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Rhox13 Is Translated in Premeiotic Germ Cells in Male and Female Mice and Is Regulated by NANOS2 in the Male[1]

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ABSTRACT

Male and female germ cells enter meiosis in response to an extrinsic cue by retinoic acid (RA), but the pathways downstream of RA signaling that regulate early gametogenesis remain uncertain. We identified a novel reproductive homeobox gene, *Rhox13*, transcribed in the prenatal ovary and testis beginning on Embryonic Day (E) 13.5. Translation of RHOX13 also begins in female germ cells on E13.5 but is suppressed in male germ cells until Postnatal Day 3. Translation of RHOX13 coincides with initiation of RA signaling in both male and female gonads in vivo but occurs precociously in neonatal testes exposed to RA in vitro or in fetal male germ cells when NANOS2 is absent in vivo. Conversely, RHOX13 translation in female germ cells is suppressed in the presence of ectopically induced NANOS2. These results strongly suggest that RHOX13 expression is regulated at a posttranscriptional step by direct interaction of NANOS2 with *Rhox13* mRNA to suppress translation.

meiosis, NANOS2, retinoic acid, Rhox13*, testis, translation*

INTRODUCTION

Meiosis is initiated in the ovary and testis at different times during development. In the female mouse, oogonia divide by mitosis until Embryonic Day (E) 13.5 and then enter the meiotic phase as preleptotene oocytes. Oocytes progress through prophase of meiosis I and then arrest at the diplotene stage of meiosis I just prior to birth [1, 2]. Some begin escaping meiotic arrest at around 3 wk of age and others escape with each estrus cycle throughout the reproductive lifespan [3]. In the human female, meiosis begins at 10–11 wk of gestation and oocytes start escaping meiotic arrest at menarche, with ovulation occurring with each menstrual cycle until menopause [4]. Instead of entering meiosis, male germ cells in the mouse undergo mitotic arrest beginning at E13.5. Mitosis is reinitiated beginning at Postnatal Day (P) 1–3, giving rise to spermatogonia, some of which enter meiosis at P6–7 while others become spermatogonial stem cells (SSCs) that self-renew and proliferate to produce spermatocytes throughout adult life [1, 5, 6].

An active form of vitamin A, retinoic acid (RA), has a central role in regulating the sex-specific timing of meiotic initiation in mice [7, 8]. However, the specific genes and pathways involved downstream towards RA signaling remain uncertain. An important advance towards understanding the role of RA in this process was the discovery that the stimulated by retinoic acid 8 (*Stra8*) gene is expressed in both male and female premeiotic germ cells in response to RA [7–11]. In females lacking the *Stra8* gene, germ cells fail to undergo premeiotic DNA replication and initiate meiosis, while in males lacking the *Stra8* gene, germ cells undergo DNA replication and initiate meiosis, but fail to progress through leptotene [12–14]. However, the molecular mechanism by which STRA8 promotes meiotic initiation in both sexes remains unknown, although a role in transcription has been suggested [15].

The delay in entry into meiosis in males is thought to occur because RA is degraded by CYP26B1 in Sertoli and peritubular myoid cells in the fetal testis, effectively blocking RA signaling in male germ cells [7, 8, 15, 16]. Supporting this conclusion is the observation that male germ cells enter meiosis by E13.5 in mice lacking the *Cyp26b1* gene [16]. However, in vitro studies have suggested that nonretinoid factors produced by testicular somatic cells induce arrest of meiosis and mitosis during fetal and neonatal life [17] and that CYP26B1 may prevent the onset of meiosis by metabolizing a non-RA substrate controlling *Stra8* expression [18].

The *Nanos2* gene also is involved in regulating meiotic entry in male germ cells. This gene is expressed in male germ cells beginning at E12.5 and continues in a subpopulation of spermatogonia in adults [19–21]. Male *Nanos2*-null mice are infertile and lack germ cells, while females are fertile [21]. Male germ cells undergo mitotic arrest at E14.5 in *Nanos2*-null mice, but they fail to maintain the arrest and enter M-phase from E15.5 and die by apoptosis [22]. The *Nanos2* gene is downregulated in E13.5 testes in *Cyp26b1*-null embryos, probably in response to RA, which is present in the absence of CYP26B1 [19, 22]. Conditional disruption of the *Nanos2* gene postnatally depletes SSC reserves, indicating NANOS2 is a regulator of SSC maintenance and self-renewal [20]. The level of *Stra8* message increases in *Nanos2*-null male mice, suggesting that NANOS2 represses *Stra8* mRNA levels in male germ cells [22]. Recently, FGF9 was shown to act in the fetal male gonad to alternately reduce *Stra8* expression while

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increasing *Nanos2* levels, both of which result in inhibition of meiosis and promotion of the male germ cell fate [19, 23].

NANOS proteins bind to the untranslated regions (UTRs) of specific mRNAs through an interaction with PUMILIO in *Drosophila* to repress their translation [24–27]. It has been suggested that NANOS2 interacts with pumilio 2 (PUM2) in mice in both the fetal and neonatal testis [19]. Recent studies in mice have identified that NANOS2 is present in P-bodies, ribonucleoprotein (RNP) complexes containing translationally repressed mRNAs and mRNA decay machinery. These studies strongly suggest that NANOS2-interacting RNAs are recruited to P-bodies and degraded by the CCR4/NOT1 deadenylase complex [28]. In support of this, mouse NANOS2 was found in abundance in the RNP fraction following sucrose gradient polysome fractionation; however, a significant amount of NANOS2 was also found in both the ribosomal subunit and polysome fractions as well [19]. This suggests that NANOS2 may have a multifunctional role in translation regulation. Recombinant NANOS2 binds several spermatogonial mRNAs involved in germ cell differentiation [19, 28], suggesting that NANOS2 in association with PUM proteins may act as an RNA-binding complex to repress translation of mRNAs in male germ cells.

A large family of reproductive homeobox genes encoding putative transcription factors is present on the X chromosome in mice [29]. We identified the *Rhox13* gene and determined it was transcribed specifically in germ cells in the fetal testis and ovary beginning at E13.5, and the protein was detectable in differentiating spermatogonia and in preleptotene spermatocytes [30]. *Rhox13* mRNA and protein are detectable in the ovary by E13.5. In the testis, however, despite the presence of message from E13.5 throughout the remainder of fetal testis development, RHOX13 protein is not detectable until P3. We report here that *Rhox13* translation is repressed in the fetal and neonatal testis, and provide evidence that the suppression is mediated through an interaction with NANOS2 in the fetal testis. RHOX13 has the characteristics of a transcription factor and is expressed during the early differentiation of oogonia and spermatogonia, suggesting it is a regulator of genes involved in meiotic initiation.

MATERIALS AND METHODS

Animals

All the animal procedures were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the National Institute of Environmental Health Sciences. *Nanos2*-knockout and transgenic mice used in this study were maintained in the animal facility in the National Institute of Genetics, Japan. Tissues from CD-1 mice were used for all of the analyses of endogenous *Rhox13* expression as well as for the neonatal testis explant experiments. The morning after a vaginal plug was detected was designated E0.5, and the day of birth was designated P0.

Immunohistochemistry and Immunoblotting

Tissues were collected, fixed for 4 h to overnight (depending on their size) in Bouin solution (Polysciences, Inc.) or 4% paraformaldehyde (PFA), pH 7.4, washed thrice in 1× PBS, dehydrated in 70% ethanol, and embedded in paraffin using standard procedures. Anti-RHOX13 was diluted (1:400) [30] and applied to 6-μm sections without antigen retrieval followed by a biotinylated secondary goat anti-rabbit antibody (Vector Laboratories). A minimum of two gonads (from different mice) were present on each slide, and immunohistochemistry (IHC) was repeated a minimum of three times for each developmental time point. For peptide blocking, 0.01 μg of immunizing peptide for anti-RHOX13 [30] was preincubated with anti-RHOX13 for 30 min at room temperature prior to IHC. A Vectastain Elite ABC avidin-biotin-peroxidase kit (Vector Laboratories) was used for detection according to the manufacturer's instructions. Hematoxylin was used to counterstain the sections, which were then mounted with Permount (Sigma-Aldrich) and visualized using an Olympus BX-50 upright microscope with an OLY-750 video camera (Olympus). Immunoblotting was performed using standard methods as previously described [30] except that 10 μg of soluble protein was loaded, and antibody dilutions were 1:1500 for anti-RHOX13 and 1:5000 for anti-ACTB (A5441; Sigma-Aldrich).

Neonatal Testis Explant Culture

In three independent experiments, neonatal (P1) testes from three different mice were detunicated and incubated in DMEM media containing 10% fetal bovine serum (FBS) (Invitrogen) with or without all-trans-RA (Sigma-Aldrich) added to a final concentration 0.7 μM for 24 h [10]. Retinoic acid was dissolved in ethanol, and ethanol was added as the vehicle control to the RA-minus (−RA) explant sample. After culturing, explants were washed thrice in 1× PBS. Two cultured explants from separate animals were prepared for indirect immunofluorescence (IIF) and RNA isolation, respectively. RNA was isolated using TRIzol reagent (Invitrogen) according to manufacturer's instructions.

Immunofluorescence on Fetal Testis Explants and Nanos2 Transgenic Fetal Ovaries

Explants were fixed in 4% PFA for 4 h, washed thrice in PBS, and embedded in optimal cutting temperature compound. Frozen sections (8 μm) were cut and washed thrice with PBS, and blocking was performed with 3% skim milk for 60 min at room temperature. RHOX13 antiserum (1:1000) was added and incubation was for 1 h at room temperature, followed by three washes with PBS. Alexa Fluor 488 anti-rabbit immunoglobulin G (IgG) (1:500; Invitrogen) was then added for 1 h at room temperature. DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired on a Nikon E600 fluorescence microscope with an Orca II CCD camera (Hamamatsu) and Metamorph image analysis and acquisition software (Universal Imaging Corporation).

NANOS2-expressing female gonads were obtained by crossing transgenic mice, CAG-lox-CAT-lox-3xFLAG-*Nanos2* [22] and *Pou5f1*/*Oct4*-CreERT2 transgenic mouse established at the National Institute of Genetics, Japan. Tamoxifen (Sigma-Aldrich) was administered at E10.5 (3 mg) to induce the expression of NANOS2. Female gonads at E12.5 and E14.5 were fixed with 4% PFA, washed with PBS, and prepared for cryosectioning as described above. Antibodies were used with the following dilutions: anti-DMC1 (1:200, C-20; Santa Cruz Biotechnology), anti-RHOX13 (1:200), anti-NANOS2 (1:100), and Alexa Fluor 488/594 anti-goat/rabbit IgG (1:1000; Invitrogen). Olympus BX61 fluorescent microscopy was used for signal detection (Olympus).

RNA Immunoprecipitation

Immunoprecipitation (IP), RNA purification, and RT-PCR were performed as previously described [28] with E14.5 male gonads of transgenic mice, which expressed Flag-tagged NANOS2. The gonads were homogenized in five volumes of ice-cold extraction buffer, and the lysates were precleared with protein A-agarose for 30 min at 4°C. The precleared lysates were then divided into two tubes and incubated with anti-Flag IgG-agarose and IgG-agarose (Sigma-Aldrich), respectively, for 3 h at 4°C. Input and immunoprecipitated mRNAs were isolated using an RNeasy mini kit (Qiagen Inc.) and were then used as templates for RT-PCR.

Semiquantitative and Quantitative RT-PCR

Primer pairs were designed to amplify across an intron/exon junction to avoid the possibility of genomic DNA amplification giving erroneous results (except for *Nanos2*, which is an intronless gene). For quantitative RT-PCR (qRT-PCR) of *Nanos2*$^{+/-}$ and *Nanos2*$^{-/-}$ fetal testes, cDNAs were synthesized with Superscript III (Invitrogen) and oligo dT primer from total RNAs of E13.5–E15.5 fetal testes. Quantitative PCR analyses were performed with the Kapa SYBR FAST qPCR Kit (Kapa Biosystems) and Thermal Cycler Dice Real Time System Single (Takara), according to the manufacturers' instructions. Relative message levels of *Rhox13* were normalized to those of *Gapdh*. Primer sequences were: *Gapdh* (5′-ACCACAGTCCATGCCATCAC and 5′-TCCACCACCCTGTTGCTGTA) and *Rhox13* (5′-ACCGCCATTC CACTTCGCAC and 5′-ATTGGGCACAGAGGTTGC). Total RNA was purified from 5 to 10 gonads and combined as one sample, and sample numbers and replicates for qRT-PCR were four and two times, respectively.

For qRT-PCR of RNA from cultured neonatal testis explants, 100 ng of RNA from two cultured explants was reverse-transcribed and amplified in triplicate using iScript One-Step RT-PCR kit with SYBR green (Bio-Rad

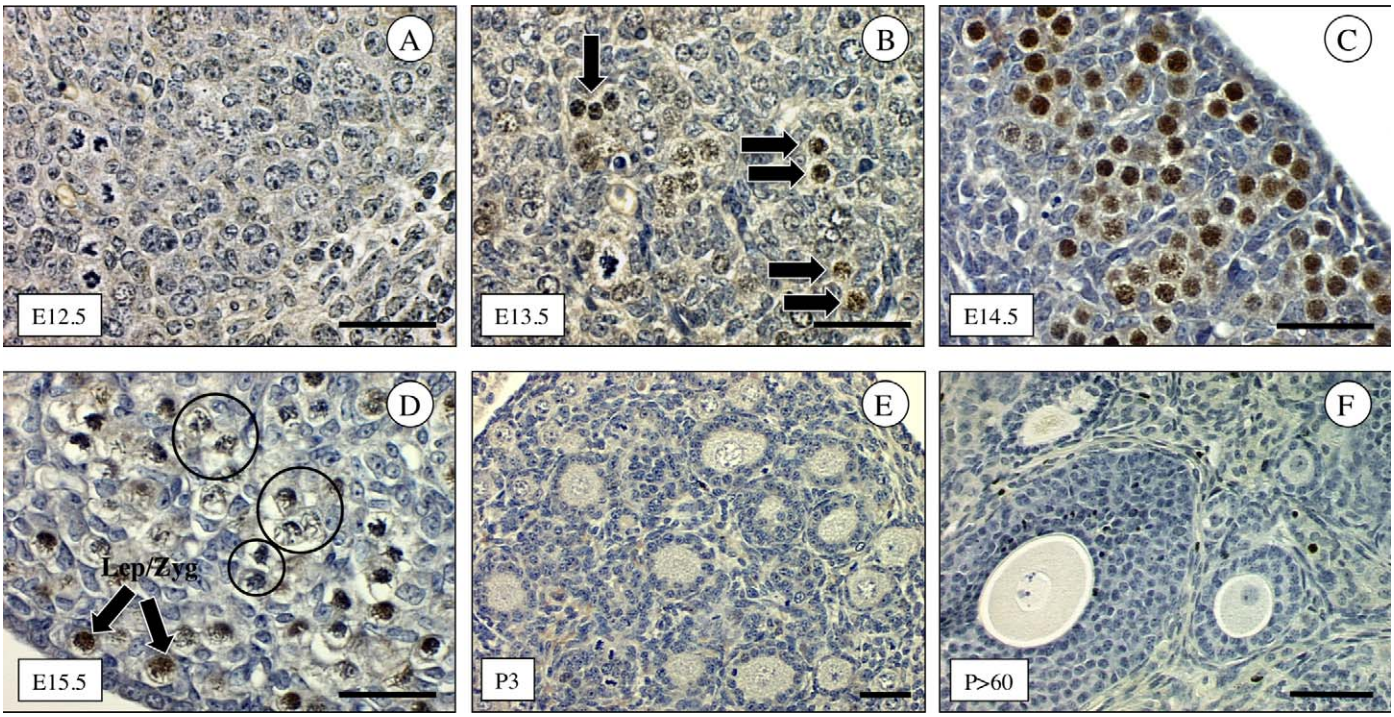

FIG. 1. Expression of RHOX13 in the fetal, neonatal, and adult ovary. **A**–**F**) Immunohistochemistry was performed on gonadal sections of the ovaries at the ages indicated using anti-RHOX13. Light immunostaining of RHOX13 was first detectable in nuclei of a subset of oogonia at E13.5 (**B**, indicated by black arrows), which increased in E14.5 preleptotene oocytes (**C**). RHOX13 was faintly detectable at E15.5 (**D**) in leptotene/zygotene oocytes (Lep/Zyg, indicated by arrows), but no signal was present in pachytene oocytes (examples are circled) at E15.5. No RHOX13-positive cells were seen after initiation of folliculogenesis by P3 (**E**) or in the adult ovary (**F**). Bars = 50 μm.

Laboratories) according to the manufacturer's instructions on a Bio-Rad iQ5 real-time PCR detection system (Bio-Rad Laboratories). Primer sequences used for amplification were: *Nanos2* (5′-GACCAGGCTCATACACTCAAG and 5′-GGAGGGTGTGGGTTGTG), *Rhox13* (5′-ACCCAGTACCCGGATTTGCT TACA and 5′-TCTGCTTCGCTCTCCGATTGGTAA), *Rpl19* (5′-GAAATCGCCAATGCCAACTC and 5′-TCTTAGACCTGCGAGCCTCA), and *Stra8* (5′-GAGGTCAAGGAAGAATATGC and 5′-CAGAGACAATAG GAAGTGTC). Ct values were normalized to *Rpl19* (60S ribosomal protein L19), and relative mRNA levels were calculated by using the delta-delta Ct method. Student *t*-test was used for all the statistical analyses, and significant differences are indicated with an asterisk ($P < 0.05$).

For semiquantitative RT-PCR, cDNA from fetal and neonatal testes were synthesized and amplified for 30 cycles as previously described [30]. The primers used were: *Rhox13* (5′-ACCGCCATTCCACTTCGCAC and 5′-ATTGGGCACAGAGGTTGC), *Stra8* (5′-TCACAGCCTCAAAGTGGCAGG and 5′-GCAACAGAGTGGAGGAGGAGT), and *Actb* (5′-TCCGATGCCCT GAGGCTCTTTTC and 5′-CTTGCTGATCCACATCTGCTGGAA).

RESULTS

RHOX13 Is Expressed in the Fetal Ovary and Neonatal Testis

Immunohistochemistry (IHC) was used to define the pattern of expression of RHOX13 protein during the development of the fetal and postnatal ovary and testis. Premeiotic and meiotic germ cells in the fetal ovary were identified by the characteristic appearance of their chromatin [31, 32]. Immunostaining for RHOX13 first was seen at low levels in the nuclei of a subset (~20/ovarian section) of differentiating oogonia at E13.5 (Fig. 1B) and then at higher levels in all preleptotene oocytes at E14.5 (Fig. 1C). It was seen at declining levels in the nuclei of leptotene and zygotene oocytes (Fig. 1D), but undetectable in pachytene oocytes at E15.5 (Fig. 1D) or in follicular oocytes at P3 and in adults (Fig. 1, E and F).

RHOX13 protein was not detected by IHC in gonocytes in the fetal testis from E13.5 to P0 (Fig. 2, A–C). However, it was detectable at low levels in nuclei of some gonocytes transitioning to spermatogonia at P3 (Fig. 2D, black arrows), and at increased levels and frequency in differentiating spermatogonia from P4 onward (Fig. 2, E–H). Approximately 50% of spermatogonia were RHOX13-positive during the interval from P4-P6. The larger, RHOX13-negative spermatogonia (Fig. 2G, white arrows) have nuclei that measure approximately 10–11 μm in diameter, while the smaller, RHOX13-positive spermatogonia nuclei are 6–7 μm in diameter (Fig. 2, E–H). The expression of RHOX13 continued in preleptotene spermatocytes in juvenile and adult mice, but decreased drastically in leptotene and zygotene spermatocytes, and was not detectable in pachytene spermatocytes or spermatids (data not shown) [30]. Immunoblotting of lysates from neonatal testes confirmed that RHOX13 was faintly detectable by that method by P3–P4 (Fig. 2I).

Rhox13 *Is Translated in Response to RA*

RHOX13 protein expression was detectable in analogous differentiating cell populations in both sexes. We realized that the temporal appearance of RHOX13 protein coincides with the onset of RA signaling in the ovary and testis [12, 13]. Since *Stra8* is robustly transcribed in response to RA in vivo and in vitro in both male and female germ cells [7–11], we amplified *Stra8* mRNA by RT-PCR to better define when RA signaling is initiated in the neonatal testes of the CD-1 mice used for our developmental expression studies. While *Stra8* messages were barely detectable in the fetal testis at E15.5, their levels increased dramatically between P3 and P4 (Fig. 3A), indicating this as the interval when gonocytes respond to the RA signal in the CD-1 mouse testis.

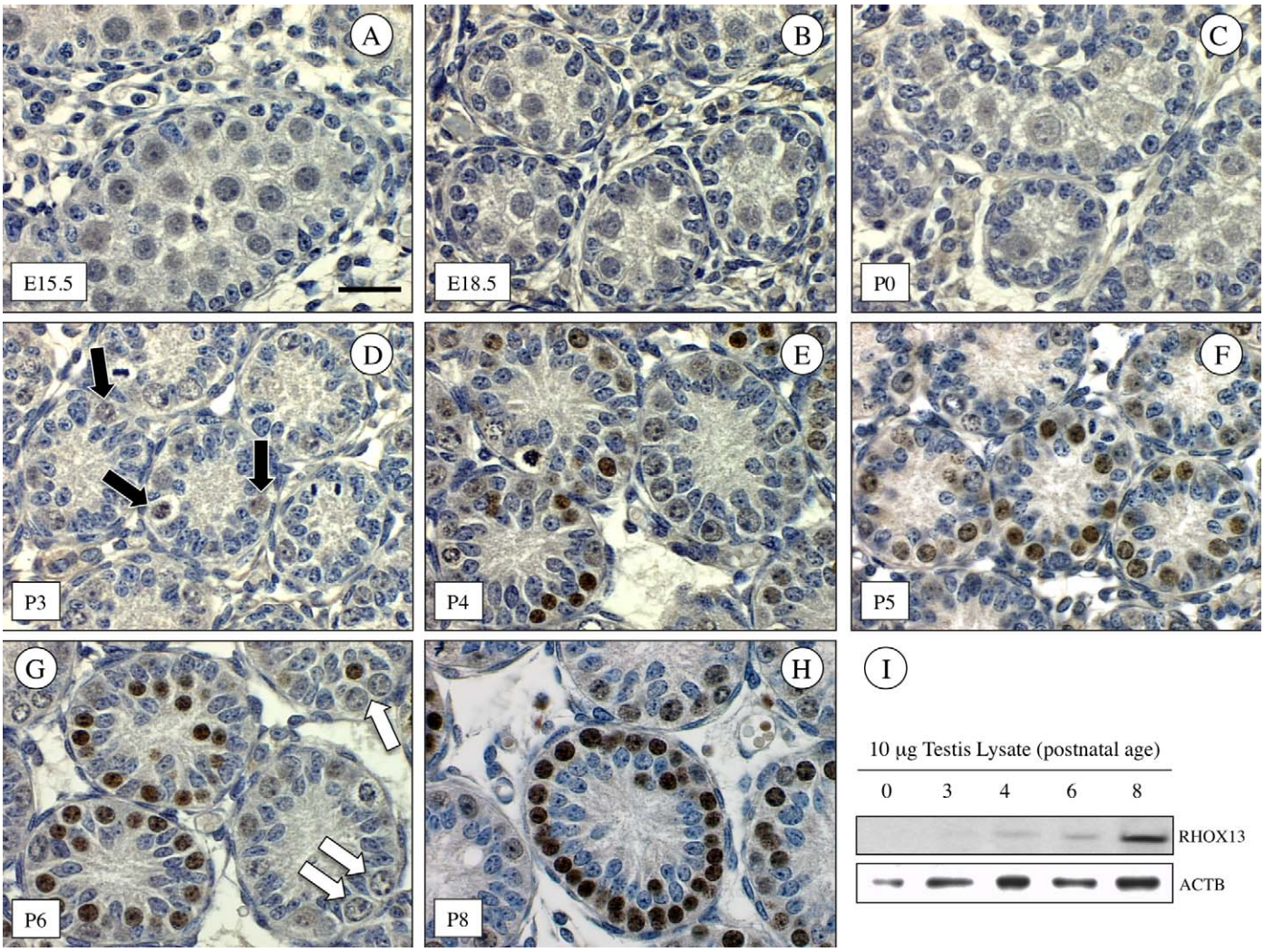

FIG. 2. Expression of RHOX13 in the pre-, neo-, and postnatal testis. **A**–**H**) Ages are indicated on each section. Immunohistochemistry reveals that RHOX13 protein is first found in a subset of spermatogonia at P3 (**D**, indicated by black arrows). By P5–P6, approximately 50% of spermatogonia express RHOX13, are smaller in diameter, and have the nuclear morphology of differentiating type B spermatogonia (**F** and **G**). The larger type A spermatogonia are RHOX13-negative and consistently located at the periphery of the testis cords (**G**, white arrows). **I**) Developmental immunoblot showing that RHOX13 protein is first detectable at P3–P4, confirming the results from IHC. ACTB was utilized as a loading control. Bar = 25 μm.

To test whether RA is involved in overcoming suppression of translation of *Rhox13* leading to RHOX13 expression in the neonatal testis, neonatal (P1) testis explants were incubated in the presence or absence of all-trans-RA for 24 h (Fig. 3B). Quantitative RT-PCR revealed that *Stra8* message levels increased nearly 6-fold in response to RA, while there was no change for *Rhox13* or *Nanos2* (Fig. 3C). While very low levels of RHOX13 protein were detected in a small proportion of gonocytes in explants grown without added RA (Fig. 3E), robust RHOX13 protein expression occurred in the majority of germ cells in explants cultured in the presence of RA (Fig. 3F). This result, combined with the observation that *Rhox13* mRNA levels do not significantly change during the interval from P0 to P5 in vivo suggests that RA is involved in relieving *Rhox13* translational suppression in the neonatal testis.

RHOX13 Is Translated in Nanos2*-Null Fetal Testes*

Because it has been shown that meiotic initiation is induced in *Nanos2*-null male gonocytes, we used IHC to examine the possible role of NANOS2 in regulating translation of RHOX13. Mice heterozygous for a mutation in *Nanos2* were fertile and phenotypically indistinguishable from wild-type (WT) mice [21], and RHOX13 was not detected in male germ cells at E15.5 (Fig. 4A). However, in *Nanos2*-null testis at E15.5, the nuclei of germ cells were stained robustly for RHOX13 (Fig. 4, B and C), and this immunostaining was blocked by the cognate peptide (Fig. 4D). In addition, we detected a small population of RHOX13-positive gonocytes in the adjacent mesonephros (Fig. 4C). The mislocalization of gonocytes outside of the testis cords was previously reported in *Nanos2*-null mice [21]. These results strongly suggest that NANOS2 directly or indirectly suppresses translation of *Rhox13* in germ cells in the fetal testis.

NANOS2 was found to reside, at least partially, in P-bodies in association with deadenylase activity and has been suggested to participate in degradation of specific mRNAs [28]. This did not appear to be the case for suppression of *Rhox13* translation because mRNA levels persisted at similar levels throughout fetal testis development [30]. To test whether NANOS2 might be degrading *Rhox13* mRNA, we assessed mRNA levels by qRT-PCR in *Nanos2*$^{-/-}$ fetal testes. If NANOS2 targeted *Rhox13* for degradation, we would expect to detect increased levels of *Rhox13* mRNA in *Nanos2*$^{-/-}$ fetal testes. Although

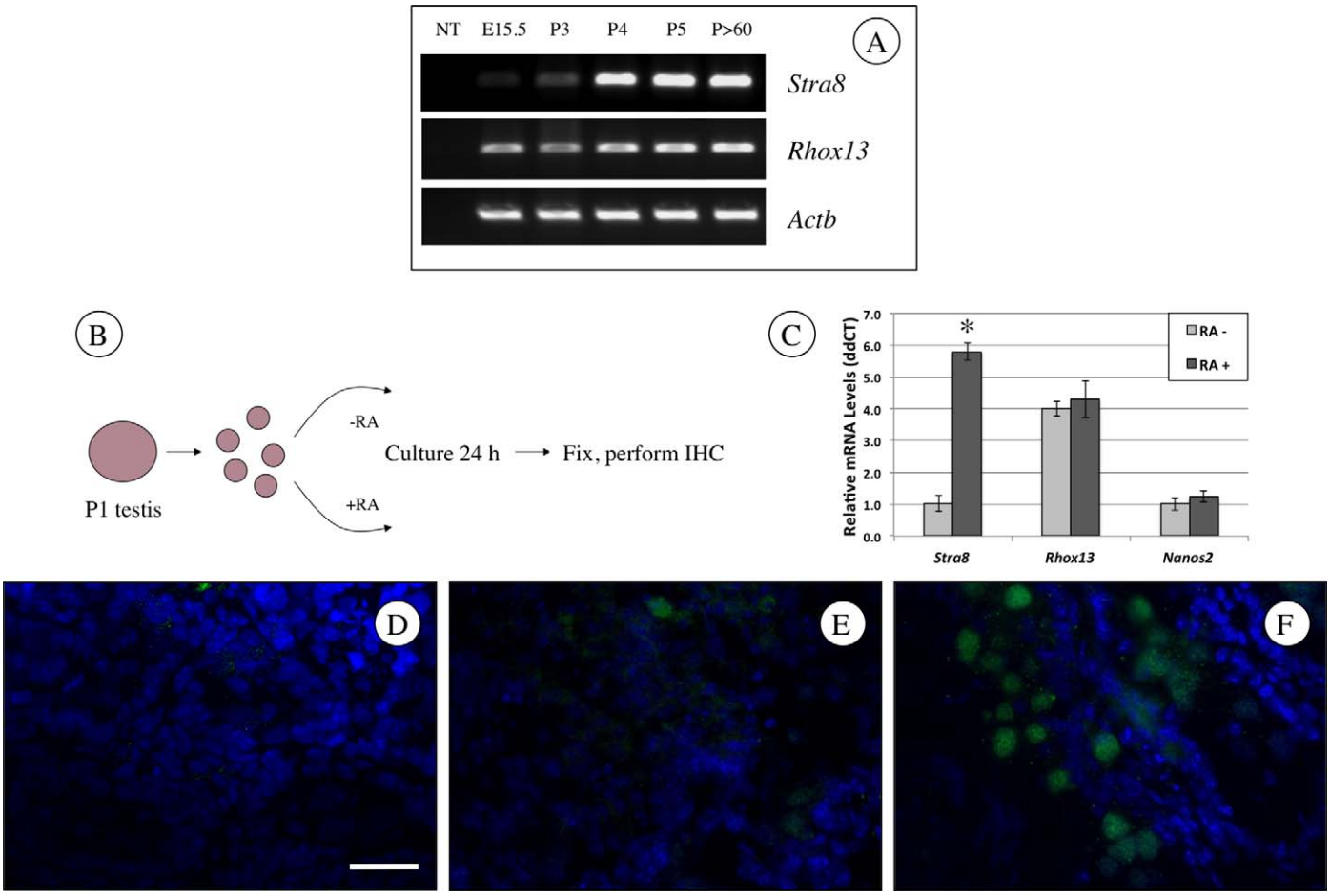

FIG. 3. RA induction of RHOX13 expression in P1 testis explants. **A**) RT-PCR was performed using primers to amplify *Stra8*, *Rhox13*, and *Actb* from testes at E15.5, P3, P4, P5, and P>60. NT = no template control. **B**) For explant culture, testes were dissected from neonatal mice, cut into small pieces, and cultured for 24 h without RA (−RA) or with RA (+RA). Retinoic acid was dissolved in ethanol, and an equivalent volume of ethanol was added to the −RA sample. **C**) Quantitative PCR was performed to quantitate *Stra8*, *Rhox13*, and *Nanos2* mRNA levels from explants grown with (RA+) or without (RA−) added RA. **D**–**F**) Positive staining was present in gonocytes in P1 testis explants cultured in the presence of RA (**F**, cells in green). Primary antibody was excluded from the negative control (**D**). Bar = 25 μm.

there was a statistically significant increase in *Rhox13* message levels in the absence of NANOS2 at E14.5 (Fig. 4E), the upregulation was only 1.58-fold. This was much lower than the 5- to 10-fold upregulation previously seen for other mRNAs that are repressed by NANOS2-mediated degradation [28]. In addition, RHOX13 protein was detectable in the absence of NANOS2 at E15.5 (Fig. 4B) but not in the presence of NANOS2 (Fig. 4A), a time when a similar amount of message was detected (Fig. 4E), which strongly suggests that NANOS2-mediated *Rhox13* regulation is primarily not mediated at the level of message abundance (e.g., through degradation).

NANOS2 Suppresses RHOX13 Expression in Fetal Ovaries

RHOX13 protein was precociously expressed in gonocytes of the fetal testis in the absence of NANOS2 (Fig. 4, B and C). We next wanted to determine whether introduction of NANOS2 was capable of suppressing translation of *Rhox13* mRNA in a dominant manner. To test this, we expressed NANOS2 in fetal oocytes using a transgenic approach and analyzed whether RHOX13 was expressed as in the WT (Fig. 1, A–C). We utilized a line of conditional transgenic mice, CAG-lox-CAT-lox-3xFLAG-*Nanos2* [22], that expresses NANOS2 following Cre recombinase-mediated excision of a blocking cassette. These mice were bred with a transgenic line harboring a *Pou5f1/Oct4*-CreERT2 transgene that expresses Cre recombinase in germ cells upon tamoxifen administration. We confirmed NANOS2 induction at E12.5 in more than 50% of female germ cells in response to tamoxifen at E10.5 (Fig. 5, A–C). We decided to examine E14.5 ovaries because RHOX13 is robustly expressed in all oocytes at this time (Fig. 1C). Since both antibodies (anti-NANOS2 and anti-RHOX13) were generated in rabbits, we indirectly examined RHOX13 expression in NANOS2-expressing female germ cells. To do this, the nuclear meiosis-specific recombinase DMC1 [33, 34] was used as a marker for meiotic oocytes. NANOS2 expression effectively repressed expression of DMC1 (Fig. 5, D–F). As noted, we can distinguish two distinct populations in female germ cells with regards to RHOX13 expression (Fig. 5H). Strong RHOX13 expression coincided with strong expression of DMC1, and cells showing weak or no expression of RHOX13 basically lacked DMC1 expression (Fig. 5, G and I), indicating that NANOS2 effectively suppressed RHOX13 expression.

Rhox13 *mRNA Is Bound by NANOS2 in the Fetal Testis*

Based on its role as an RNA-binding protein, we hypothesized that NANOS2 suppresses translation of *Rhox13* mRNA in the fetal and neonatal testis by a direct interaction.

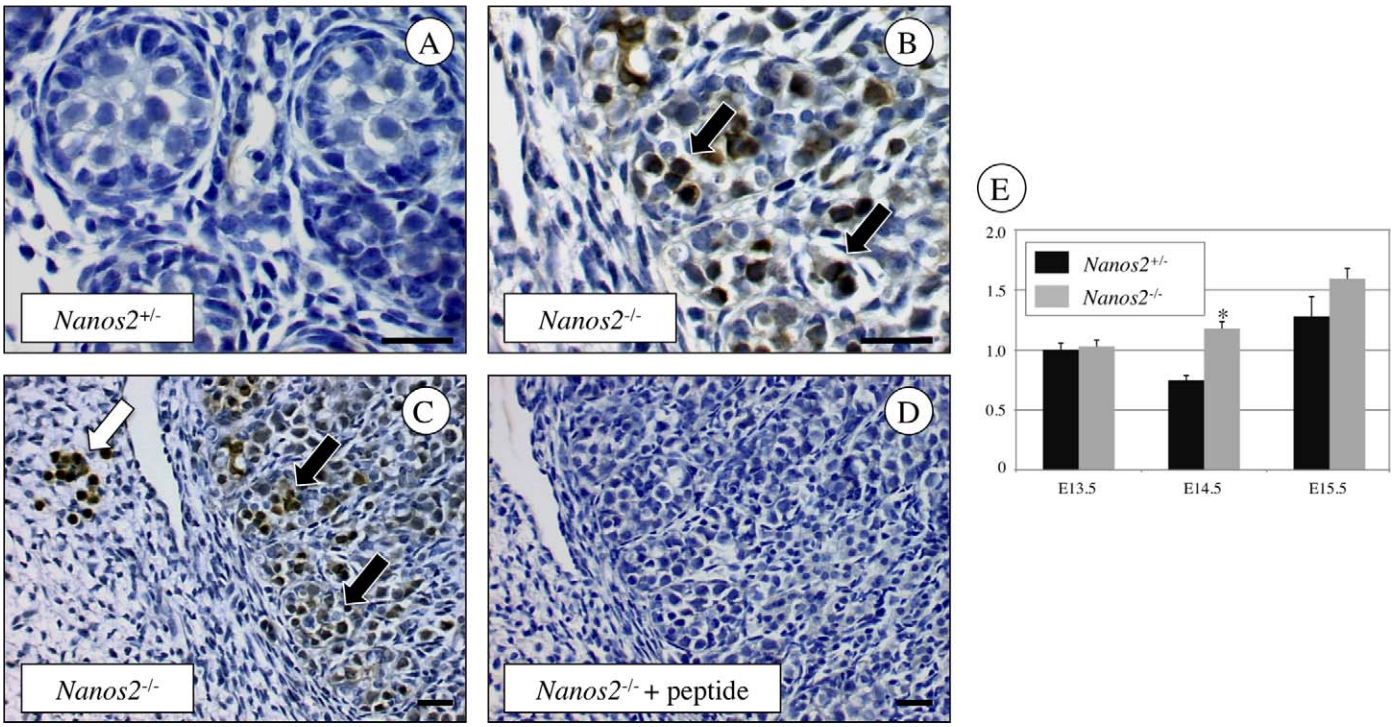

FIG. 4. RHOX13 is precociously translated in *Nanos2*$^{-/-}$ fetal testes. **A**) Immunohistochemistry was performed on E15.5 testis sections using anti-RHOX13, and no signal was evident in *Nanos2*$^{+/-}$ gonocytes. **B**–**C**) A strong signal was detected in gonocytes in spermatic cords (black arrows) as well as in the adjacent mesonephros (white arrow) in the *Nanos2*$^{-/-}$ testis. **D**) Preincubation of anti-RHOX13 with the immunizing peptide resulted in loss of signal in the *Nanos2*$^{-/-}$ testis. Bars = 25 μm. **E**) *Rhox13* mRNA levels were assessed by qRT-PCR in *Nanos2*$^{+/-}$ and *Nanos2*$^{-/-}$ testes at E13.5, E14.5, and E15.5. *P* values (Student *t*-test) for each stage at E13.5, E14.5, and E15.5 are 0.12, 0.00, and 0.11, respectively. Statistically significant differences are indicated by an asterisk (E14.5).

To test this, NANOS2 was immunoprecipitated from E14.5 fetal testes extracts, and the pellets were assayed for *Rhox13* mRNA (Fig. 6). *Taf7l* mRNA served as a positive control because it was previously reported to bind to NANOS2 [19, 28]. *Rhox13* and *Taf7l* mRNAs were both detectable in the input material from both WT and FLAG-NANOS2 transgenic mice, as expected. Immunoprecipitation using anti-FLAG resulted in a substantial enrichment of both messages in extracts from transgenic FLAG-NANOS2 fetal testes, but not WT fetal testis (Fig. 6). This revealed a direct interaction between NANOS2 and *Rhox13* mRNA in fetal testes. Taken together, these data suggest that NANOS2 suppresses *Rhox13* translation and synthesis of RHOX13 in the fetal testis by binding and maintaining its mRNA in an inactive form.

DISCUSSION

Our studies demonstrate that RHOX13 is expressed in an analogous subset of differentiating germ cells in both the ovary and testis prior to their entry into meiosis. In fetal oocytes, the initiation of *Rhox13* transcription is quickly followed by translation of RHOX13 protein. In contrast, *Rhox13* mRNA is present in the testis from E13.5 onward, but protein is not detected in spermatogenic cells until P3, when gonocytes transition to spermatogonia. Because the appearance of RHOX13 protein coincides temporally with the initiation of RA signaling in the testis and ovary, we tested and confirmed that RA can induce precocious translation of *Rhox13* in an explant culture system. In addition, we have shown that NANOS2 directly interacts with *Rhox13* mRNAs in the fetal testis, and our data suggests that it is suppressed through this interaction by a mechanism that involves storage rather than degradation. Our results highlight the presence of a posttranscriptional repression system in the fetal and neonatal testis that we believe may function to delay germ cell differentiation and meiotic initiation in gonocytes in the testis until P3–P4.

The timing of germ cell differentiation in the postnatal testis and the establishment of the SSC population are both currently topics of great interest within the field of reproductive biology. In the postnatal testis, gonocytes begin to reinitiate mitotic activity from P1–P3 [1, 5, 35] and transition to generate a population of spermatogonia shortly thereafter. Postnatal Day 8 has traditionally marked the first appearance of differentiated or type B spermatogonia [36]. However, recent studies have suggested that a subset of spermatogonia arising from gonocytes at P3–P4 bypass the undifferentiated stem cell stage and proceed to enter meiosis and ultimately generate spermatozoa capable of fertilization [10, 37]. Our results indicate that RHOX13 indeed marks a subset of a clearly heterogeneous population of spermatogonia by P4 (Fig. 2E).

We utilized an explant culture system and found that RA was capable of inducing RHOX13 protein expression (Fig. 3). In our explant culture system, *Stra8* mRNA levels increased dramatically in response to exogenous RA, as expected. However, we found that *Rhox13* and *Nanos2* mRNA levels were unaffected by the addition of RA to the culture medium. This indicates that the increase in RHOX13 translation was not due to an increase in *Rhox13* mRNA abundance, which supports our hypothesis that the appearance of RHOX13 in the postnatal testis is regulated at the level of translation. We did detect a small number of RHOX13-positive gonocytes in explants grown in the absence of added RA in PFA-fixed tissues. When we initially performed these experiments, we fixed explants in Bouin solution for detection of RHOX13 using IHC (data not shown), and did not detect any RHOX13

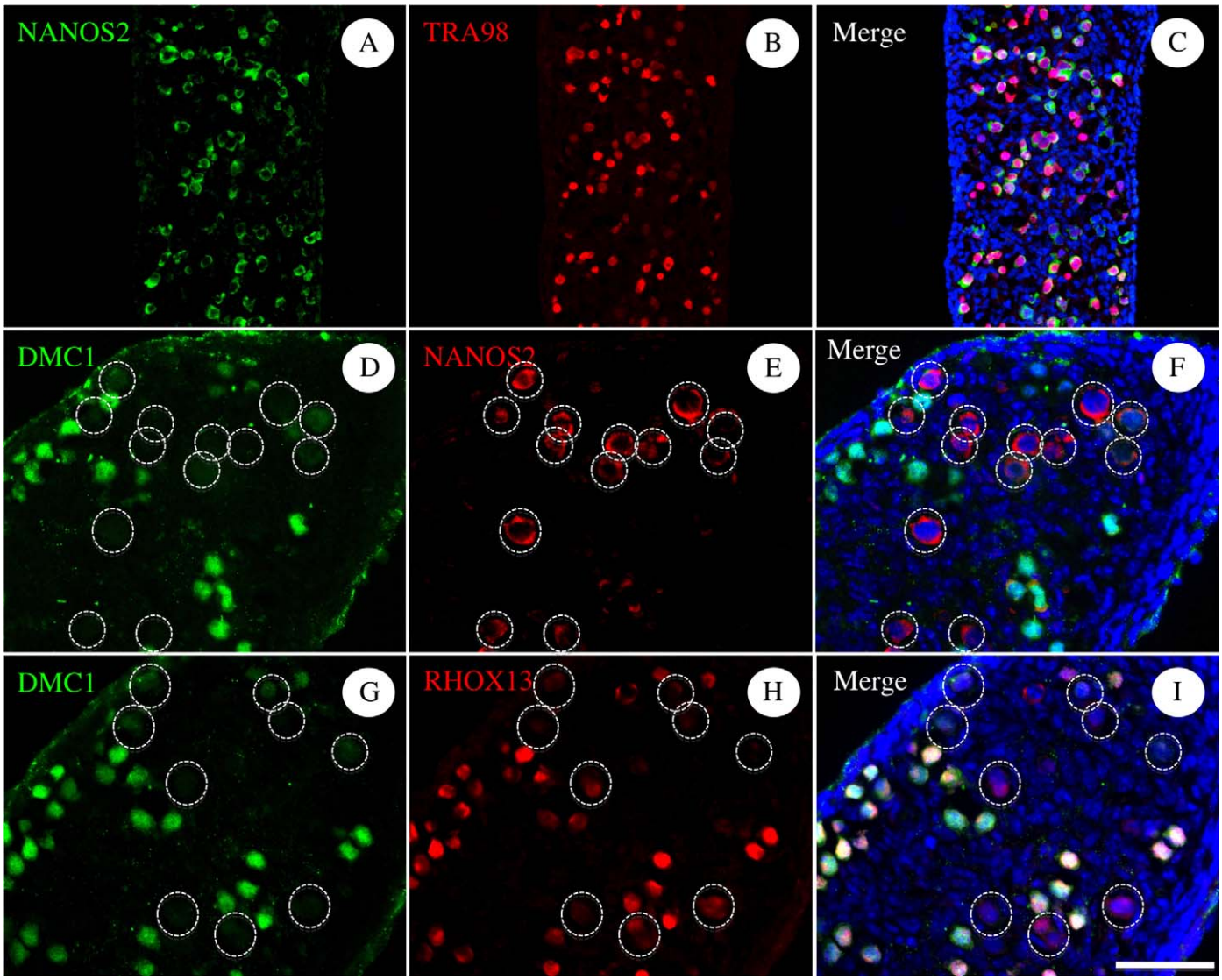

FIG. 5. Transgenic NANOS2 inhibits RHOX13 expression in oocytes. Tamoxifen was injected at E10.5 to pregnant female double transgenic mice harboring both *CAG-lox-CAT-lox-FLAG-Nanos2* and *Pou5f1-CreERT2,* and ovaries were prepared at E12.5 (**A**–**C**) and E14.5 (**D**–**I**). Ovaries from E12.5 mice were sectioned and coimmunostained with anti-NANOS2 and anti-TRA98 (**A**–**C**). Ovaries from E14.5 mice were sectioned and coimmunostained with anti-DMC1 and either anti-NANOS2 (**D**–**F**) or anti-RHOX13 (**G**–**I**). **D**–**F**) White dashed lines encircle germ cells that express NANOS2 (**E**), and all have reduced levels of DMC1 (**D**). **F**) A merged image of **D** and **E** is presented, indicating that there is minimal coexpression. **G**–**I**) Germ cells that have NANOS2-induced reduction of DMC1 (**G**) also do not express RHOX13 (**H**), indicating that NANOS2 is capable of repressing meiosis and RHOX13 expression. **I**) A merged image of **G** and **H** indicates that cells that do not express DMC1 also do not express RHOX13; cells that express both DMC1 and RHOX13 are yellow. Nuclei were stained with DAPI and can be seen in blue (**C**, **F**, **I**). Bar = 100 μm for **A**–**C** and 50 μm for **D**–**I**.

in the absence of RA. Tissues fixed in PFA often retain epitopes better than those fixed in Bouin solution (data not shown), which likely explains the difference in our level of detection. Additionally, RA is present in FBS (final concentration in media containing 10% FBS is 3.5–35 nM), and it therefore may be responsible for stimulating the low levels of *Stra8* transcription as well as RHOX13 translation; alternatively, the very low levels of RHOX13 may represent basal levels. Since RA is capable of directing transcription of *Stra8* and *Kit* [10] as well as stimulating translation of RHOX13, it is likely that RA provides the cue that directs spermatogonia to begin this early lineage commitment. The search for the gene(s) involved in the initial decision between a SSC and differentiated spermatogonial fate might best focus on this interval between P3 to P4.

In *Caenorhabditis elegans* and *Drosophila*, translation repression regulates aspects of gametogenesis such as sex determination and the mitosis-to-meiosis switch [38]. It is apparent that some of these mechanisms have been conserved in mammalian fetal male germ cell development. In particular, the RNA-binding proteins NANOS2 and NANOS3 are required for gonocyte development during the fetal and neonatal period [21, 39]. The NANOS proteins heterodimerize with PUMILIO proteins (PUM1 and PUM2 in mammals) and are thought to act by binding to nanos-response elements (NREs), often found in 3′ UTR sequences, to repress the translation of target gene mRNAs [19, 40]. In *Xenopus*, PUM2 has been shown to bind to both a NRE in the 3′ UTR of target mRNAs and to the 5′ cap. By binding to the cap structure, PUM2 is thought to block interactions with translation initiation factors such as EIF4E and prevent the assembly of the initiation complex [41]. A sequence resembling a NANOS/PUMILIO consensus-binding sequence is located in the *Rhox13* 3′ UTR (UGUGCCAUA; [30]) and may serve to

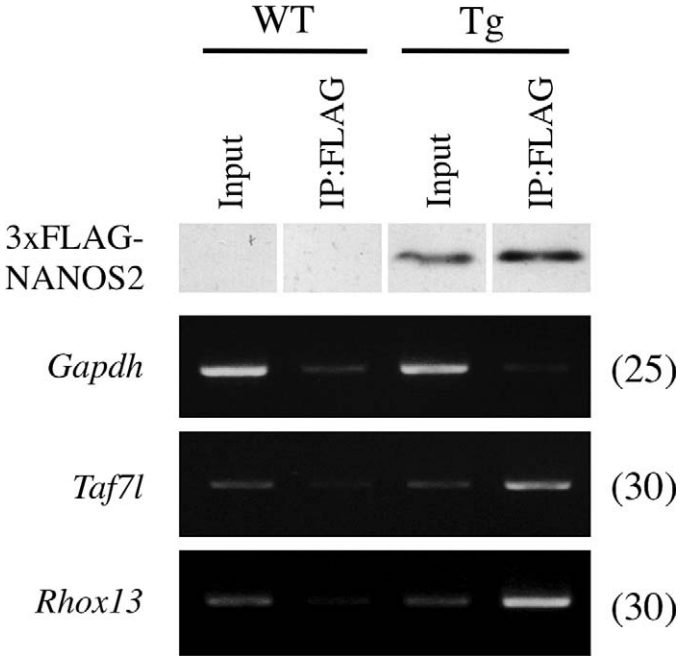

FIG. 6. *Rhox13* mRNA directly binds to NANOS2 in the fetal testis. Male gonadal extracts from wild-type (WT) and transgenic (Tg) mice expressing FLAG-NANOS2 at E14.5 were subjected to IP with anti-FLAG antibody. RNA precipitants were analyzed by RT-PCR for each of the genes indicated. The numbers of amplification cycles used for each gene are listed in parentheses to the right.

position a repressor complex on the endogenous *Rhox13* mRNA in fetal and early neonatal gonocytes. Our model proposes that binding of NANOS2/PUMILIO to *Rhox13* messages prevents their translation until RA signaling causes progression of meiotic entry, at which point RHOX13 is expressed.

Translational suppression of *Rhox13* mRNA in the fetal and neonatal testis would ensure that the timing of RHOX13 protein expression in the testis in differentiating spermatogonia at P3–P4 matches that of analogous differentiating oogonia at E13.5–E14.5. In the ovary, female germ cells enter meiosis shortly after sex determination (at E13.5), while in the male there is a protracted period of time (until P8–P10) following sex determination prior to meiotic initiation [42]. During this time, fetal gonocytes are in mitotic arrest and obtain paternal-specific DNA methylation on imprinted genes [43]. After birth, gonocytes must reenter the cell cycle, migrate to the periphery of the testis cords, and differentiate into stem and proliferating spermatogonial populations [2, 5, 43, 44]. It is unclear why the developmental expression pattern of *Rhox13* is not managed at the level of transcription in the testis, but if RHOX13 is involved in initiation of meiosis (as can be inferred from its expression in developing germ cells of both sexes), then perhaps its suppression in gonocytes is required to prevent precocious meiotic entry during the important processes described above.

Gonocytes in *Nanos2*-null fetal testes enter meiosis precociously, which is likely caused by the abnormal expression of NANOS2-repressed messages. In fetal gonocytes, NANOS2 interacts with components of the CCR4-NOT deadenylation complex in association with P-bodies [28]. This result is consistent with a role for NANOS2 in the degradation of specific mRNAs in the fetal and neonatal testis. Indeed, in the absence of NANOS2, mRNA levels increased for several meiotic markers normally expressed at very low to undetectable levels in the fetal WT testis, including *Sycp3*, *Stra8*, *Taf7l*, *Dazl*, and *Prdm9* (also known as *Meisetz,* see [28]). In another report, *Taf7l* and *Gata2* mRNAs were discovered bound to NANOS2 [19].

We and others have found that *Nanos2* mRNA and protein levels decrease in the testis from the fetal to neonatal stages [19, 45], and NANOS2 expression becomes restricted to a small subset of undifferentiated spermatogonia in the postnatal testis [20, 45]. *Nanos2* mRNA levels decline in the *Cyp26b1*-null fetal testis [22], indicating that *Nanos2* expression is negatively regulated by RA signaling either at the level of transcription or mRNA stability in the fetal testis. We found, however, that a 24-h RA treatment did not result in a decrease in *Nanos2* mRNA levels in neonatal testis explants in culture. This suggests that a decline in *Nanos2* is not responsible for RHOX13 protein expression in our explant cultures, but additional studies will be required to test the relationship between RA signaling, loss of NANOS2 in differentiated spermatogonia, and translation of *Rhox13* mRNA in the neonatal testis.

Our data supports a scenario whereby NANOS2, in addition to promoting the degradation of specific messages [28], functions in the fetal testis to store mRNAs in a translationally inert form. We found that *Rhox13* messages are bound by NANOS2 without significant degradation as evidenced by: 1) mRNA levels do not change appreciably between E13.5 (when RHOX13 protein is not detectable) and P8 (when RHOX13 protein is expressed) [30], and 2) *Rhox13* mRNA levels do not increase significantly in the *Nanos2-null* fetal testis at E15.5 when *Rhox13* mRNA is rapidly translated into protein in *Nanos2*-null gonocytes (Fig. 4, B, C, and E). In yeast, mRNAs present in large stationary phase P-bodies can move to polysomes for translation upon resumption of growth [46]. This scenario resembles that of *Rhox13* messages, which are repressed in arrested gonocytes but then become translated in dividing spermatogonia. Recently, NANOS2 was found in both RNP and light polysomal sucrose gradient fractions [19], suggesting that it might indeed play multiple roles in mRNA regulation. *Rhox13* mRNAs bound to NANOS2 may either be present in RNPs but escape degradation, or be associated with ribosomes in a situation where translation initiation or elongation is blocked. It will be of interest to determine how NANOS2 alternatively selects some messages for degradation while simultaneously preserving others.

In summary, we show that RHOX13 is expressed in germ cells just prior to entry into meiosis in both the male and female gonad. While *Rhox13* transcription and translation temporally coincide in the ovary, its translation is first blocked in the fetal testis and then promoted in the neonatal testis by components of the RA-signaling pathway. Our results provide the first bona fide target for developmental regulation by NANOS2 and implicate RHOX13 as an important participant in the program of germ cell differentiation in both sexes.

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