GAMETE BIOLOGY

Green fluorescence protein driven by the Na,K-ATPase α4 isoform promoter is expressed only in male germ cells of mouse testis

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Abstract

Purpose Expression of the Na,K-ATPase α4 isoform is required for sperm motility and fertility and is controlled by the *Atp1a4* promoter. Here, we have investigated the specific tissue, cell type and developmental regulation of expression mediated by the *Atp1a4* promoter.
Methods We have inserted the green fluorescent protein (GFP), downstream of the endogenous *Atp1a4* promoter, in place of the Na,K-ATPase α4 gene, and used it as a marker for α4 expression in mice (*Atp1a4$^{null(GFP)}$* mice).
Results Replacement of α4 by GFP completely disrupted α4 expression and activity, produced sperm morphological and functional abnormalities, and caused infertility of *Atp1a4$^{null(GFP)}$* male mice. Immunoblot analysis of *Atp1a4$^{null(GFP)}$* mouse tissues showed GFP expression in testis. This particular expression pattern was found in adult, but not in mouse embryos or in 7, 18 day old mice. In agreement with expression of GFP, adult *Atp1a4$^{null(GFP)}$* mouse testis displayed the typical fluorescence of GFP. Immunocytochemistry of testis identified GFP in more differentiated male germ cells, but not in spermatogonia, Leydig or Sertoli cells. Further analysis, using immunoblot of fluorescently sorted testis cells with cell specific markers, detected GFP only in spermatocytes, spermatids and spermatozoa. While epididymis showed GFP expression, this was confined to the spermatozoa within the epididymal tubules.

Conclusions Our results show that the *Atp1a4* promoter drives GFP expression exclusively in male germ cells of the testis, where it restricts it to post-meiotic stages of spermatogenesis. These findings highlight the exquisite spatial and temporal control of expression exerted by the *Atp1a4* promoter on Na, K-ATPase α4, which is particularly well suited to fulfill the special functions of spermatozoa.

Introduction

The Na,K-ATPase is a plasma membrane enzyme that catalyzes the active transport of Na^+ and K^+ between the cell and its environment [1, 2]. Function of the Na,K-ATPase is essential for maintaining key parameters in the cell and this is why expression and activity of this ion transporter is highly regulated [3]. In mammalian cells, the Na,K-ATPase is expressed as various isozymes, which result from the association of four different isoforms of the α subunit (α1, α2, α3 or α4) and three distinct isoforms of the β polypeptide (β1, β2 or β3) [4, 5]. Expression of these structurally and functionally different variants of the Na,K-ATPase is not redundant but rather represents a mechanism that cells use to adapt Na^+ and K^+ transport to their specific physiologic needs [3, 4, 6, 7].

The α4 polypeptide is the Na,K-ATPase isoform that is least abundantly expressed across the body. It has been found in male germ cells of the testis, where it predominates in the sperm flagellum [8, 9]. The α4 isoform has particular affinities for Na^+, K^+ and ATP [10, 11] and is important for maintaining membrane potential, pH and the intracellular Na^+ and Ca^{+2} levels of sperm [12]. Moreover, the α4 polypeptide plays a primary role in sperm motility [8, 13, 14] and it is fundamental for sperm fertility since knockout

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Capsule The Na,K-ATPase a4 promoter drives protein expression specifically in testis male germ cells after meiosis.

mice in which the α4 isoform has been deleted are completely sterile [15]. While original studies with specific antibodies identified α4 in testis male germ cells [8, 10]; more recently, a work based on RT-PCR analysis, reported the presence of Na,K-ATPase α4 mRNA in Sertoli cells, suggesting that α4 expression may not be solely confined to male germ cells, but could also extend to somatic cells [16]. This shows that other independent experimental approaches will be important to precisely understand the tissue and cell type specific distribution of Na,K-ATPase α4. In addition, while studies of RNA, protein and activity have helped follow the pattern of expression of the α4 isoform [8, 9], a complete characterization of the expression of α4 during different stages of development has not yet been accomplished. Comprehending α4 expression pattern during development is an important step in deciphering the biological relevance of this Na,K-ATPase isoform in male reproduction and fertility.

The Na,K-ATPase α4 isoform is encoded by the *ATP1a4* gene, which is regulated by DNA sequences located upstream the α4 coding sequences. These sequences, identified as the proximal promoter regions of α4 (*Atp1a4* promoter) have regulatory sites that drive the expression of the *ATP1a4* gene [17, 18]. In the present work, we have taken advantage of the endogenous *Atp1a4* promoter to drive the expression of the Green Fluorescent Protein (GFP) in mice. This genetic approach allowed us to follow GFP expression as a marker for the spatial and temporal regulation of protein expression exerted by the *Atp1a4* promoter in mice. We show that *ATP1a4* promoter dependent expression of GFP is testis specific and exclusively limited to the germ cells of the male gonad that have progressed to late stages of spermatogenesis. In addition, our knock-in strategy has established a new mouse model that will be useful for future studies related to the Na,K-ATPase α4 isoform.

Materials and methods

Preparation of knockout animals

A mouse 129/SvEv BAC (BAC ID# bMQ-247 N9) (Source BioScience) containing the *Atp1a4* genomic locus was used to generate the *Atp1a4* knockout gene-targeting vector. The targeting vector was made by subcloning a GFP reporter gene with polyadenylation signal (Clontech) into the *Atp1a4* start site. Recombineering was employed to fuse the targeting vector to the BAC *Atp1a4* locus [19]. Homologous recombination simultaneously inserted the vector and removed the *Atp1a4* start methionine in the first 62 nucleotides of exon 6, generating a frame shift mutation of the remaining DNA. The targeting vector was linearized with PmeI and electroporated into EDJ22 (ATCC) ES cells (129SvEv cell line) for homologous recombination. After neomyocin selection, isolated ES

cell clones were screened by PCR. Four clones were confirmed by Southern blotting with positive clones being further characterized by karyotyping. Targeted ES clones were microinjected into C57BL/6 blastocysts and these were implanted into pseudopregnant females. Resulting chimeras were mated to wild-type C57BL/6 mice to generate F1 heterozygous offspring and breeding continued until a founder line was established. Genotype analysis of tail biopsies was performed by PCR on isolated genomic DNA using the REDExtract-N-Amp Tissue PCR Kit (Sigma).

Sperm isolation

All experimental protocols involving animals in this work were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. Spermatozoa from wild-type and homozygous *Atp1a4*$^{null(GFP)}$ mice were obtained from the cauda of adult mice epididymides after swim-up of the cells, as previously described [15]. Sperm was resuspended in modified Tyrode's medium, containing: 100 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 5.5 mM Glucose, 0.8 mM pyruvic acid, 4.8 mM lactic acid, 20 mM Hepes (pH 7.4), counted, and used for the different assays. For some experiments, sperm was capacitated in modified Tyrode's medium, supplemented with 1.7 mM CaCl$_2$, 25 mM sodium bicarbonate and 0.5 % BSA.

Southern blot analysis

Southern blot was used to confirm the ES cell clones containing the α4 construct. DNA was first digested with Bgl II and electrophoretically separated on a 0.8 % agarose gel. After transfer to a nylon membrane, DNA was hybridized with probes targeted to the 5' external region of the Bgl II digested DNA, or to the 3' external region of the Bgl II digested DNA. After washing off the non-hybridized probe, membranes were processed for autoradiography as described [12].

Polymerase Chain Reaction (PCR) analysis

PCR was used for analysis of genomic DNA. Reactions were performed using primers directed to Na,K-ATPase α4 isoform sequences (sense 5'-TCAGACTTGCTCCCTTCACCTTTCCT-3') and antisense (5'-GTTTGCTTTGGCCATCCTCACCAT-3') and to the GFP reporter sequence (5'-TGTAGTTGCCGTCGTCCTTGAAGA-3'). Procedures were as described [9], using a first cycle of 120 s at 94 °C, followed by 35 cycles at 94 °C for 15 s, 62 °C for 15 s, and 72 °C for 30 s.

Immunoblot analysis

Tissue samples were homogenized in RIPA buffer (1 % NP-40, 0.25 % NaDOC, 1 mM Na3VO4, 1 mM NaF, 1 mM EDTA,

150 mM NaCl, 10 μM leupeptin, 10 μg/ml apotinin, 1 mM PMSF and 50 mM Tris (pH 7.4), centrifuged and the supernatant used for immunoblot analysis. Samples were subjected to SDS/PAGE (7.5 % gel) and immunoblotting, as previously described [9]. The Na,K-ATPase $\alpha 1$ and $\alpha 4$ isoforms were identified using antisera generated against specific amino acids in the N-terminal regions of the α isoforms in rabbit and chicken respectively as described [9]. Other antibodies used to probe the blots included mouse anti-PLZF (1:200 dilution; Calbiochem), rabbit anti-DDX4 (1 μg/ml; Abcam, Cambridge, MA), rabbit anti-DMRT1 (1:4000 dilution; a kind gift from Leslie Heckert, University of Kansas Medical Center), anti-KIF17b (1:1000 dilution), anti-GFP (1:2000 dilution; Abcam) and mouse anti-beta tubulin (1:1000 dilution; Sigma-Aldrich). Horseradish peroxidase conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) and chemiluminescence was used for detection.

Na,K-ATPase assay

Na,K-ATPase activity was assayed on testis homogenates, through determination of the initial rate of release of $^{32}P_i$ from $\gamma[^{32}P]$-ATP, as previously described [20]. The ATPase activity of 10 μg of total protein per sample was measured in a final volume of 0.25 mL in medium containing 120 mM NaCl, 30 mM KCl, 3 mM MgCl$_2$, 0.2 mM EGTA, 30 mM Tris–HCl (pH 7.4), 3 mM ATP with 0.2 μCi $\gamma[^{32}P]$-ATP in the presence and absence of the indicated ouabain concentrations. Curve fitting of the experimental data were performed using a Marquardt least-squares nonlinear regression computing program (Sigma Plot; Jandel Scientific).

Sperm motility assay

Sperm motility was measured on spermatozoa from wild-type, and $Atp1a4^{null(GFP)}$ mice as previously described [20]. Samples were analyzed by computer-assisted sperm analysis (CASA), using the Minitube Sperm-Vision Digital Semen Evaluation system (version 3.5, Penetrating Innovations). Total sperm motility and different parameters of sperm movement were determined using the analytical setup parameters defined before [20].

Histological and immunocytochemical analysis

Mouse testis and epididymis were dissected, fixed with Bouin's fixative solution (Ricca Chemical Company, Arlington TX), embedded in paraffin and sectioned to obtain 4 μm slices. After placing on slides, samples were stained with hematoxylin/eosin, or they were processed for immunofluorescence as described [21]. Briefly, tissue was permeabilized with 0.3 % Triton X100 in PBS. After blocking for 2 h at room temperature with 0.1 % BSA and 5 % normal goat serum in PBS, the anti-GFP antibody

was applied overnight at 4 °C at a dilution of 1:2500. For immunochemical staining, biotinylated goat anti-rabbit antiserum was used as the secondary antibody and detection was performed using the Vectastain ABC Kit from Vector laboratories (Burlingame, CA) following the manufacturers' instructions. For fluorescence labeling, an Alexafluor-488 conjugated goat anti-rabbit antiserum (Molecular Probes, Eugene, OR) was used as the secondary antibody. Digital images were obtained using a Nikon 80i microscope. Similar protocols were used to explore expression of GFP in 16 day old whole mouse embryos.

Sorting of testis cells

Testis from adult (8 weeks) wild type and Atp1a4$^{null(GFP)}$ mice were dissected, decapsulated and placed in DMEM medium supplemented with 2 mM glutamate, non-essential amino acids, 100 IU/ml penicillin, 100 ug/ml streptomycin and 15 mM HEPES (pH 7.4). Cells were dissociated following a modification of the protocol previously described. Briefly, samples were first digested with 0.03 mg/ml collagenase/dispase and 0.1 mg/ml DNase for 15 min with continuous agitation at 37 °C. Dissociated seminiferous tubules were centrifuged at 1,500 rpm for 5 min and further digested with 0.03 collagenase type IV-S for 20 min with continuous agitation at 37 °C. After centrifugation at 2200 rpm for 10 min, cells were suspended to a final density of 5×10^6 cells/ml and were filtered through a cell strainer (BD Biosciences, San Jose, CA). Dissociated cells were sorted according to their fluorescence at 488 nm using the Fluorescent-Activated Cell Sorter (FACS) Aria II cell sorter (BD Biosciences, San Jose, CA). Separated GFP positive and negative cells were collected after centrifugation and samples used for immunoblot analysis.

Results

$Atp1a4^{null(GFP)}$ mice express GFP in place of the Na,K-ATPase $\alpha 4$ isoform

To determine the temporal and spatial pattern of expression of the $\alpha 4$ subunit, we have used a knock in strategy to insert GFP in place of the $Atp1a4$ gene in mice ($Atp1a4^{null(GFP)}$ mice). The reporter GFP gene with polyadenylation signal was introduced in the $Atp1a4$ start site in mouse embryonic stem (ES) cells. This left GFP under the control of the $Atp1a4$ promoter and disrupted the $Atp1a4$ locus by removing the remaining downstream sequence of exon 1 through exon 6 by homologous recombination (Fig. 1a). ES cell clones were screened by Southern blot analysis and positive clones were confirmed following the strategy depicted in Fig. 1b. One of the positive clones was chosen to make the chimeric mice. Once this was generated, a
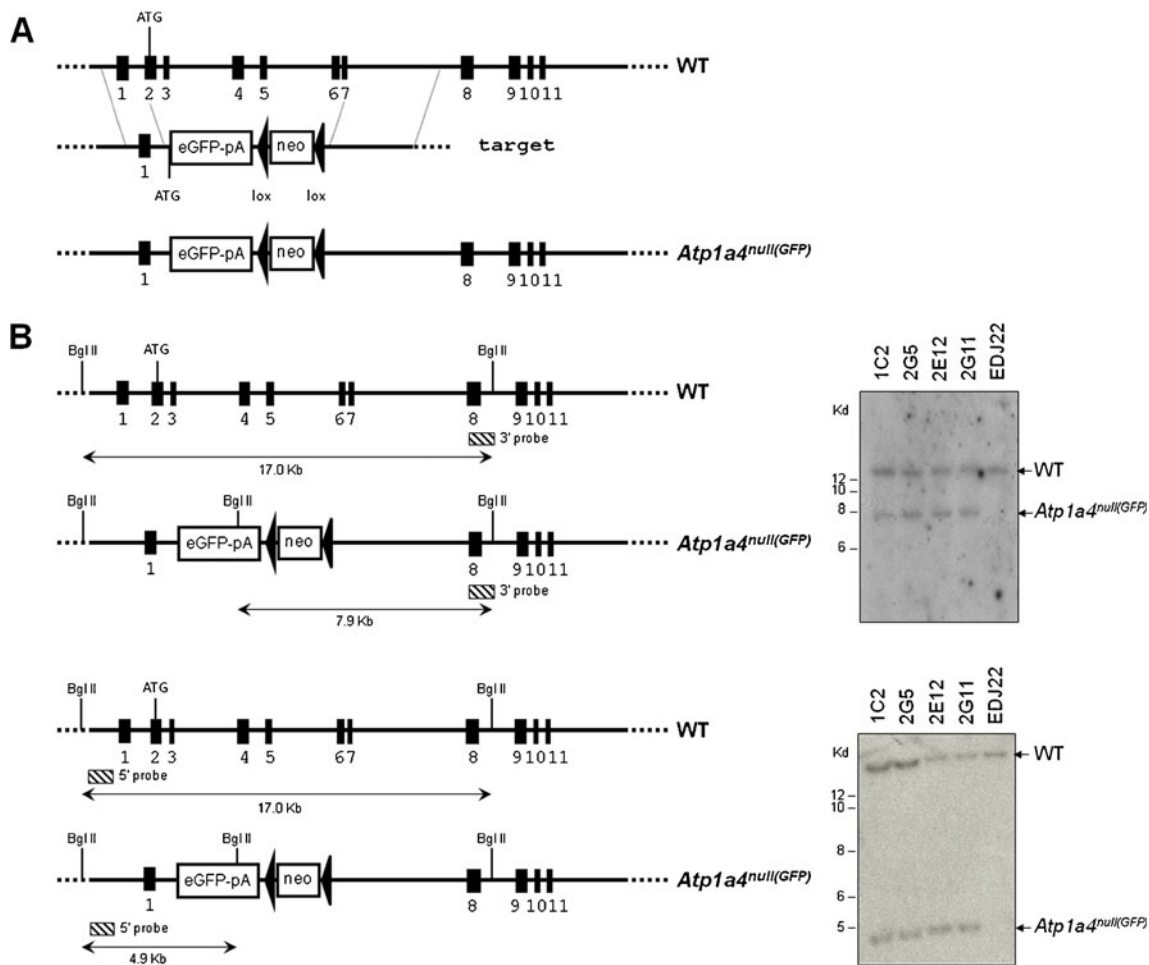
Fig. 1 Knock in of GFP into the Na,K-Atpase *Atp1a4* gene. **a** Scheme showing the targeting strategy used to introduce GFP into the *Atp1a4* locus. **b** Southern blot strategy employed to screen ES cell clones. Identification of positive ES cell clones was performed using probes that specifically hybridized to regions flanking the targeted α4 region.

Wild-type (WT) and α4-knockin clones (*Atp1a4^null(GFP)^*) exhibited the predicted DNA bands of 17.0 kb and 7.9 kb for the 3' end and 17.0 kb and 4.9 kb for the 5' end. 1C2, 2 G5, 2E12 adn 2 G11 represent the different cell clones. As negative controls, genomic DNA from the parental line, EDJ22, was used

founder line was established and the desired genotype of offspring was verified by PCR (Fig. 2a). Further validation of the absence of the Na,K-ATPase α4 isoform and its substitution by GFP was performed by immunoblotting of mouse sperm proteins. As shown in Fig. 2b, genetically modified mouse homozygous for the allele, completely lacked the α4 polypeptide and expressed GFP instead. The null allele inheritance of mated heterozygous F1 mice (15 litters) followed Mendelian ratios (27 wild-type, 52 heterozygous, and 29 homozygous mice). While *Atp1a4^null(GFP)^* mice lacked expression of the α4 isoform, levels of α1, the other catalytic subunit of sperm Na,K-ATPase showed no change (Fig. 2b). Altogether, these results show that the *Atp1a4* promoter is able to efficiently express GFP in place of the α4 polypeptide and demonstrate that the effect of our genetic manipulation was Na,K-ATPase isoform specific.

*Atp1a4^null(GFP)^* mice are devoid of Na,K-ATPase α4 isoform activity

As shown previously, the α4 isoform has a characteristically high affinity for the inhibitor ouabain and is important for sperm morphology and motility [15, 20]. To ascertain that insertion of GFP in the *Atp1a4^null(GFP)^* mice produced the functional phenotype corresponding to disruption of the *Atp1a4* gene, we performed Na,K-ATPase activity and motility assays on sperm from the genetically engineered mice. Determination of ouabain inhibition profiles of Na,K-ATPase activity in testis homogenates from wild type mice showed a biphasic dose–response curve, with IC_{50} values in the nanomolar ($2.04 \pm 0.31 \times 10^{-9}$ M) and micromolar ($2.34 \pm 0.63 \times 10^{-5}$) range (Fig. 3a). This kinetic behavior corresponds to the activities of the highly ouabain sensitive α4 and ouabain resistant α1 isoforms, respectively [4]. In
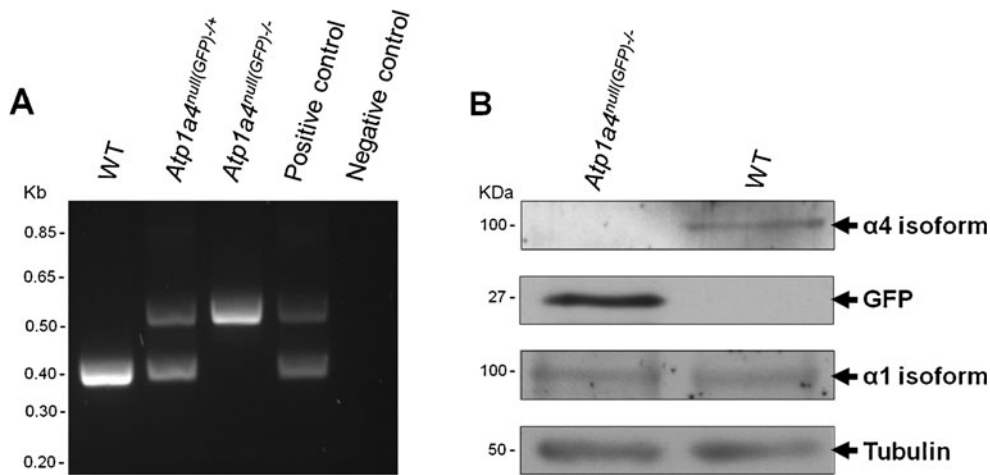
Fig. 2 Characterization of founder lines of the *Atp1a4^null(GFP)* mice. **a** PCR genotyping of mice was performed on genomic DNA extracted from tail clips of the mice. Distinct products of 405 and 531 bp correspond to wild-type and *Atp1a4^null(GFP)* mice respectively. Samples containing wild-type DNA spiked with the targeting vector was used as a positive control, whereas samples with no DNA were used as a negative control. **b** Immunoblot analysis of GFP expression in sperm lysates from wild type and homozygous *Atp1a4^null(GFP)* mice. Anti-α4 antiserum generated in chicken and anti-α1 antiserum generated in rabbit, followed by horseradish peroxidase conjugated secondary antibodies and chemiluminescence were used for detection. Tubulin was used as a loading control

contrast, inhibition of Na,K-ATPase by ouabain in *Atp1a4^null(GFP)* mice testis showed a monophasic response, lacking the enzymatic component characteristic of α4 and presenting a single IC_{50} value of $4.41 \pm 1.10 \times 10^{-5}$ M, which indicated the presence of only α1 in the sample (Fig. 3a). In agreement with the absence of α4, total Na,K-ATPase activity was lower in *Atp1a4^null(GFP)* than in wild type sperm (Fig. 3b). These functional data

further support the lack of expression of the α4 isoform in *Atp1a4^null(GFP)* mice.

Atp1a4^null(GFP) male mice are infertile

Atp1a4^null(GFP) mice were overall phenotypically similar to wild-type mice. However, while homozygous and heterozygous female mice and heterozygous male mice were fertile,
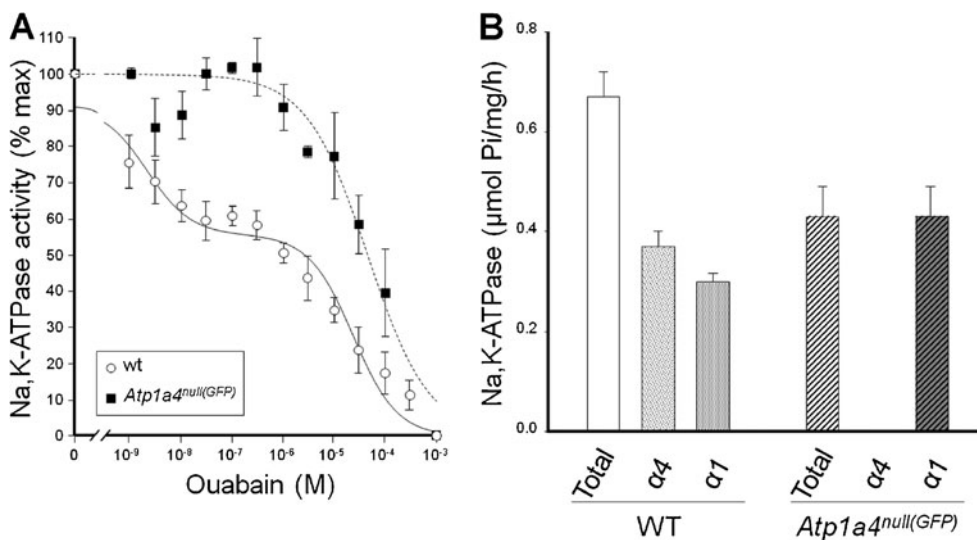

Fig. 3 Sperm from *Atp1a4^null(GFP)* mice lack Na,K-ATPase α4 activity. **a** Dose–response curves for the inhibition of Na,K-ATPase activity by ouabain. Assays were performed on testes homogenates, using 10 μg total protein, at saturating concentrations of all physiological ligands of the Na,K-ATPase, in the presence of the indicated concentrations of ouabain. The curves represent the best fit of the experimental data to highly ouabain-sensitive and ouabain-resistant α4 and α1 isoforms of the Na,K-ATPase. Each value is the mean±SEM of three experiments. **b** Maximal Na,K-ATPase activity for α4 and α1 isoforms in wild type and *Atp1a4^null(GFP)* mice. Activity of the α4 and α1 isoforms was distinguished based on the difference in the ouabain affinity that characterizes these isoforms. Total Na,K-ATPase activity corresponded to that inhibitable by 10^{-3} M ouabain. Hydrolysis of ATP by α4 was determined as that sensitive to 10^{-6} M ouabain. Activity of α1 corresponded to the difference in ATP hydrolysis obtained at 10^{-6} and 10^{-3} M ouabain

producing normal litter numbers and sizes, homozygous *Atp1a4*^{null(GFP)} male mice were completely sterile. Controlled matings of homozygous null males with wild type females yielded no pregnancies during a period of 3 months. This was not due to the inability of the mice to mate, as noted by the presence of vaginal plugs in the females that have been mated with *Atp1a4*^{null(GFP)} mice. Testis size, testis general morphology and testis to body weight ratios were similar in the *Atp1a4*^{null}^(GFP) and wild type mice (data not shown). This male specific abnormal reproductive phenotype resembles that previously found in α4 null mice [15] and suggests first, that disruption of the *Atp1a4* gene by GFP was efficient and second, it supports the important role that the Na,K-ATPase α4 isoform has in male fertility.

Atp1a4^{null(GFP)} mice show sperm morphological and functional abnormalities

When examined by CASA and under non capacitating media, sperm from *Atp1a4*^{null(GFP)} mice showed a drastic reduction in total and progressive sperm motility compared to wild-type spermatozoa (Fig. 4a and b). Other parameters of sperm motility, such as straight line, curvilinear and average path velocities, and amplitude of lateral head displacement were also decreased (data not shown). Moreover, when sperm was incubated in capacitating medium, containing BSA and bicarbonate, the hyperactivation pattern of motility that sperm normally achieves was drastically reduced in *Atp1a4*^{null}

Fig. 4 Sperm from *Atp1a4*^{null}^(GFP) mice show morphological and functional abnormalities. **a-c** Sperm from *Atp1a4*^{null(GFP)} mice are asthenoospermic. Sperm in modified Tyrode's medium was subjected to CASA analysis to determine sperm total **a**, progressive **b** and hyperactive motility. Bars represent the mean±SEM of three experiments. Values significantly different from the controls are indicated with an asterisk, with *P* values ranging between 0.05 and 0.001. **d** Sperm from *Atp1a4*^{null(GFP)} mice present flagellar bending. Sperm was fixed in buffered formalin phosphate, permeabilized with Triton X-100 and labeled with anti-GFP antibody and Alexa 488 conjugated goat anti-rabbit IgG. Images were obtained using a Nikon Eclipse 80i scope (Nikon Instruments, Melville, NY) under a 40X objective
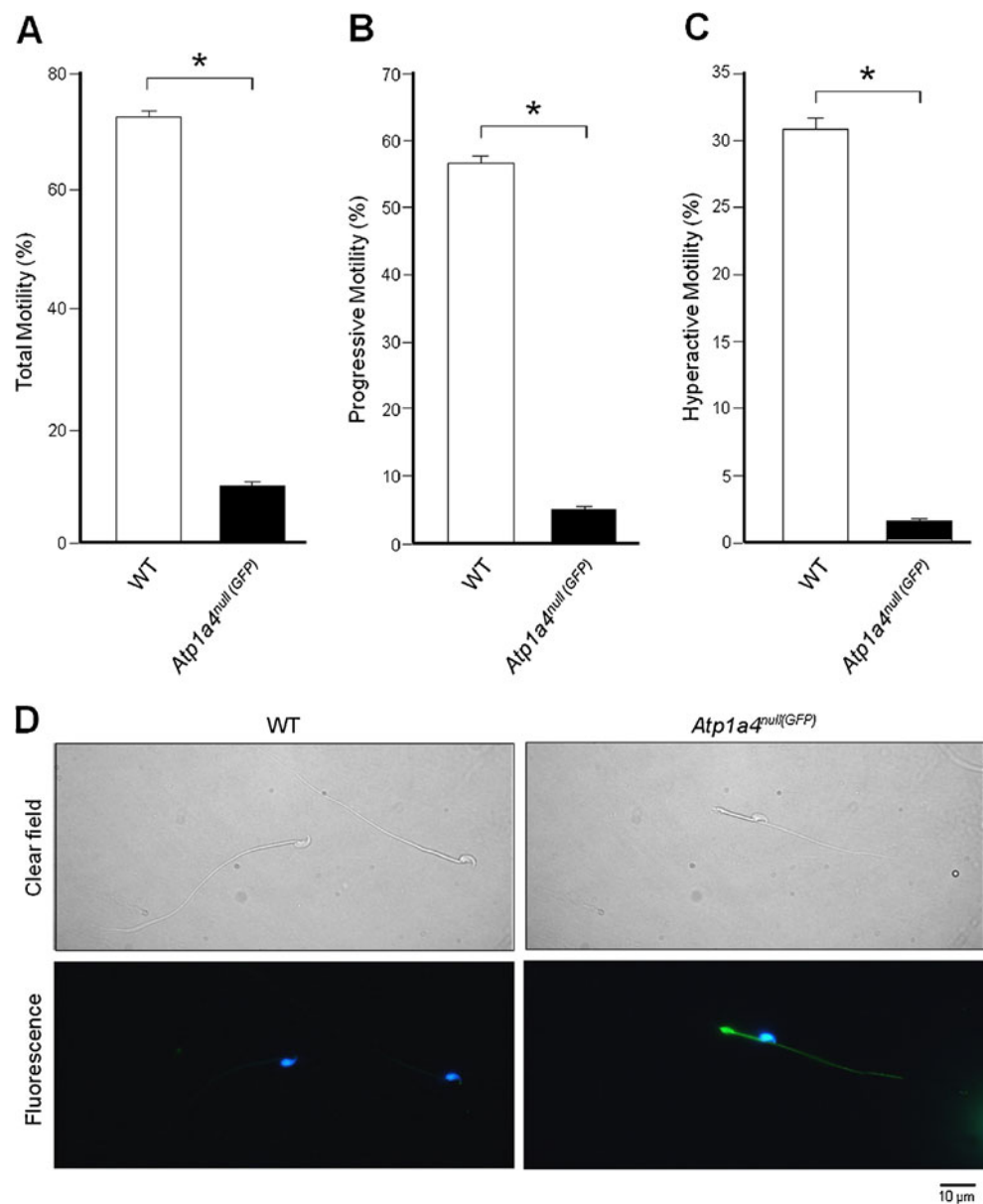
(GFP) mice (Fig. 4c). In addition to the defects in motility, sperm from $Atp1a4^{null(GFP)}$ mice also displayed abnormal morphology, consisting in a bend between the mid- and the principal piece of the sperm flagellum (Fig. 4d). This corresponds to the phenotype previously seen in α4-null sperm and is consistent with alterations in osmolarity of the cell cytoplasm resulting from lack of the α4 isoform [15]. Altogether, these data indicate that insertion of the GFP in place of the $Atp1a4$ gene results in the phenotypical defects of sperm that are typical of the absence of the α4 isoform [15].

GFP expression in $Atp1a4^{null(GFP)}$ mice is developmentally regulated and organ specific

Because GFP was placed under the $Atp1a4$ promoter, $Atp1a4$-$^{null(GFP)}$ mice provided the opportunity to use GFP as a substitute marker for the expression of the Na,K-ATPase α4 isoform. Therefore, we examined expression of GFP at the protein level in testis and a series of other major organs of $Atp1a4^{null(GFP)}$ mice at different stages of development. Three time points in the sexual maturation of mice were chosen, since they represent milestones in the development of male germ cells in the testis:

Fig. 5 GFP is expressed in testis of $Atp1a4^{null(GFP)}$ mice. **a-c** Immunoblot analysis of GFP in different tissues from adult, 18 dpp and 7 dpp $Atp1a4^{null}$ $^{(GFP)}$ mice. A total of 50 μg of protein per sample was loaded onto a SDS-PAGE gel, transferred to a nitrocellulose membrane and analyzed with anti-GFP antibody. Tubulin was used as a loading control. Representative gels are shown. **d** Immunochemical analysis of GFP in embryos. Whole body sections from 16 day old wild type and $Atp1a4^{null(GFP)}$ mouse embryos were fixed, embedded in paraffin and sectioned. GFP was detected by immunocyto-chemistry, using anti-GFP antibody, followed by biotinylated goat anti-rabbit antiserum the Vectastain ABC Kit. Hematoxylin was used as a counterstain
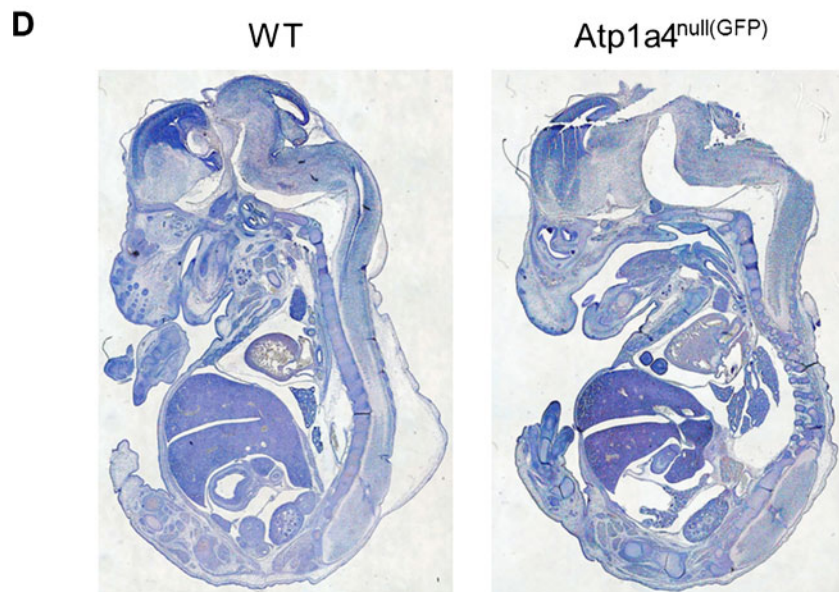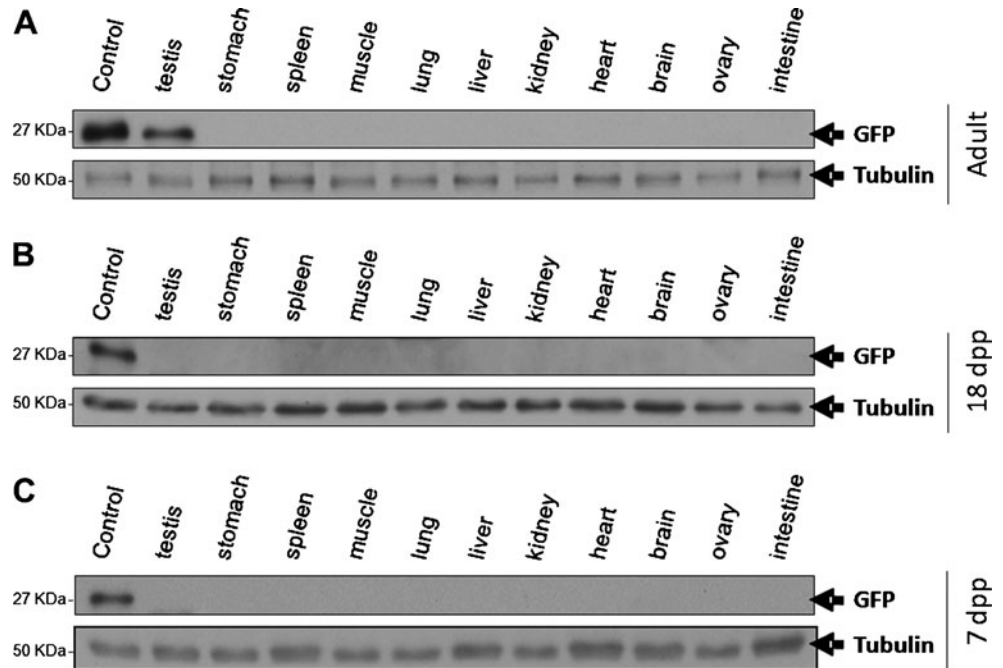
a) 7 days post partum (7 dpp), when the testes contain germ cells at the stage of spermatogonia, b) 18 dpp, when preleptotene, leptotene, and pachytene spermatocytes are present, but the gonad has no haploid spermatids, and c) adult animals, in which cells at all stages of spermatogenesis are found and spermatozoa are fully differentiated [22]. Immunoblot analysis using an antibody against GFP identified this protein exclusively in testis of adult mice (Fig. 5a, b and c). No GFP could be detected in any of the tissues tested at 7 or 18 dpp. In addition, we explored expression of GFP in the mouse embryo, using whole body sections of wild type and $Atp1a4^{null(GFP)}$ mice at embryonic day 16 day. As shown in Fig. 5d, no GFP reactivity was found in either the wild type or $Atp1a4^{null(GFP)}$ embryos. These results show that the $Atp1a4$ promoter drives expression of GFP in a tissue specific manner, being present in the testis and only when mice have reached adulthood.

GFP fluorescence is present in testis and epididymis of adult $Atp1a4^{null(GFP)}$ mice

Another indication of $Atp1a4$ promoter driven expression of GFP in the male gonad during development was obtained by directly following the fluorescence of whole testis samples at

488 nm. Testis from 7 ddp, 18 ddp and adult $Atp1a4^{null(GFP)}$ mice were dissected and analyzed under regular and fluorescent light. As shown in Fig. 6a, no specific label corresponding to GFP was observed in wild type and $Atp1a4^{null(GFP)}$ mouse testis at either 7 dpp or 18 dpp. Also, no GFP fluorescence was observed in wild type adult testis. In contrast, significant label was detected in testis of adult $Atp1a4^{null(GFP)}$ mice expressing GFP (Fig. 6a). In addition, we also studied direct fluorescence of wild type and $Atp1a4^{null(GFP)}$ mouse epididymis. Similar to testis, GFP fluorescence was also apparent in epididymis of adult $Atp1a4^{null(GFP)}$ mice (Fig. 6b). These observations further indicate that expression of GFP under the $ATP1a4$ promoter is seen in both testis and epididymis of the adult $Atp1a4^{null(GFP)}$ mice.

GFP expression driven by the Atp1a4 promoter has a developmental and cell type specific pattern of expression within $Atp1a4^{null(GFP)}$ mouse testis

To further study expression of GFP within the testis, immunocytochemistry of testis sections was performed, using an anti GFP antibody. As shown in Fig. 7a, testis from wild type mice showed no label for GFP either at 7 dpp, 18 dpp
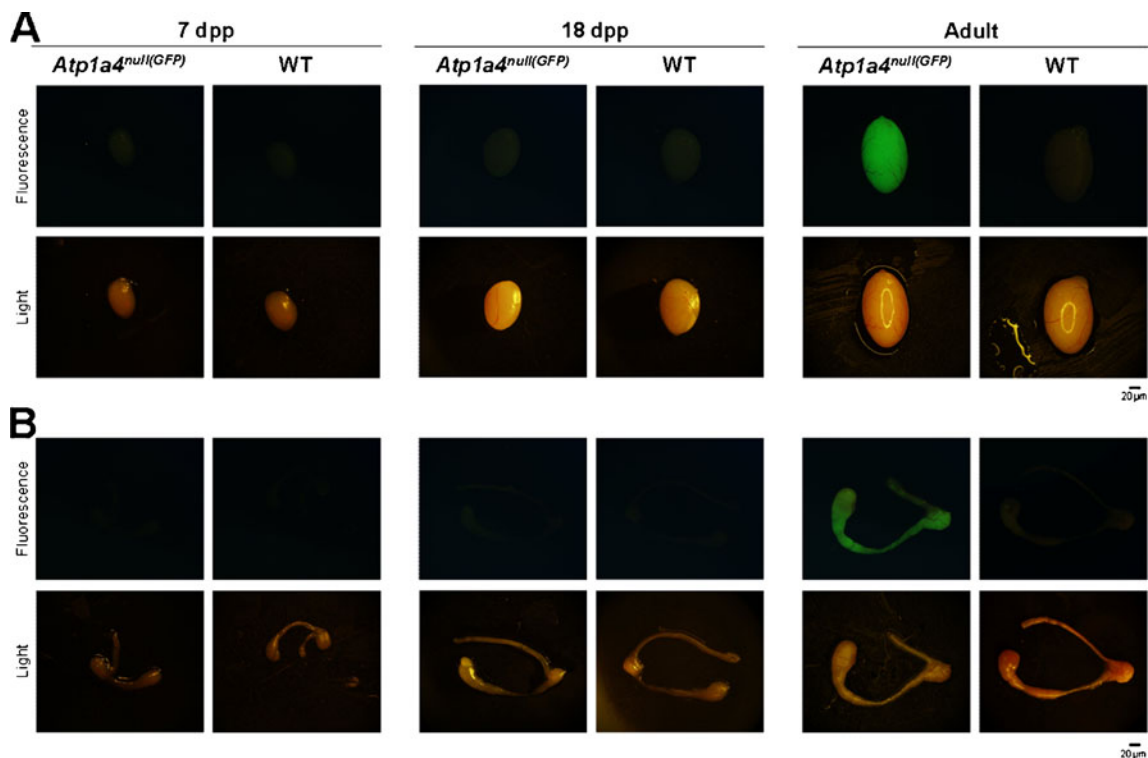

Figure 6 Testis and epididymis of $Atp1a4^{null(GFP)}$ mouse show GFP fluorescence. Tissues were dissected and whole mount light and fluorescent microscopy for testis **a** and epididymis **b** from 7 dpp, 18 dpp and adult mice was determined. Samples were examined using a Leica MZFLIII stereomicroscope (Leica Microsystems, Buffalo Grove, IL) and images were captured with an Olympus DP72 digital camera
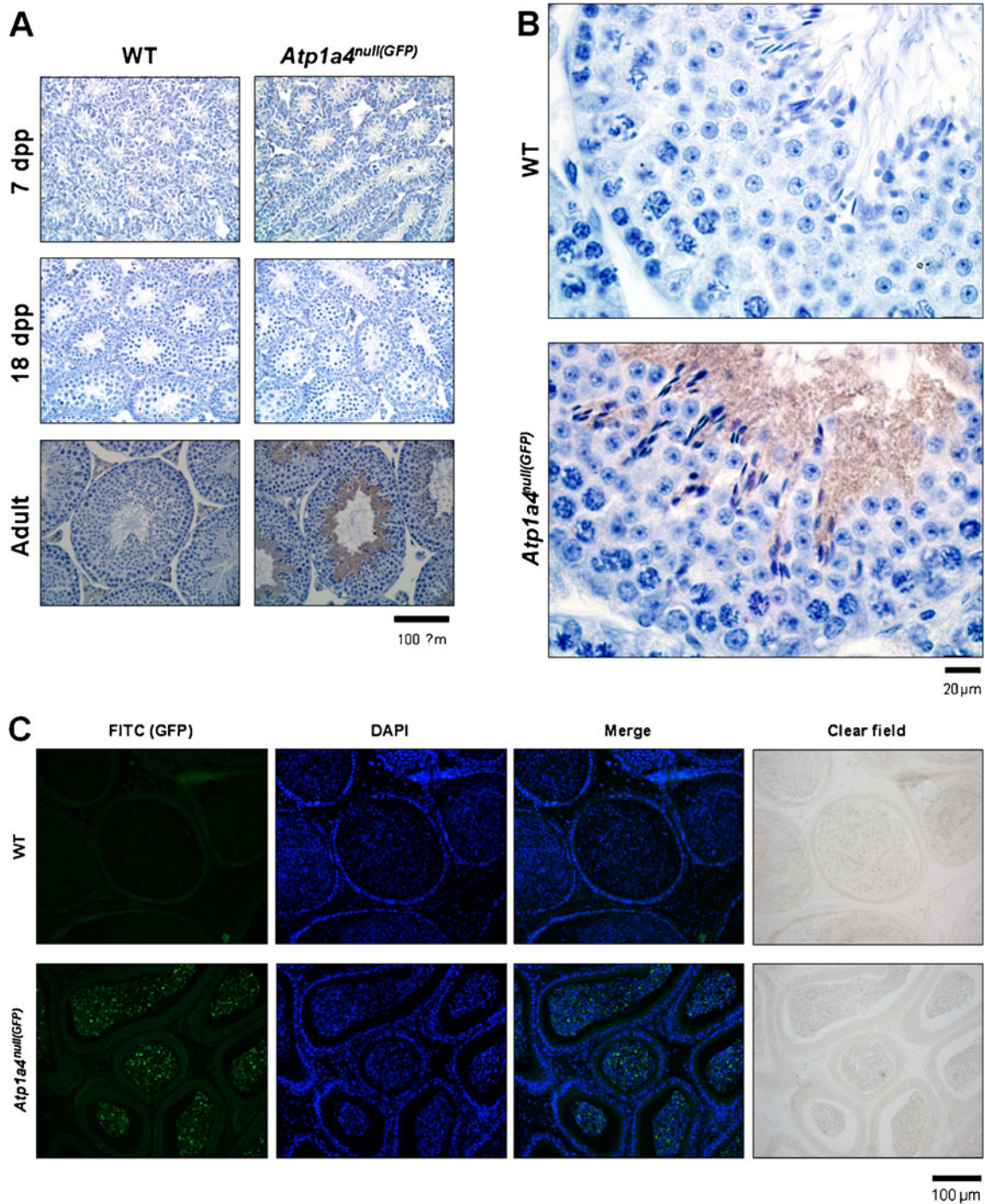
Fig. 7 GFP is expressed in male germ cells of the testis and in spermatozoa in the epididymis of *Atp1a4*^{*null(GFP)*} mice. **a, b** Testis from 7 dpp, 18 dpp and adult mice were fixed overnight in Bouin's fixative and embedded in paraffin. Tissue section was performed using a Leica CM3050S cryostat. Samples were hydrated, permeabilized with 0.3 % Triton X-100, and quenched with 3 % H_2O_2. Samples were labeled with anti-GFP overnight followed by incubating with a biotinylated goat anti-rabbit IgG. Staining and detection was performed following manufactures instruction (Vectastain ABC Kit, Vector laboratories, Burlingame, CA). Samples were counter stained with hematoxylin, dehydrated, and mounted in Permount Mounting Media (Sigma Chemical Co, St. Louis, MO). Images were captured using a Nikon Eclipse 80i scope (Nikon Instruments, Melville, NY), using a 40 X **a** or 100 X **b** objective. **c** Epididymis from wild type and *Atp1a4*^{*null(GFP)*} mice were dissected and processed for immunocytochemistry, using an anti-GFP antibody and fluorescently labeled secondary antibodies. DAPI was included to stain the cell nuclei. Clear field images were obtained using regular light microscopy

or adulthood. Similarly, GFP was not identified in *Atp1a4-null(GFP)* mouse testis at 7 and 18 dpp. In contrast, Atp1a4^{null} ^(GFP) adult mouse testis presented GFP, which was localized to the inner region of the seminiferous tubules (Fig. 7a). When samples were viewed at higher magnification, GFP staining was observed specifically at the later stages of male germ cell development (Fig. 7b). No staining was observed in less differentiated male germ cells, such as spermatogonia and early spermatocytes. In addition, the anti-GFP antibody did not label the Sertoli or the Leydig cells (Fig. 7b). These results support the idea that *Atp1a4* promoter dependent expression of GFP takes place mainly in male germ cells and not somatic cells of the testis and that it is limited to late stages of spermatogenesis.

Using also immunocytochemical analysis, we followed GFP expression in the epididymis from adult *Atp1a4^{null(GFP)}* mice. As shown in Fig. 7c, specific label for GFP was identified in this organ and it was only localized to the spermatozoa present in the lumen of the epididymal tubules. No specific GFP reactivity was found in the epithelial cells lining the tubules. This indicates that GFP expression controlled by the *Atp1a4* promoter is confined to the differentiated male germ cells stored in the epididymal tubules.

GFP expression in adult *Atp1a4^{null(GFP)}* mouse testis is limited to post meiotic male germ cells

To further determine the association of GFP expression with a particular cell type population in the testis, we isolated testis cells from *Atp1a4^{null(GFP)}* mice based on their fluorescence at 488 nm, by FACS and subjected the isolated cell populations by immunoblot, using antibodies against cell type specific markers. We used the Dsx- and *mab-3*-related transcription factor 1, DMRT1 to label Sertoli cells [23], the transcriptional repressor protein PLZF for spermatogonia [24], the DEAD family protein DDX4 as a marker for spermatocytes and round spermatids [25] and the kinesin KIF17b as a postmeiotic marker of male germ cells and spermatozoa [26]. Also we included GFP as a control for cell sorting and tubulin as a loading control. As shown in Fig. 8a, before being subjected to cell sorting, dissociated cells from wild type mice were positive for all cell markers, except for GFP. Dissociated cells from *Atp1a4^{null(GFP)}* mice showed the additional expression of GFP (Fig. 8a). In cells from *Atp1a4^{null(GFP)}* mice, cell sorting generated a GFP positive population that displayed the presence of GFP and were enriched in DDX4 and KIF17b, indicating the presence of spermatocytes, round spermatids and spermatozoa, but had almost undetectable levels of DMRT1 and PLZF, characteristic of Sertoli cells and spermatogonia (Fig. 8b). In contrast, the cells that showed no fluorescence after sorting had no GFP, low levels of DDX4, no KIF17b and high expression of DMRT1 and PLZF (Fig. 8b). Altogether, these
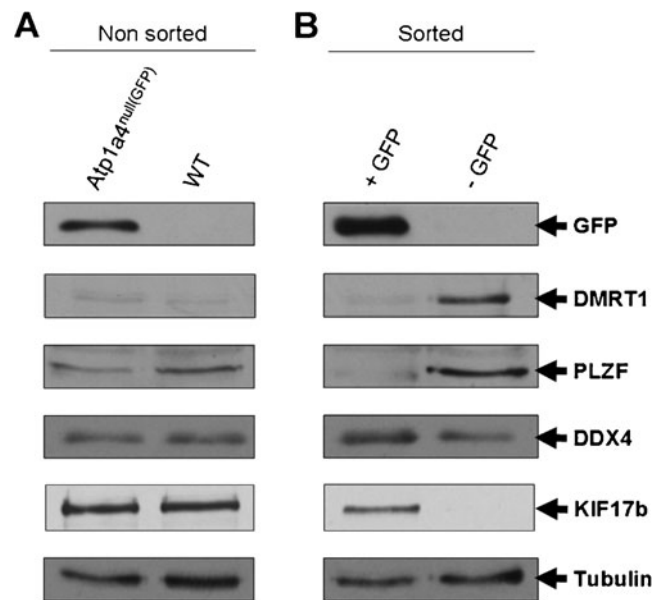

Fig. 8 GFP is expressed in male germ cells of the testis late in spermatogenesis. Cells were isolated from adult testis of wild type and *Atp1a4^{null(GFP)}* mice and subjected to immunoblot analysis, before **a** or after sorting **b**, based on their GFP fluorescence at 488 nm using FACS. Blots were probed using antibodies against GFP, Dmrt1, PLZF, DDX4 and KIF17b. Tubulin was also determined in the samples as a control for gel loading

results suggest that the *Atp1a4* promoter drives expression of GFP in the testis seminiferous epithelium and specifically in the male germ cells that have reached more differentiated stages of spermatogenesis.

Discussion

In this work, we have used the expression of GFP, driven by the *Atp1a4* promoter, as a reporter for the expression of the Na,K-ATPase α4 isoform in mice. Different from previous studies, which detected α4 using RT-PCR and immunoblotting techniques, this genetic approach provided us with an alternative powerful means to define the tissue pattern of expression of α4 during development and its presence in different cell types of the male gonad. In addition, knock in of GFP under the *Atp1a4* promoter further proved the function of α4 via disruption of the *ATP1a4* gene. Introduction of GFP efficiently blocked expression of the α4 isoform and eliminated the highly ouabain sensitive Na,K-ATPase activity typical of α4. Moreover, replacement of α4 with GFP resulted in a phenotype that reproduced that of the α4 null mice [15]. This consisted in the *Atp1a4^{null(GFP)}* male mice being completely infertile and exhibiting several sperm alterations, including a bend in the sperm flagellum and a drastic reduction in sperm motility. While expression of GFP in place of α4 affected sperm morphology and function, it did not alter the size or macroscopic and histological morphology of the testis and epididymis. This

suggests that expression of α4 is not necessary in defining testis and epididymal structure or the development of either somatic or male germ cells in those tissues. The similarity in phenotype between the α4 knock out mice [15] and the present $Atp1a4^{null}$ $^{(GFP)}$ mice show that targeting of the Na,K-ATPase α4 isoform gene was appropriate and suggests that the resulting defects in the mice are a consequence of the absence of the α4 isoform rather than expression of GFP. Additional support for this are our previous data in which over-expression of GFP as a fusion protein with the rat α4 isoform in mice increased sperm motility without affecting fertility of the α4-GFP mice [20]. The deficiency in α4 in $Atp1a4^{null(GFP)}$ mice was not compensated through up-regulation of expression and activity of the other α isoform of the Na,K-ATPase of testis, the α1 polypeptide. This lack of compensation agrees with our previous observations in α4 null mice [15] and provides further evidence that Na,K-ATPase isoform diversity is a biologically relevant event, with the α4 isoform being specifically suited to fulfill the particular requirements of sperm function.

Concurrent with deletion of the α4 isoform, $Atp1a4^{null(GFP)}$ mice efficiently expressed GFP as detected by the increase in intrinsic fluorescence and appearance of reactivity to the anti-GFP antibody in immunoblots and histological sections of testis and epididymal samples. We found that GFP expression was uniquely limited to the testis and epididymis. This protein localization coincides with previous observations that found α4 RNA and protein in rat and human testis and in the mouse epididymis [14, 27–29]. The adequate expression of GFP under the $Atp1a4$ promoter further allowed us to follow the developmental changes of GFP as an indicator of α4 temporal regulation of expression. GFP expression in $Atp1a4^{null(GFP)}$ mice was only observed in testis and epididymis of adult mice, being undetectable in whole body sections from $Atp1a4^{null(GFP)}$ mouse embryos, or in testis and epididymis from 7 and 18 day old pre-pubertal mice. These results agree with previous observations, which showed that α4 expression and hydrolysis of ATP dependent on α4 are significantly up-regulated with testis development in rats [8, 9]. Those studies however, showed differences regarding the precise onset of α4 expression and while one of them detected low levels of α4 protein in testis at 7 and 18 days after birth [9], the other identified α4 mRNA and protein in the male gonad at four and six weeks of age respectively [8]. In agreement with this last report, our present data indicate a late start for GFP expression by the $Atp1a4$ promoter, taking place at post pubertal stages of testis development.

Our immunochemical and GFP based cell sorting experiments showed that the $Atp1a4$ promoter directed expression of GFP specifically in male germ cells and not in Leydig or Sertoli cells of $Atp1a4^{null(GFP)}$ mouse testis. These results differ from those of Konrad and coworkers, which reported expression of the α4 isoform in Sertoli cells [16]. In that study, however, the α4 isoform was identified at the mRNA level using RT-PCR and not at the protein level. In addition, that work was performed in a rat-derived Sertoli cell line and in Sertoli cells isolated from rat testis, in which cell homogeneity of the preparation depended on the purification steps performed. Besides these differences, an alternative explanation that may account for the finding of α4 message in Sertoli cells is the possibility that while Sertoli cells can transcribe the α4 DNA into RNA, they are unable to translate the α4 message into polypeptide molecules. Further experiments are needed to ascertain this last possibility. On the other hand, in agreement with a specific male germ cell localization of the α4 isoform in testis are previous observations which have used antibodies against the Na,K-ATPase α4 isoform [8, 9].

Our current data also show that the $Atp1a4$ promoter induces expression of GFP in male germ cells at late stages of spermatogenesis. This is apparent from the immunocytochemical labeling of testis sections with GFP and from the expression in GFP positive cells of DDX4 and KIF17b, markers for spermatocytes, spermatids and spermatozoa respectively. However, while expression of GFP could be barely detected in spermatocytes through the immunochemical analysis of testis sections (Fig. 7), it could be identified in spermatocytes after cell sorting based on their GFP fluorescence. The finding of GFP expression earlier in spermatogenesis (i.e. spermatocytes) using FACS as compared to immunocytochemistry may depend on the higher sensitivity that the cell sorting approach has over the immunocytochemical analysis. These results also suggest that expression of GFP in spermatocytes is low and less than in spermatids and spermatozoa. Another indication that GFP expression in spermatocytes is low comes from our immunoblot analysis, which could not detect GFP in testis of 18 dpp mice. Our previous analysis of the α4 isoform expression in rat male germ cells separated via unit gravity sedimentation showed that α4 is up-regulated during spermatogenesis and while α4 mRNA increases in spermatocytes, α4 protein rises in spermatids [9]. Therefore, it appears that the $ATP1a4$ promoter does already drive low levels of GFP expression in spermatocytes. The $ATP1a4$ promoter driven expression of GFP at late stages of spermatogenesis is consistent with our previous observations, which showed that the proximal 5' untranslated region of the human Na,K-ATPase $ATP1a4$ promoter contains CRE binding elements that respond to the testis specific activator of transcription CREMτ [17], a transcription factor that is a master controller of post-meiotically activated genes of male germ cells [30]. Therefore, our current data support the notion that the $ATP1a4$ promoter forms part of the transcription regulatory machinery that is up-regulated after meiosis to serve important roles in the mature spermatozoa.

Our immunoblot and immunocytochemical data show that GFP expression is high in the differentiated spermatozoa, which are clearly labeled by the anti-GFP antibody in the testis, epididymis and after swim up of the cells. Lack of GFP labeling in prepubertal mouse testis, which is devoid of spermatozoa also supports the notion that most of the protein

expression driven by the *Atp1a4* promoter is aimed to the differentiated spermatozoa. Expression of GFP is primarily located to the mid-piece of the sperm flagellum, a site that corresponds to that previously described for the α4 isoform [9, 10, 14]. However, some staining for GFP also appears in other segments of the sperm tail and little GFP appears to be even present in the sperm head as seen in the immonocytochemical labeling of GFP in testis and epididymis (Fig. 7). This wider localization of GFP compared to α4 is probably not surprising, since GFP may not contain the structural determinants within its sequence to target this protein to the appropriate domain on the sperm plasma membrane.

In conclusion, this new mouse model that we have generated shows that the *Atp1a4* promoter directs protein synthesis specifically in male germ cells of the testis at late stages of spermatogenesis. This particular spatial and temporal transcriptional control of expression exerted by the *Atp1a4* promoter is consistent with the need of α4 for motility and fertility of the male gamete. In addition, this mouse line represents a valuable tool for future studies of Na,K-ATPase α4 isoform expression and function.

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