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NANOS2 promotes male germ cell development independent of meiosis suppression

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ABSTRACT

NANOS2 is an RNA-binding protein essential for fetal male germ cell development. While we have shown that the function of NANOS2 is vital for suppressing meiosis in embryonic XY germ cells, it is still unknown whether NANOS2 plays other roles in the sexual differentiation of male germ cells. In this study, we addressed the issue by generating *Nanos2*/*Stra8* double knockout (dKO) mice, whereby meiosis was prohibited in the double-mutant male germ cells. We found that the expression of male-specific genes, which was decreased in the *Nanos2* mutant, was hardly recovered in the dKO embryo, suggesting that NANOS2 plays a role in male gene expression other than suppression of meiosis. To investigate the molecular events that may be controlled by NANOS2, we conducted a series of microarray analyses to search putative targets of NANOS2 that fulfilled 2 criteria: (1) increased expression in the *Nanos2* mutant and (2) the mRNA associated with NANOS2. Interestingly, the genes predominantly expressed in undifferentiated primordial germ cells (PGCs) were significantly selected, implying the involvement of NANOS2 in the termination of the characteristics of PGCs. Furthermore, we showed that NANOS2 is required for the maintenance of mitotic quiescence, but not for the initiation of the quiescence in fetal male germ cells. These results suggest that NANOS2 is not merely a suppressor of meiosis, but instead plays pivotal roles in the sexual differentiation of male germ cells.

Introduction

The germline is established as a singular cell lineage that transmits genetic information to the next generation during early embryonic stages in both vertebrates and invertebrates. In the mouse embryo, primordial germ cells (PGCs) are formed at the base of the allantois by embryonic day (E) 7.25 (Saitou and Yamaji, 2010; Kurimoto et al., 2008). They then migrate through the hindgut toward the genital ridge. PGCs become competent to initiate meiosis after colonizing the gonad, regardless of their sex chromosome constitution (Lin et al., 2008). However, once gonadal sex is determined, the PGCs commence sex-specific differentiation by responding to factors derived from somatic cells in the developing gonads. In the female gonads, PGCs immediately initiate meiosis and enter meiotic prophase I. By contrast, in male gonads, the PGCs cease cell division, enter quiescent G0/G1 mitotic arrest and do not enter meiosis during the embryonic stage (Spiller and Koopman, 2011).

In the female gonad, retinoic acid (RA) produced and secreted by the mesonephros acts as an inducer of meiotic initiation via activation of the RA responsive gene *Stimulated by retinoic acid gene 8* (*Stra8*) in PGCs (Koubova et al., 2006; Bowles et al., 2006; Anderson et al., 2008). STRA8 is required for premeiotic DNA replication and the subsequent events of meiotic prophase I (Baltus et al., 2006). In male development, fibroblast growth factor 9 (FGF9) secreted from Sertoli cells induces PGCs to progress through the male pathway, thereby leading to the production of spermatogonial precursors (Bowles et al., 2010). In addition, cytochrome P26B1 (CYP26B1) is also implicated as an important somatic factor required for suppression of meiosis in XY PGCs via the degradation of meiosis-inducing substances, including RA (Koubova et al., 2006; Bowles et al., 2006; Maclean et al., 2007). Once germ cells receive the appropriate signals in the male gonad, they start to express NANOS2, which is an intrinsic factor that is essential for the promotion of the male pathway (Tsuda et al., 2003; Suzuki and Saga, 2008).

Abbreviations: 5-bromo-2′-deoxyuridine, BrdU; double knockout, dKO; embryonic day, E; immunoprecipitation, IP; retinoic acid, RA; reverse transcription, RT; phosphate-buffered saline, PBS; polymerase chain reaction, PCR; primordial germ cell, PGC; RNA immunoprecipitation, RIP

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Nanos is an evolutionarily conserved RNA-binding protein containing 2 CCHC-type zinc finger motives. Nanos proteins are involved in post-transcriptional RNA metabolism via their binding to target mRNAs in germ cells (Curtis et al., 1997; Kadyrova et al., 2007; Lai et al., 2010). *Nanos2* is one of 3 Nanos family genes (*Nanos1*, *2*, and *3*) in mice that play essential roles in the survival of embryonic male germ cells and the maintenance of spermatogonial stem cells after birth (Tsuda et al., 2003; Sada et al., 2009). In the embryo, NANOS2 is required for the sexual differentiation of XY PGCs because *Nanos2*-deficient XY germ cells fail to express male-type genes and abnormally initiate meiosis (Suzuki and Saga, 2008). In addition, the ectopic expression of NANOS2 in female gonads causes germ cells to express male-type genes and suppresses the meiotic program (Suzuki and Saga, 2008). Thus, NANOS2 directs both the suppression of meiosis and promotion of male gene expression. Recently, we showed that NANOS2 associates with the deadenylation complex, thereby suggesting that NANOS2 induces PGCs to differentiate into male gonocytes via the RNA-degradation pathway (Suzuki et al., 2010). However, it is still unknown whether the suppression of meiotic genes is crucially required for male-type gene expression or whether NANOS2 independently regulates these gene expressions.

To clarify the roles of NANOS2, we adopted a genetic approach whereby meiosis was prevented in *Nanos2*-deficient male germ cells via the deletion of the *Stra8* gene together with *Nanos2*. The results indicate that NANOS2 is required for male-type gene expression independent of the suppression of meiosis. The gene expression and immunoprecipitation analyses predicted putative NANOS2 targets, which implied the possible involvement of NANOS2 in the termination of the characteristics of PGCs. Lastly, we showed that NANOS2 is required for the maintenance of mitotic quiescence, but not for entry into this state. These results suggest that NANOS2 is essential for male germ cell differentiation irrespective of its ability to suppress meiosis.

Materials and methods

Mice

The *Nanos2*-knockout mouse line used in this study has been previously described (Tsuda et al., 2003). The *Stra8*-knockout mouse line was established in our laboratory using the strategy shown in the Supplementary Material, Fig. S1A. The targeting vector was constructed to knock in the *EGFP* cDNA in-frame at the translational initiation site, with a long 6.6-kb homology arm and a short 1.7-kb arm just upstream and downstream of the site, respectively. The vector (25 μg) was electroporated into TT2 embryonic stem cells (Yagi et al., 1993) and correct homologous recombinants were aggregated with MCH (a closed ICR colony established at CLEA Japan Inc., Tokyo, Japan) 8-cell-stage embryos and then transferred into pseudopregnant female recipients. The resulting chimeric mice were bred with MCH females to obtain the *Stra8*$^{+/-}$ mice.

RNA isolation

XX and XY gonads from E12.5 to E15.5 were dissected in ice-cold phosphate-buffered saline (PBS) and deposited in RNAlater (Ambion, Life Technologies, Carlsbad, USA) at −80 °C until required for total RNA extraction. Samples of 6–20 gonads were used for each RNA analysis. Total RNA was purified using an RNeasy mini kit (Qiagen, Venlo, Netherlands) and TURBO DNA-free (Ambion). Quantification and qualitative analysis of purified RNAs were performed using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and a Bioanalyzer 2000 (Agilent, SantaClara, CA, USA), respectively.

Microarray analysis

For each hybridization assay, 500 ng of total RNA was labeled with Cy3 and hybridized to a Whole Mouse Genome Oligo Microarray (G4122F; Agilent) in accordance with the manufacturer's protocol for the Low RNA Input Linear Amplification Kit, One Color (Agilent), and Gene Expression Hybridization Kit (Agilent), respectively. Arrays were scanned using a Microarray Scanner System (G2565BA; Agilent) and the generated images were processed using Feature Extraction, version 9.1 (Agilent) software to generate signal values for each probe set. Two independent datasets were obtained for each collation. The processed data were analyzed using Genespring GX software, version 7.3.1 (Agilent). Each data set was normalized. Specifically, measurements of less than 5 were set to 5 for data transformation, per chip normalization to the 50th percentile, and per gene normalization to the median. The microarray data reported in this study were registered in the Genome Network Platform (http://genomenetwork.nig.ac.jp/download/experimental_data/MicroArray/Micro-Array_mouse_111102_e.html) and in the Gene Expression Omnibus (GEO) database (GSE37720).

RT-qPCR

cDNAs were synthesized from the total RNA of mouse fetal gonads using Superscript III (Invitrogen, Life Technologies, Carlsbad, CA, USA) and oligo dT or random primers. Quantitative PCR analysis was then performed using a KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Woburn, MA, USA) and a Thermal Cycler Dice (Takara, Otsu, Japan). The primer sets are listed in the Supplementary Material, Table S1.

Purification of NANOS2-associated RNAs

NANOS2-associated RNAs were obtained as previously described (Suzuki et al., 2010) with some modifications. The E14.5 XY gonads of the transgenic mice, which expressed FLAG-tagged NANOS2, were dissected in ice-cold PBS and stored at −80 °C until required for RNA-immunoprecipitation (IP). Samples of 80–100 gonads were used per one precipitation reaction. The tissues were homogenized in 5 volumes of ice-cold extraction buffer, and the lysates were pre-cleared using protein A-agarose (Sigma, St. Louis, USA) for 30 min at 4 °C. The pre-cleared lysates were then divided into 2 tubes and incubated with either anti-FLAG IgG-agarose or IgG-agarose (Sigma) for 3 h at 4 °C. Inputs and immunoprecipitated mRNAs were isolated using an RNeasy mini kit (Qiagen) and were then used as templates to generate Cy3-labeled RNA probes to hybridize to the microarrays.

Immunohistochemistry

XY gonads were dissected in ice-cold PBS, fixed with 4% paraformaldehyde in PBS at 4 °C for 2 h. 5-Bromo-2′-deoxyuridine (BrdU) was administered to pregnant mice at 100 μg/g body weight 2 h before dissection. Fixed embryos were embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), frozen and sectioned at 8 μm using a cryostat. Frozen sections were incubated with the primary and secondary antibodies: rat TRA98 (a gift from Y. Nishimune, Osaka University); rabbit anti-DNMT3L (a gift from S. Yamanaka, Kyoto University); rabbit anti-SYCP3 (a gift from S. Chuma, Kyoto University); rabbit anti-KI67 (Neomarkers, Thermo Scientific, Waltham, MA, USA); rabbit anti-PRB1 (Cell Signaling Technologies, Danvers, MA, USA); mouse anti-BrdU (BD Biosciences,

San Joe, CA, USA); donkey anti-rat and anti-rabbit IgG conjugated with Alexa Fluor 594 (Invitrogen); and donkey anti-goat and anti-rabbit IgG conjugated with Alexa Fluor 488 (Invitrogen).

Results

Nanos2 is required for male gene expression independent of Stra8 suppression

We previously reported that male-type gene expression was suppressed in *Nanos2*$^{-/-}$ XY germ cells, in which a putative trigger of meiosis, *Stra8*, was abnormally up-regulated (Suzuki and Saga, 2008; Baltus et al., 2006; Anderson et al., 2008). The observation led us to assess the possibility that ectopic induction of meiosis disturbs normal male-type gene expression in *Nanos2*$^{-/-}$ XY germ cells.

To test the proposal, we generated a *Stra8*-null mouse line (Fig. S1A) and assessed whether male-type genes are activated in *Nanos2* and *Stra8* double knockout (dKO) XY germ cells. First, we confirmed that our *Stra8* mutant mice showed defective progression of meiosis in embryonic XX germ cells (Fig. S1B–E) as previously reported (Baltus et al., 2006). Then, we generated dKO mice and examined the expression of representative meiotic genes, *Sycp3* and *Dmc1* (Yuan et al., 2000; Pittman et al., 1998), and a gene involved in oogenesis, *Figla* (Soyal et al., 2000),which are abnormally activated in *Nanos2*$^{-/-}$ XY germ cells, to confirm that the meiotic pathway was suppressed in the dKO gonads. As we expected, the expression of these genes in the dKO was decreased to the level of control gonads (Fig. 1A), indicating that the ablation of *Stra8* successfully prevents meiotic gene expression.

To investigate whether male-type genes were recovered in dKO male germ cells, we performed RT-qPCR of the representative male-specific genes *Dnmt3l*, *Piwil4/Miwi2*, and *Tdrd9* (Shovlin et al., 2007; Aravin et al., 2008; Shoji et al., 2009, respectively), which are involved in DNA methylation and transposon silencing. Surprisingly, the results showed that these gene expressions were still decreased even in the dKO gonads (Fig. 1B). The results of the RT-qPCR were further confirmed by immunostaining for DNMT3L. Consistent with the RNA expression levels, DNMT3L was not recovered in the dKO germ cells and its expression level was nearly identical to that measured in the *Nanos2*$^{-/-}$ cells at E15.5 (Fig. 1C–E). These results suggest that ectopic activation of STRA8

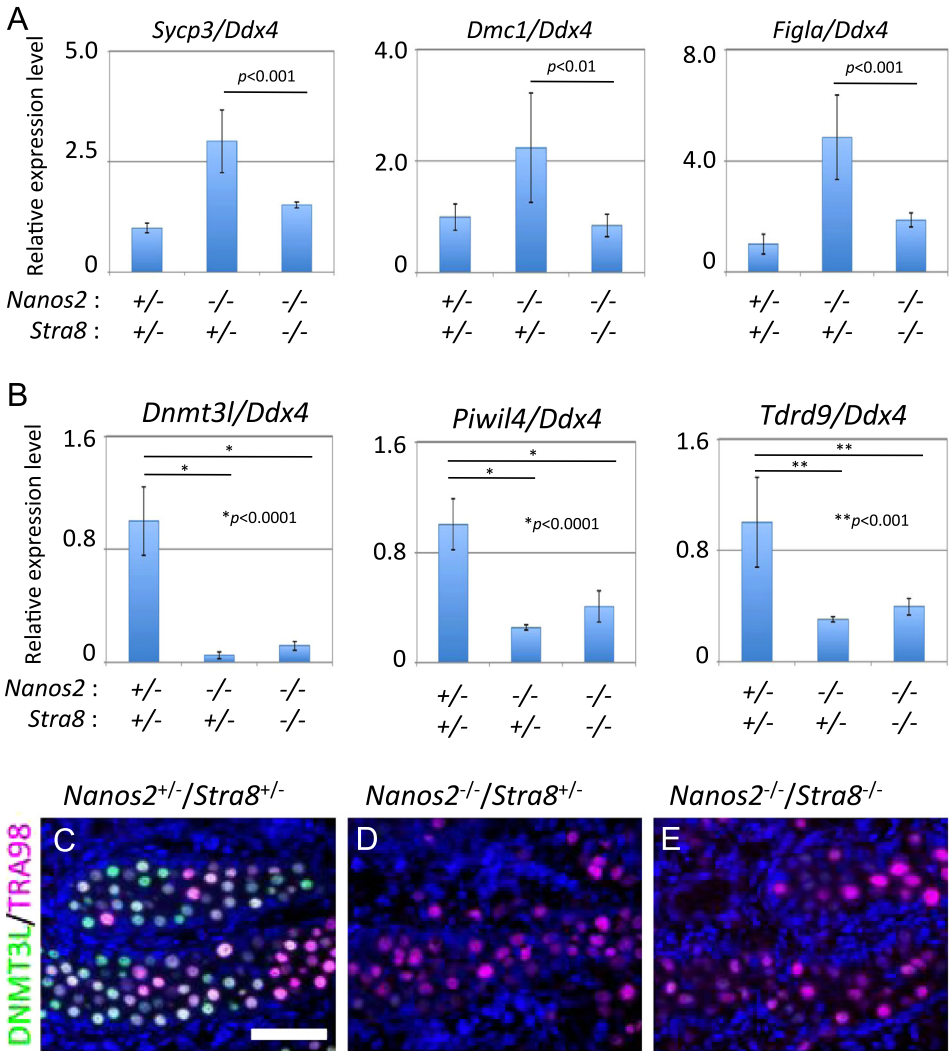

Fig. 1. *Stra8* is not responsible for the suppression of male gene expression in *Nanos2*$^{-/-}$ gonads. Graphs showing relative expression levels of meiotic/female germ cell markers, *Sycp3*, *Dmc1*, and *Figla* (A), and male germ cell markers, *Dnmt3l*, *Piwil4, and Tdrd9* (B), to *Ddx4* in *Nanos2*$^{+/-}$/*Stra8*$^{+/-}$, *Nanos2*$^{-/-}$/*Stra8*$^{+/-}$, and *Nanos2*$^{-/-}$/*Stra8*$^{-/-}$ XY gonads at embryonic day (E)15.5. The data are shown as the average ± standard deviation ($n=4$). p values as determined by a Student's t-test. Immunohistochemical detection of DNMT3L (green) in *Nanos2*$^{+/-}$/*Stra8*$^{+/-}$ (C), *Nanos2*$^{-/-}$/*Stra8*$^{+/-}$ (D), and *Nanos2*$^{-/-}$/*Stra8*$^{-/-}$ (E) XY gonads at E15.5. Magenta, TRA98 (germ cell marker); blue, Hoechst 33342 (nuclei marker). Scale bar, 50 μm.

is not responsible for the suppression of male-specific genes in the *Nanos2*$^{-/-}$ XY germ cells.

Involvement of NANOS2 in the termination of the undifferentiated state of PGCs

The results of the *Nanos2*/*Stra8* double mutant analyses indicate that NANOS2 is required for male-type gene expression independent of the suppression of meiosis. This prompted us to explore the targets of NANOS2 that may be involved in the differentiation of male germ cells. We first determined the genes that were affected in the *Nanos2*$^{-/-}$ embryo by comparative expression analyses using microarrays for the XY gonads of *Nanos2*$^{+/-}$ and *Nanos2*$^{-/-}$ embryos from E12.5 to E15.5. The subtraction procedure is described in Table 1. Conspicuous expression changes were apparent in the *Nanos2*$^{-/-}$ embryos from E14.5, which is just one day after the initiation of NANOS2 protein expression (Suzuki et al., 2007). Specifically, 578 probe sets (1.95% of the total "present" probe sets) showed more than 2-fold changes in their normalized signal intensities. The expression patterns of the 136 probe sets that were increased (Gene list 1) and 442 that were decreased (Gene list 2) among the 578 probes are shown by heat maps (Fig. 2A and B). The number of altered transcripts in the *Nanos2*$^{-/-}$ embryo increased to 1757 probes (5.62% of the total "present", 396 increased and 1361 decreased) at E15.5 (Table 1). Because the impaired male-type gene expression was already observed from E14.5 (Fig. 2B and Gene list 2), we concentrated our search on the expression changes at E14.5. To assess the involvement of NANOS2 in the male-type gene expression, we selected 366 probes that were predominantly expressed in male germ cells and were sequentially increased from E13.5 to E15.5 (Gene list 3). In the selected male-type genes, 279 (76.23%) probes were decreased more than 2-fold in the *Nanos2*$^{-/-}$ embryo at E14.5, indicating the significant role of NANOS2 in male-type gene expressions. To further examine the effect of ablation of *Stra8* for male-type gene expressions in the *Nanos2*$^{-/-}$ embryo, we performed microarray analysis using the dKO gonads at E14.5. The 279 probes decreased *Nanos2*$^{-/-}$ embryos were found to be significantly decreased even in the dKO male gonads, though the expression level was slightly recovered (Fig. S2 and Gene list 3). These results are consistent with the results of RT-qPCR (Fig. 1B), further supporting our idea that STRA8 is not responsible for the suppression of male-type genes.

Next, we performed RNA-immunoprecipitation (RIP) analysis to identify the RNAs that could associate with NANOS2 using a transgenic line carrying FLAG-tagged NANOS2 (Suzuki et al., 2007). Embryonic XY gonads at E14.5 were lysed and FLAG-tagged NANOS2 was precipitated using the anti-FLAG antibody. The precipitated RNAs were subsequently subjected to microarray analysis. While many probes showed higher association with NANOS2 (more than 4-fold normalized intensity to input), we focused on the 578 probe sets whose expressions were altered in the *Nanos2*$^{-/-}$ embryo. We found that 46 out of 136 up-regulated probe sets (33.8%, 40 genes) were highly associated with NANOS2 (Fig. S3). The microarray data was confirmed by RT-PCR (Fig. 2C). Among the 46 probes, 23 probes (50.0%, 21 genes) showed higher expression by E13.5 and declined thereafter (Fig. S3, category I and II). We noticed that 8 genes [*Cdh1* (Di Carlo and De Felici, 2000), *Sox2* (Western et al., 2005), *Nanog* (Yamaguchi et al., 2005), *Nr5a2* (Gu et al., 2005), *Zic3* (Kurimoto et al., 2008), *Tcfap2c* (Kurimoto et al., 2008; Weber et al., 2010), *Six4* (Kurimoto et al., 2008) and *Wnt3* (Ohinata et al., 2009)] were reported to be expressed in the undifferentiated PGCs, which included representative PGC marker genes, *Sox2* and *Nanog*. Fourteen probes (13 genes) showed increased expression at E13.5 than those at E12.5 and retained the higher expression until E14.5, which includes 2 cell cycle regulators, *Hus1b* (Hang et al., 2002) and *Rb1* (Western et al., 2008; Spiller et al., 2010), and 2 meiotic genes, *Sycp1* (Kerr et al., 1996) and *Hormad2* (Wojtasz et al., 2009) (Fig. S3, category III). The rest of genes showed lower expressions at E12.5 and peaked expressions at E14.5 or E15.5 (Fig. S3, category IV and V). We next examined the association of NANOS2 with the down-regulated genes. Surprisingly, 66 out of 442 down-regulated probe sets (14.9%) were also highly associated with NANOS2 (Gene list 4). Although the proportion was lower than the up-regulated genes, this result raises a possibility that NANOS2 plays a role not only in gene suppression but also in the promotion or stabilization of the target mRNAs. However, most of male-type genes was indirectly decreased in *Nanos2*$^{-/-}$, since only 9 probes in the 66 probe sets were included in the male-type genes (3.2%, 9/279).

To verify that these RNAs were post-transcriptionally suppressed in male germ cells, we adopted RT-qPCR using primer sets amplifying unspliced or spliced transcripts based on the assumption that spliced, but not unspliced transcripts, are specifically increased in the *Nanos2*$^{-/-}$ gonads. The results showed that 6 out of 10 genes tested (*Cdh1, Tcfap2c, Rb1, Zic3, Sycp1* and *Nr5a2*) were increased only in the spliced RNAs in the mutant gonads (Fig. 2D), thereby supporting the idea that NANOS2 is involved in post-transcriptional suppression of these RNAs. Although the mutant gonads showed elevated *Taf7l* and *Nkx2-4* levels (Kimmins et al., 1998) in both unspliced and spliced RNAs, the levels were much higher in the spliced RNA. It is likely that these genes may also be targets of NANOS2 although their expression was induced at the transcriptional level. In contrast, *Nanog* and *Hormad2* were similarly increased in both the unspliced and spliced RNAs in a similar pattern to that observed for the negative control, *Stra8* (Suzuki and Saga, 2008), thereby indicating that these genes were indirectly upregulated in the mutant. Based on this evaluation, 8 out of the 10 selected genes may be direct targets of NANOS2.

The enhanced enrichment of the genes predominantly expressed in PGCs suggests that NANOS2 may play a role in the termination of the undifferentiated properties during sexual differentiation of male germ cells. However, the expression levels of the representative PGC markers *Sox2* and *Nanog* were decreased at E15.5 in the *Nanos2* single mutant gonads, although they retained higher expression levels until E14.5 (Fig. 2E). We suspect that this NANOS2-independent reduction of the genes could be caused by entry into the meiotic pathway. To test the possibility, we further examined the expression of the genes in the *Nanos2*/*Stra8* dKO gonads. The results showed that both *Sox2* and *Nanog* expressions were retained until E15.5 in the dKO gonads (Fig. 2E).

Table 1
Microarray data and the subtraction procedure.

Subtraction procedure	E12.5 ♂	E13.5 ♂	E14.5 ♂	E15.5 ♂
All probe sets	41,326	41,326	41,326	41,326
Present in *Nanos2*+/− vs−/− [a]	27,192	27,737	29,636	28,033
T-Test, $p<0.05$ in *Nanos2*+/− vs−/− [b]	1,789	1,097	5,962	5,670
2 fold change in *Nanos2*+/− vs−/− [c]	51	69	844	2,019
Raw value more than 50 [d]	9	50	578	1,757
Increased in *Nanos2*−/−	0	15	136	396
Decreased in *Nanos2*+/−	9	35	442	1,361
(Ratio of *b*/*c*)	0.03%	0.18%	1.95%	5.62%

The genes affected by the lack of NANOS2 were extracted by filtering steps for each stage.

[a] "Present" either in *Nanos2*$^{+/-}$ or *Nanos2*$^{-/-}$;

[b] Significant differences between *Nanos2*$^{+/-}$ and *Nanos2*$^{-/-}$ according to the Student's *t*-test ($p<0.05$)

[c] 2-Fold difference in the normalized signals from *Nanos2*$^{+/-}$ and *Nanos2*$^{-/-}$.

[d] A raw value of >50. Ratio of *d*/*a* is shown.

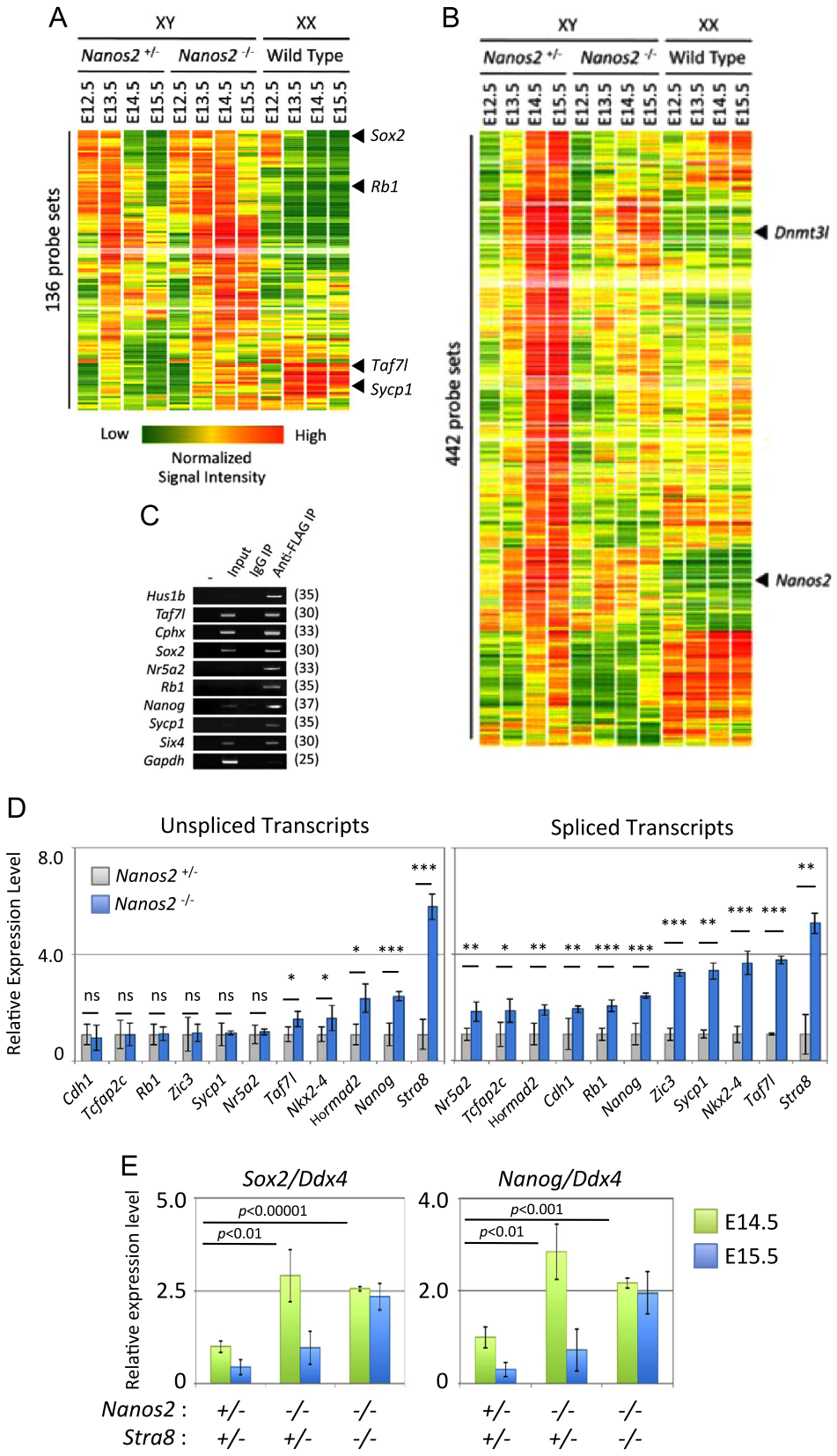

Fig. 2. Identification of NANOS2-target mRNAs in the embryonic XY gonad. Heat maps showing the expression patterns of 136 (A) and 442 (B) probe sets (upregulated and downregulated in E14.5 *Nanos2*$^{-/-}$, respectively) in the XY gonads and wild-type XX gonads from E12.5 to E15.5. The position of the representative genes *Sox2*, *Rb1*, *Taf7l*, and *Sycp1* (A), and *Dnmt3l* and *Nanos2* (B) are indicated by arrowheads. (C) Confirmation of enriched mRNAs in immunoprecipitation with FLAG-NANOS2 by RT-PCR. The number of PCR cycles is shown in parenthesis. (D) Graphs showing relative expression levels of unspliced (left) and spliced (right) transcripts of indicated genes in the E14.5 XY gonad as determined by RT-qPCR. *$p < 0.03$, **$p < 0.01$ and ***$p < 0.001$ as determined by Student's *t*-test. (E) RT-qPCR analyses of *Sox2* and *Nanog* at E14.5 (green bars) and E15.5 (blue bars) in each genotype. The data are shown as the average ± standard deviation ($n=4$).

These results imply that the male pathway is compromised even in the dKO gonads because of the prolonged expression of PGC-associated genes.

NANOS2 is required for the maintenance of mitotic cell cycle arrest, but not for the initiation

Arrested mitosis at G0/G1 is one of the characteristics of male germ cell differentiation. We previously showed that the mitotic marker pH3 was detectable from E15.5 in *Nanos2*$^{-/-}$ germ cells (Suzuki and Saga, 2008). We initially considered that the apparent cell cycle progression reflected entry into meiotic prophase because a meiotic maker SYCP3 was also elevated at E15.5. However, it is possible that NANOS2-null male germ cells resume the mitotic cell cycle and then enter meiosis following the upregulation of STRA8. The results of the microarray analysis supported this possibility because one of the typical cell cycle regulators *Rb1*, which functions in G1/S progression, was identified in the list of putative NANOS2 targets (Fig. S3). Therefore, we decided to examine the cell cycle state of *Nanos2*-null XY germ cells by comparison with *Nanos2*/*Stra8* dKO mice.

First, we ascertained whether *Nanos2*-null XY germ cells actually entered G0/G1 quiescence. The results of the immunofluorescent analysis using KI67, which is a marker of mitotically proliferating cells, and phosphorylated RB1 (Ser807/811; PRB1) antibodies showed that signals for these proteins were barely detectable in the *Nanos2*$^{-/-}$and control germ cells at E14.5 (Fig. 3A, B, E and F); however, these proteins were significantly increased at E15.5 in the *Nanos2*$^{-/-}$ germ cells (Fig. 3C, D, G and H). These results indicate that *Nanos2*-null XY germ cells indeed enter mitotic arrest at E14.5 but cell cycle was resumed at E15.5.

To determine whether the resumed cell cycle reflects mitosis or meiosis, we further analyzed the cell cycle markers in the *Nanos2*/*Stra8* dKO germ cells. Both the KI67 and PRB1 signals were detected in the dKO germ cells, suggesting that the loss of NANOS2 function causes abnormal cell cycle progression independent of the suppression of meiosis (Fig. 4A–F). Because STRA8 is required for premeiotic DNA replication (Baltus et al., 2006), we next investigated whether DNA replication occurs in dKO male germ cells by incorporating BrdU in dKO male germ cells. As expected, BrdU was incorporated not only into the *Nanos2*$^{-/-}$ male germ cells but also in the dKO male germ cells (Fig. 4G–I). These results indicate that *Nanos2* is required for the maintenance of mitotic arrest, irrespective of the suppression of meiosis.

Discussion

Using *Nanos2*/*Stra8* dKO mice, we showed that NANOS2 is required for the differentiation of male germ cells independent of the suppression of meiosis. Comparative microarray analysis indicated that the prolonged retention of the genes predominantly expressed in undifferentiated PGCs occurred in *Nanos2*-null male germ cells. Subsequent RIP analysis further demonstrated that some of these RNAs were associated with NANOS2, suggesting that one of the functions of NANOS2 may be to terminate the undifferentiated state of PGCs. Furthermore, abnormal resumption of the cell cycle in the absence of *Nanos2* was not repressed even in the *Nanos2*/*Stra8* double mutant, indicating that NANOS2 plays a role in the maintenance of mitotic quiescence independent of the suppression of meiosis.

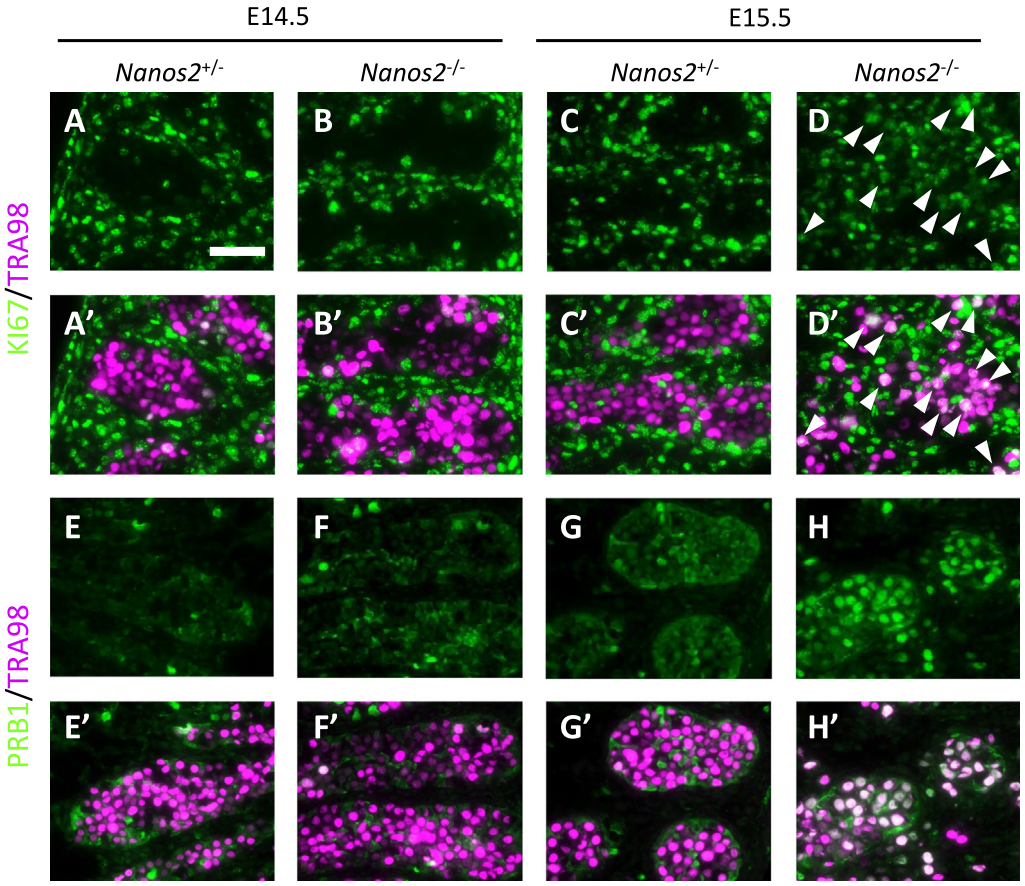

Fig. 3. NANOS2 is essential for the maintenance of mitotic cell cycle arrest. Immunohistochemical detection of KI67 (green in A–D and A′–D′) and PRB1 (green in E–H and E′–H′) in *Nanos2*$^{+/-}$ (A, A′, C, C′, E, E′, G, and G′) and *Nanos2*$^{-/-}$ (B, B′, D, D′, F, F′, H, and H′) germ cells at E14.5 (A, A′, B, B′, E, E′, F, and F′) and E15.5 (C, C′, D, D′, G, G′, H, and H′). The germ cell marker TRA98 is shown in magenta. Arrowheads indicate KI67-positive (D and D′) germ cells. Scale bar, 50 μm.

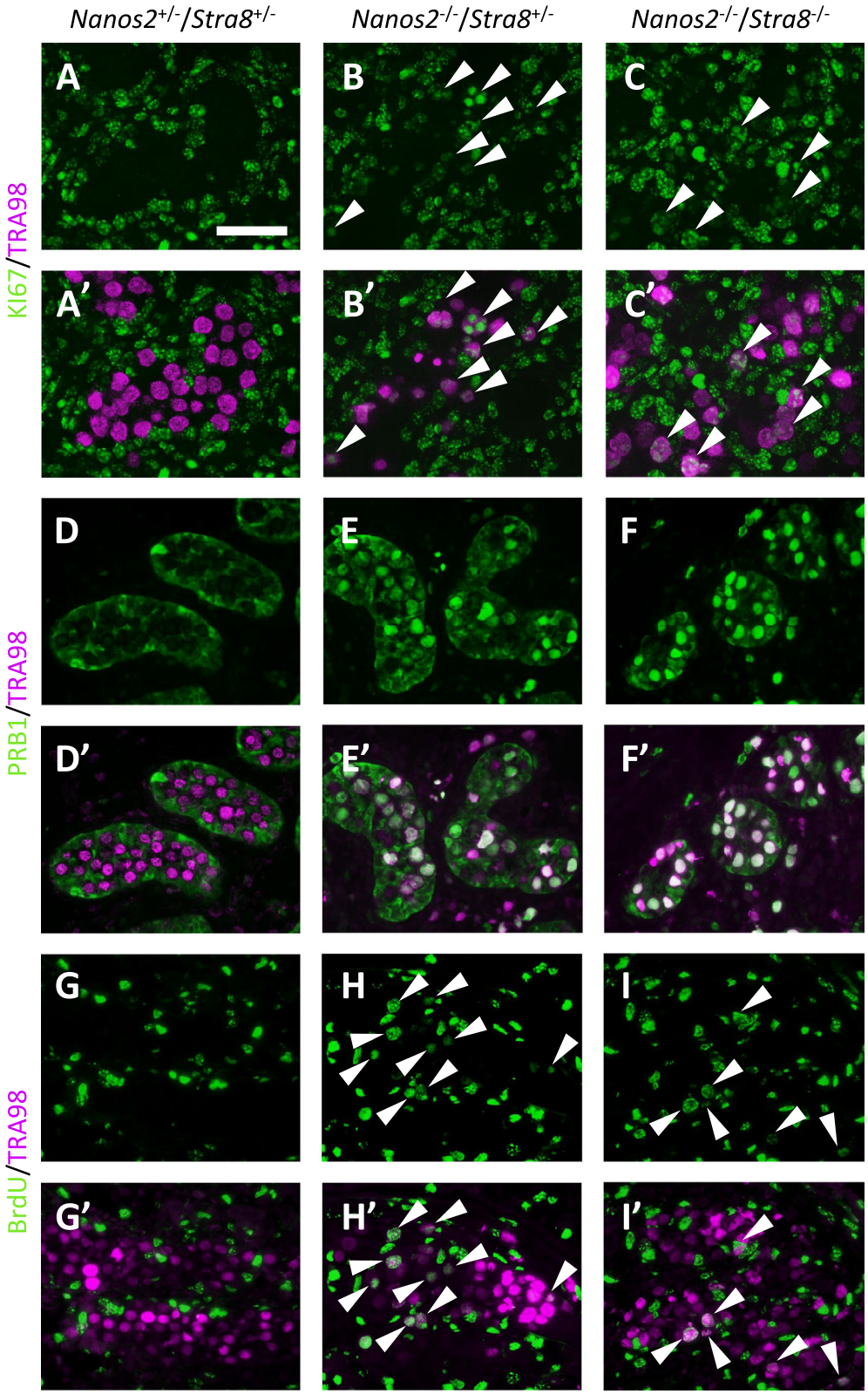

Fig. 4. *Stra8* is not responsible for the resumption of mitosis in *Nanos2*$^{-/-}$ XY germ cells. Immunohistochemistry of KI67 (green in A–C and A′–C′), PRB1 (green in D–F and D′–F′), and BrdU (Green in G–I and G′–I′) in *Nanos2*$^{+/-}$/*Stra8*$^{+/-}$ (A, A′, D, D′, G and G′), *Nanos2*$^{-/-}$/*Stra8*$^{+/-}$ (B, B′, E, E′, H and H′), and *Nanos2*$^{-/-}$/*Stra8*$^{-/-}$ (C, C′, F, F′, I and I′) XY gonads at E16.5. Magenta, TRA98 (germ cell marker). Arrowheads indicate KI67-positive (B, B′ C, and C′) and BrdU-positive (H, H′, I and I′) germ cells. Scale bar, 50 μm.

Sexual differentiation of male germ cells involves dynamic changes in genetic pathways, whereby proliferating PGCs enter into the mitotic quiescent state concomitantly with the elevation of male-specific gene expression. *Nanos2* is an early germ cell-intrinsic gene that responds to the signaling cues from the surrounding somatic cells (Bowles et al., 2010). Since we previously found that NANOS2 is required for the suppression of meiosis in embryonic XY germ cells (Suzuki and Saga, 2008), we speculated

that male germ cell development could be restored to some extent if meiosis was further prohibited in *Nanos2* mutant male germ cells. Surprisingly, however, male germ cell development was not rescued in the *Nanos2/Stra8* double mutant germ cells, indicating that ectopic induction of meiosis is not a cause but an effect of *Nanos2*-deficiency. Although we could not specify the primary defect in the *Nanos2* mutant, our results clarified the requirement and possible involvement of NANOS2 in multiple cellular events that occur in the sexually differentiating male germ cells.

A deduced function of NANOS2 relates to its ability to terminate PGC properties. *Sox2* and *Nanog*, which are representative PGC markers, are upregulated in human testicular germ cell tumors (Korkola et al., 2006), and their abnormal retention in embryonic XY germ cells increases the risk of teratoma after birth (Cook et al., 2011; Western et al., 2010). Therefore, it is reasonable to assume that germ cells have several defense systems to reduce this risk. NANOS2-mediated post-transcriptional suppression of PGC marker genes might be one of them. Specifically, transition from undifferentiated PGCs to prospermatogonia might require accurate timing. After induction of NANOS2, epigenetic systems including DNA methylation may lock their expression at the transcriptional level concomitant with genome-wide *de novo* methylation (Western et al., 2010). Although germ cells mostly disappeared and tumor-like cells were not observed in the *Nanos2/Stra8* dKO testis at 1 week after birth as well as *Nanos2* mutant testis (data not shown), this might depend on genetic background. Moreover, prolonged retention of PGC properties might repress male-specific genes in the *Nanos2* mutant male germ cells. However, it is unlikely that the failure to terminate PGC properties is the sole cause of repression of male-specific genes, because SOX2 and NANOG were heterogeneously expressed in both the *Nanos2* KO and *Nanos2/Stra8* dKO mutant (data not shown). Rather, other abnormalities, such as resumption of the cell cycle, may also be involved in the repression of male-specific genes.

Another function of NANOS2 clarified in this study is the maintenance of mitotic quiescence. Although we could not exclusively conclude that cell cycle resumption in the *Nanos2*$^{-/-}$ mice reflects mitosis, the results obtained in the dKO mice strongly support the idea. In *Drosophila*, NANOS inhibits pole cell division by suppressing the translation of *cyclin B*, which controls the arrest of pole cells at the G2 phase (Kadyrova et al., 2007). Analogous cell cycle regulation might be involved in the mouse male germ cell, and NANOS2 may exert this function via RNA degradation. In addition, we demonstrated that NANOS2 was not required for entry into mitotic arrest, despite the crucial requirement of NANOS2 for the maintenance of mitotic quiescence. Recently, it was suggested that another RNA binding protein, DND1, promotes the entry of male germ cells into mitotic arrest through its interaction with mRNAs of negative cell cycle regulators including $p21^{Kip}$ (*Cdkn1b*), $p27^{Cip}$ (*Cdkn1a*), *Rb1*, and *Lats2* (Cook et al., 2011). Our microarray analysis showed that the expression of these molecules, with the exception of *Rb1*, was not significantly changed in the *Nanos2*$^{-/-}$ XY gonads at E14.5. This may explain the normal entry of male germ cells into mitotic quiescence in the *Nanos2*$^{-/-}$ mice. However, the *Dnd1* levels were decreased in the mutant (2.44-fold reduction) at E15.5, which may have induced resumption of the cell cycle. How NANOS2 regulates the maintenance of mitotic arrest is an open question that requires further experiments to reveal the molecular mechanisms involved.

Although the molecular cascade that promotes male germ cell differentiation remains to be determined, it is clear that NANOS2 plays an important role in switching from PGCs to prospermatogonia. The expression of *Nanos2* mRNA declines after E15.5, but the protein expression is maintained until birth (Suzuki et al., 2007), indicating that it orchestrates the transcriptome throughout the embryonic stage. Identification of the upstream factors involved in the induction of *Nanos2* and functional analysis of specific components of the important regulatory pathways downstream of *Nanos2* will provide new clues to the molecular mechanisms that control the sexual development of the mammalian germline.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.10.018.

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