Reduced Sperm Count and Normal Fertility in Male Mice with Targeted Disruption of the ADP-Ribosylation Factor-Like 4 (*Arl4*) Gene

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The ADP-ribosylation factor-like protein 4 (ARL4) is a 22-kDa GTP-binding protein which is abundant in testes of pubertal and adult rodents but absent in testes from prepubertal animals. During testis development, ARL4 expression starts at day 16 when the spermatogenesis proceeds to the late pachytene. In the adult testis, the ARL4 protein was detected in pre- and postmeiotic cells, spermatocytes, and spermatids, but not in spermatogonia and mature spermatozoa. Mouse *Arl4*-null mutants generated by targeted disruption of the *Arl4* gene were viable and grew normally; male as well as female *Arl4*$^{-/-}$ mice were fertile. However, inactivation of the *Arl4* gene resulted in a significant reduction of testis weight and sperm count by 30 and 60%, respectively, without reduction of litter size or frequency. It is suggested that the disruption of *Arl4* produces a moderate retardation of germ cell development, possibly at the stage of meiosis.

ADP-ribosylation factor-like protein 4 (ARL4) is a 22-kDa GTP-binding protein which belongs to the extended family of ADP-ribosylation factors (18). ADP-ribosylation factors (ARFs) are critical components in eukaryotic vesicular trafficking pathways and activators of phospholipase D (6, 17). The ARF-like proteins ARL1 to ARL7 and ARFRP1 exhibit all typical sequence characteristics of ARFs (3, 4, 9, 19) but lack their activity to enhance the cholera toxin-catalyzed ADP-ribosylation. ARL4 and two closely related proteins (ARL6 and ARL7) represent a subgroup of the ARL family, characterized by rapid nucleotide exchange and a nuclear localization signal (9). Data from *Saccharomyces cerevisiae* two-hybrid and in vitro protein interaction assays indicated that ARL4 interacted with importin-α in a GTP-independent manner. In addition, ARL4 was detected in the nucleus and also in the nucleolus by indirect immunofluorescence (13).

Among the ARF-like GTPases, ARL4 is particularly interesting because of its differential expression in the 3T3-L1 preadipocyte cell line (18). ARL4 mRNA was undetectable in undifferentiated 3T3-L1 cells and abundant in differentiated cells. In addition, ARL4 has been shown to be developmentally expressed during mouse embryogenesis. mRNA of ARL4 appears transiently at days 8.5 to 10.5 in a rostro-caudal direction, suggesting that ARL4 is involved in somite formation and central nervous system differentiation (13). ARL4 mRNA was also detected in several adult tissues, e.g., testis, muscle, spleen, and brain. In testis, ARL4 mRNA was exclusively detected in the pubertal and adult glands, but not in the prepu-

bertal gland (8). Interestingly, the strong expression in adult testis is driven by a separate, testis-specific promoter (8).

The aim of the present study was to investigate the role of ARL4 in embryonic development, germ cell proliferation and differentiation. We identified the time point of ARL4 induction during testicular development and the localization of ARL4 in the seminiferous tubule. In addition, an *Arl4*-null mutant was generated and characterized with regard to fertility and sperm count. The data indicate that disruption of the *Arl4* gene produces a significant reduction of sperm count without affecting fertility. In addition, the data indicate that ARL4 is dispensable for embryonal development or adipogenesis.

MATERIALS AND METHODS

Northern blot analysis. Total RNA was prepared from testes of mice at ages of 12 to 30 days and from different tissues of *Arl4*$^{+/+}$, *Arl4*$^{+/-}$, and *Arl4*$^{-/-}$ mice as described previously (5). Samples (15 µg) were separated by electrophoresis on 1% (wt/vol) agarose gels containing 3% (vol/vol) formaldehyde and were transferred onto nylon membranes (Hybond N$^+$; Amersham-Pharmacia, Freiburg, Germany). Probes were generated with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP by random oligonucleotide priming. The nylon membranes were hybridized at 42°C, and blots were washed twice with 0.8% SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate.

Immunohistochemical detection of ARL4 in testis. Immunolocalization of ARL4 was performed with a polyclonal rabbit antiserum against the C terminus of ARL4. Frozen tissue sections were dried and fixed with 4% paraformaldehyde for 15 min. Endogenous peroxidase was blocked with 3% H$_2$O$_2$. Nonspecific antibody binding was blocked with 50% normal goat serum (Dianova, Hamburg, Germany). Testis sections were incubated with anti-ARL4 antibody in a dilution of 1:150 for 2 h at 37°C, and immunostaining was performed with a peroxidase-conjugated goat anti-rabbit immunoglobulin in a dilution of 1:100 (Dianova). The sections were counterstained with Mayer's hematoxylin.

Construction of the *Arl4*-targeting vector and generation of *Arl4*-mutant mice. A neomycin resistance cassette was inserted into the *Xmn*I and *Xho*I sites of exon 2 of the *Arl4* gene, thereby introducing diagnostic *Eco*RI and *Nco*I sites. Mouse embryonic stem (ES) cells were transfected by electroporation with the linear-

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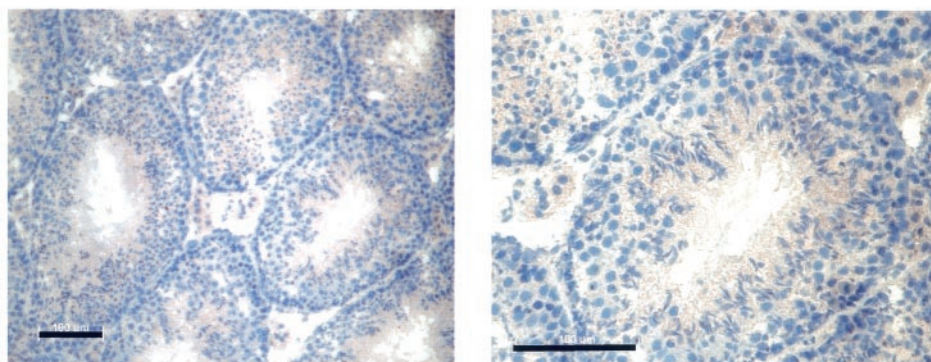
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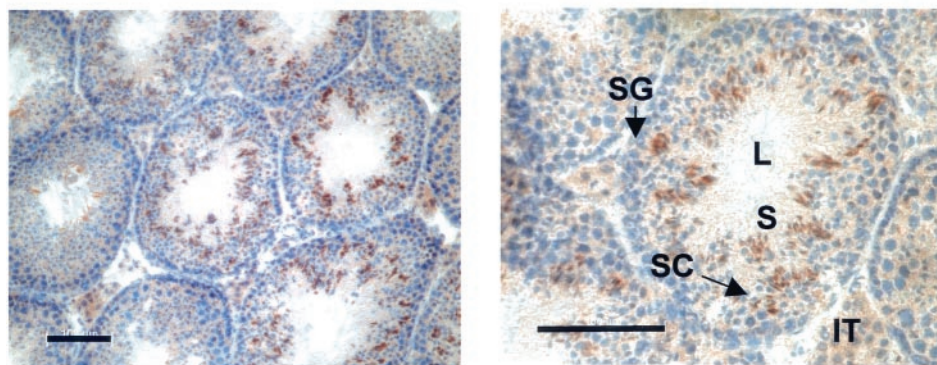
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FIG. 1. Expression of ARL4 in seminiferous tubules. Frozen sections of testis from adult mice were fixed with formaldehyde and incubated with an antiserum against the C terminus of ARL4. The immunostaining was performed with peroxidase-conjugated goat anti-rabbit immunoglobulin and analyzed at a magnification of ×400. The sections were counterstained with Mayer's hematoxylin for 10 min. IT, interstitial tissues; L, lumen; S, spermatides; SC, spermatocytes; SG, spermatogonia.

ized *Arl4* targeting vector and were then cultured in the presence of 335 µg of G418 (Geneticin) per ml. Eighty-four neomycin-resistant ES clones were picked, and their genomic DNA was isolated and digested with *Eco*RI and *Nco*I. Correctly targeted clones were identified by Southern blot analysis with a 5′ external probe (nucleotides −2503 to −1803). One ES cell clone that had incorporated the targeting vector by homologous recombination was used for microinjection of C57BL/6J blastocyts, which were subsequently transferred into a pseudopregnant foster mouse. Chimeric males were mated with C57BL/6J females. Heterozygote offspring were mated, and genomic DNA was isolated from tail biopsies, digested with *Eco*RI and *Nco*I, and analyzed by Southern blotting using the 5′ external probe or by PCR using primers for amplification of both the *neo* gene and *Arl4* (upstream, 5′-CAATCGCGTAGCCCGATCAC-3′; downstream, 5′-AGGTAC TGCCATTCATCTCTTC-3′; nucleotides 2104 to 2123 and 2784 to 2805 in the *Arl4* genomic sequence).

Preparation of spermatozoa. Caudae epididymidis and vasa deferentia were excised and then rinsed with medium containing 150 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 30 mM HEPES, 10 mM glucose, 10 mM lactic acid, and 1 mM pyruvic acid (pH 7.4). After transfer to 1 ml of medium supplemented with 5 mg of bovine serum albumin per ml and 15 mM NaHCO$_3$, semen was allowed to exude (15 min at 37°C, 5% CO$_2$) from three to five small incisions. Cells were diluted to 4 ml and collected twice by sedimentation (400 × *g*; 5 min [20]). The sperm count and motility of the sperm cells were analyzed with a CASA (computer-assisted sperm analyzer) as described by Krause (10).

RESULTS

Localization of ARL4 in seminiferous tubules of adult mouse testis. A polyclonal antiserum against a peptide corresponding with the C terminus of ARL4 was used for immunohistochemistry in order to identify the germ cells expressing the protein. As is shown in Fig. 1 (upper panels), ARL4 was detected in spermatocytes, and in round, elongating, and condensating spermatides. The protein was not detected in spermatogonia and mature spermatozoa. Furthermore, no immunoreactivity was detected with the preimmune serum within the seminiferous tubules (Fig. 1, lower panels).

Time course of ARL4 expression during testicular development. Since previous results had indicated that ARL4 is expressed in germ cells of pubertal and adult testes, but not in prepubertal testis (8), we investigated the expression of ARL4 during testicular development in more detail. Total RNA from testes of mice at 10 to 30 days of age was prepared and analyzed by Northern blotting. Expression of ARL4 mRNA was started at day 16 and reached a maximum at day 24 (Fig. 2A).
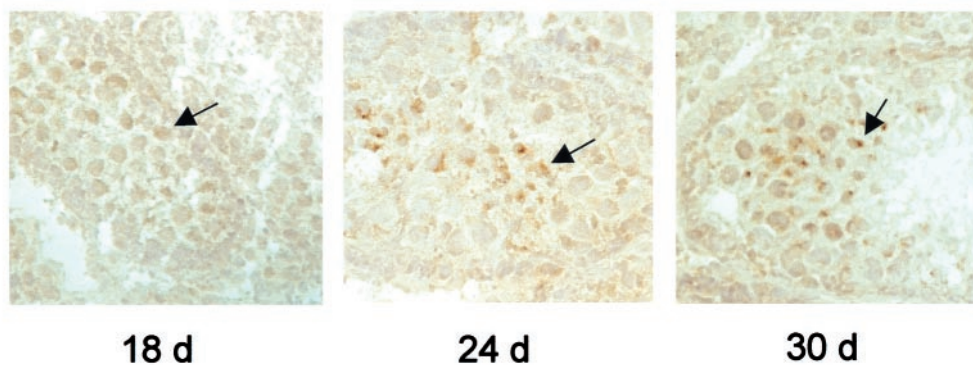
A

B

18 d   24 d   30 d
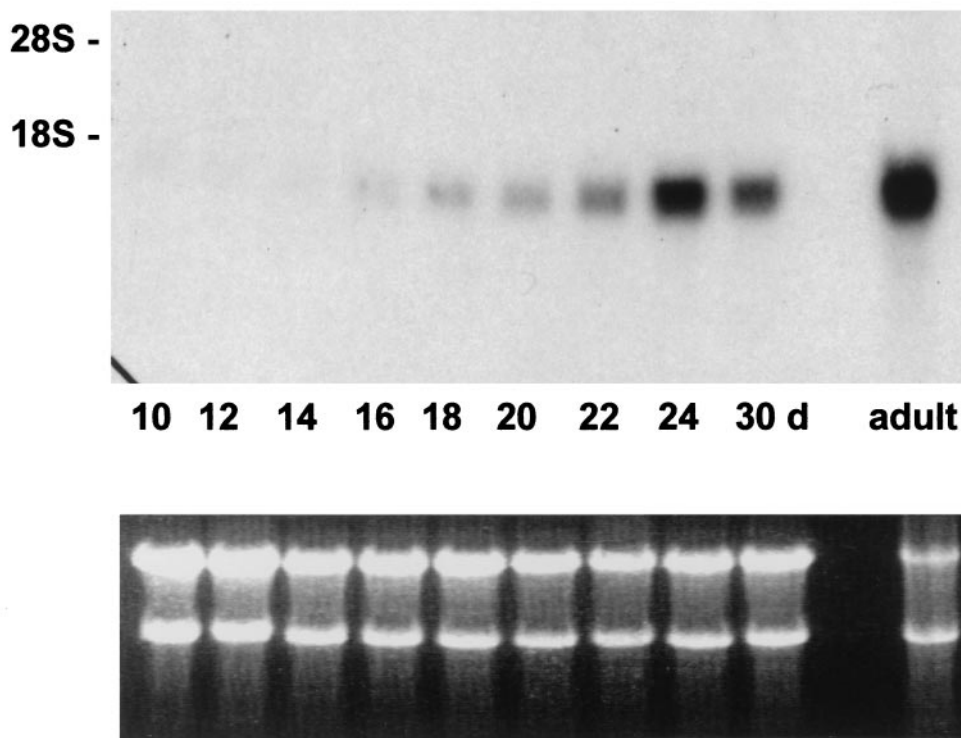
FIG. 2. Time course of expression of ARL4 during testis development. (A) Northern blot analysis of ARL4 expression in testes from mice of different ages. Total RNA was isolated from testes of mice at 10 to 30 days of age and analyzed in Northern blots with a probe derived from the full-length cDNA of rat ARL4. (B) Immunohistochemical detection of ARL4 in testis tissues from 18-, 24-, and 30-day-old mice. Frozen sections of mouse testes at the ages indicated were fixed with formaldehyde and immunostained with the ARL4 antiserum as described for Fig. 1. For better visualization of immunostaining, counterstaining with Mayer's hematoxylin was performed only for 1 min.

Immunochemical detection of the ARL4 protein confirmed the results obtained by Northern blot analysis. A very weak signal of ARL4 protein was detected in tubules from 18-day-old mice. Thereafter, the abundance of the protein increased and reached a maximum at day 24 (Fig. 2B). These data show that the expression of ARL4 in mice testis starts during meiosis, when spermatogenesis proceeds to the late pachytene stage, and before spermatides or mature spermatozoa are present (15).

Targeted disruption of the *Arl4* gene. For generation of *Arl4*-null mutants, a targeting construct in which the whole coding region of *Arl4* was exchanged for a neomycin resistance cassette was designed (Fig. 3A). The construct contained 4.5 and 3 kb derived from the 5′ and 3′ side flanking regions of the *Arl4* gene, respectively.

Transfection of embryonic stem cells with the targeting construct generated one homologous recombination in 85 transformants. Homologous recombination was ascertained by
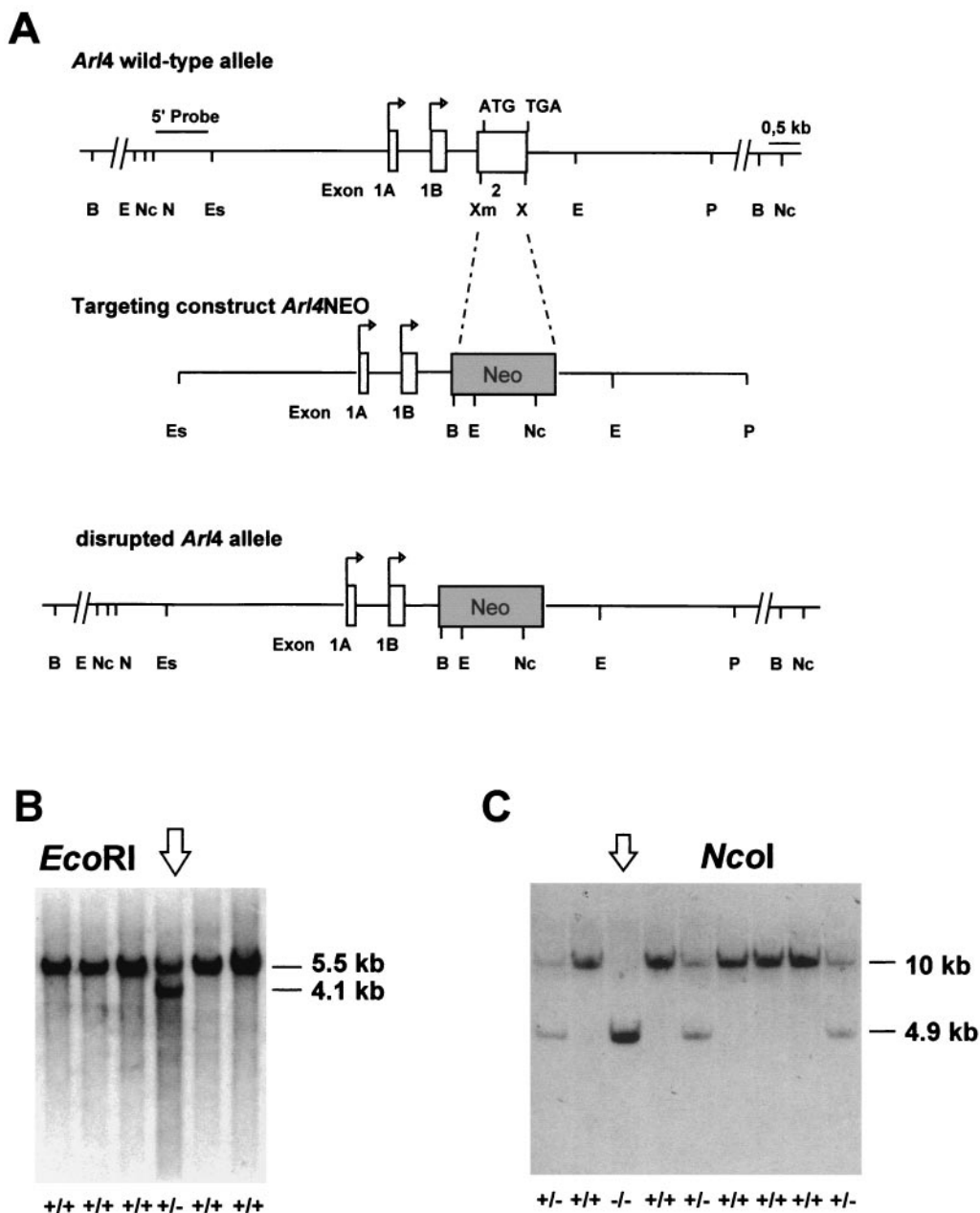
FIG. 3. Targeted disruption of the *Arl4* gene by homologous recombination. (A) Organization of the *Arl4* gene, targeting construct, and organization of the disrupted *Arl4* allele. The *neo* cassette was inserted into the *Xmn*I and *Xho*I sites of exon 2, introducing diagnostic *Eco*RI and *Nco*I sites. Relevant restriction sites: B, *Bam*HI; E, *Eco*RI; Nc, *Nco*I; Es, *Esc*I; Xm, *Xmn*I; X, *Xho*I; P, *Pst*I. The position of the 5′ probe for Southern blot analysis is indicated (bp −2503 to −1803). (B) Southern blot of several ES cell clones. DNA was digested with *Eco*RI and analyzed with the 5′ probe. The arrow designates a clone that incorporated the targeting vector by homologous recombination; this clone was used for injection into blastocysts. (C) Southern blot analysis of genomic DNA from the F_2 progeny (neonate tail DNA). The DNA was digested with *Nco*I and hybridized with the 5′ probe. The positions of the wild-type allele (10 kb) and the targeted allele (4.9 kb) are indicated.

Southern blot analysis with an external probe (Fig. 3B). The stem cell clone carrying the homologous recombination was injected into blastocysts of C57BL/6 mice. Male chimeric mice were mated with C57BL/6 females, and F_1 progeny carrying the transgene were interbred. From a total number of 108 F_2 progeny we obtained 38.5% $Arl4^{+/+}$, 46.2% $Arl4^{+/-}$, and 15.4% $Arl4^{-/-}$ mice, indicating that the disruption of the *Arl4* gene did not result in embryonic lethality.

Arl4 mRNA was absent in the testis, spleen, and intestine tissues of *Arl4*-null mutants and was significantly reduced in heterozygotes ($Arl4^{+/-}$) (Fig. 4A). In order to demonstrate the absence of the protein, we analyzed sections of testes from wild-type and $Arl4^{-/-}$ mutants by immunohistochemistry with antiserum against a C-terminal peptide of ARL4. Figure 4C illustrates that no ARL4 immunoreactivity was detected in sections of testes from $Arl4^{-/-}$ mice (Fig. 4C, right panel).
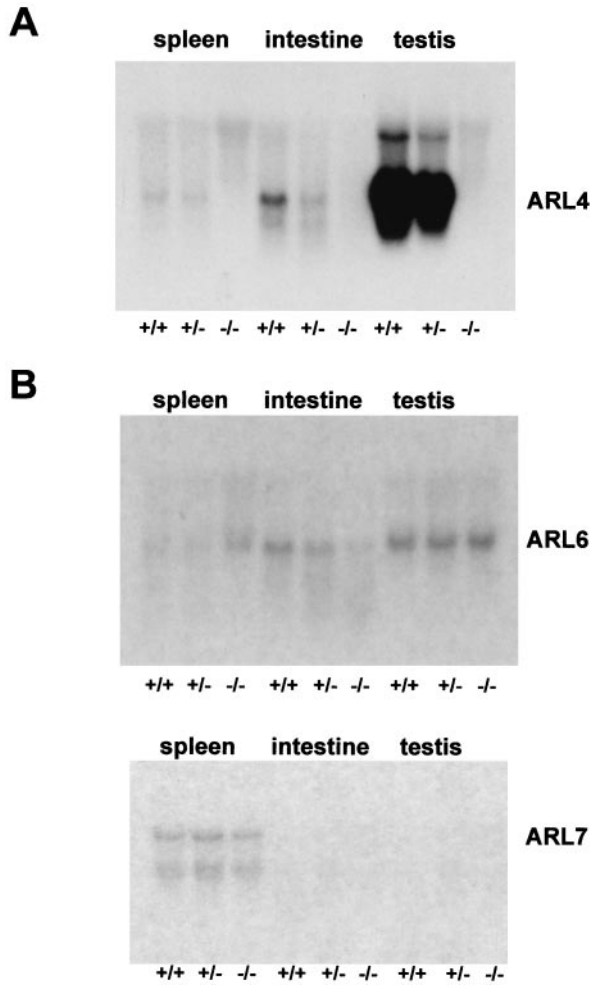
FIG. 4. Expression of ARL4 mRNA and ARL4 protein. (A) Northern blot analysis of the expression of ARL4 in different tissues of $Arl4^{+/+}$, $Arl4^{+/-}$, and $Arl4^{-/-}$ mutants. Northern blots of total RNA (10 µg) from the indicated tissues were hybridized with a probe derived from full-length rat ARL4 cDNA. (B) Northern blot analysis of the expression of ARL6 and ARL7 in spleen, intestine, and testis tissues of $Arl4^{+/+}$, $Arl4^{+/-}$, and $Arl4^{-/-}$ mutants. Northern blots of total RNA (10 µg) from the indicated tissues were hybridized with a probe derived from ARL6 or ARL7 cDNA. (C) Immunohistochemical detection of ARL4 in seminiferous tubules. Frozen sections of testes from adult $Arl4^{+/+}$ (left panel) or $Arl4^{-/-}$ (right panel) mice were fixed with formaldehyde and incubated with the ARL4 antiserum. The immunostaining was performed with peroxidase-conjugated goat anti-rabbit immunoglobulin and analyzed at a magnification of ×400. The sections were counterstained with Mayer's hematoxylin.
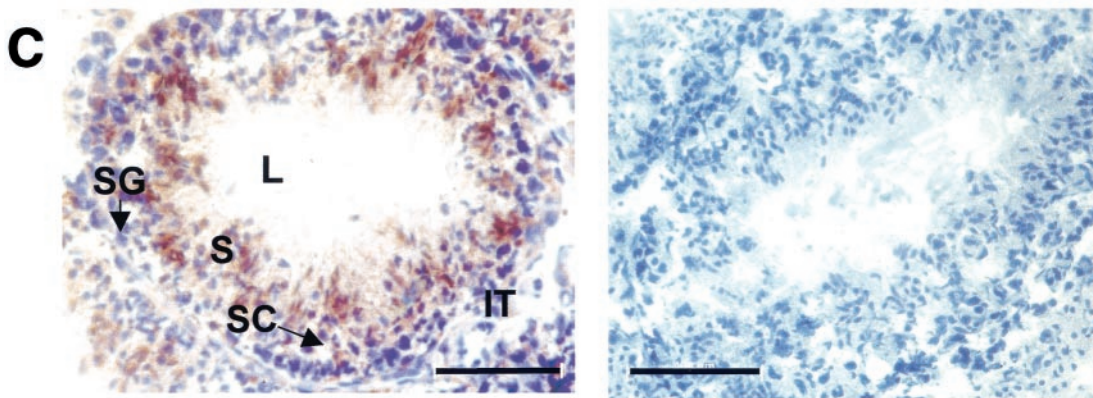
In order to test the possibility that the lack of *Arl4* is compensated by an increased expression of related genes, blots were probed with cDNA of *Arl6* and *Arl7* (9). As illustrated in Fig. 4B, mRNA levels of *Arl6* were slightly increased in spleen but not altered in intestine and testis by disruption of the *Arl4* gene. In addition, *Arl7* mRNA levels were unaltered in spleens from $Arl4^{-/-}$ mice and were not detectable in testes and intestines from wild-type or *Arl4* knockout mice.

Characteristics of $Arl4^{-/-}$ mice. $Arl4^{-/-}$ mice were viable, with normal growth and no apparent abnormality. In sections from several tissues (brain, thymus, spleen, intestine, fat) from 3- and 12-month-old male and female $Arl4^{-/-}$ mice, no morphological or pathological abnormalities were observed (data not shown). Body weights and body lengths of wild-type and knockout mice were identical. In contrast, the weights of testes were significantly reduced in $Arl4^{-/-}$ mice (Table 1).

TABLE 1. Body weight and length and reproductive data
of $Arl4^{-/-}$ male mice

Genotype	Body wt[a,b] (g)	Body length[a] (cm)	Testis wt[a,b] (mg)	Litter size[c] (no. of young)
$Arl4^{+/+}$	35.03 ± 1.3	10.87 ± 0.03	293 ± 20	6.6 ± 1.15
$Arl4^{-/-}$	36.33 ± 1.1	11.03 ± 0.12	214 ± 27[d]	7.4 ± 0.59

[a] Values are means ± SEM from at least seven animals.
[b] Data obtained from 18-month-old $Arl4^{+/+}$ and $Arl4^{-/-}$ male mice.
[c] Data are means of 18 litters obtained from six breading pairs for each genotype.
[d] $P > 0.005$, t test.

Sperm production in $Arl4^{-/-}$ mice. In order to characterize the spermatozoa of $Arl4$ mutants, we excised epididymal tissue of $Arl4^{+/+}$, $Arl4^{+/-}$, and $Arl4^{-/-}$ mice, isolated the spermatozoa, and analyzed them as described in Materials and Methods. The sperm count of $Arl4^{-/-}$ males was reduced by 60%

compared with wild-type and $Arl4^{+/-}$ mice (Fig. 5A, left panel). In contrast, no differences in sperm motility between $Arl4^{+/+}$, $Arl4^{+/-}$, and $Arl4^{-/-}$ mice were observed (Fig. 5A, right panel). Furthermore, we confirmed the effect of reduced sperm count by a comparison of the numbers of spermatozoa in tubular cross sections of testes from wild-type and $Arl4^{-/-}$ mice. In a blinded study, the numbers of mature spermatozoa in sections from testes of 9-month-old wild-type and $Arl4^{-/-}$ animals were counted and normalized per number of spermatogonia. The number of spermatozoa from $Arl4^{-/-}$ mutants was reduced by 25% compared with that of wild-type animals (Fig. 5B). Furthermore, $Arl4^{-/-}$ testis sections contained only 20.4 ± 3.3 (mean ± standard error of the mean) spermatozoon-containing tubuli per 100 tubuli, whereas in the sections from wild-type testis 36.3 ± 5.4 spermatozoon-containing tubuli per 100 tubuli were counted (Fig. 5B). These results indicate that the develop-
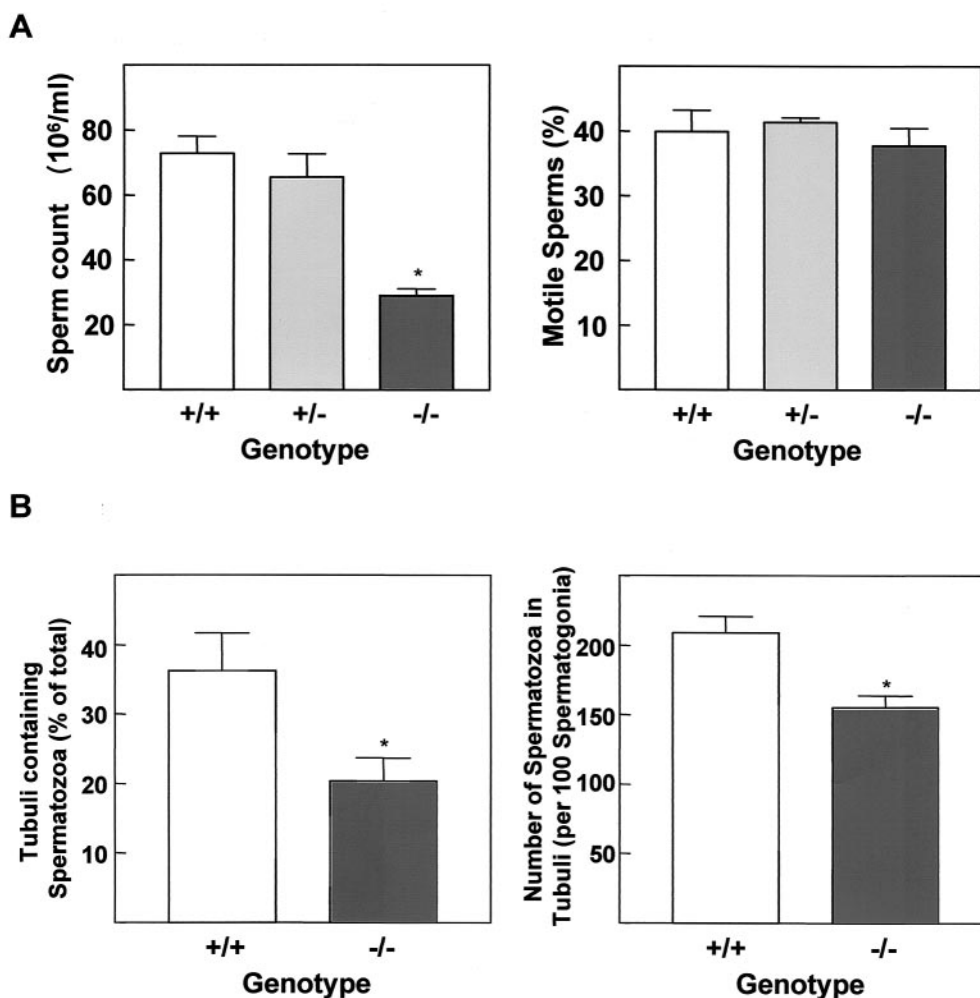

FIG. 5. Sperm count and sperm motility from $Arl4^{+/+}$, $Arl4^{+/-}$, and $Arl4^{-/-}$ mice. (A) Spermatozoa were isolated from epididymal tissues of 6-month-old $Arl4^{+/+}$, $Arl4^{+/-}$, and $Arl4^{-/-}$ mice and counted (left panel), and their motility (right panel) was analyzed as described in Materials and Methods (means ± SEM from six $Arl4^{+/+}$ and $Arl4^{-/-}$ mice and from three $Arl4^{+/-}$ mice). (B) Sections of testes from 9-month-old mice were stained with Mayer's hematoxylin. Tubules containing spermatozoa were counted and normalized per 100 tubules (left panel); spermatozoa were counted and normalized per 100 spermatogonia (right panel; means ± SEM from five animals are shown). Asterisks indicate statistical significance ($P < 0.001$, one-way analysis of variance (panel A) or t test (panel B).

TABLE 2. Adipose tissue weight, serum metabolite, and blood cell characteristics of $Arl4^{-/-}$ mice[a]

Genotype	Wt of epididymal fat pad (g)	Amt of serum metabolite			No. of blood cells		
		Blood glucose mg %	Serum cholesterol (mmol/liter)	Serum triglycerides (mmol/liter)	Leukocytes ($10^3/\mu$l)	Erythrocytes ($10^6/\mu$l)	Thrombocytes ($10^6/\mu$l)
$Arl4^{+/+}$	1.07 ± 0.31	7.50 ± 0.1	2.52 ± 0.1	1.77 ± 0.12	6.8 ± 0.33	9.94 ± 0.13	1.022 ± 0.045
$Arl4^{-/-}$	0.91 ± 0.27	6.98 ± 0.49	2.14 ± 0.08	1.86 ± 0.13	7.7 ± 0.48	10.23 ± 0.12	1.014 ± 0.038

[a] Values are means ± SEMs. Epididymal fat pads were dissected from 18-month-old $Arl4^{+/+}$ and $Arl4^{-/-}$ mice ($n = 7$). Blood glucose, serum cholesterol, serum triglycerides, and the numbers of leukocytes, erythrocytes, and thrombocytes were measured from 6- to 12-month-old mice ($n = 31$) by autoanalyzer.

ment of male germ cells was markedly retarded in *Arl4*-deficient mice.

The reduction in the amount of spermatozoa had no apparent effect on the fertility of $Arl4^{-/-}$ mice. We observed that all $Arl4^{-/-}$ mice in our breeding program proved capable of siring litters; there was no reduction in litter size (Table 1).

Adipose tissue weight, serum metabolites, and blood cell count in *Arl4* mutants. Since we have previously shown that ARL4 is differentially expressed in 3T3-L1 adipocytes (18), we expected a defect in the development of adipose tissues. We therefore analyzed the weights of epididymal fat pads and determined several metabolic parameters of $Arl4^{+/+}$ and $Arl4^{-/-}$ mice. Epididymal fat pad weights obtained from $Arl4^{-/-}$ mice were not significantly different from those of the $Arl4^{+/+}$ littermates (Table 2). In addition, we failed to detect any differences in blood glucose levels, serum cholesterol, serum triglycerides, or blood cell counts (Table 2).

DISCUSSION

The present data suggest that the ADP-ribosylation factor-like GTPase ARL4 is involved in the development of male germ cells. In the course of spermatogenesis, spermatogonia differentiate to spermatocytes, which undergo a meiotic division and subsequently differentiate to mature spermatozoa (1, 2, 16). The expression of ARL4 mRNA started at days 16 to 18, when late pachytene spermatocytes appear (15). In adult animals, the protein was detected in spermatocytes and spermatides, but not in spermatogonia and mature spermatozoa. Furthermore, the deletion of the *Arl4* gene produced a significant reduction of testis weight and number of spermatozoa without affecting their motility or reproductive function. These data exclude that ARL4 is involved in the division of spermatogonia and render it unlikely that the GTPase is required for the function of the mature spermatozoa. Rather, the data are consistent with the conclusion that ARL4 is required for progression of cells through meiosis and that its deletion causes a retardation of the formation of the haploid spermatides.

Several knockout mice with reduced sperm count but normal fertility have been described previously. Mice lacking the centromere protein B exhibit a phenotype which is very similar to that of the *Arl4*-null mutant. Centromere protein B is a DNA binding protein required for meiosis; its deletion caused a reduction in testis weight by 30% and reduction in the sperm count by 40% without a reduction in fertility (7). Furthermore, mice lacking the follicle-stimulating hormone β (FSHβ) are fertile despite reduced testis size and number of Sertoli cells (11). Activin II receptor A (ActRcIIA)-null mutants exhibit reduced serum FSH levels, decreased testis size, and reduced

Sertoli cell number but are also fertile (14). Even the double mutants lacking both FSHβ and ActRcIIA are fertile, although their numbers of spermatozoa were even lower than those of the single FSHβ or ActRcIIA gene knockout mice (12).

The cellular function of ARL4 is largely unknown. The protein carries a nuclear localization signal in its C terminus and is predominantly located in the nucleus (8, 13). Furthermore, it appears to shuttle between the nucleus and intracellular organelles (endoplasmic reticulum and/or Golgi) depending on its GTP/GDP-binding status (A. Schürmann and H.-G. Joost, unpublished data). Thus, it appears possible that ARL4 is involved in the regulation of protein transport from or to the nucleus during the division or differentiation of cells.

The next relatives of ARL4 are the ARF-like proteins ARL6 and ARL7 (9). Both GTPases have the C-terminal nuclear localization motif and exhibit GTP-binding and exchange characteristics similar to those of ARL4 (9). ARL7 is expressed in spleen but not in testis, whereas high levels of ARL6 mRNA are found in testis. ARL4 and ARL6 share 61% identical amino acids with stretches of 80% identity. Thus, ARL6 is a potential candidate to compensate a loss of function of ARL4. Since *Arl6* mRNA levels in spleen tissues of *Arl4*-null mutants were higher than those of control animals, *Arl6* appears to be upregulated in this tissue, suggesting that ARL4 and ARL6 play a specific role in spleen. In contrast, no upregulation of mRNA levels of ARL7 in spleen and of ARL6 in all other tissues was detected. However, the possibility cannot be excluded that the normal transcript levels of ARL6 and ARL7 are sufficient to partially compensate the lack of ARL4.

Based on its differential expression in 3T3-L1 adipocytes (18) and on its specific localization of expression during embryogenesis (13), we had anticipated that mice lacking *Arl4* would exhibit developmental abnormalities and/or an impairment of adipogenesis. Although we cannot rule out a subtle phenotype that escaped our preliminary characterization, the present data strongly argue against an essential role of ARL4 in adipogenesis or neurogenesis and suggest that its main function is related to the development of male germ cells.

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