

# Opposing effects of retinoic acid and FGF9 on *Nanos2* expression and meiotic entry of mouse germ cells

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## Summary

In the mouse, three genes that are homologous to the *Drosophila Nanos (Nos)* gene have been identified. Deletion of one of these genes, *Nanos2*, results in male sterility, owing to loss of germ cells during fetal life. Before apoptosis, *Nanos2*-null gonocytes enter meiosis, suggesting that *Nanos2* functions as a meiotic repressor. Here, we show that *Nanos2* is continuously expressed in male germ cells from fetal gonocytes to postnatal spermatogonial stem cells. We observed that the pro-meiotic factor AtRA, an analog of retinoic acid (RA), downregulates NANOS2 levels, in both fetal and postnatal gonocytes, while promoting meiosis. Interestingly, FGF9, a growth factor crucial for sex differentiation and survival of fetal gonocytes, upregulates levels of NANOS2 in both male and female primordial germ cells (PGCs) and in premeiotic spermatogonia. This effect was paralleled by an impairment of meiotic entry, suggesting that FGF9 acts as an inhibitor of meiosis through the upregulation of *Nanos2*. We found that NANOS2 interacts with PUM2, and that these two proteins colocalize in the ribonucleoparticle and polysomal fractions on sucrose gradients, supporting the notion that they bind RNA. Finally, we found that recombinant NANOS2 binds to two spermatogonial mRNAs, *Gata2* and *Taf7l*, which are involved in germ-cell differentiation.

**Key words:** NANOS2, FGF9, RA, *Gata2*, *Taf7l*, Meiosis, Spermatogenesis

## Introduction

*Nanos* is an evolutionarily conserved protein that is essential for the survival of primordial germ cells (PGCs). It has been shown to act as a putative RNA-binding protein involved in germ cell development in both vertebrates (Koprunner et al., 2001; Tsuda et al., 2003) and invertebrates (Fujii et al., 2006; Lehmann and Nusslein-Volhard, 1991; Pilon and Weisblat, 1997; Subramaniam and Seydoux, 1999). In the mouse, three genes encoding *Nanos* have been identified (Haraguchi et al., 2003). Although *Nanos1* is predominantly expressed in the neural system (Haraguchi et al., 2003), *Nanos2* and *Nanos3* are exclusively expressed in fetal and postnatal germ cells (Tsuda et al., 2006; Tsuda et al., 2003). The expression pattern of *Nanos2* is restricted to gonocytes within fetal testis undergoing mitotic arrest, and is then maintained in spermatogonia after birth. Deletion of *Nanos2* results in male sterility, due to germ-cell loss during fetal life (Tsuda et al., 2003). *Nanos2*-null male fetal germ cells enter meiosis before undergoing apoptosis at around 15.5 days post coitus (d.p.c.) (Suzuki and Saga, 2008). Furthermore, misexpression of *Nanos2* in female fetal germ cells prevents meiotic entry, strongly suggesting that *Nanos2* is an anti-meiotic gene (Suzuki and Saga, 2008).

The mechanisms that trigger meiosis have been a subject of debate, but it is now clear that retinoic acid (RA) produced by the mesonephros induces meiosis in female PGCs (Bowles et al., 2006; Koubova et al., 2006), whereas the same action in the fetal testis is prevented by the presence of the retinoid-degrading enzyme CYP26B1 (Bowles et al., 2006; MacLean et al., 2007). The role of RA as a meiotic inducer is also conserved postnatally, because it is able to induce the meiotic entry of differentiating, KIT-positive spermatogonia (Pellegrini et al., 2008). An inverse correlation

between levels of RA and NANOS2 exists in *Cyp26b1*-null fetal testis – a condition in which RA is elevated (MacLean et al., 2007), gonocytes enter meiosis and NANOS2 levels are low (Suzuki and Saga, 2008). The crucial role of RA in inducing meiosis in both female and male is played by *Stra8* (stimulated by retinoic acid gene 8) (Anderson et al., 2008; Mark et al., 2008); indeed, *Stra8*-null males and females show impairment of gametogenesis owing to failure of meiotic commitment (Anderson et al., 2008; Mark et al., 2008). However, meiotic commitment is aberrantly induced, not only in *Nanos2*-null mice, but also in *Fgf9* knockouts (Colvin et al., 2001).

*Fgf9* is known to be expressed by somatic cells in XX and XY gonads at 11.5 d.p.c., but it becomes XY restricted by 12.5 d.p.c. and is then expressed within the testis cords (Schmahl et al., 2004). *Fgf9*-null mice die shortly after birth owing to defects in lung formation, but XY embryos show phenotypic male-to-female sex-reversal, and the surviving gonocytes are found in meiosis (Colvin et al., 2001). The consequences of *Fgf9* ablation on male germ cells are probably independent of the proliferative and differentiative effect on fetal Sertoli cell, because it has been shown that FGF9 is able to promote survival of male germ cells in vitro (DiNapoli et al., 2006).

We report that NANOS2 is continuously expressed in male germ cells from fetal to postnatal development. In particular, we investigated the role of RA and FGF9 on the regulation of NANOS2 in correlation with their ability to influence meiosis. We found that AtRA represses NANOS2 expression, whereas FGF9 upregulates *Nanos2* mRNA levels in female and male germ cells and inhibits meiosis in both sexes. Moreover, we show evidence that NANOS2 associates with ribonucleoparticles (RNPs) and polysomes, both in

fetal and postnatal germ cells. Finally, by GST-RNA pull-down assay, we found that NANOS2 shows affinity for two RNA transcripts, *Gata2* and *Taf7l*, which are involved in spermatogonial differentiation, strengthening the hypothesis that NANOS2 can act as a meiotic inhibitor through a post-transcriptional regulation mechanism.

## Results

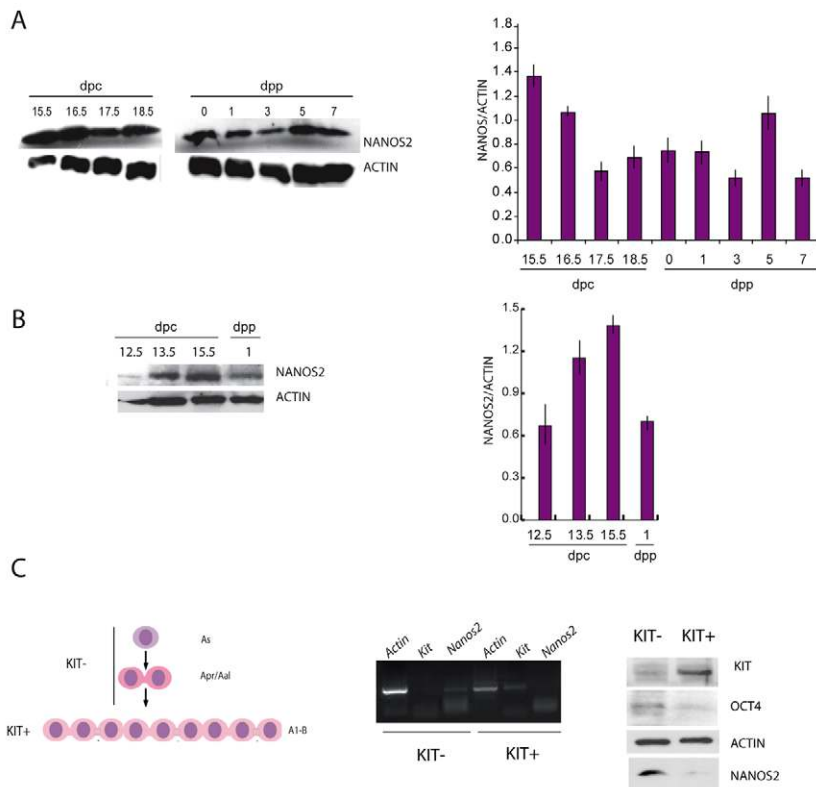
### Developmental expression of *Nanos2* in the male gonad

It is known that *Nanos2* mRNA starts to be expressed in male PGCs at around 12.5 d.p.c. (Suzuki et al., 2007). Furthermore, by immunofluorescence analysis, it has been very recently shown that undifferentiated spermatogonia type  $A_{\text{single}}$  ( $A_s$ ) and  $A_{\text{paired}}$  ( $A_{\text{pr}}$ ) are the testicular cells that express NANOS2 in the adult testis (Suzuki et al., 2009). However, it is not clear whether NANOS2 levels drop before birth, or remain elevated in the early postnatal testis. To clearly address this question, we performed a western blot analysis on male germ cells isolated at various stages of fetal and postnatal development. As shown in Fig. 1A we found that NANOS2 protein levels were high at 15.5 d.p.c. and then decreased up to birth. Since NANOS2 levels were already high at 15.5 d.p.c., to better define the fetal period when they start to increase, we analyzed protein extracts obtained from germ cells isolated at 12.5, 13.5, 15.5 d.p.c. and 1 day post partum (d.p.p.). As shown in Fig. 1B, we found that NANOS2 levels were low at 12.5 d.p.c., but they consistently increased up to 15.5 d.p.c. We also found that NANOS2 was expressed after birth and its levels peaked at 5 d.p.p. and then decreased at 7 d.p.p., when differentiating premeiotic spermatogonia (KIT positive) and preleptotene spermatocytes are found (Fig. 1A,B). Isolated pachytene spermatocytes and spermatids did not express *Nanos2*, either at the RNA or protein level (not shown). Thus, to determine which spermatogonial cell type expressed

*Nanos2* in the prepuberal testis, we isolated pure populations of undifferentiated or differentiating spermatogonia by immunomagnetic cell sorting. KIT-negative (undifferentiated) and KIT-positive (differentiating) spermatogonia were obtained by using anti-CD117 (Kit)-coated beads and analyzed for *Nanos2* expression by semiquantitative RT-PCR. As shown in Fig. 1C, *Nanos2* amplification was obtained only in KIT-negative spermatogonia, whereas KIT-positive cells did not show any signal. Purity of the cell fractions was assessed by western blot analysis probing extracts for KIT and OCT4 (Fig. 1C) or RT-PCR amplification of PLZF and OCT4 (not shown), as previously reported (Lolicato et al., 2008).

### Retinoic acid downregulates *Nanos2* expression in fetal gonocytes and postnatal spermatogonia

RA has been proposed as a meiosis-inducing substance, both in fetal female germ cells and in postnatal male germ cells (Bowles et al., 2006; Koubova et al., 2006; Pellegrini et al., 2008). In *Cyp26b*-null fetal testis, gonocytes enter meiosis and *Nanos2* mRNA levels are low (Suzuki and Saga, 2008). This suggests that, apart from *Stra8* induction, the pro-meiotic action of RA might also result from the downregulation of *Nanos2* expression. To test this hypothesis, we cultured testes from 12.5 d.p.c. embryos, the developmental stage at which *Nanos2* starts to be expressed, in the presence of 0.3  $\mu\text{M}$  AtRA for 48 hours. As shown in Fig. 2A, AtRA completely abolished the expression of *Nanos2* mRNA, whereas it upregulated *Stra8* mRNA levels. The same effect was observed when we cultured disaggregated 13.5 d.p.c. testes for 24 hours in the presence of AtRA (Fig. 2A, see also Fig. 3A). Using chromosomal spreads stained for SCP3, we found that about 33% of male germ cells were in the early meiotic leptotene stage in the presence of AtRA whereas no meiotic germ cells were present in the control. Using the same



**Fig. 1. Developmental expression of NANOS2.**

(A) Western blot analysis of NANOS2 from fetal (15.5 d.p.c.) throughout postnatal life (7 d.p.p.). Male germ cells express NANOS2 at 15.5 d.p.c. and the protein levels decrease around birth, and peak again at 5 d.p.p. to decrease later at 7 d.p.p. Densitometric analysis of western blots from three separate experiments is shown on the right. Bars represent mean  $\pm$  s.d.;  $P < 0.001$ . (B) Western blot analysis of NANOS2 expression starting at earlier developmental stages. Densitometric analysis of the western blots from three independent experiments is shown on the right. Bars represent mean  $\pm$  s.d.;  $P < 0.001$ . (C) Schematic representation of spermatogonia development in the prepuberal testis (7 d.p.p.) is shown on the left. Two populations can be distinguished: the undifferentiated spermatogonia, that do not express KIT, and the differentiated KIT-positive spermatogonia. Adapted from de Rooij and Mizrak (de Rooij and Mizrak, 2008). *Nanos2* expression is restricted to the undifferentiated spermatogonia population as shown by semiquantitative RT-PCR (middle panel) or by western blot analysis (right panel).

culture conditions, in the presence of AtRA about 85% female germ cells were found in leptotene and leptozygotene compared with 75% of the control (Fig. 2A, see also Fig. 3B).

We, and others, have previously demonstrated that G0-arrested gonocytes and undifferentiated KIT-negative spermatogonia are not able to enter meiosis in vitro upon stimulation by AtRA (Pellegrini et al., 2008; Trautmann et al., 2008). Since NANOS2 expression is high in these cells (see Fig. 1) we tested whether AtRA was also able to downregulate NANOS2 at these developmental stages. The results showed that 48 hours of AtRA treatment actually downregulated the levels of *Nanos2* mRNA and protein in 15.5 d.p.c. and 4 d.p.p. isolated male germ cells (Fig. 2B). This effect was accompanied by upregulation of STRA8 (Fig. 2B) but, as expected, did not result in meiotic induction (Fig. 2C).

### FGF9 upregulates *Nanos2* expression and represses meiosis in both male and female germ cells

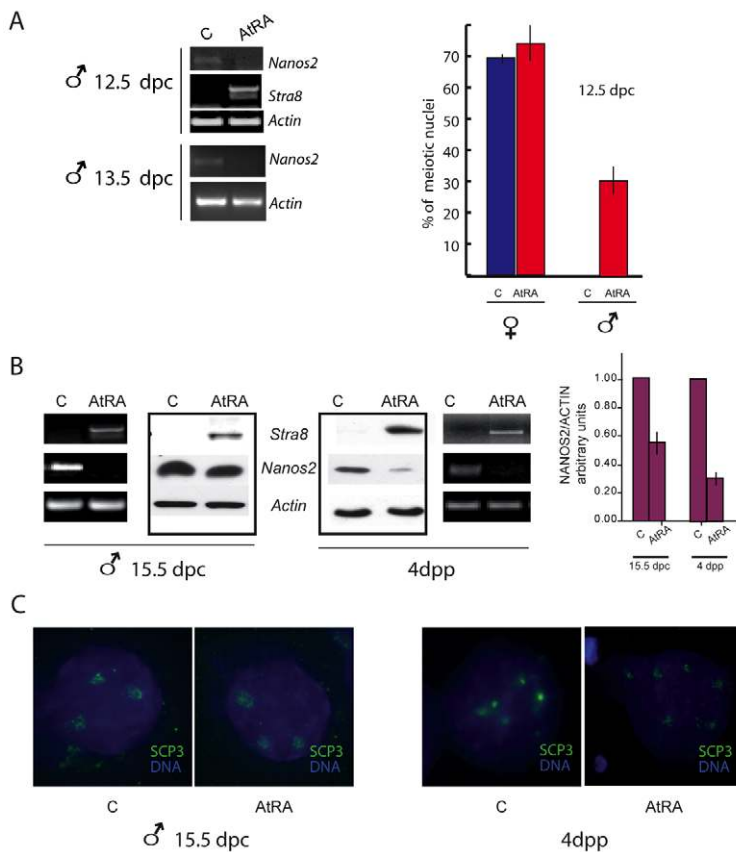
XY *Fgf9*-null gonocytes enter meiosis before undergoing apoptosis, suggesting that FGF9 prevents meiosis in XY germ cells (DiNapoli et al., 2006). Since this phenotype resembles that of *Nanos2*-null gonocytes, we hypothesized that FGF9 could directly or indirectly upregulate *Nanos2* expression in germ cells. To test this possibility, we chose a developmental stage at which *Nanos2* mRNA levels were low in the fetal testis and were obviously absent in the fetal ovary. Thus, 12.5 d.p.c. male or female gonads were cultured for 48 hours in the presence or absence of 25 ng/ml FGF9. By semiquantitative RT-PCR, we found that levels of *Nanos2* mRNA were upregulated by treatment with FGF9 in fetal testes and, interestingly, *Nanos2* mRNA expression was stimulated also in fetal ovaries (Fig. 3A,B). The same effect was observed when disaggregated 13.5 d.p.c. gonads were cultured for only 24 hours

in the presence of FGF9 (Fig. 3A). A control PCR minus RT was run for *Nanos2* mRNA to exclude genomic contamination of the cDNA samples (Fig. 3A). Since the expression of NANOS2 in female germ cells was found to be detrimental for meiotic entry (Suzuki and Saga, 2008), we prepared SCP3-stained nuclear spreads from fetal ovaries after 48 hours of culture with FGF9. The results showed that whereas in the control most of the female germ cells were in leptotene and leptozygotene (75%), less than one third of them entered meiosis in the presence of FGF9 (22%; Fig. 3B). The nuclear morphology of the non-meiotic nuclei was similar to that of G0-arrested gonocytes (Fig. 3B, compare also with Fig. 2C) and showed faintly stained chromatin using DAPI stain.

Finally, we investigated whether FGF9 was able to affect meiosis also in 7 d.p.p. spermatogonia. We found that FGF9 reduced the number of meiotic cells after 48 hours of culture, as judged by chromosomal spread counts (40% in the control versus 20% in the FGF9 treated cells, Fig. 4A) and by the decrease of SCP3 levels (Fig. 4B). The role of FGF9 in meiotic inhibition was not mediated by the decrease of STRA8 levels, because they did not change after FGF9 stimulation. To understand which cell population was responsive to FGF9, we then isolated KIT-positive and KIT-negative spermatogonia by immunomagnetic cell sorting, and stimulated them with the factor for 24 hours. FGF9 sensitivity was present in both cell types, because it induced upregulation of NANOS2 in KIT-negative and KIT-positive spermatogonia, as shown by RT-PCR and by western blot analyses (Fig. 4C).

### NANOS2 associates with RNPs and polysomes and interacts with PUM2

In *Drosophila* germline progenitor pole cells, NANOS has been shown to interact with pumilio (PUM) to repress Cyclin B (*CCNB1*)



**Fig. 2. AtRA downregulates *Nanos2* in both fetal and postnatal male germ cells.** (A) Male fetal gonads isolated at 12.5 d.p.c. cultured in vitro for 48 hours in the presence of AtRA. RT-PCR analysis shows that *Stra8* and *Nanos2* mRNA expression are oppositely modulated by AtRA treatment (top left). Testes from 13.5 d.p.c. embryo were collected, disaggregated, and kept in culture for 24 hours in the presence of AtRA. RT-PCR analysis shows that AtRA strongly reduces *Nanos2* expression also at this developmental stage (bottom left). The histogram represents the percentage of meiotic nuclei scored in control or in AtRA-treated cultures of 12.5 d.p.c. gonads. Three independent experiments performed in triplicate are represented. Bars show mean  $\pm$  s.d.;  $P < 0.001$ . (B) Isolated male germ cells of 15.5 d.p.c. and 4 d.p.p. were stimulated in vitro with AtRA for 48 hours. Western blots and semiquantitative RT-PCR analyses show that, concomitantly with STRA8 upregulation, NANOS2 is downregulated in AtRA-treated cells. The right panel represents the densitometric analysis of the western blots from three independent experiments performed in triplicate. Bars represent mean  $\pm$  s.d.;  $P < 0.001$ . (C) Nuclear spreads from 15.5 d.p.c. (left) or 4 d.p.p. (right) spermatogonia were prepared after 48 hours of culture in the presence or absence of AtRA and probed for SCP3. Merged images of SCP3 (not corresponding to correctly assembled synaptonemal complexes) and DAPI are shown.



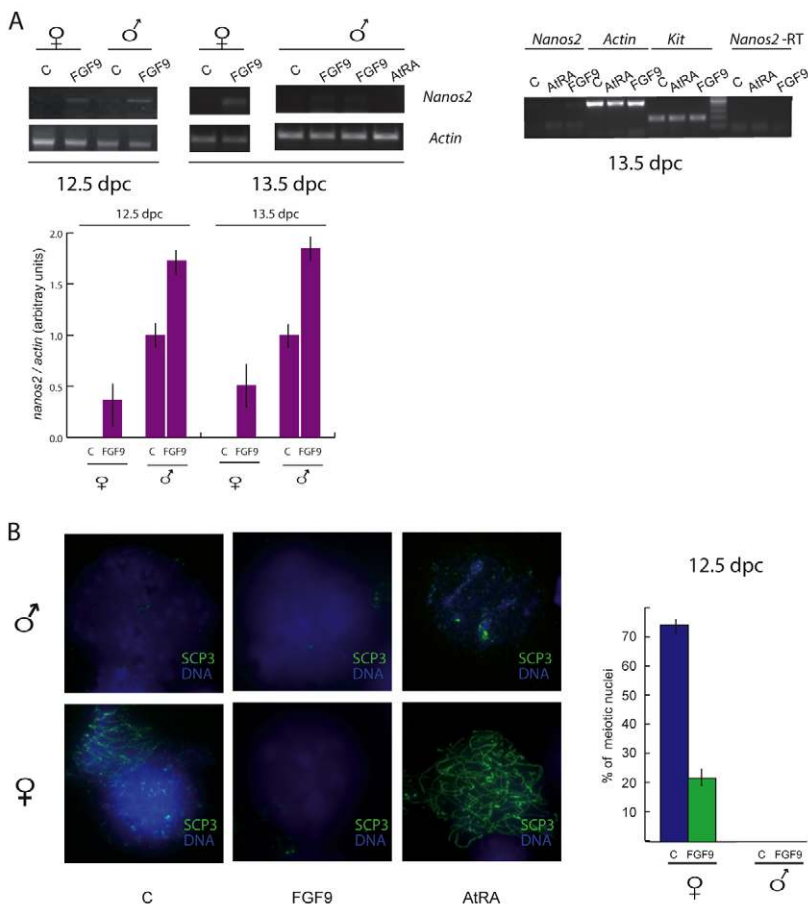
mRNA translation (Kadyrova et al., 2007). To date, none of the mammalian NANOS homologs has been demonstrated to act as a translational repressor, but we have recently shown that NANOS3 is able to interact with PUM2 and associates with the RNPs, in an heterologous system (Lolicato et al., 2008). To test whether NANOS2 might be involved in the control of translation, a *Myc*-tagged *Nanos2*-expressing vector was transfected into HEK293T cells. By immunofluorescence analysis using anti-MYC antibodies, we found that NANOS2-MYC was distributed both in the cytoplasmic and the nuclear compartments of transfected cells (Fig. 5A). Cytoplasmic extracts were then fractionated on a sucrose gradient and analyzed by western blot. Fig. 5B shows that NANOS2-MYC was found mostly in the RNP fractions but also in the ribosomal and light polysome fractions. We then followed the distribution pattern of endogenous NANOS2 on sucrose gradients using 16.5 d.p.c. or 7 d.p.p. spermatogonia extracts (Fig. 6A). Similarly to what was observed with transfected cells, NANOS2 colocalized with the RNP fractions, as well as with those containing the ribosomal subunits and light polysomes. The sedimentation pattern of PUM2 on the sucrose gradients mirrored that of NANOS2. However, TIAR, another RNA-binding protein known to be a translational repressor (Mazan-Mamczarz et al., 2006) that is essential for germ-cell survival (Beck et al., 1998), was found to be exclusively present in the RNP fractions (Fig. 6A). The finding that NANOS2 also associates with polysomes was further verified by fractionating 7 d.p.p. spermatogonial extracts on sucrose gradients in the presence of EDTA, to disrupt polysomes. As shown in Fig. 6, association of NANOS2 with the light polysomal fractions

was completely reverted toward the ribosomal subunits and the RNPs in the presence of EDTA, as occurs for DAZL, an RNA-binding protein that has been shown to be present both in the RNP and in the polysomal fractions (Tsui et al., 2000).

### NANOS2 interacts with PUM2 and binds to target mRNAs

We have previously shown that PUM2 is able to bind not only NANOS3 but also NANOS2 when used as a bait in pull-down experiments (Lolicato et al., 2008). Since the sedimentation profile of NANOS2 and PUM2 suggested that they might associate in vivo (Fig. 6), we tested the ability of NANOS2 to interact with the endogenous PUM2 from germ-cell extracts in a pull-down assay. We prepared protein extracts from fetal gonocytes (16.5 d.p.c.) or (4 d.p.p.) postnatal spermatogonia and incubated them with a GST- or a GST-NANOS2-agarose coupled resin (Fig. 7A). We found that PUM2 from both fetal and postnatal cell extracts was able to interact with GST-NANOS2, whereas a faint background band was observed in the presence of GST (Fig. 7B).

Preliminary data obtained by microarray analysis reported that significant alterations occur in the transcriptome of the developing male gonad of *Nanos2*-null mice (Saba, 2009). To verify whether murine NANOS2 works as an RNA-binding protein, we undertook a GST-RNA pull-down approach, by probing GST or GST-NANOS2 agarose beads with ribonucleoprotein extracts from postnatal spermatogonia. RNA molecules bound to GST-NANOS2 or to GST were then extracted and reverse transcribed to obtain cDNAs. Using primers that spanned the 3'UTR of the putative target genes, we looked for mRNAs whose expression was altered in the



**Fig. 3. FGF9 stimulates *Nanos2* expression and inhibits meiosis in fetal gonads.** (A) Semiquantitative RT-PCR from control and FGF9-treated cultures of 12.5 d.p.c. whole gonads (48 hours) or disaggregated 13.5 d.p.c. (24 hours) gonads. The expression of *Nanos2* is upregulated upon FGF9 stimulation in both sexes, at both stages and in both culture conditions. A control PCR analysis without reverse transcriptase was run with cDNAs from 13.5 d.p.c. male disaggregated gonads cultured with and without FGF9 or AtRA for 24 hours (right). Densitometric analysis of three independent experiments is shown below. Bars represent mean  $\pm$  s.d.;  $P < 0.001$ . (B) Nuclear spreads of the corresponding cultures, immunostained for SCP3 to detect the presence of synaptonemal complexes. Graphical representation of meiotic chromosome counts both in fetal male and female gonads, from three independent experiments, is shown on the right. Bars represent mean  $\pm$  s.d.;  $P < 0.001$ .

*Nanos2* knockouts and/or for transcripts that displayed a sexually dimorphic expression pattern during fetal gonad development. Specifically, we screened the cDNAs obtained from the RNA pull-down for *Stra8*, *Taf7l*, *Kit*, *Gcnf1* and *Gata2*, by PCR analysis. We found that *Gata2* and *Taf7l* mRNA was retained specifically by GST-NANOS2 beads (Fig. 7C), whereas we did not observe any specific signal for *Kit*, *Stra8* or *Gcnf1* mRNAs (Fig. 7C). We actually found that perfect consensus binding sites for PUM2 [UGUANAUA(A/G)NNNN(C/G/U)(C/G/U)(C/G/U)(C/G/U)(C/G)CC] (White et al., 2001) are located within the 3'UTR of both *Gata2* and *Taf7l* mRNAs, supporting the notion that a complex of NANOS-pumilio is essential for mRNA binding. When looking in the Geo Profile database for *Taf7l* and *Gata2* expression in germ cells, we found that these two genes were not only differentially regulated in the developing male and female gonads, but their levels were higher in the differentiating type B compared with type A spermatogonia (GDS2223, GDS2390). Interestingly, *Gata2* mRNA levels were found to be significantly increased in the postnatal testis in a period in which high levels of *Kit* are also found (GDS401).

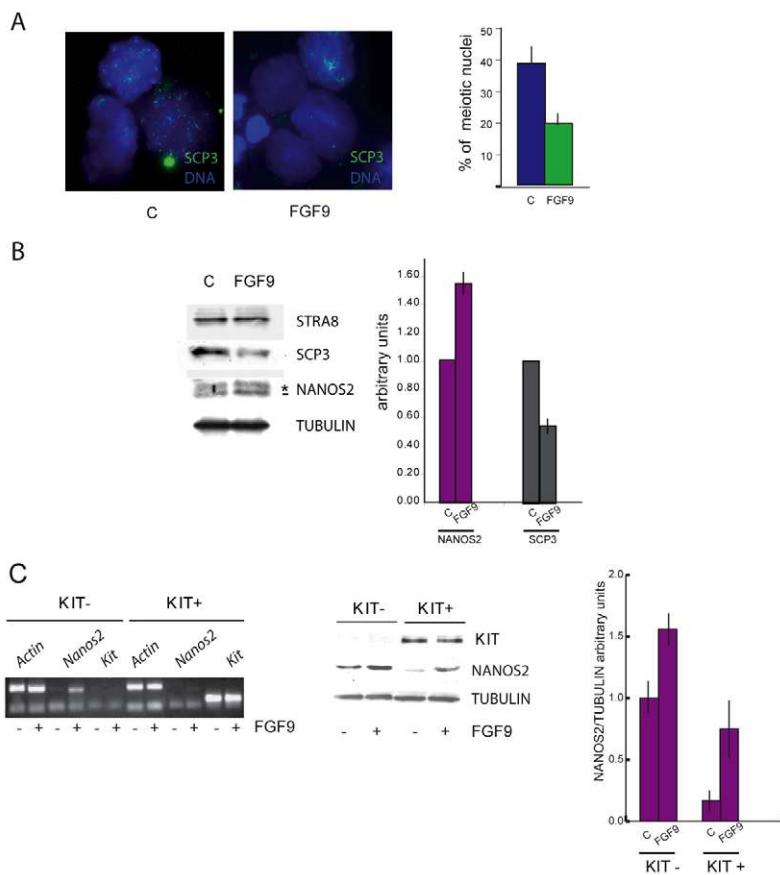
**Discussion**

NANOS2 is a potential RNA-binding protein that functions cell autonomously to control germ-cell sexual differentiation during fetal development. It has been proposed to act as an antimeiotic gene, because its deletion in mice is associated with abortive meiosis in fetal gonocytes, before they undergo apoptosis. Furthermore, its misexpression in female PGCs prevents meiosis and makes the nuclear morphology of these cells similar to that of G0-arrested gonocytes (Suzuki and Saga, 2008).

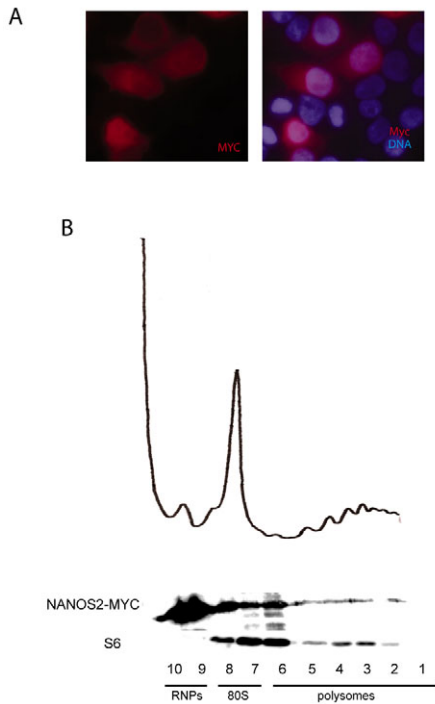
In the present paper, we used fetal germ cells and postnatal spermatogonia as models to understand the function of NANOS2 and its relationship with the beginning of meiosis.

**NANOS2 expression and meiotic commitment**

By analyzing the developmental profile of NANOS2 expression in the testis during the perinatal period, we found that NANOS2 is continuously expressed from the fetal to the postnatal period and it shows two peaks of expression: one at around 15.5 d.p.c. and another one at around 5 d.p.p. The first peak temporally corresponds to the mitotic block period, which male germ cells undergo prenatally. In this period, gonocytes do not enter meiosis and retain stem cell features, as demonstrated by their ability to complete spermatogenesis when transplanted into adult testes (Ohta et al., 2004). The second peak corresponds to a period in which cells expressing neurogenin-3 (NGN3) (a marker for undifferentiated spermatogonia) (Nakagawa et al., 2007; Yoshida et al., 2004) start differentiation into KIT-positive cells (Pellegrini et al., 2008). During this period, the spermatogonial stem cell factor PLZF (promyelocytic leukemia zinc finger) tightly controls *Kit* mRNA expression in stem cells (Filipponi et al., 2007), and they are completely lost when *Kit* is not repressed (Buaas et al., 2004; Costoya et al., 2004). By purifying KIT-positive and KIT-negative spermatogonia, we found that NANOS2-expressing cells were present only within the KIT-negative spermatogonia population, which corresponds to PLZF-expressing spermatogonia at 4-5 d.p.n., indicating that *Nanos2* is a spermatogonial stem-cell-expressed gene. In agreement with our results, during the revision of this paper, two studies have shown that NANOS2 is mostly expressed in A<sub>s</sub>



**Fig. 4. FGF9 stimulates *Nanos2* expression and inhibits meiosis in postnatal spermatogonia.** Spermatogonia from prepubertal testes (7 d.p.p.) were cultured in the presence or absence of FGF9 for 48 hours. (A) Nuclear spreads of the corresponding cultures stained for SCP3 and DAPI. The histogram represents the percentage of meiotic nuclei scored in control or in FGF9-treated cultures from three independent experiments. Bars show mean ± s.d.; *P*<0.001. (B) Western blot analysis for NANOS2 (Abnova antibody), STRA8, SCP3 and tubulin. NANOS2 levels are increased by FGF9 whereas SCP3 levels are decreased; STRA8 is not affected. The bar indicates the specific NANOS2 band, used for densitometry, whereas asterisk denotes a non-specific one. Densitometric analysis of the western blots from three separate experiments is shown on the right. Bars represent mean ± s.d.; *P*<0.001. (C) Immunomagnetic-sorted KIT-positive and KIT-negative spermatogonia analyzed for FGF9 sensitivity. Semiquantitative RT-PCR on mRNAs (left) or western blot on protein extracts (middle) from KIT-positive and KIT-negative spermatogonia were probed for NANOS2, actin and KIT. Densitometric analysis of the western blots from three separate experiments is shown on the right. Bars represent mean ± s.d.; *P*<0.001.

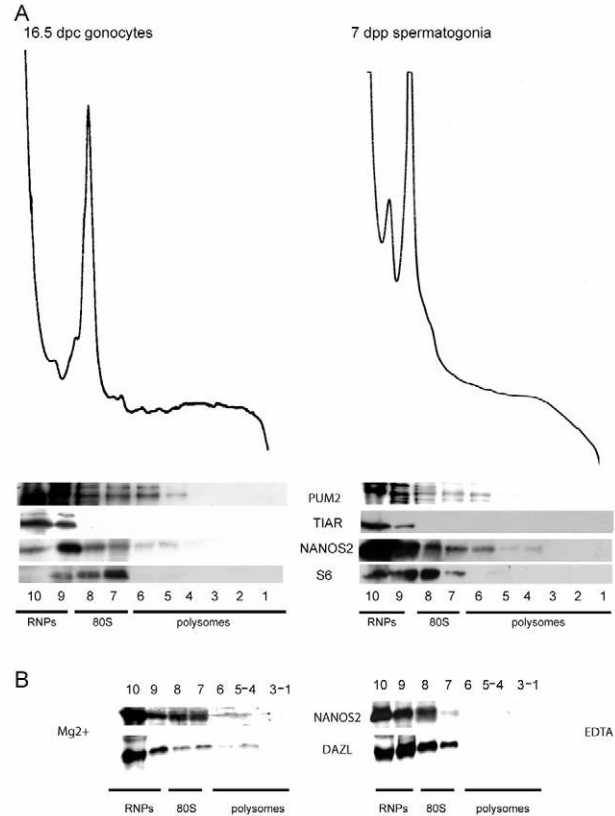


**Fig. 5. NANOS2 co-sediments with RNPs and polysomes in HEK293T.**

(A) Transfection of *Myc*-tagged *Nanos2* in HEK293T cells. Immunofluorescence analysis with anti-MYC antibodies shows that NANOS2 is distributed mainly in the cytoplasm, but also in the nucleus. (B) RNP-polysome fractionation on sucrose gradients of HEK293T cells transfected with *Myc-Nanos2*. Western blot analysis of the collected fractions shows that NANOS2 sediments mainly in the light fraction of the gradient (fraction 10, RNPs) but it is also present through the ribosomal subunits (fractions 7-9) and to a lesser extent in the polysomes (fractions 3-6). S6 pattern and UV absorbance profile demonstrate the correct fractionation assay.

and  $A_{pr}$  spermatogonia from the adult testis and that its conditional deletion in adulthood leads to spermatogonia stem cell loss (Sada et al., 2009; Suzuki et al., 2009).

Evidence from genetic studies suggests that NANOS2 acts as a suppressor of meiosis in male fetal germ cells (Suzuki and Saga, 2008). We found that AtRA, a derivative of RA and a well-known promeiotic factor (Bowles et al., 2006; Pellegrini et al., 2008), was able to downregulate *Nanos2* at both mRNA and protein levels in male PGCs (12.5 d.p.c.) and in undifferentiated spermatogonia. In mitotically arrested gonocytes (15.5 d.p.c.), however, we observed that the strong decrease of *Nanos2* mRNA levels induced by AtRA was paralleled only by a less-intense decrease of the protein levels. Since this different behaviour was consistently reproduced, we hypothesize that NANOS2 protein stability is higher in mitotically arrested germ cells than in proliferating germ cells. We found that AtRA increased the percentage of proliferating male PGCs entering meiosis, but it was not effective on mitotically arrested gonocytes or on undifferentiated spermatogonia. For arrested gonocytes, this discrepancy can be explained by the existence of a narrow window for meiotic competence, which corresponds to the period in which NANOS2 starts to be expressed (12.5 d.p.c.) and is needed to repress the meiotic fate (Saga, 2008; Suzuki and Saga, 2008). After this period, high levels of NANOS2, as a result of increased protein stability and/or enhanced synthesis stimulated by factors such as

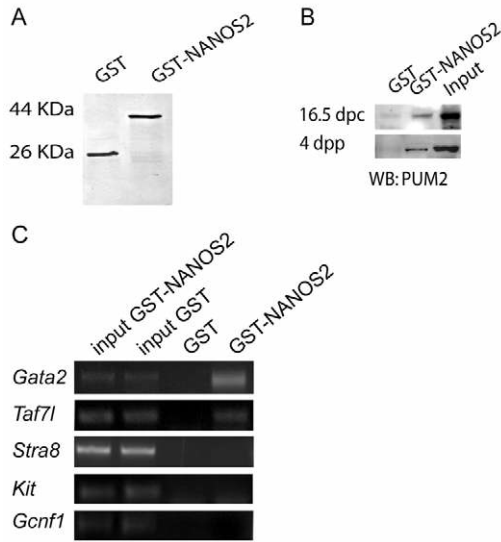


**Fig. 6. NANOS2 associates with RNPs and polysomes of fetal and postnatal male germ cells.** (A) Fetal (16.5 d.p.c.) and postnatal (7 d.p.p.) male germ cells were collected and cytoplasmic extracts were fractionated on sucrose gradients. Western blot analysis of the collected fractions shows that NANOS2 sediments with the RNP fraction, the ribosomal subunits and the polysomal fractions. PUM2 showed a sedimentation pattern similar to NANOS2, suggesting that both proteins can interact in vivo. By contrast, the translational repressor TIAR is found exclusively in the RNPs fraction in both fetal and postnatal stages. S6 and UV absorbance are presented as controls of correct fractionation. (B) Untreated ( $Mg^{2+}$ ) or EDTA-treated (EDTA) 7 d.p.p. spermatogonial extracts were fractionated on sucrose gradients and analyzed by UV spectrometry (not shown) and western blotting for NANOS2 and DAZL.

FGF9 (see below), ‘masculinize’ gonocytes. The evidence that AtRA does not trigger meiosis in undifferentiated spermatogonia, notwithstanding the modulation of NANOS2 and STRA8, suggests that these cells lack other molecular factors required for meiotic entry. One of these factors could be the KIT tyrosine kinase receptor, whose downstream signalling is essential for meiotic entry of differentiating spermatogonia in vitro (Pellegrini et al., 2008). In line with this hypothesis is the observation that *Kit* is expressed in proliferating fetal germ cells of both sexes up to 12.5–13.5 d.p.c. (Manova and Bachvarova, 1991) when the ‘meiotic window’ is active, and is then re-expressed in the male only postnatally, in differentiating spermatogonia – the meiosis-competent cells of the testis.

In a search for factors that could upregulate NANOS2 levels and negatively influence meiosis, we found that FGF9 was a good candidate. Fibroblast growth factors (FGFs) have a role in the proliferation and survival of many cell types (Ornitz and Itoh, 2001). *Fgf9* is expressed in the undifferentiated gonads of both sexes at





**Fig. 7. NANOS2 interacts with PUM2 to regulate target mRNAs.** Protein and RNA pull-downs were performed to investigate the interaction between NANOS2 and PUM2. (A) Coomassie blue staining of GST and GST-coupled NANOS2 used for pull-down experiments. (B) Western blot of cell extracts from fetal (16.5 d.p.c.) and postnatal (4 d.p.n.) male germ cells loaded onto GST or GST-NANOS2-agarose beads and probed for PUM2. A specific band for PUM2 is observed in the presence of GST-NANOS2 in both fetal and postnatal cell extracts. (C) RT-PCR for potential target mRNAs of NANOS2.

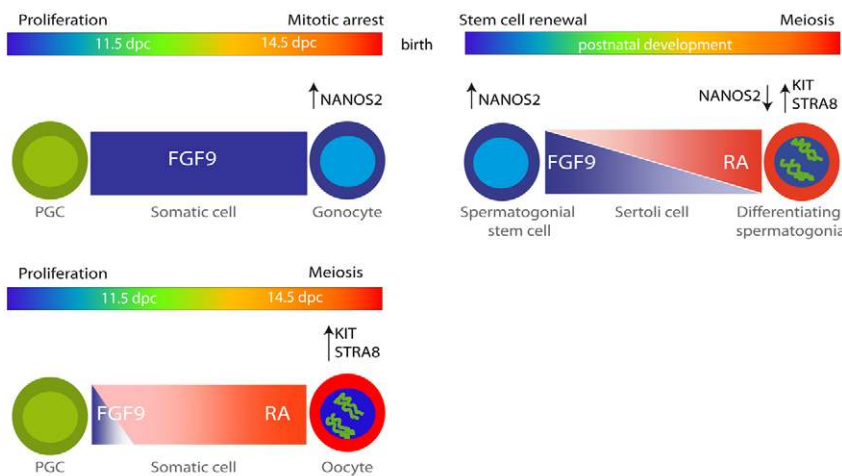
11.5 d.p.c., but it becomes XY restricted by 12.5 d.p.c. and is maintained in the testis cords (Schmahl et al., 2004). Sertoli cell precursors fail to proliferate in *Fgf9* mutants and testis differentiation is disrupted (Schmahl et al., 2004). The majority of germ cells are lost at 12.5 d.p.c.; however, the surviving gonocytes within the *Fgf9*-null gonads are found in meiosis (DiNapoli et al., 2006), suggesting that FGF9 normally promotes survival and prevents meiosis of fetal male germ cells. Indeed, we found that FGF9 upregulated NANOS2 and strongly inhibited meiosis in female PGCs in vitro. This observation is in line with the evidence that *Nanos2* misexpression in female germ cells inhibits meiosis and masculinizes germ-cell nuclei (Suzuki and Saga, 2008). FGF9 also upregulated NANOS2 in postnatal spermatogonia, and such an increase was only observed

after a long incubation time (not shown), suggesting that a transcriptional mechanism probably mediates this effect. Sensitivity to FGF9 was present in the undifferentiated and in the differentiating spermatogonia, because both these cell types express an FGF9 receptor, as confirmed by microarray analysis (Rossi et al., 2008). We also observed a negative effect of FGF9 on meiotic entry in differentiating spermatogonia, suggesting a common mechanism shared between female and male germ cells in the control of the mitotic-meiotic switch. It has been hypothesized that a possible role for NANOS2 in the prevention of meiosis is to repress STRA8 expression during the period in which CYP26B1 levels decrease in the male gonad and RA is increased in the mesonephros. In our studies we did not observe STRA8 downregulation after FGF9 stimulation of postnatal spermatogonia in vitro, suggesting that NANOS2 might act independently or downstream of STRA8, even if we cannot exclude that other mechanisms are active in vivo.

**NANOS2 subcellular localization and molecular interactions**

In contrast to what we observed for NANOS3 (Lolicato et al., 2008), NANOS2 was found to be associated not only with RNPs, but also with the ribosomal and polysomal fractions. This localization is uncommon for translational repressors, but it has been recently shown that translational repression can also occur at the polysomal level, during protein synthesis, through a miRNA repressive mechanism (Cannell et al., 2008; Gebauer and Hentze, 2004; Petersen et al., 2006). Furthermore, the possibility cannot be ruled out that NANOS2, and eventually PUM2, might function both as translational repressors and activators, as has been shown for FMRP (Zalfa et al., 2006); they could also function at other levels of post-transcriptional regulation of mRNA expression, such as the control of mRNA stability.

When transfected in HEK93T cells, NANOS3 associates with PUM2 and is found within the RNPs (Lolicato et al., 2008). Since it has been shown that NANOS2 can complement the NANOS3 defect in *Nanos3*-null mice, we reasoned that it could also recognize the same partners in male germ cells. Indeed, we found that NANOS2 was able to bind PUM2 expressed either in fetal gonocytes or in postnatal spermatogonia, suggesting that PUM2 can interact in vivo with NANOS2, probably both acting in post-transcriptional mRNA regulation. In the attempt to discover potential NANOS2 targets, we looked for genes showing a sexually



**Fig. 8. Opposing effects of RA and FGF9 on *Nanos2* expression and meiotic entry of mouse germ cells.** The niche between Sertoli cell precursors and fetal gonocytes might be equivalent to the stem cell niche of the postnatal testis. Fetal gonocytes and spermatogonia stem cells could have an equivalent cell fate signed by the expression of *Nanos2*. Such fate would be lost when spermatogonia are committed to meiosis. In the fetal testis, Sertoli cell precursors secrete FGF9 to induce *Nanos2* expression and masculinization of PGCs (that become gonocytes). FGF9 upregulates *Nanos2* and prevents meiosis. Postnatally, the balance between FGF9 and RA secreted by Sertoli cells regulates the levels of *Nanos2* expression in undifferentiated spermatogonia (NANOS2-positive cells) and in differentiating spermatogonia (STRA8- and KIT-positive cells). In the fetal ovary, FGF9 secretion from somatic cells drops early during gonadogenesis and as a consequence PGCs are not masculinized. High RA levels ensure meiotic entry through induction of *Stra8* and possibly *Kit*.

dimorphic expression pattern during gonadal differentiation and/or that were more abundantly expressed in differentiating spermatogonia. Among several genes tested (*Kit*, *Taf7l*, *Gata2*, *Stra8* and *Gcnfl*), *Gata2* and *Taf7l* mRNAs were found to interact with GST-NANOS2. GATA2 is a zinc finger transcription factor expressed in fetal oocytes up to 15.5 d.p.c., but not in fetal gonocytes (Siggers et al., 2002). There are no studies on *Gata2* expression in postnatal gonads; however, its mRNA levels are strongly increased in differentiating spermatogonia, according to the microarray analysis database (Geo Profiles) at NCBI. Although the role of GATA2 in germ cells is unknown, it is well established that in hematopoietic stem cells, this factor controls *Kit* expression at the transcriptional level (Orkin, 1992). *Taf7l* is a component of the transcriptional machinery specific to preleptotene spermatocytes and haploid spermatids (Pointud et al., 2003) and its deletion leads to reduced male fertility (Cheng et al., 2007). Thus, the identification of two transcription factors involved in the differentiation of spermatogonia as targets of post-transcriptional control by NANOS2 is in line with the evidence that NANOS2 negatively regulates differentiation of spermatogonia stem cells (Sada et al., 2009). Studies to identify other NANOS2 mRNA targets, whose post-transcriptional regulation is determinant for spermatogonia differentiation, will be important to understand how commitment of stem cell occurs in the initial phase of spermatogenesis.

In conclusion, our results can be interpreted as schematically depicted in Fig. 8. RA and FGF9 produced locally by the somatic cells of the gonads act in opposite ways to regulate *Nanos2* expression and meiotic entry both in fetal and postnatal germ cells. In the fetal ovary, FGF9 levels sharply decrease at 11.5 d.p.c. in parallel with a rise of circulating levels of RA, which stimulates *Stra8* expression in PGCs and their commitment to meiosis. In the fetal testis, FGF9 levels are kept high, and circulating RA is degraded by CYP26B1 (not depicted) in somatic cells. High levels of FGF9 without RA signalling in male PGCs upregulate *Nanos2*, which determines their transition into mitotically arrested gonocytes. In the postnatal testis, *Nanos2* is expressed exclusively in SSCs and Sertoli cells to safeguard the delicate balance between stem cell self-renewal and meiotic commitment of spermatogonia by modulating the levels of FGF9 and RA.

## Materials and Methods

### Cell isolation and culture

Postnatal male germ cells (0–7 d.p.p.) were obtained as previously reported, by sequential enzymatic digestion of testes from CD1 albino mice (Pellegrini et al., 2008). After preplating of cell suspension, germ cells were cultured in modified Earle's medium (MEM, Gibco) with 20 mM glutamine (Gibco), 2 mM pyruvic acid (Sigma), 1 mM lactic acid (Sigma), non-essential amino acids (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco), without serum supplementation.

To obtain enriched fetal germ-cell suspensions, fetal testes and ovaries were collected at different developmental stages and digested with trypsin (Gibco) and DNaseI (Sigma). Cell suspensions were pre-plated in the same medium as postnatal germ cells supplemented with 10% fetal bovine serum, (FBS) and recovered after 3 hours of culture, to allow most of the somatic cells to adhere to the plastic dishes. In the case of organ culture, fetal gonads were cultured for 48 hours onto 0.8% agarose blocks pre-equilibrated with MEM and 10% FBS.

All-*trans*-retinoic acid (ATRA, Sigma) was dissolved at a concentration of 10 mM in ethanol and diluted to a final concentration of 0.3 µM (Pellegrini et al., 2008) in culture medium. Control cultures received the corresponding doses of diluted ethanol. FGF9 (from Società Italiana Chimici) was dissolved in PBS 1 mg/ml BSA and used to a final concentration of 25 ng/ml. All the factors were replaced in the culture medium after 24 hours. Separation of KIT-positive spermatogonia from KIT-negative spermatogonia was performed by magnetic-activated cell sorting (MACS) with CD117 conjugated microbeads (Miltenyi Biotech, Germany) as previously described (Pellegrini et al., 2008).

### Definition of the germ-cell stages

Fetal male and female germ cells are defined as PGCs as long as they reach the gonads and proliferate. After gonadal colonization, female PGCs start entering meiosis (at around 13.5 d.p.c.) and are then defined as oocytes. Male PGCs, however, enter mitotic block after gonadal colonization and are then defined as gonocytes. This definition is retained up to birth, when gonocytes resume mitosis and spermatogonial stem cells can be identified. KIT-negative (OCT4- and PLZF-positive) spermatogonia represent the stem cell population of the testis whereas KIT-positive spermatogonia are the differentiating mitotic germ cells which are committed to enter meiosis.

### Polysome-RNP fractionation

Prepuberal spermatogonia and fetal gonocytes were subjected to polysome-RNP fractionation on sucrose gradients as previously described (Lolicato et al., 2008). In brief, 600 µg cytoplasmic extracts in polysome buffer were loaded onto 15–50% sucrose gradients. Ten fractions were collected and protein precipitation from each fraction was performed with 72% trichloroacetic acid (TCA) and subsequently resuspended in 2× sample buffer and processed for SDS-PAGE. Absorbance at 260 nm was continuously recorded while collecting the fractions. EDTA treatment was performed as previously described (Grivna et al., 2006).

### Recombinant proteins, transfection, GST protein and RNA pull-down

*Nanos2-Myc* was produced by RT-PCR using primers using a proofreading polymerase (Pfu, Stratagene) containing *Bam*HI-*Eco*RI sites at the extremities. The amplified band was cloned in *pcDNA3-Myc* (Invitrogen, Milan, Italy) or in *pGEX4T1* (GE Healthcare, Milan, Italy). For transient transfections, HEK293T cells were transfected with *pcDNA3Myc-Nanos2* by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Pull-down experiments were performed using GST fusion proteins purified from bacterial lysates on glutathione-agarose (Sigma). In the case of protein pull-down, cell extracts (600 µg total proteins) of 16.5 d.p.c. gonocytes or 4 d.p.p. spermatogonia, precleared for 1 hour with glutathione-agarose in lysis buffer, were added to 2 µg GST protein or GST-NANOS2 fusion protein adsorbed on glutathione-agarose in lysis buffer supplemented with 0.05% BSA. After incubation for 90 minutes at 4°C under constant shaking, beads were washed three times with the same buffer, adsorbed proteins were eluted in SDS sample buffer and resolved on a 12% SDS-PAGE for western blot analysis. Input represents 1/10 of the initial samples.

For RNA pull-down experiments 1 mg cytoplasmic extracts in polysome buffer were obtained from 7 d.p.p. spermatogonia and processed as described (Keene et al., 2006). Extracts containing RNA and protein were incubated with GST or GST-NANOS2 agarose beads for 4 hours at 4°C. RNA-protein complexes were washed four times in NT2 buffer and RNA was eluted from the beads by incubation at 55°C for 30 minutes with 30 µg proteinase K. After Trizol extraction (Invitrogen) and DNaseI treatment (Zymo Research, Rome, Italy), RNA was reverse transcribed as described in the following section.

### RT-PCR

Total RNA from whole fetal gonads and fetal and postnatal germ cells was extracted with Trizol reagent and treated with DNase I to avoid potential contamination by genomic DNA. DNA-free RNA was reverse transcribed using SuperScript First Strand Synthesis kit (Invitrogen) according to the manufacturer's instructions. Primers used for PCR were as follows: *Nanos2* F, AGTGGCATGGACCTACCGCCCTTT and *Nanos2* R, TCTCAATTATCGCTTGACTCTGC; *c-Kit* F, GCCACGTCT-CAGCCATCTG and *c-Kit* R, GTCGGGATCAATGCACGTCA; *Stra8* F, TAGGATCCATGGCCACCCTGGAGAAG and *Stra8* R, GAATCTTACAGATCGTCAAAGGTCTCCA; *Actin* F, GGCTGTATTCCCCTCCATCG and *Actin* R, CCAGTTGGTAAACAATGCCATGT. For semiquantitative RT-PCR 25 cycles were performed for the amplification of *Actin* and all other genes, 30 cycles for *Nanos2* amplification from male germ cells or 40 cycles from female germ cells.

RNA from RNA pull-down experiments was reverse transcribed using SuperScript First Strand Synthesis kit (Invitrogen) according to the manufacturer's instructions. Primers for putative targets were designed, when possible, to span a region between the last two exons. Primers were the following: *Gata2* F, CAAGCTGCACAATGTTAACAGGC and *Gata2* R, ATTCACAGTAATGGCGGCACAAGG; *Taf7l* F, GGAGGAGGAAGAGACAGACAATTC and *Taf7l* R, GTAGAGGGA-CAGAAGTATGTGGTTCCAC; *Stra8* F, TGCCGGACCTCATGGAATTC and *Stra8* R, GAATCTTACAGATCGTCAAAGGTCTCCA; *Gcnfl* F, GGATGGAGG-TGATTGAACGACT and *Gcnfl* R, AGTCTCCATCTTGGTCTCTGGCT. PCR conditions were: 95°C for 20 seconds, 58°C for 30 seconds, 72°C for 30 seconds, for 40 cycles.

### Immunofluorescence

Transfected HEK293T cells were adhered onto poly-L-lysine glass slides and fixed for 10 minutes at room temperature in 2% paraformaldehyde. After washing with PBS, cells were permeabilized for 10 minutes with PBS containing 0.1% Triton X-100, and incubated for 30 minutes at room temperature with PBS containing 0.5% BSA. Samples were then incubated overnight at 4°C in a humidified chamber with anti-MYC antibody from Santa Cruz (sc-2048) at a final concentration of 2 µg/ml, then for 1 hour at room temperature with cyanin 3 (AP 180 C; Chemicon). Slides were washed and mounted in 50% glycerol in PBS and immediately examined by



fluorescence microscopy. Nuclei were counterstained with 1 µg/ml Hoechst 33342 (Sigma). Control experiments were performed using non-immune immunoglobulins instead of the specific antibody.

#### Western blotting

For western blot analysis, germ cells were harvested and washed three times with ice-cold PBS. Cell lysis was performed with 10 mM HEPES, pH 7.9, 1% Triton X-100, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT), 10 mM β-glycerophosphate, 0.1 mM sodium vanadate and protease inhibitor cocktail (Sigma). 40 µg protein extracts were separated by SDS-PAGE in 4-20% gradient gels (Serva, Milan, Italy) or uniform gels of 15% polyacrylamide and transferred to nitrocellulose membrane (Amersham, Piscataway, NJ). The membrane was blocked in phosphate saline buffer with 0.1% Triton (PBST) and 5% fat-free milk powder for 1 hour at room temperature. Incubation with primary antibodies was carried out at 4°C overnight in PBST-5% BSA. Appropriate horseradish-peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) was used at a 1:5000 dilution in PBST for 1 hour at room temperature. Anti-NANOS2 serum (in this study we used both one obtained from Abnova, Taipei, Taiwan, and a gift from Yumiko Saga, Sokendai, Shizuoka, Japan; in the figure legends we indicated when the commercial antibody was used) was used at a 1:300 dilution in PBST with 5% BSA. Anti-KIT rabbit polyclonal antibody (Albanesi et al., 1996) was diluted at 1:1000. Anti-PUM2 rabbit polyclonal (Abcam) was diluted at 1:3000. Anti-TIAR mouse monoclonal antibody (a kind gift from P. Anderson, Harvard, MA) was diluted at 1:1000. Anti-S6 rabbit polyclonal antibody (Cell Signaling, Beverly, MA) was diluted 1:1000. Anti-Actin rabbit polyclonal (A2066, Sigma) was diluted 1:1000. Anti-MYC antibody was from Santa Cruz (sc-2048) and diluted 1:1000. Mouse monoclonal antibody anti human DAZL was from AbD Serotec, Milan Italy and used at a 1:1000 dilution. Anti-STRA8 rabbit polyclonal from Abcam (Ab 49602) was used at a 1:1000 dilution. Anti-SCP3 rabbit polyclonal (1:1000) was from Novus. Anti-OCT4 mouse monoclonal (1:1000) was from Santa Cruz (sc-5279). The horseradish peroxidase conjugate was detected by chemiluminescence with an ECL Kit (Santa Cruz) and autoradiography. Densitometric analysis was performed by ImageQuant™ TL software (GE Healthcare).

#### Spreads

Cultured spermatogonia or single cell suspensions from cultured fetal gonads were prepared and stained essentially as previously described (Romanienko and Camerini-Otero, 2000). Slides were washed twice in PBS, and incubated overnight at 4°C with anti-SCP3 rabbit polyclonal antibody (Novus) diluted in blocking solution (1:100 in 10% goat serum, 3% BSA, 0.5% Triton X-100 in PBS). After washing, secondary antibody was added for 1 hour at 37°C. The slides were washed and allowed to dry. Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) was added and the slides were viewed using a Leica microscope. Spreads analysis was performed in three independent experiments.

#### Statistical analysis

Continuous variables were summarized as means ± s.d. All tests were two-sided and were determined by Monte Carlo's significance. A significance value threshold of 0.05 was used for the current analysis. Student's *t*-test was used to test for differences between two independent groups, whereas one-way ANOVA was used to test for differences among three or more independent groups. For multiple comparisons the Tukey's HSD test was carried out. All statistical tests were carried out using the SPSS statistical analysis software package, version 10.0.

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