Published in final edited form as:
J Pathol. 2013 May ; 230(1): . doi:10.1002/path.4161.

Left-sided cryptorchidism in mice with Wilms Tumour 1 gene deletion in gubernaculum testis

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Abstract

A significant number of patients with germline mutations in the Wilms tumour 1 (*WT1*) gene, a transcriptional factor essential for early renal and gonadal development, displays cryptorchidism or non-scrotal testis position. We show here that WT1 is expressed during development in the mouse gubernacular ligament connecting the testis to the abdominal wall. Conditional inactivation of *Wt1* in the gubernaculum (GU-WT1KO animals) resulted in abnormal differentiation of the gubernacula during development and in about 40 % adult males, unilateral, always left-sided, cryptorchidism. At birth the right testis was positioned above the processus vaginalis and eventually moved into the developing scrotal pouch. In affected mutants the left testis was displaced from the normal position and the left processus vaginalis failed to form. The analysis of testicular descent at different stages of postnatal development suggests that the unilateral cryptorchidism might be caused by the asymmetry in the position of abdominal organs providing a higher degree of mobility for the left testis. Spermatogenesis in GU-WT1KO animals was blocked in cryptorchid testes located in a high pararenal position but was maintained in testes located in a low abdominal position. Conditional inactivation of both *Wt1* and androgen receptor (*Ar*) genes in gubernaculum led to a bilateral asymmetrical cryptorchidism in all mutant males with the left testis again located higher than the right one. The malformations induced by WT1 and AR deficiency in gubernaculum and processus vaginalis, in combination with mechanical constraints on testis descent, determine the final position of the testes. In summary, our data indicate that WT1 is directly involved in gubernaculum differentiation. Taken together the results of the study underline the complex nature of testicular descent with an involvement in this process of several genetic factors and developmental events.

Keywords

WT1; androgen receptor; testicular descent; cryptorchidism

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Conflict of interest statement: All authors declare the absence of conflict of interest in this work.

Statement of author contributions

AIA, EMK, conceived the experiments, collected and analysed data, searched the literature and generated figures; GN conducted the experiments and collected data; VH analysed data. All authors were involved in writing the paper and had final approval of the submitted version.

Introduction

Cryptorchidism or non-scrotal testis position is one of the most common congenital abnormalities with an estimated 1-4% incidence in term-born boys [1]. In most cases cryptorchidism is presented as an isolated abnormality, but it is also often linked with more complex syndromes affecting the normal development of urogenital system, sex determination and testis function. The degree of testicular maldescent varies from a high intraabdominal position, associated with the complete disruption of germ cell development, to more mild manifestation of high scrotal testis position. Unilateral testicular maldescent occurs more frequently than bilateral [2].

The initial descent of the male gonads from a high pararenal position into the scrotal region is directed by the differentiation of inguinoscrotal ligaments, or gubernacula, derived from the mesenchymal cells of the metanephros, and the regression of cranial suspensory ligaments which run between the cranial pole of the internal gonad and the dorsal abdominal wall [1]. Mouse transgenic experiments demonstrated that this phase of testicular descent is controlled by insulin-like 3 (INSL3) hormone, produced in testicular Leydig cells, and signalling through its cognate receptor RXFP2 (relaxin family peptide receptor 2) which is expressed in the gubernaculum [3-7]. During the second stage, the descent of the testes to the scrotum is mediated by complex developmental events involving further differentiation of gubernacula, shortening of the gubernacular cord and anchoring of the testis to the region above the internal inguinal ring. This is followed by the formation of the processus vaginalis (PV) through an invagination of the gubernacular bulb, development of the cremasteric sac, and myogenesis leading to the differentiation of cremaster muscles [1]. The second stage of testicular descent is controlled by androgens signalling through the androgen receptor (AR) in target organs [8]. Testis descent is complete at birth in humans and by postnatal day 12 (P12) in mice [8].

Wilms tumour 1 (WT1) is a transcription factor expressed in early embryonic development that plays a major role in the differentiation of renal and reproductive systems [9]. Human patients with germline mutations of the *WT1* gene develop syndromes associated with Wilms tumour and/or nephropathy, such as WAGR (Wilms tumour, Aniridia, Genitourinary abnormalities, mental Retardation), Denys–Drash and Frasier syndrome. A significant number of such patients have an abnormal development of reproductive organs ranging from XY sex reversal and gonadal dysgenesis to less severe genital abnormalities. Among other congenital defects, cryptorchidism or a non-scrotal testis position is often noted. In mice, homozygous *Wt1* deletion causes embryonic lethality with a failure of kidney and gonad development [10-12]. In such animals the cells of the metanephric blastema undergo apoptosis, the gonadal ridges fail to differentiate, and there is no metanephric kidney. All these events occur before testicular descent, and thus, the systemic ablation of *Wt1* in mice makes it difficult to separate the direct effects of *Wt1* on the development of anatomical structures involved in testicular descent from the indirect effects due to an abnormal Leydig cell development and potentially decreased hormone production in mutant testis. It was suggested however that the occurrence of testicular maldescent in patients with WT1 mutations might not be a consequence of genital defects [13].

WT1 and AR are coexpressed in cells of human gubernaculum [14], and several reports suggest that AR gene expression can be regulated by WT1 in cells of urogenital linage [14, 15]. As AR signalling plays a central role in the second phase of testicular descent [8], it was proposed that the cryptorchidism in WT1 mutants might be the result of abnormal masculinization [14]. Here we report that the targeted inactivation of *Wt1* in the mouse gubernaculum results in left-sided cryptorchidism, thereby demonstrating that WT1-associated cryptorchidism is not secondary to the failed masculinization of gonads, but a

direct result of abnormal gubernacular differentiation. The possible mechanisms involved in the asymmetry in testes descent in these mutants were investigated.

Materials and methods

Animal breeding

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at FIU and conducted in accordance with the National Academy of Science Guide for Care and Use of Laboratory Animals. Conditional inactivation of the floxed *Wt1*^{fl} allele [12] was achieved by interbreeding with *Rarb-cre (Tg(Rarb-cre)1Bhr*) [16] to produce *Wt1*^{fl}*/Wt1*^{fl}*, Rarb-cre* males (GU-WT1KO thereafter). Mice with floxed *Ar*^{fl} allele (*Ar*$^{tm1.1Verh}$) were described previously [17]. *Wt1-cre (Wt1*$^{tm1(EGFP/cre)Wtp/J)}$ [18] and ROSA26-LacZ reporter mice (*Gtrosa26*^{tm1Sor}) [19] were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were genotyped by PCR using allele-specific primers described in the original publications.

Histology, immunohistochemistry and LacZ staining

The mouse embryos, proximal body segments of P1, P3, and P7 pups, or adult organs were collected, fixed, embedded in paraffin, and 7 μm frontal and sagittal sections were cut. Immunohistochemistry (IHC) was performed overnight at 4°C using the following antibodies: WT1 (1:1000; Dako North America, Carpinteria, CA), AR (1:800; Santa Cruz Biotechnology, Santa Cruz, CA); desmin (1:1500; Sigma-Aldrich, St. Louis, MO). Detection was performed using a Vectastain ABC (avidin–biotin–peroxidase) kit (Vector Laboratories, Burlingame, CA) as recommended. The colour was developed with diaminobenzidine (DAB) as chromogen. Samples were counterstained with Harris Hematoxylin. To evaluate expression of the Cre transgene in neonatal gubernaculum, we crossed females homozygous for ROSA26-LacZ reporter with *Rarb-cre* transgenics. Male newborn pups were frozen in Tissue-Tek OTC medium, sectioned at 12-15 μm, stained using a β-galactosidase kit (Cell Signaling Technology, Inc., Danvers, MA), and counterstained with eosin.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from target tissues using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. cDNA was synthesized using an oligo(dT) primer and RETROscript kit (Ambion, Austin, TX). A Q-PCR SybrGreen real time assay on an Eppendorf Mastercycler ep realplex instrument (Eppendorf, Westbury, NY) was used for the real time quantitative RT-PCR (qRT-PCR). β-Actin gene expression was used for normalization of the data. The relative fold change in mRNA level was calculated by the comparative C_t ($2^{-\Delta\Delta C_t}$) method. Data from at least 5 samples for each genotype were statistically assessed with 2-tailed Student's *t* test using GraphPad software (La Jolla, CA). All PCR primer sequences are available upon request.

Flow cytometry of mouse testis cells

The testicular cell suspensions were prepared as described previously [20]. After counting, cells were fixed in 70% ice-cold ethanol and stored at 4°C until flow cytometry analysis. The cells were stained with propidium iodide and analysed in an AccuriC6 flow cytometer (Becton-Dickinson Immunocytometry, San Jose, MI). The fluorescent signals were recorded and a histogram of DNA intensity versus cell count was used to compare cell populations from different samples. A total of 500, 000 events were recorded for each histogram. The relative numbers of cells (1N = haploid, 2N = diploid, 4N = tetraploid, S-phase) were calculated. Three animals were analysed for control and mutant groups. Student *t*-test for

two group comparisons was used to assess significance of differences. The analysis was performed using the GraphPad software.

Results

Conditional inactivation of *Wt1* gene in gubernaculum causes unilateral left-sided cryptorchidism

The IHC analysis of WT1 expression at early stages of gubernacular development (E13.5, embryonic day 13.5) showed a strong signal in the proximal part of the gubernaculum, the gubernacular cord, and to a lesser degree in the distal part, the gubernacular bulb (Figure 1Aa). As expected, the strong expression of WT1 was detected in Sertoli cells (Figure 1Ab). In newborn gubernaculum (P1, postnatal day 1) the WT1 expression was detected both in the mesenchymal cells within the ligament and in the epithelial cells lining the gubernaculum (Figure 1Ba). To delete *Wt1* in the gubernaculum we used *Rarb-cre* transgene, in which Cre recombinase, under the control of the retinoic acid receptor β2 promoter, is expressed in tissues of the urogenital system beginning from day E9.5 [16]. Analysis of Cre expression using LacZ reporter in *Rarb-cre*, ROSA26-LacZ revealed strong blue staining in the gubernacular cord of P1 males (Figure 1Bb) in addition to the gubernacular bulb expression as we have shown previously [8]. We concluded that the *Rarb-cre* transgene can be used for targeting *Wt1* in the gubernaculum. Using a two generation breeding scheme we produced mice homozygous for $Wt1^{fl}$ allele with or without *Rarb-cre* (GU-WT1KO and control). Both GU-WT1KO males and females were obtained in the expected ratios indicating their normal viability. No differences in body weight were observed in 2-4 month old males (GU-WT1KO, 26.0±0.5g vs control, 26.9±0.8g). All external and internal reproductive organs in mutants were fully developed suggesting normal masculinization (Figure 1C). Dissection of 54 GU-WT1KO and 22 control males at 2 month and older revealed that 39% of mutants (21/51) had left testis located abdominally (Figure 1C), whereas all control and 61% mutants had scrotal testes. The majority of males with cryptorchidism (17) had a left testis located at the same level or slightly above the bladder (Type-a, Figure 1Cb,c). The remaining 4 animals had a left testis at high pararenal position (Type-b, Figure 1Cd). PCR of genomic DNA isolated from adult cremaster muscles demonstrated the presence of the deleted allele on both descended and undescended sides (see Supporting information, Figure S1). Analysis of 2-4 month old males revealed no significant difference in the weight of the left and right control testes, or the right or left testes from the 61% of GU-WT1KO mice with normal testicular descent (Figure 1D). In contrast, the weight of the cryptorchid left testis in Type-a GU-WT1KO males was reduced comparing with the descended right testis (73.1±3.7 mg vs 93.3±3.0 mg, $p<0.0001$, n=11). The size of the cryptorchid Type-b testis was reduced four times vs right one (24.7±0.9 mg vs 98.7±3.2 mg, $p<0.0001$, n=3). Normal spermatogenesis was observed in control testes, in right and left descended testes, and in the left testes from Type-a GU-WT1KO males (Figure 1E). However, germ cell development was severely disrupted in left Type-b cryptorchid testis (Figure 1Ed). The left Type-a epididymis was attached to the small poorly developed non-inverted cremaster muscle through a long cord (Figure 1D). The left Type-b epididymis was reduced in size and attached through the long fascia to the body wall; the cremaster muscle was not developed (Figure 1D). When crossed to wild-type females no differences in fertility of GU-WT1KO mutant males with unilateral cryptorchidism was found, suggesting that the descended testis compensated for any deficiency of the undescended one. None of the mutant animals developed gonadal or other malignancies.

Abnormal gubernacular development and the delay of PV in GU-WT1KO males during the inguinal stage of testicular descent

In contrast to the 39% of adult GU-WT1KO males that displayed unilateral cryptorchidism, 91% (10/11) of newborn GU-WT1KO males displayed clear abnormal testicular position (Figure 2A). No abnormalities were found in 9 littermate controls. The initiation of PV formation was delayed on both sides in GU-WT1KO males. The right testis, however, was always situated in the same distal position above the developing external inguinal ring as in the wild-type controls. The left testis was often misplaced, positioned more proximally, and in extreme cases located in the dorsal abdominal region leftward to the bladder. Both left and right mutant gubernacula had a small size and abnormally long cords (Figure 2C). The long gubernacular cords and small gubernacular bulbs on both sides were also clearly visible in mutants at prenatal stage E17.5 (data not shown). No cranial suspensory ligaments were detected in mutant males at this stage.

At P3 43% (3/7) of analysed GU-WT1KO males displayed an abnormal left testis position with the long gubernacular cord. At P7 50% (4/8) of GU-WT1KO males had the left testis remaining in the abdominal cavity with the left PV not properly formed (Figure 3). At this stage in control animals the gubernacular bulb was inverted and differentiated into PV and crematerіc sac. The left gubernacular bulb in affected mutants failed to undergo an inversion, increased in size and muscle content (Figure 3Bc). Mutant gubernacular cord was often attached to the side of the PV with the epididymis located below the point of attachment (Figure 3Bb,c), whereas the wild-type epididymis was always positioned above the gubernacular cord (Figure 3Ab,c). Developing cremaster muscles were thinner in mutant mice than in controls. The left testes of the affected mutants were found in dorsal abdominal region (Figure 3Bd).

To determine whether WT1 ablation in the GU-WT1KO mice affected AR expression, we performed IHC on mutant and control sections at day P7. As we have reported [8], at this stage in wild-type mice AR-staining was observed predominantly in the non-differentiated fibroblasts in the base of gubernacular cord (Fig. 4). There was some reduction of AR-positive cells in *Wt1* mutants, but not as much as reported for GU-ARKO males [8]. The WT1 deletion affected myogenesis in PV as revealed by staining with desmin, a marker of muscle cells (Figure 4). Importantly, both sides were affected in mutants. Quantitative RT-PCR analysis of the left (cryptorchid) and right (normal) gubernacula from P3 mutant males and the gubernacula from littermate controls did not detect significant differences in the relative *Ar, Esr1*, or *Rxfp2* gene expression; the expression of the genes involved in myogenesis (*Pax7, Acta1, Acta2*); or in the expression of genes important for reproductive organs and gubernacular differentiation (*Sox9, Ctnnb1, Wnt4, Wnt5A, or Sfrp1*) (Supporting information, Figure S2). Previously it was shown that the deletion of the floxed exon in $Wt1^{fl}$ allele did not affect its expression [12]. We also did not find differences in the expression of *Wt1* gene between left and right side in mutant or wild-type pups (data not shown).

Conditional inactivation of WT1 and AR in gubernaculum leads to a bilateral cryptorchidism

WT1 is expressed in numerous urogenital organs that are also the targets of androgen signalling. Inactivation of AR using an Cre transgene knocked-in into the *Wt1* locus led to a testicular feminization phenotype of XY males which displayed external female organs and small intraabdominal testes (Supplemental information, Figure S3). This again suggested that a deficiency of AR signalling in cells where WT1 was inactivated could be a contributing factor in causing cryptorchidism in GU-WT1KO males.

As previously noted, ablation of AR results in defective testicular descent [21]. Because of this and the reports that WT1 can regulate AR expression, we investigated whether ablation of both AR and WT1 would result in a more dramatic phenotype than ablation of either gene alone. We produced mice with simultaneous conditional inactivation of both *Wt1* and *Ar* genes using the same *Rarb-cre* transgene (Figure 5). All fourteen 3 month old double knockout GU-WT1KO, GU-ARKO males analysed had bilateral cryptorchidism (Figure 5A). One male had a symmetric position of low abdominal testes (Type-c) similar to GU-ARKO males [8], one had a slightly higher position of the left testis (Type-ca) and the remaining twelve males (Type-cb) had left testis in high intra-abdominal position (Figure 5A,B). In all 14 double mutant mice, the right cremaster muscle was poorly developed and the right testis stayed above external inguinal ring (Figure 5A, 4B). Left cremaster muscle was affected even more severely and the left small epididymis was attached through a long collagenous ligament to the body wall (Figure 5B). The deletion of both genes had therefore an additive effect on testis maldescent.

All stages of spermatogenesis including elongated spermatids and spermatozoa were present in control and in the right low abdominal testes of double knockout males (Figure 5Ca-c). Germ cell development was severely disrupted in the left high intraabdominal testes (Figure 5Cd). DNA staining and fluorescence activated cell sorting (FACS) was performed to analyse quantitatively the changes in different germ cells in mutant testis. The FACS histogram showed that the testicular cell populations of haploid, diploid, S-phase and tetraploid cells were not different between control and Type-a GU-WT1KO mutant males (Figure 6). In contrast, the FACS histogram demonstrated a dramatic reduction of haploid cells in left high intraabdominal GU-WT1KO, GU-ARKO cryptorchid testes, but not in the right descended testis, confirming the histological observations.

Discussion

WT1 gene encodes a transcriptional factor involved in early differentiation of urogenital organs. In addition to Wilms tumour and nephropathy, human male patients with germline *WT1* mutations often have genital abnormalities and cryptorchidism. Having demonstrated that *Wt1* is expressed in embryonic gubernaculum, we subsequently conditionally abated it in derivatives of metanephric mesenchyme, which includes the gubernaculum ligament critical for normal testicular descent. About forty per cent of adult mutant males displayed unilateral left-sided cryptorchidism, suggesting that WT1 plays a direct role in the differentiation of structures involved in testicular descent. The *Rarb-cre* transgene used in these studies is highly expressed even before testicular descent in metanephric mesenchyme [16] and hence causes deletion of *Wt1* at early embryonic stages. Nevertheless, the neonatal testes in mutant animals were located in a low abdominal position, suggesting that the initial, transabdominal phase of testes descent that is regulated by INSL3/RXFP2 was fully completed in mutants. Indeed, we showed that the expression of *Rxfp2, Ctnnb2*, or some *Wnt* genes was not affected, suggesting that INSL3/RXFP2 signalling was fully active in mutants and thus did not require WT1. The second phase of testicular descent is androgen dependent [8]. We showed that the deletion of AR in cells expressing *Wt1*caused full testicular feminization suggesting that the function of two genes might be linked, as suggested previously [14]. Indeed, while we did not find significant changes in relative expression of *Ar* in neonatal gubernaculum, the absolute number of AR-positive cells was visibly reduced in the base of GU-WT1KO gubernacula on both sides. Previously, we have suggested [7, 8] that these cells might be responsible for gubernaculum inversion and the PV formation, the events delayed or deficient in affected GU-WT1KO mutants. The reduction of AR signalling does not fully explain, however, the cryptorchidism of GU-WT1KO males. *Ar* knockout using the same *Rarb-cre* (ARKO mice) results in bilateral cryptorchidism with the testes located in a suprascrotal position [8], whereas the GU-WT1KO mice displayed

unilateral cryptorchidism with left testes remaining higher up in the body cavity. When *Ar* and *Wt1* were inactivated together we observed a phenotype that combined the features of both mutations: bilateral cryptorchidism, as observed in the ARKO mouse, with the left testis located in higher position as in GU-WT1KO mutants. Thus while there might be some overlap in WT1 and AR signalling, the abnormalities caused by the deletion of the two genes are distinct.

The question arises, why only the left side is affected in mutant mice? When mutant males were analysed at embryonic or neonatal stages, before the initiation of inguinoscrotal descent, the same abnormalities were found in the development of the left or right gubernacula in almost all newborn mutant males. On both sides the gubernacular cords were abnormally long providing an increased mobility to the testes. Additionally no differences in expression of different markers, associated with testicular descent, were detected between two sides. The right testis however was always positioned above the developing scrotum sac, whereas the left one was often displaced. We hypothesize that in addition to the bilateral anatomical abnormalities observed on both *Wt1*-deficient gubernacula, the asymmetrical position of internal organs creates mechanistic restrictions on the movements of gonads. It appears that the left testis had a higher degree of freedom to move behind the bladder that was positioned more to the left from the midline. Thus the left testis was moved from its normal position and remained there during development. The displacement of gonads was correlated with the failure of PV development, suggesting that the presence of testis or epididymis above the scrotal area was required for the invagination. Thus, several factors, such as poor gubernaculum differentiation combined with the absence of cranial suspensory ligaments, mechanistic restrictions, and perhaps different abdominal pressure on two gonads led to the shift of the position of the epididymis and testis at the beginning of inguinoscrotal descent, and eventually caused abnormal scrotal sac development and testicular maldescent. Such combination of genetic and developmental causes appears to be unique for this mutant, as no other animal models show preferential unilateral cryptorchidism [22].

The abnormalities in spermatogenesis correlated with the relative position of the cryptorchid testis, but not with the specific gene ablation. The testis weight was slightly smaller in undescended testes located in a low abdominal position, but the size decreased dramatically for testes located in high abdominal position. The spermatogenesis was defective in small high intraabdominal testes, but appeared normal in an abdominal mutant testes positioned lower. This contrasts with the suppression of germ cell development in older GU-ARKO mice described previously [8] or in the left testis of double GU-WT1KO, GU-ARKO males described here. It is possible that some additional sites of *Rarb-cre* expression cause AR deficiency within the cryptorchid testes affecting normal spermatogenesis in such mutants. Alternatively, the variations in testis position caused some slight variations in the testis temperature, the amount of abdominal fat surrounding the gonads, or other changes that might provide less permissive conditions for maintenance of germ cell development. Further analysis of these two models might provide the clues to the link between cryptorchidism and infertility.

Cryptorchidism is a common congenital defect in humans and other mammals. We showed, that early transcriptional factor WT1, involved in gonadal and reproductive tract differentiation, plays a crucial role in early gubernacular differentiation. Analysis of testicular descent in GU-WT1KO males demonstrated a complex interplay of different genetic, anatomical, and developmental factors in testicular descent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Drs. Richard Behringer (University of Texas MD Anderson Cancer Center, Houston, TX, USA), Guido Verhoeven and Karel De Gendt (Katholieke Universiteit Leuven, 3000 Leuven, Belgium) for providing *Tg(Rarb-cre)* and *Ar*-floxed mice. We are thankful to Mr. Agustin Barbara for technical support. This work was supported by the Eunice Kennedy Shriver National Institute of Child Health & Human Development grant R01HD37067 to AIA and National Cancer Institute CA34936 grant to VH.

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List of abbreviations

AR	androgen receptor
CSL	cranial suspensory ligament
GU-ARKO	mice with conditional inactivation of androgen receptor gene in gubernaculum
GU-WT1KO	mice with conditional inactivation of Wilms tumour gene in gubernaculum
IHC	immunohistochemistry
PV	processus vaginalis
qRT–PCR	quantitative real-time polymerase chain reaction
WT1	Wilms tumour gene

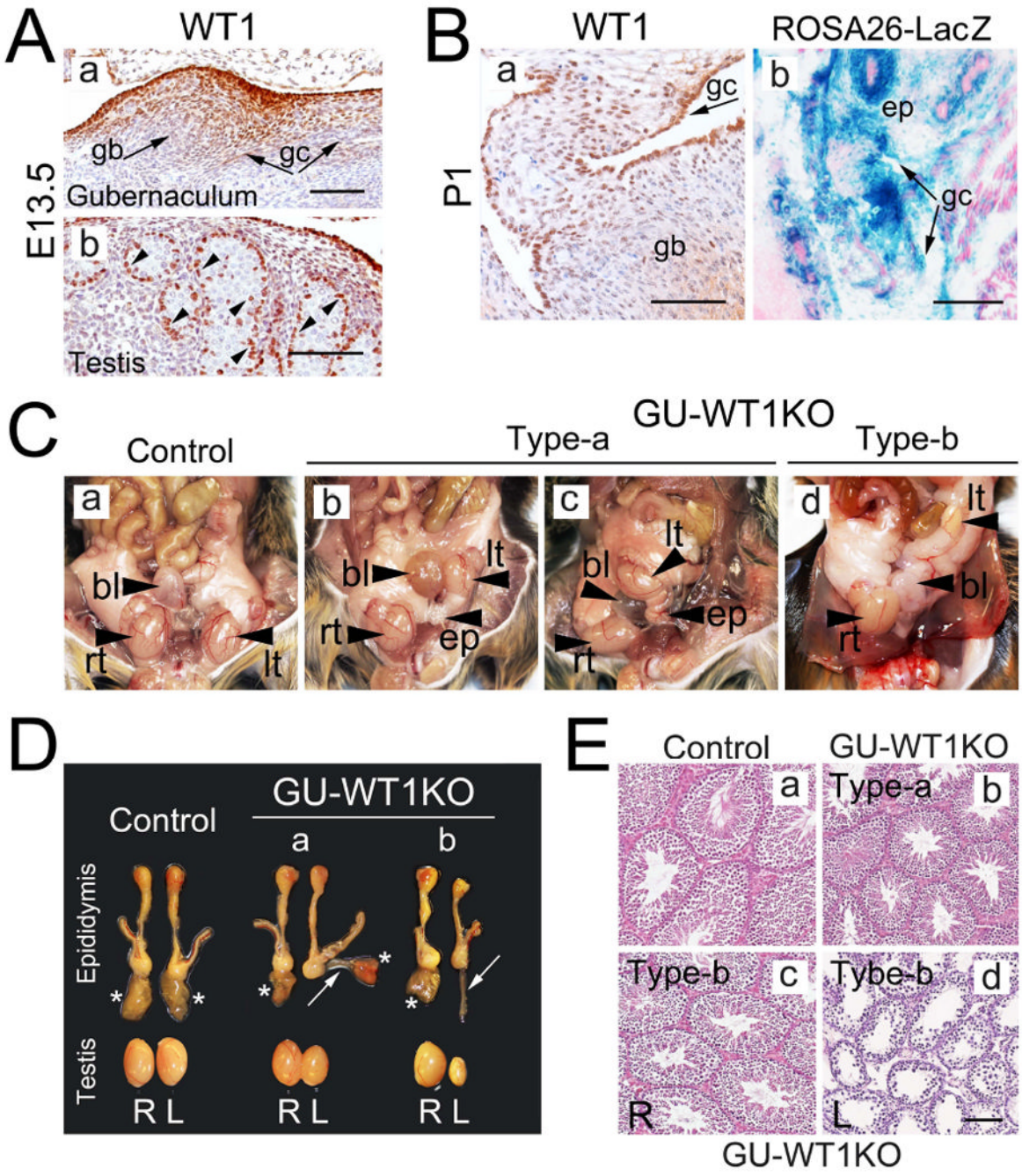

Figure 1.

Unilateral left-sided cryptorchidism in GU-WT1KO (*Wt1*^{fl}/*Wt1*^{fl}, *Tg(Rarb-cre)*) male mice. (Aa) Immunohistological detection of WT1 expression in gubernacular bulb (gb) and gubernacular cord (gc) of E13.5 male. (Ab) The WT1 expression in Sertoli cells in E13.5 testis was used as a positive control. (Ba) WT1 expression in P1 (newborn) gubernaculum. (Bb) Expression analysis of the Cre transgene in a *Tg(Rarb-cre), Gtrosa26*^{tm1Sor} gubernaculum at day P1. The expression of Cre recombinase leads to an activation of β-Gal activity of the ROSA26 allele and blue staining. Strong expression was detected in gubernacular cord and epididymis (ep). (C) Position of the right (rt) and left (lt) testes in control and GU-WT1KO males. In Type-a unilateral cryptorchidism (Cb,c) left testis is

located at the bladder (bl) level. In Type-b unilateral cryptorchidism (Cd) left testis is at high intraabdominal position. In euthanized males the left cryptorchid testes cannot be manually pushed into the scrotum in GU-WT1KO males. (D) Morphology of epididymides, cremaster muscle (marked with *) and testes from 14 week old control and GU-WT1KO males. Note the decreased left testis size in cryptorchid Type-a (a) and Type-b (b) males. The cremaster muscle in Type-a is poorly differentiated and connected to cauda epididymis by a long ligament (arrow). The small left epididymis in Type-b males connected to the abdominal wall with the thin fascia (arrow). The left cremaster muscle is not differentiated. (E) H&E staining of control and GU-WT1 three month old testes showing the presence of all germ cells in control (Ea), left Type-a (Eb) and right Type-b (Ec) testes. The spermatogenesis in left Type-b testis (Ed) is severely affected. Scale bar, 20 μm.

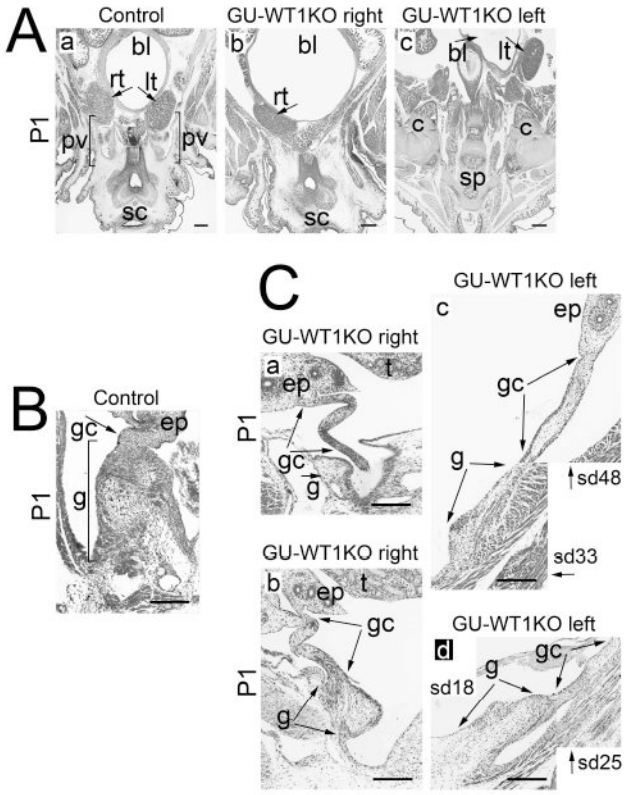

Figure 2.

Abnormal development of gubernacula and PV in GU-WT1KO males before inguinoscrotal testicular descent. (A) Asymmetrical testes position in newborn (P1) GU-WT1KO males. (Aa) Both right (rt) and left (lt) testes are located in the same position below the bladder (bl) in control males. (Ab,c) In GU-WT1KO the right testis is located in correct position (Ab), whereas the left testis is positioned more dorsally, behind the bladder (bl) (Ac). sc, scrotum sac; pv, processus vaginalis; sp, spine; c, coxa. (B) Gubernaculum in control newborn male. Note the well-developed gubernacular bulb (g) and short gubernacular cord (gc) connected to the epididymis (ep). (C) Representative images of mutant right (Ca,b) and left (Cc,d) gubernacula in GU-WT1KO males. Both gubernacular cords are long; the gubernacular bulbs do not undergo complete swelling as in controls. The presented images in (Cc) and (Cd) were consolidated from two serial sections of the same two block. The section numbers (sd) are indicated. Scale bar, 20 μm.

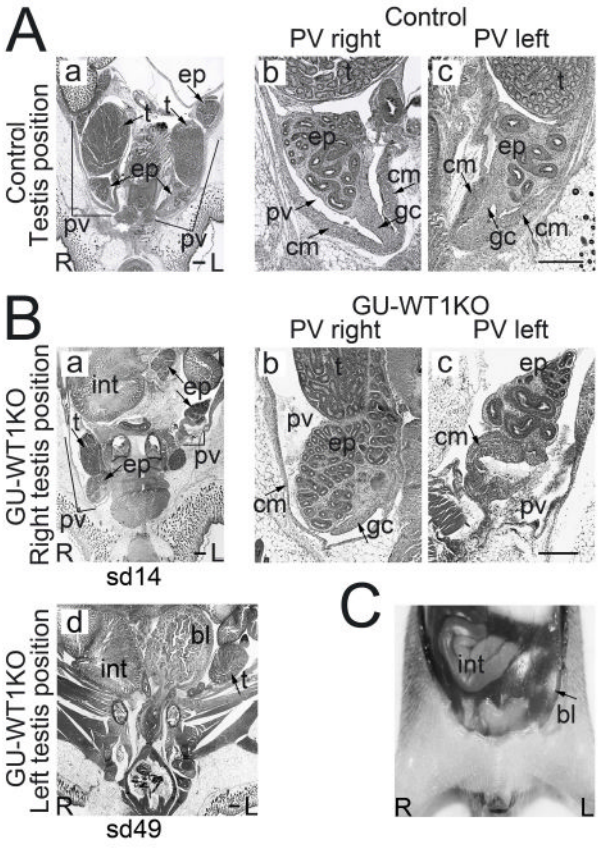

Figure 3.

Inguinoscrotal testicular descent in GU-WT1KO males at postnatal day 7. (A) Symmetrical testicular descent in control males. The abbreviations are the same as in Figure 2. PVs (strait lines) on both sides (Ab,c) are deep and contain epididymides, and parts of the testes (t). Cremaster muscles (cm) form the walls of PV. (B) Testicular descent in GU-WT1KO males. (Ba,d) The right testis in cryptorchid GU-WT1KO male is located in a normal position, whereas the left undescended testis is located in an abdominal dorsal space behind the bladder. No left PV was found. The section (sd14 and sd 49) are made from the same block. (Bb,c) The right PV is developed, whereas the left PV is not. Note an inward growth of left cremaster muscle (cm) and the asymmetrical position of internal organs. (C) Asymmetrical abdominal organs' position might be responsible for increased mobility of the left testis in GU-WT1KO males. int, intestines; bl, bladder. Scale bar, 20 μm.

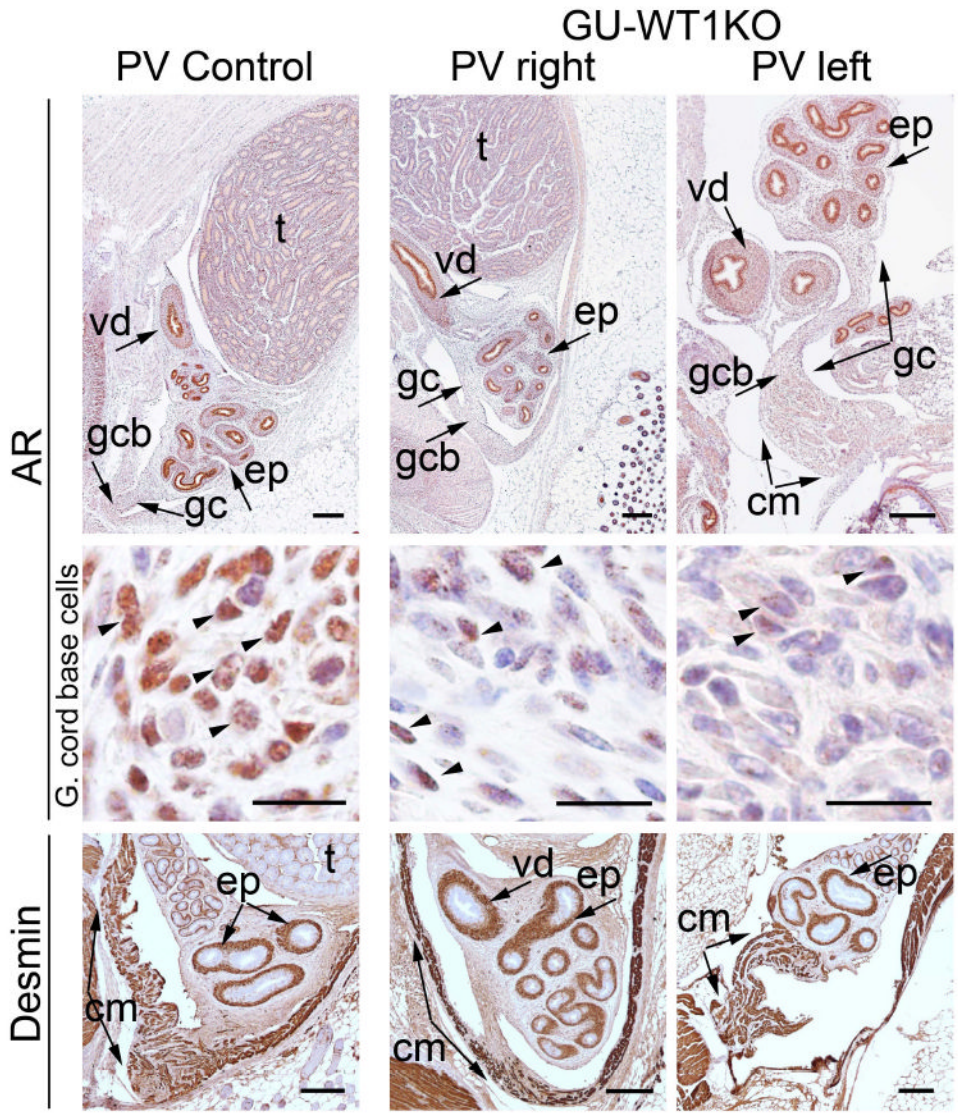

Figure 4.

Expression of androgen receptor (AR) and desmin in the gubernacula and PVs of P7 GU-WT1KO cryptorchid males. IHC with anti-AR antibody shows strong AR expression in epididymis (ep), testis (t), and vas deferens (vd). Middle row shows the reduced number of AR-positive cells (arrowheads) cells in the base of gubernacular cord (gcb) in GU-WT1KO. Muscle cell marker desmin shows well developed muscle layer (cm) of the control PV (bottom left), but much thinner layer in the right GU-WT1KO PV (bottom center). Note the desmin staining in inward growing left gubernaculum in GU-WT1KO.

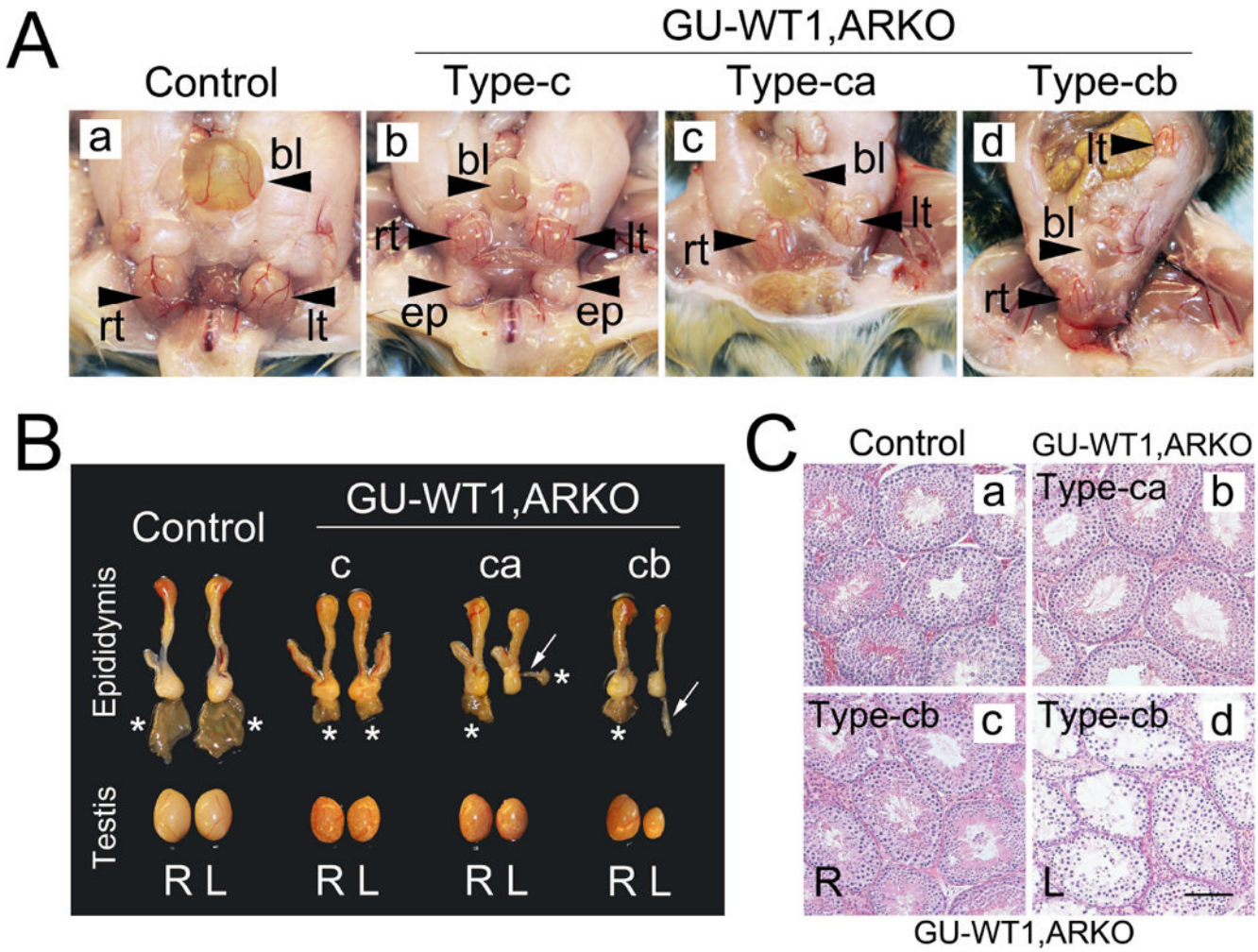

Figure 5.

Bilateral cryptorchidism in male mice with conditional inactivation of AR and WT1. (A) Scrotal position of right (rt) and left (lt) testis in control (Aa) males. Three types of cryptorchidism were detected in double mutants at 3 month of age. In all cases cryptorchid testes cannot be manually pushed into the scrotum in euthanized males. (Ab) Type-c bilateral symmetrical low intraabdominal cryptorchidism found in one out of fourteen mutant animals. Both testes are located above external inguinal ring at the same level. (Ac) In Type-ca (1/14 animals) the left testis is located slightly higher and more dorsally than the right testis. (Ad) In most of the double mutants (12/14) the left testis was located in high intraabdominal position imbedded in fat. (B) Dissection of male reproductive organs in control and mutant animals. Note the decreasing size of testis, epididymis and cremaster muscle (marked with *) in mutants with different testis position. In Type-ca small cremaster muscle is connected to the cauda epididymis with long ligament (arrow). In Type-cb left cremaster muscle is not developed, the epididymis is connected to the abdominal wall with a thin collagenous ligament (arrow). (C) H&E staining of control and GU-WT1, ARKO (double mutant) three month old testes showing the presence of all germ cells in control (Ca), Type-ca (Cb) and right Type-cb (Cc) testes. The spermatogenesis in left Type-cb testis (Cd) is severely affected. Scale bar, 20 μm.

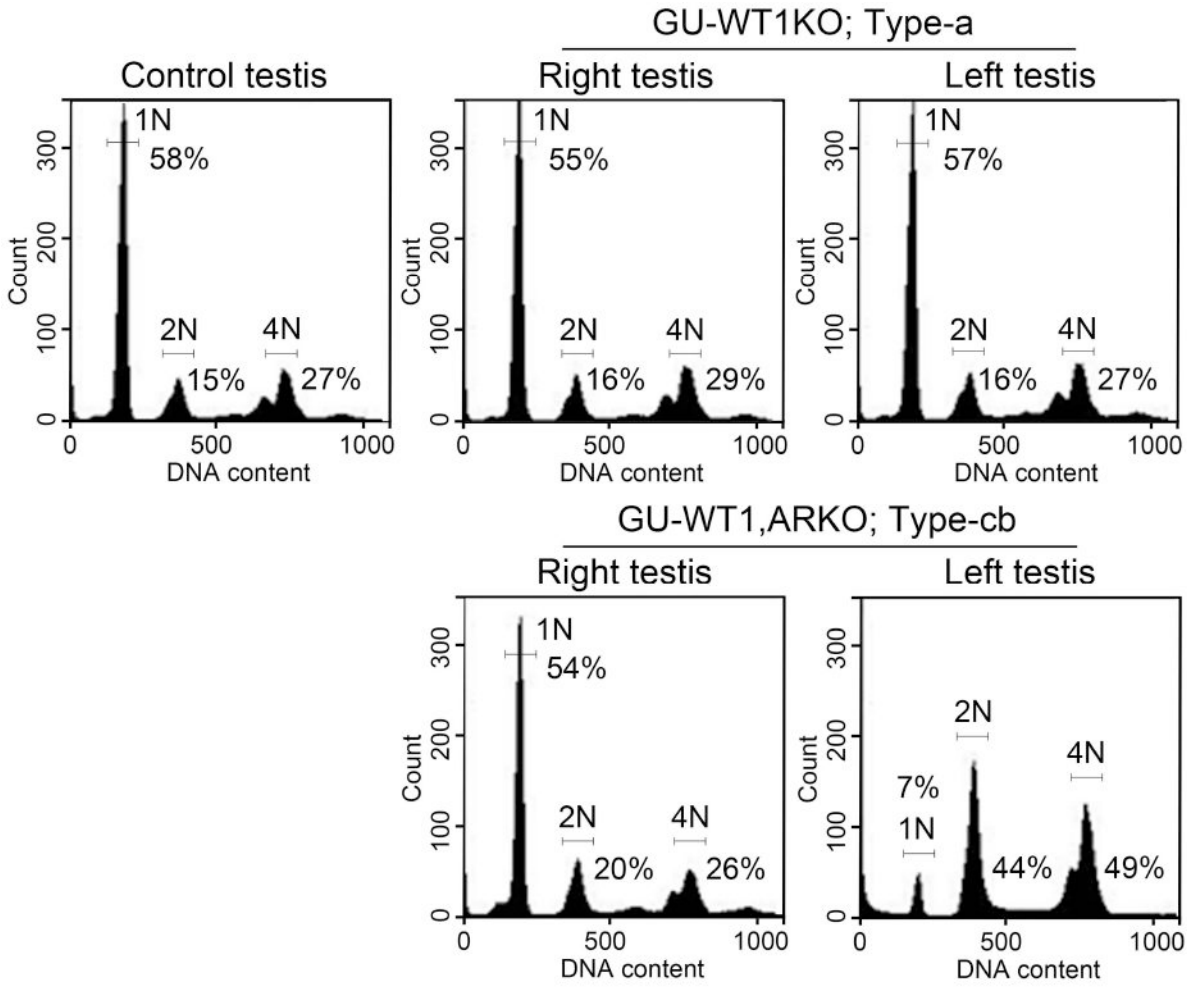

Figure 6.

Flow cytometry analysis of testis from cryptorchid GU-WT1KO males. Flow cytometry histograms of the control testis shows the per cent of haploid (1N), diploid (2N), and S-phase + tetraploid (S+4N) cells. The Y axis is the cell count and the X axis represents the DNA intensity. The representation of each cell population is indicated by %. Significant reduction of haploid cell population in high intraabdominal testis in double mutant GU-WT1KO, GU-ARKO is detected (7% vs 58% in control, $p<0.001$) consistent with the H&E histology.