time RT-PCR

For the immunoprecipitation – realtime RT-PCR analysis, 60 male gonads from E15.5 embryos of either wild-type or Nanos2-ΔN10 transgenic mice were homogenized on ice in 200 μl of Buffer A (25 mM HEPES-KOH [pH 7.4], 250 mM sucrose, 75 mM β-glycerophosphate, 1 mM DTT, 0.05% NP-40, 2×Complete Mini (Roche) containing 400 units/ml of RNase inhibitor (Toyobo) and 1/100 volume of phosphatase inhibitor cocktail 1 (Sigma, St Louis, MO), and spun at 10,000 g for 10 min at 4°C. NaCl (5 M) was then added to the supernatants to a final concentration of 150 mM. The samples were then mixed with 20 μl of anti-FLAG M2 affinity gel (Sigma) and incubated on a rotator for 3 h at 4°C. After 5 washes with Buffer A containing 150 mM NaCl, co-precipitated RNAs were purified using the RNeasy Mini Kit (Qiagen). After synthesis of first-strand cDNAs with 200 U SuperScript III reverse transcriptase (Invitrogen) and 100 pmol (dT)20 primer, real-time RT-PCR analyses were carried out according to manufacturer’s instruction. The level of G3pdh control mRNA was set at 1 and the levels of each mRNA were calculated (each mRNA/ control mRNA was set at 1 and the levels of each mRNA were calculated (each mRNA/ control mRNA was set at 1 and the levels of each mRNA were calculated (each mRNA/ control mRNA was set at 1 and the levels of each mRNA were calculated (each mRNA/G3pdh)).

Supporting Information

File S1 This file includes Materials and Methods for amplification of cnot genes and Realtime RT-PCR. (DOCX)

Figure S1 Conservation of Nanos proteins. (A) Amino acid sequence alignment of putative NANOS2 proteins among different vertebrate species. The overall sequence identity values in comparison with the mouse NANOS2 protein are shown at the end of each sequence. Three highly conserved regions are indicated in frame. Red and blue circles indicate conserved CCHC residues in the former and latter zinc finger motifs, respectively. (B) Schematic structure of the NANOS2 protein indicating the conserved zinc finger motif. NR, N-terminal region; CR, C-terminal region. (TIF)

Figure S2 GST pull-down assay. E. coli extracts expressing GST-fused CNOT1-1, 1-2, 1-3, 2, 3, 4, 6, 6L, 7, 8, 9, 10, or
D1Bwg0212e were mixed with MBP-NANOS2 and subjected to a GST pull-down assay. CNOT proteins that precipitated with Glutathione Sepharose were visualized by CBB staining whereas co-precipitated MBP-NANOS2 was detected by western blotting. Note that only CNOT1-3 precipitates large amount of MBP-NANOS2.

**Figure S3 RT-PCR analyses of Nanos2 mRNA.** (A) Schematic representations of endogenous Nanos2, 3xFlag-tagged full-length Nanos2 and 3xFlag-tagged Nanos2-A110 mRNAs. Red arrows indicate the primer pair used to measure the total Nanos2 mRNA level (C, D), whilst the blue arrows indicate a primer pair designed to discriminate between endogenous and exogenous Nanos2 mRNA (B). (B) Semi-quantitative RT-PCR analysis of Nanos2 mRNA in E14.5 male gonads from wild-type, transgenic mice expressing full-length Nanos2 and Nanos2-A110. (C, D) Comparison between the total Nanos2 mRNA levels in the E14.5 male gonads of wild-type and transgenic mice expressing full-length Nanos2 (B) or Nanos2-A110 (C) by real-time RT-PCR analysis. (TIF)

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**Author Contributions**
Conceived and designed the experiments: AS YS. Performed the experiments: AS KM YM. Analyzed the data: AS RS. Contributed reagents/materials/analysis tools: RS. Wrote the paper: AS YS. Microarray data was analyzed: AS RS. Microarray analysis was performed: RS. Corresponding authors: AS YS.

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