AUTHOR(S)
Junnila, A., Zhang, F.-P., Martínez Nieto, G., Hakkarainen, J., Mäkelä, J.-A., Ohlsson, C., Sipilä, P., & Poutanen, M.

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HSD17B1 COMPENSATES FOR HSD17B3 DEFICIENCY IN FETAL MOUSE TESTIS BUT NOT IN ADULTS

Arttu Junnila[1], Fu-Ping Zhang[1,2], Guillermo Martínez Nieto[1,2], Janne Hakkarainen[1,#], Juho-Antti Mäkelä[1], Claes Ohlsson[3], Petra Sipilä[1, 2], Matti Poutanen[1, 2, 3]

[1]Institute of Biomedicine, Research Centre for Integrative Physiology and Pharmacology, and [2]Turku Center for Disease Modeling (TCDM), Institute of Biomedicine, University of Turku, FI-20520 Turku, Finland; [3] Department of Internal Medicine and Clinical Nutrition, Institute of Medicine, the Sahlgrenska Academy, Gothenburg University, 41345 Gothenburg, Sweden; [#]Current affiliation: Organon R&D Finland, Turku, Finland.

Correspondence: Matti Poutanen, Institute of Biomedicine, Research Centre for Integrative Physiology and Pharmacology, Institute of Biomedicine, University of Turku, Kiinamyllynkatu 10, FI-20520 Turku, Finland. Email: matti.poutanen@utu.fi, ORCID ID: 0000-0002-8953-1734

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ABSTRACT

Hydroxysteroid (17β) dehydrogenase (HSD17B) enzymes convert 17-ketosteroids to 17beta-hydroxysteroids, an essential step in testosterone biosynthesis. Human XY-individuals with inactivating *HSD17B3* mutations are born with female-appearing external genitalia due to testosterone deficiency. However, at puberty their testosterone production reactivates, indicating HSD17B3-independent testosterone synthesis. We have recently shown that *Hsd17b3* knockout (3-KO) male mice display a similar endocrine imbalance, with high serum androstenedione and testosterone in adulthood, but milder undermasculinization compared to humans. Here, we studied whether HSD17B1 is responsible for the remaining HSD17B-activity in the 3-KO male mice by generating a Ser134Ala point mutation that disrupted the enzymatic activity of HSD17B1 (1-KO), followed by breeding *Hsd17b1*/*Hsd17b3* double-KO (DKO) mice. In contrast to 3-KO, inactivation of both HSD17B3 and HSD17B1 in mice results in a dramatic drop in testosterone synthesis during the fetal period. This resulted in a female-like anogenital distance at birth, and adult DKO males displayed more severe undermasculinization compared to 3-KO, including more strongly reduced weight of seminal vesicles, *levator ani*, epididymis, and testis. However, qualitatively normal spermatogenesis was detected in the adult DKO males. Furthermore, similar to 3-KO mice, a high serum testosterone was still detected in the adult DKO mice, accompanied by upregulation of various steroidogenic enzymes. The data show that HSD17B1 compensates for HSD17B3 deficiency in fetal mouse testis but is not the enzyme responsible for testosterone synthesis in adult mice with inactivated HSD17B3. Therefore, other enzymes are able to convert androstenedione to testosterone in the adult mouse testis and presumably also in the human testis.

INTRODUCTION

The proper differentiation and development of male reproductive organs depends on the male sex hormone testosterone and its more potent metabolite dihydrotestosterone (DHT). For the reproductive organs, timely testosterone exposure is required for the stabilization of the Wolffian ducts, development of the internal genitalia, initiation and maintenance of spermatogenesis, and, after local conversion to DHT, the development of the external genitalia (1–4).

The family of hydroxysteroid (17β) dehydrogenases (HSD17B) consists of 14 currently identified enzymes that are categorized so based on their ability to either reduce 17-ketosteroids or oxidate 17β-hydroxysteroids. The preferred reaction direction, involved cofactors, and utilized substrates are highly variable within the family (5,6). HSD17B3 is considered to be the main enzyme catalyzing the 17β-reduction of androgens. Most importantly, the enzyme is involved in testosterone synthesis in the testis in both humans and rodents by converting the inactive androgen androstenedione to active testosterone (7,8). Human HSD17B1 is considered to be primarily involved in the corresponding step in ovarian estradiol production – by converting estrone to estradiol – but can also accept androgens as substrate, although with a higher K_m (9). Rodent HSD17B1, while most highly expressed in the ovary, catalyzes the conversion of estrone to estradiol and androstenedione to testosterone with similar catalytic efficacy (9,10).

Although fetal Leydig cells of rodents express other enzymes of steroid biosynthesis and produce androstenedione, they do not express HSD17B3 (11,12). Instead of Leydig cells, HSD17B3 and HSD17B1 are expressed in the fetal Sertoli cells responsible for the final conversion of androstenedione to testosterone (11,12). Postnatally, the expression of HSD17B3 disappears from the testis until puberty. After puberty, HSD17B3 is expressed in adult Leydig cells and is part of the enzymatic chain producing testosterone (12). A recent study has shown a similar pattern of expression in humans (13).

Expectedly, a deficiency of HSD17B3 activity due to inactivating germ line mutations drastically affects testosterone synthesis in XY-individuals. As a result, there is a developmental deficiency of active androgens leading to a disorder of sex development (DSD) with unvirilized or poorly virilised external genitalia, and the human individuals are often assigned female sex at birth, if not diagnosed at the time (7,14). However, the undiagnosed condition often becomes evident during puberty, due to both primary amenorrhea and progressive virilisation (7,15). The latter is caused by a sudden rise in serum testosterone, combined with greatly increased androstenedione produced by the testes (16). Testosterone is also produced to some extent during fetal development in these individuals. This is demonstrated by the presence of differentiated Wolffian duct-derived internal genitalia, distinguishing the condition from the phenotype of a complete androgen insensitivity syndrome (CAIS) caused by the

complete inactivation of the androgen receptor (AR) (16–18). It is, therefore, clear that an HSD17B3-independent pathway of testosterone production exists in men. However, due to the relative rarity of HSD17B3 deficiency, the compensatory enzyme involved in testosterone production during early development and after puberty has remained unknown (19).

We have previously generated and characterized a mouse line with a deletion of the *Hsd17b3* gene (3-KO) (20). The male mice showed endocrine imbalance similar to that in humans with HSD17B3 deficiency. In particular, XY mice displayed clear signs of androgen deficiency during the fetal period, followed by high levels of both androstenedione and testosterone after puberty, along with high LH and a reduced testosterone/androstenedione ratio. However, the *Hsd17B3* mutant male mice presented with a milder undermasculinization compared to the drastic DSD phenotype observed in humans. Similar endocrine imbalance, but an even lesser degree of undermasculinization, have been also observed by others using an unrelated *Hsd17b3* knockout model (21). The male mouse genitalia, thus, seem to be able to differentiate with only a low androgen exposure (20,22,23).

The fact that the adult 3-KO mice produce testosterone in the presence of the excess of androstenedione, just like human XY-individuals with *HSD17B3* inactivating mutations, suggests a possibility for a similar compensatory mechanism in mice and human. In addition to HSD17B3, several other enzymes with considerable HSD17B-activity converting androstenedione to testosterone have been characterized, including AKR1C3 (HSD17B5) and HSD17B1, but their role in testosterone biosynthesis in HSD17B3 deficiency has not been tested yet (8,24). A close ortholog for AKR1C3 does not exist in the mouse, but mouse HSD17B1 is similar to the human enzyme, sharing 63% amino acid identity (9). In addition, its ability for sex steroid biosynthesis has been well characterized both in humans and mice (5).

In the present study, we hypothesized that HSD17B1 is the enzyme responsible for the compensation of testosterone production in 3-KO mice and generated double knockout mice with inactivated *Hsd17b1* and *Hsd17b3* (DKO). DKO male mice were born feminized due to a drastic reduction in testosterone production during the fetal period, and compared to 3-KO males, DKO males showed greater changes in parameters dependent on fetal androgen action. However, the adult endocrine phenotype was similar in the 3-KO and DKO males, showing elevated androstenedione and testosterone in circulation and in the testes. These data indicate that in the fetal mouse testis, HSD17B1 is able to partially compensate for the absence of HSD17B3.

MATERIALS AND METHODS

Animals

We have previously described the generation of the HSD17B3KO mouse line (3-KO)(20). To generate *Hsd17b1*/*Hsd17b3* double-knockout (DKO) mice, we established a mouse line with a specific *Hsd17b1* Ser143Ala point mutation (1-KO), using the CRISPR/Cas9 gene editing system. This substitution has been shown to result in a near complete inactivation of the enzyme *in vitro* and in cell culture (25). In brief, a single guide RNA (sgRNA; 5'-GTGCTGGTGACCGCGAGTGT-3') was synthesized with GeneArt Precision gRNA Synthesis Kit (ThermoFisher Scientific, California, 92008, USA) and purified using GeneJET RNA Cleanup and Concentration Micro Kit (ThermoFisher Scientific). The quality and concentration of the sgRNA were assessed using NanoDrop spectrophotometer and Qubit RNA BR assay kit (ThermoFisher Scientific). Subsequently, we microinjected the sgRNA (25 ng/µl), Cas9 mRNA (50 ng/µl; obtained from IDT, Integrated DNA Technologies, Inc., Iowa, USA), and a repair template in the form of single-stranded DNA (ssDNA, 50 ng/µL; TCTGCCAGACATGAAGAGGCGCCACTCTGGGCGTGTGCTGGTGACCGCGGCAGTGGGAGGCTTGATGGGTGA GCGAAAGGGACAGAGTAAGAGGTTCCGA)(IDT, Integrated DNA Technologies) into C57BL/6NCrl mouse zygotes. One hundred zygotes were transferred to pseudopregnant recipient females. Thirteen pups were born from the implantations, 5 of which were positive for the mutation and used for further breedings.

DKO mice for the present experiments were obtained by mating mice heterozygous for both alleles. The mice were genotyped by PCR from genomic DNA extracted from ear biopsies, used to identify the individual mice. The primer pairs used were capable of distinguishing the wild type (WT) and knock-in allele of *Hsd17b1* (Hsd17b1KiSe; 5'- GCTTGGGACCATTCGGATGC -3', Hsd17b1KiAs; 5'-GCTGGGGTTCCCAAGCTTTA -3') after Cfr42I (SacII) restriction enzyme (Thermo Fisher Scientific) digestion of the PCR products, and the WT and deleted alleles of *Hsd17b3* were detected as described previously (20). The sex of the animals was confirmed by analyzing the presence of sex-determining region of Chr Y (*Sry*) from genomic DNA using primers SrySe (5'-TCTTAAACTCTGAAGAAGAGAC-3') and SryAs (5'-GTCTTGCCTGTATGTGATGG-3').

Mice were housed under a controlled environment (12h light cycle, temperature 21 ± 3°C, humidity 55% ± 15%, specific pathogen-free) at the Central Animal Laboratory of University of Turku. Soy-free SDS-RM3 chow (Special Diets Service, Witham Essex, United Kingdom) and tap water were available *ad libitum*. All animal experiments were approved by the Finnish Animal Ethics Committee (licenses no. ESAVI/7487/04.10.07/2013, ESAVI/41729/2019, and ESAVI/23322/2023) that also fully met the requirements as defined by the U.S. National Institutes of Health guidelines on animal experimentation.

For experiments *in vitro*, mice were sacrificed at the predetermined age by carbon dioxide asphyxiation, followed by collection of blood via cardiac puncture and cervical dislocation. Tissues (testis, epididymis, seminal vesicle, kidney, and *levator ani* muscle) and serum were collected from WT (n = 10), 1-KO (n = 8), 3-KO (n = 6) and DKO (n = 8) male mice, weighed, snap frozen in liquid nitrogen and stored at -80°C until used for analyses or fixed for histology. Newborn pups (amounting to 9 WT, 12 1-KO, 10 3-KO, and 14 DKO male pups) were sacrificed by inducing hypothermia by indirect exposure to ice, followed by decapitation, dissection, and similar freezing or fixing of testes.

Anogenital distance

The anogenital distance of the mice was determined by measuring the distance between the external genitalia and the anus with a digital caliper (Hogetex, Germany). The measurements were done from neonatal animals and at the age of 3 months.

Steroid measurements

The concentrations of serum androstenedione (A-dione), testosterone (T), dihydrotestosterone (DHT), progesterone, estrone (E1), and estradiol (E2) were analyzed by a validated GC-MS/MS, with the quantification limits of 12 pg/ml, 8 pg/ml, 2.5 pg/ml, 74 pg/ml, 0.5 pg/ml and 0.5 pg/ml, respectively. The method was also used to measure intra tissue steroid concentrations after homogenizing the tissues in sterile deionized water 1:10 (w/v) using an Ultra-Turrax homogenizer (IKA-Werke, Staufen im Breisgau, Germany), as previously described (26).

HSD17B-activity measurement

HSD17B activity was measured from tissue homogenates of newborn 1-KO, 3-KO, DKO, and WT testes (n = 4 per group). The testes were homogenized in 10 mM KH_2PO_4 (pH 7.4), 1 mM EDTA, and 20% glycerol, with cOmplete EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland), pooling 4 testes of each group in 200 µl of buffer. 20 µl/ml of homogenate was used for each reaction, and androstenedione (Sigma-Aldrich, St. Louis, MO, USA) was added to a concentration of 10 nM. The cofactor (NADPH, Sigma-Aldrich) was added to a final concentration of 1.4 mM to initiate the reaction, the samples were incubated at +37°C for 3 h, and the reaction was terminated by snap freezing in liquid nitrogen. The steroids were then measured using a previously described method on GC-MS/MS(26). The reaction rate was calculated based on the amount of product per reaction time per amount of protein in reaction.

Histological analyses

Testes and epididymides were collected for histological analysis from newborn and 3-month-old mice. Testes were fixed in Bouin fixative (Sigma-Aldrich) for approximately 24 hours at room temperature. Epididymides were fixed in 10% neutral buffered formalin (FF-Chemicals, Haukipudas, Finland) for approximately 24 hours at RT. The samples were then dehydrated, embedded in paraffin, 5 µm sections were prepared, deparaffinized and rehydrated, stained with hematoxylin and eosin (HE), and analyzed via light microscopy. The cross-sectional area of seminiferous tubules in the newborn testes was analyzed from 10 randomly selected orthogonal tubule cross-sections each from 4 WT and 4 DKO testes sections with CaseViewer software (3DHISTECH, Budapest, Hungary).

mRNA expression analyses

Total RNA was extracted from the testes of 3-month-old males (WT, n=6; DKO, n=8) with Trisure (Bioline, London, UK) following manufacturer's instructions. The RNA integrity of the samples was confirmed with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). One µg of RNA was treated with DNase Amplification Grade Kit (Thermo Fisher Scientific, Waltham, MA, USA) and used for cDNA synthesis (SensiFast, Bioline). The cDNA was used to quantify gene expression by qPCR (CFX96 Real-Time PCR detection system; Bio-Rad, Hercules, CA, USA) with the DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific). The primers used are shown in Table 1. Data were normalized to the expression of housekeeping genes *L19* and *Ppia,* and expression relative to the WT levels was calculated from Ct values using the ΔΔCt method (27).

Statistical analyses

Statistical analyses were done using GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, USA). The normality of the data was evaluated on the basis of a Shapiro-Wilk normality test. Heavily skewed data from the steroid concentration measurements underwent $\log_2$ transformation for analyses. Statistical difference between two groups was determined by two-tailed Student's t-test or Mann-Whitney test for normally and non-normally distributed data, respectively. For comparison of multiple groups, one-way ANOVA and Tukey's multiple comparisons test, or Kruskal-Wallis test and Dunn's multiple comparisons test were used for normally and non-normally distributed data, respectively. Numerical values in text are reported as means ± SD. Significance was determined as $p \leq 0.05$, and results are shown as individual values, with a center line indicating the mean unless stated otherwise.

RESULTS

Generation of *Hsd17b1* Ser143Ala mutation in mice

We have previously shown that disrupting the mouse *Hsd17b1* gene by replacing the gene with a lacZ -cassette results in a severe down-regulation of the *Naglu* gene located in the immediate proximity of

Hsd17b1 with a distance of < 1 kb, complicating the analysis of the phenotype (29). Therefore, in the present study, we generated 1-KO mice with a Ser143Ala point mutation (known to inactivate the catalytic activity of the enzyme) in mouse *Hsd17b1* gene by CRISPR/Cas gene editing (Fig.1A-B). Mice were genotyped with primers resulting in either a 319 bp PCR product for the WT allele or 240 and 79 bp products for the mutant allele after *Sac II* digestion (Fig. 1C). The Ser143Ala point mutation had no effect on the level of *Hsd17b1* mRNA expression, as determined by the RT-qPCR of the adult ovary (Fig. 1D).

Inactivation of both HSD17B1 and HSD17B3 in mice results in a markedly reduced testosterone synthesis in the fetal mouse testis

The fetal mouse testis co-expresses *Hsd17b1* and *Hsd17b3* mRNAs, both peaking between E18.5 and birth, with *Hsd17B1* mRNA peaking 1-2 days earlier than *Hsd17b3* (Fig. 2A). At birth, neither 3-KO nor 1-KO showed a reduced testosterone synthesis as demonstrated by intratesticular steroid concentrations. However, compared to WT mice (1348 ± 869 ng/g), the DKO mice showed a 7-fold reduction (182 ± 70 ng/g) in the intratesticular testosterone ($p \leq 0.01$), associated with a nearly 4-fold increase in androstenedione concentration (WT: 2075 ± 1418 ng/g, DKO: 7520 ± 841 ng/g; $p \leq 0.01$) (Fig. 2B-C). A clear difference was also observed in the HSD17B-activity in the neonatal testes, with a 20-fold decrease in the ability of the 3-KO testes homogenates to convert androstenedione to testosterone compared to WT or 1-KO testes ($p \leq 0.001$), and an even greater 250-fold decrease in the activity was observed in the DKO ($p \leq 0.001$) (Fig. 2D).

The steroid data indicate that while the expression of either of the two enzymes is enough for proper testosterone production in the fetal testis, the lack of both cannot be effectively compensated. It is of interest that even though HSD17B3 has much greater activity converting androstenedione to testosterone in the *in vitro* activity assay, the low amount of HSD17B1-activity can bring testosterone production to a normal range without a significant increase in androstenedione concentration *in vivo* (Fig. 2B-D). However, measurable levels of testosterone were also present in the DKO mouse testis, indicating that a low amount of testosterone is still being produced.

As expected due to the observed fetal testosterone deficiency, the newborn DKO males had an anogenital distance comparable to female littermates, whereas 3-KO and *Hsd17b1* mutant mice (1-KO) were indistinguishable from WT males (Fig. 2E). Despite the effect on anogenital distance, the testes of the newborn DKO males showed typical macroscopic structure and normal transabdominal descent, and the testis histology was comparable to WT males (Fig. 2F). In a closer histological analysis, no difference was seen in the cross-sectional area of seminiferous tubules (Fig. 2G).

Hsd17b1-Hsd17b3 double-KO mice show a feminized reproductive phenotype but high circulating testosterone in adulthood

The macroscopic appearance of the urogenital region of the adult DKO males demonstrated a deficiency in androgen action. All the Wolffian duct -derived structures (epididymides, vasa deferentia and seminal vesicles) had developed but were small in size, and especially the seminal vesicles were severely underdeveloped (Fig. 3A). The existence of the Wolffian duct -derived structures indicates that some residual testosterone production, however, takes place in DKO males during the fetal development.

Significant reduction in serum testosterone concentration at the fetal age and the reduced anogenital distance shown at birth was associated with a markedly reduced anogenital distance of the DKO mice also at the age of 3 months (Fig. 3B). The appearance of the external genitalia was also feminized significantly, and the presence of the penis and scrotum was not evident in the adult males. Many adult DKO males also had visible nipples (data not shown). The hypoandrogenic state was further evidenced by the reduced weight of the seminal vesicles, epididymides, testes, kidneys and *levator ani*, all being androgen-sensitive organs in mice measured at the age of 3 months (Fig 3C-D). Notably, these parameters were more severely affected in the DKO males than in the 3-KO males that show a milder undermasculinized state, according with our previous results (Fig. 3). The adult 1-KO males did not differ from the WT males.

While in the fetal testis a dramatic drop in testosterone production was seen to result from the concurrent lack of both HSD17B3 and HSD17B1 activity, no such effect was observed at the age of 3 months. Instead, the results with the DKO males closely resembled the endocrine imbalance of the 3-KO mice. Alongside high serum androstenedione (WT: 0.1 ± 0.1 ng/ml, DKO: 4.4 ± 1.4 ng/ml; $p \leq 0.001$), serum testosterone in the DKO males (15.0 ± 3.2 ng/ml) was also higher than in WT (1.1 ± 1.2 ng/ml; $p \leq 0.001$) or in 1-KO mice (Fig 4A-B). A similar increase in serum DHT was also observed (WT: 61.4 ± 69.8 pg/ml, DKO: 344.5 ± 69.5 pg/ml in DKO; $p \leq 0.001$), likely reflecting the rise in testosterone. The serum testosterone/androstenedione ratio was dramatically reduced in the DKO compared to the WT and 1-KO males ($p \leq 0.01$), mainly due to the marked increase in the androstenedione concentration (Fig. 4C).

In DKO males, intratesticular concentrations of androstenedione (1445 ± 357 ng/g) were seen to be increased in comparison with WT (133 ± 135 ng/g; $p \leq 0.001$) similarly as in 3-KO, and testosterone reached normal WT levels (Fig. 4D and E). However, the testis to serum ratio of testosterone was reduced in both 3-KO and DKO due to the larger increase in circulating testosterone compared to the rise in testes (Fig. 4F). The tissue/serum ratio of testosterone indicated a 12-fold higher concentration

in the testis of the DKO, indicating that the testes are still a net producer of testosterone in the DKO males.

We then analysed the testicular expression of mRNAs encoding the proteins involved in steroid synthesis. An upregulation of the steroidogenic pathway steps preceding HSD17B activity – *Star*, *Cyp11a1, Cyp17a1* and *Hsd3b1* – was observed in both 3-KO and DKO, with a greater increase in the latter (Fig. 4G). This likely explains the observed overproduction and accumulation of androstenedione.

The lack of HSD17B1 and HSD17B3 impairs Leydig cell maturation to a similar degree as the lack of Hsd17b3 alone

In the 8 DKO males analysed, 15 out of 16 testes were descended to the inguinal area, although could not descend to the unformed scrotum. The histology of the testes was identical to that previously shown for the 3-KO males, showing normal spermatogenesis but an abnormal appearance of the Leydig cells in the hematoxylin-eosine -stained formalin-fixed sections (Fig. 5A). Likewise, the expression of markers of mature adult Leydig cells (*Insl3*, *Nr5a1*, *Sult1e1*, *Ptgds*, and *Vcam1*) were downregulated similarly to that in the 3-KO males, indicating a comparable defect in Leydig cell maturation (Fig. 5B). Similar to WT, the cauda epididymides of DKO males were filled with spermatozoa (Fig. 5C). The active spermatogenesis confirms that the androgen action in DKO testes is sufficient for the maintenance of qualitatively normal spermatogenesis post-pubertally.

DISCUSSION

HSD17B3 is the major HSD17B-enzyme responsible for androstenedione to testosterone conversion in the testes of both rodents and primates, including men (7,8). Recent evidence indicates that the expression of HSD17B3 during testicular development is similar in humans and mice: HSD17B3 is expressed in the Sertoli cells of the fetal testis, and the expression appears in Leydig cells after the development of the adult-type Leydig cells (13). While various other enzymes with HSD17B-activity *in vitro* have been identified, their role in testosterone production in a physiological setting *in vivo* is not known (5). However, the continuing testosterone production in HSD17B3 deficient men and mice demonstrates that alternative enzymes exist (20,30). At least in conditions with supraphysiological levels of androstenedione, enzymes with only a low efficacy are also able to produce significant amounts of testosterone (31).

In addition to HSD17B3, HSD17B1 is also expressed in fetal Sertoli cells in both humans and mice (29,32). In this study, we demonstrate that the HSD17B1 activity compensates for the absence of HSD17B3 in fetal mouse testosterone production. Compared to mice lacking HSD17B3 only (20), the

lack of both HSD17B1 and HSD17B3 led to a much greater degree of feminization of the male pups. The activity of HSD17B1 is sufficient to reach normal concentrations of androstenedione and testosterone in newborn mice, and androstenedione starts to build up in neonates only when both HSD17B1 and HSD17B3 are inactive. The drastically reduced testosterone production in newborn DKO males implies that no other enzymes have significant compensatory activity during the fetal period. However, despite normal androgen levels in newborn 3-KO males, our previous data indicated a mild under-masculinization of adult 3-KO males, suggesting that HSD17B1 cannot fully compensate for the lack of HSD17B3 (20).

Although previous studies have shown that the endocrine phenotype of the 3-KO is close to that in humans with HSD17B3 deficiency, the outward phenotype and the degree of DSD were much milder in mice than in humans (20,21). The present data show that the phenotype of the DKO is a closer phenocopy of the human phenotype with the HSD17B3 inactivation, which often presents as complete feminization of external genitalia and marked hypoplasia of internal genitalia (14). The need to inactivate both HSD17B1 and HSD17B3 in mice to more accurately replicate the effects of human HSD17B3 deficiency confirms differences in fetal testosterone biosynthesis and action between the mouse and human. Similar differences between human and mouse development are seen, for example, in the mouse KO model of 5α-reductases, where again the defect of masculinization is much milder in mice than the DSD observed in humans due to 5α-reductase deficiency (23). The human HSD17B1 enzyme has a greater preference for estrone over androstenedione as a substrate, which makes it unable to compensate for the lack of HSD17B3 effectively (9). Still, we have previously demonstrated the capacity of human HSD17B1 to produce testosterone, indicated by the masculinization of female fetuses in transgenic mice over-expressing the human enzyme under Ubiquitin-C promoter (33). Thus, it is possible that HSD17B1 is involved in the low-level testosterone biosynthesis responsible for the fetal differentiation of internal genitalia in XY-individuals with HSD17B3 deficiency.

While mice lacking HSD17B1 are unable to compensate for the lack of HSD17B3 during the fetal period, this is not true in the adult animals, having a similar steroid profile regardless of whether they are missing only HSD17B3 or both HSD17B1 and HSD17B3. In both cases, the males present with a high intratesticular and serum androstenedione and testosterone concentrations. Our results confirm that yet another enzyme is capable of testosterone biosynthesis in adult male mice and that the testes are still the likely site for the remaining activity, indicated by the high testis/serum testosterone ratio. Both 3-KO and DKO mice experience a rise in testosterone after puberty, which is similar to what is observed in humans with HSD17B3 deficiency. This suggests that the compensatory mechanism is likely the same across the two species. However, the enzymes responsible for this mechanism are yet to be identified.

Several candidates exist in the families of short-chain dehydrogenase/reductase (SDR) and aldo-keto-reductase (AKR) families, composing together of nearly a hundred enzymes in humans alone, of which substrate specificities are only superficially known (34,35).

A recent study demonstrated that stimulating the testis by human chorionic gonadotropin, an LH analog, further increased the level of already elevated precursor steroids in male mice with inactivated HSD17B3, but testosterone was not affected (21). This suggests that the enzymes compensating for the lack of HSD17B3 are not under the regulation by LH. This is also the case for HSD17B3 itself, which is up-regulated by a feed-forward mechanism of androgens, thus differing from many of the steroidogenic enzymes regulated by LH (36,37). Even so, a large increase in serum LH has been previously observed in adult 3-KO mice despite the high circulating testosterone, and similarly, high LH is also seen in human patients (17,20). Thus, the lack of HSD17B3 seems to desensitize the regulation of the hypothalamic-pituitary-gonadal axis to testosterone by an unknown reason. A previous study on an *Hsd17b3* knockout mouse model has further demonstrated that reintroducing active HSD17B3 in testes normalized the concentrations of both androstenedione and circulating LH (21), indicating an active pituitary response and the revisability of the high serum LH. In humans with inactivating *HSD17B3* mutations the testosterone levels at adulthood tend to reach only the lower limits of reference values, whereas in DKO mice circulating testosterone is higher than in WT mice. This further indicates that there are differences between human and mice, either in the compensatory enzymes involved or their relative catalytic properties (38,39).

Similar to our previous observations in 3-KO, the decreased weights of seminal vesicles, *levator ani*, epididymis, and testis due to fetal steroidogenic defects were not compensated for by the high circulating testosterone in adulthood (20). Thus, the development of these organs depends on timely androgen action, despite the fact that, e.g., the kidneys and seminal vesicles are highly responsive to both testosterone deficiency and supplementation later in life (40,41). The lack of fetal testosterone exposure results in these organs being less sensitive to androgen action in later life. Such an effect has been previously shown for rodent reproductive organs, but not for other androgen-sensitive tissues (2).

Leydig cell development in the DKO was affected, resulting in cells retaining morphological and gene expression characteristics similar to those previously associated with the impaired Leydig cell maturation observed in 3-KO (20). Nevertheless, the greater defect in fetal testosterone production in the DKO compared to 3-KO males did not result in a more drastic phenotype of the adult Leydig cells, as was seen in many other parameters. The normal spermatogenesis in the adult DKO males is in line with the data obtained in LH receptor KO mice, which have very low testosterone levels during

development, but complete spermatogenesis can be restored by testosterone treatment post-pubertally (42). Thus, spermatogenesis is not dependent on early developmental priming by testosterone. In humans with HSD17B3 deficiency, the pubertal rise in testosterone does not initiate normal spermatogenesis, although the failure likely results from cryptorchidism often observed in humans but not in the mouse mutant (43). Despite the active spermatogenesis, the developmental defects of the external genitalia likely prevent the DKO males from reproducing.

In contrast to the current data, spermatogenesis was severely affected in our previous study where the whole *Hsd17b1* gene was replaced with a *lacZ*-cassette. However, as previously shown, deleting the genomic region spanning the entire *Hsd17b1* gene broadly affected the function of the genomic locus, demonstrated by the silencing of the neighboring gene (*Naglu*) (29). Thus, it is possible that the spermatogenic failure in those mice is an off-target effect caused by a mis-expression of a gene other than *Hsd17b1*. Nevertheless, the possibility that the protein also possesses a function not related to enzymatic HSD17B activity cannot be ruled out.

In conclusion, we have shown that in mice, expression of HSD17B1 in the testis efficiently compensates for the lack of HSD17B3 in converting androstenedione to testosterone during fetal development, but not at adulthood. This is likely the reason why the phenotype of DKO more closely resembles the DSD present in humans with inactivating *HSD17B3* mutations, as compared to 3-KO males. However, an enzyme other than HSD17B1 must be responsible for testosterone production in the adult 3-KO and DKO male mice.

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Author contributions

A. Junnila, P. Sipilä and M. Poutanen designed the main line of the research and carried out majority of the studies, with the essential contribution by FP. Zhang (generation of HSD17B1KI and HSD17B3KO mouse models), G. Martinez Nieto and J. Hakkarainen (enzyme activity analyses), J-A. Mäkelä (testis biology and analyses), and C. Ohlsson (steroid biology and analyses); and A. Junnila, P. Sipilä and M. Poutanen wrote first draft of the article, which was edited by all authors.

Data availability

The data generated during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Figure legends

Fig 1. Generating the *Hsd17b1* point mutation (1-KO) mice A) The Ser143Ala point mutation in the mouse genome was achieved by targeting the region of interest in exon 3 of the mouse *Hsd17b1* gene by co-injecting the guide RNA (gRNA), *Cas9* mRNA, and repair template ssDNA to the pro-nuclei of mouse zygotes. B) The point mutation of the codon coding for serine (Ser) to alanine (Ala) was confirmed by sequencing the germ line DNA. C) The genotypes of the mice were confirmed via PCR of the genomic DNA, followed by restriction enzyme digestion of the products, resulting in a 319 pb product for the WT allele and 240 and 79 bp products for the point mutation allele. D) The expression of *Hsd17b1* mRNA was not affected by the point mutation in the adult ovary, analyzed by RT-qPCR.

Fig 2. HSD17B1 compensates for the lack of HSD17B3 for the testosterone production in fetal mouse testis. A) Both *Hsd17b1* and *Hsd17b3* mRNAs are expressed in the testes during fetal development, with *Hsd17b1* peaking earlier and dropping drastically after birth, normalized to lowest values at 21 days of age. B) Disrupting both the *Hsd17b1* and *Hsd17b3* genes (DKO) in mice leads to a significant drop in intratesticular testosterone concentration in neonatal animals. In contrast, no differences are observed with disrupting either *Hsd17b1* (1-KO) or *Hsd17b3* (3-KO) gene alone. C) Likewise, androstenedione is elevated only in the DKO testis, whereas no difference is seen between wild-type (WT), 1-KO and 3-KO testis. This indicates that HSD17B1 and HSD17B3 enzymes compensate for the lack of each other in fetal mouse testis. D) Conversion of androstenedione to testosterone per mg of

protein is markedly reduced in 3-KO testes compared to the WT controls, but further reduced significantly in DKO testis. No difference is observed between WT and 1-KO testis. E) The fetal androgen deficiency is reflected in the anogenital distance of newborn DKO male mice compared to the WT males, being comparable to females. No difference was observed in WT, 1-KO and 3-KO males. F) The histology of newborn DKO testes is comparable to WT controls. G) The cross-sectional area of the seminiferous tubules in newborn mice does not differ between the genotypes. In Fig. A the data labels represent means, and whiskers indicate SD. In Figs B to E the mean is shown by a dotted line. Values in Fig. B-C are presented as Log_2-transformed. Values in Fig. D are untransformed but presented on a log_{10} scale. The scale bar in Fig. F is 50 µm. * = $p \leq 0.05$ *** = $p \leq 0.001$.

Fig 3. Adult male mice with inactivated *Hsd17b1* and *Hsd17b3* genes present with feminized reproductive phenotype. A) At the age of 3 months, the anatomy of the urogenital system in the male mice with inactivated *Hsd17b1* and *Hsd17b3* genes (DKO) is fully developed and is comparable to the wild-type mice (WT), but the internal genitalia are reduced in size. B) The anogenital distance of adult DKO males is reduced more than is observed in males with disrupted *Hsd17b3* gene (3-KO). C) Compared to WT mice, the testes and epididymides of DKO mice are reduced in size, while there was no difference in the weights between WT and *Hsd17b1* mutant (1-KO) and 3-KO mice. D) The weight of the seminal vesicles, as well as non-reproductive androgen-sensitive tissues such as kidneys and the levator ani muscle is also reduced in the DKO mice compared to WT mice. A: K = kidney, SV = seminal vesicle, T = testis, E = epididymis. B-D: Lines indicate means. * = $p \leq 0.05$; ** = $p \leq 0.01$ *** = $p \leq 0.001$

Fig 4. Testosterone is produced in adult male mice with inactivated *Hsd17b1* and *Hsd17b3* genes. A, B) At the age of 3 months, the circulating androstenedione (50-fold) and testosterone (3-fold) are both elevated in adult mice with inactivated *Hsd17b1* and *Hsd17b3* genes (DKO) compared to the wild-type (WT) mice. Similar elevations are observed in the males with inactivated *Hsd17b3* (3-KO), while no difference was observed between WT mice and those with inactivated *Hsd17B1* gene (1-KO). C) Serum testosterone/androstenedione ratio (T/A) is reduced equally in adult 3-KO and DKO males. D) The intratesticular testosterone and androstenedione concentration in DKO is elevated compared to both WT and 3-KO males. F) Compared to WT and 1-KO, the testis/serum testosterone ratio is low in both 3-KO and DKO males. G) The expression of mRNAs for *Star* and the enzymes responsible for the first steps of steroidogenesis (*Cyp11a1*, *Cyp17a1* and *Hsd3b1*), are upregulated in the testis of 3-KO and DKO males compared to WT males. Values in Fig. A-F are presented as Log_2-transformed. Lines indicate means. * = $p \leq 0.05$ ** = $p \leq 0.01$ *** = $p \leq 0.001$

Fig 5. Leydig cell maturation is similarly impaired in adult mice with inactivated *Hsd17b3* gene and those with inactivated both *Hsd17b1* and *Hsd17b3* genes. A) The histology of HE-stained testis reveals

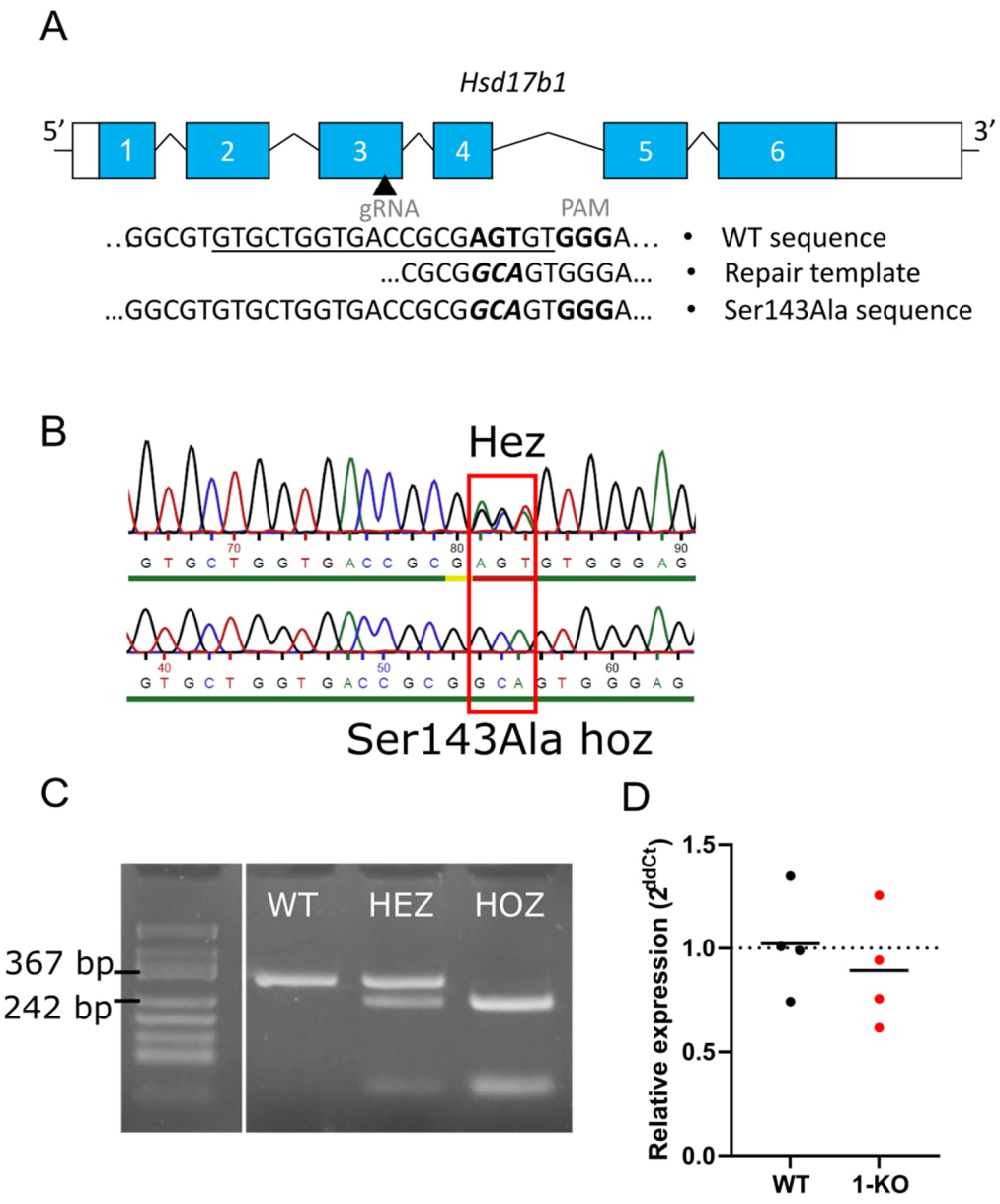
abnormal Leydig cell staining in male mice with inactivated *Hsd17b1* and *Hsd17b3* genes (DKO). A similar staining pattern is seen in mice with disrupted *Hsd17b3* (3-KO). Both mice show prominent eosinophilic areas near the nucleus. Equivalent staining is not observed in wild-type mice (WT) or in mice with inactivated *Hsd17B1* (1-KO). B) Compared to WT mice, the expression of markers of mature adult type Leydig cells is similarly downregulated in 3-KO and DKO testes. C) The cauda epididymis contains abundant mature spermatozoa in both WT and DKO mice. Scale bars in Fig. A and C are 50 µm. B: Lines indicate means. * = $p \leq 0.05$ *** = $p \leq 0.001$

Table 1. *Primers used for qPCR analysis*

Gene	Forward primer sequence	Reverse primer sequence	Gene accession number
Cyp11a1	AGATCCCTTCCCCTGGCGACAATG	CGCATGAGAAGAGTATCGACGCATC	NM_019779.3
Cyp17a1	CAAGCCAAGATGAATGCAGA	AGGATTGTGCACCAGGAAAG	NM_007809.3
Hsd3b1	CAGGAGCAGGAGGGTTTGTG	GTGGCCATTCAGGACGAT	NM_008293.4
Hsd17b1	TTTATGCCACATTGCGGGAC	CACCCACAGCGTTCAATTCA	NM_010475.2
Insl3	AGACATCTCCTGCACGCG	CAACAGGTCTTGCTGGGTG	NM_013564.7
L19	GGACAGAGTCTTGATGATCTC	CTGAAGGTCAAAGGGAATGTG	NM_009078.2
Nr5a1	CGCACAGTCCAGAACAACAA	ACATGGGCCCAAACTTGTTC	NM_139051.3
Ppia	CATCCTAAAGCATACAGGTCCTG	TCCATGGCTTCCACAATGTT	NM_008907.1
Ptgds	GGAGGCCAACTATGACGAGT	GGTTGGGGCAGGAAAACAAT	NM_008963.3
Star	CAGGGAGAGGTGGCTATGCA	CCGTGTCTTTTCCAATCCTCTG	NM_011485.5
Sult1e1	GGAGTGCAGAAACGAAGACC	AGAAACGGCGACATCTTTGG	NM_023135.2
Vcam1	ACTAAACGCGAAGGTGAGGA	TGTCTGGAGCCAAACACTTG	NM_011693.3

Full names of genes: Cytochrome p450, family 11, subfamily A, polypeptide 1; Cytochrome p450, family 17, subfamily A, polypeptide 1; 3β-Hydroxysteroid dehydrogenase/Δ^{5-4} isomerase 1; Hydroxysteroid (17β) dehydrogenase 1; Insulin-like 3; Ribosomal protein L19; Nuclear receptor, subfamily 5, group A, member 1; Peptidylprolyl isomerase A; Prostaglandin D2 synthase; Steroidogenic acute regulatory protein; Sulfotransferase family 1E member 1; Vascular cell adhesion molecule 1

588 Figure 1



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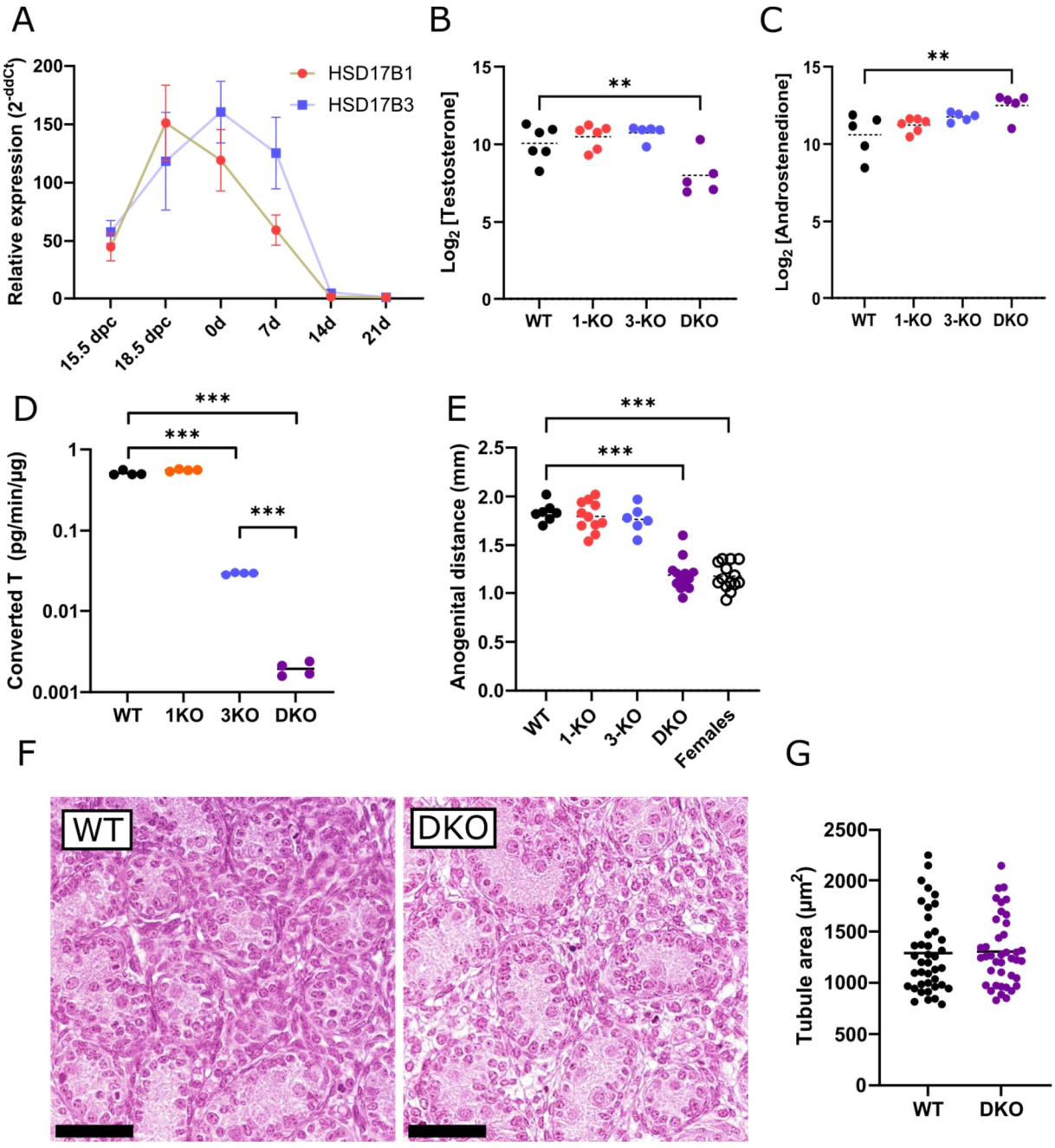
591 Figure 2



592

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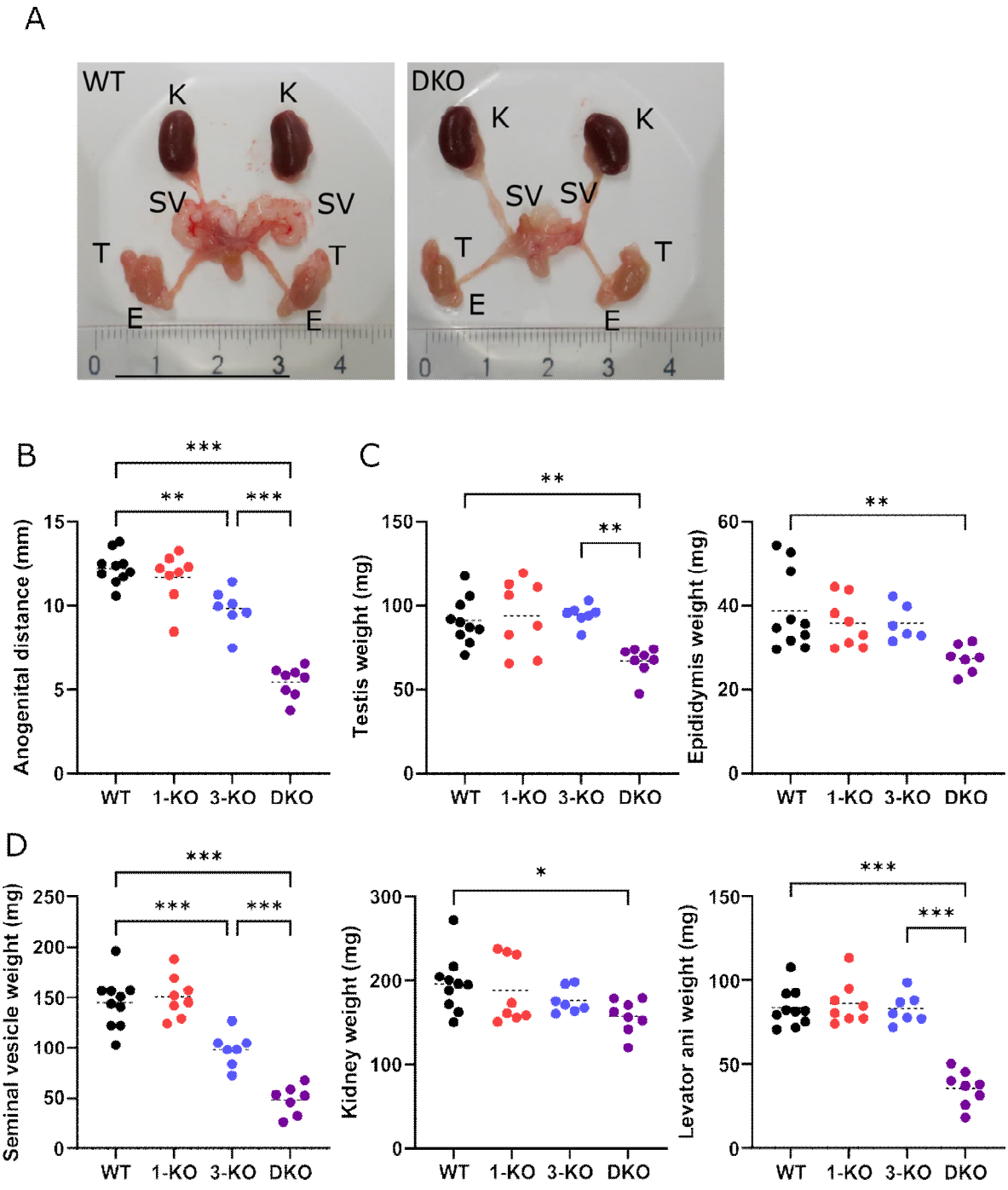
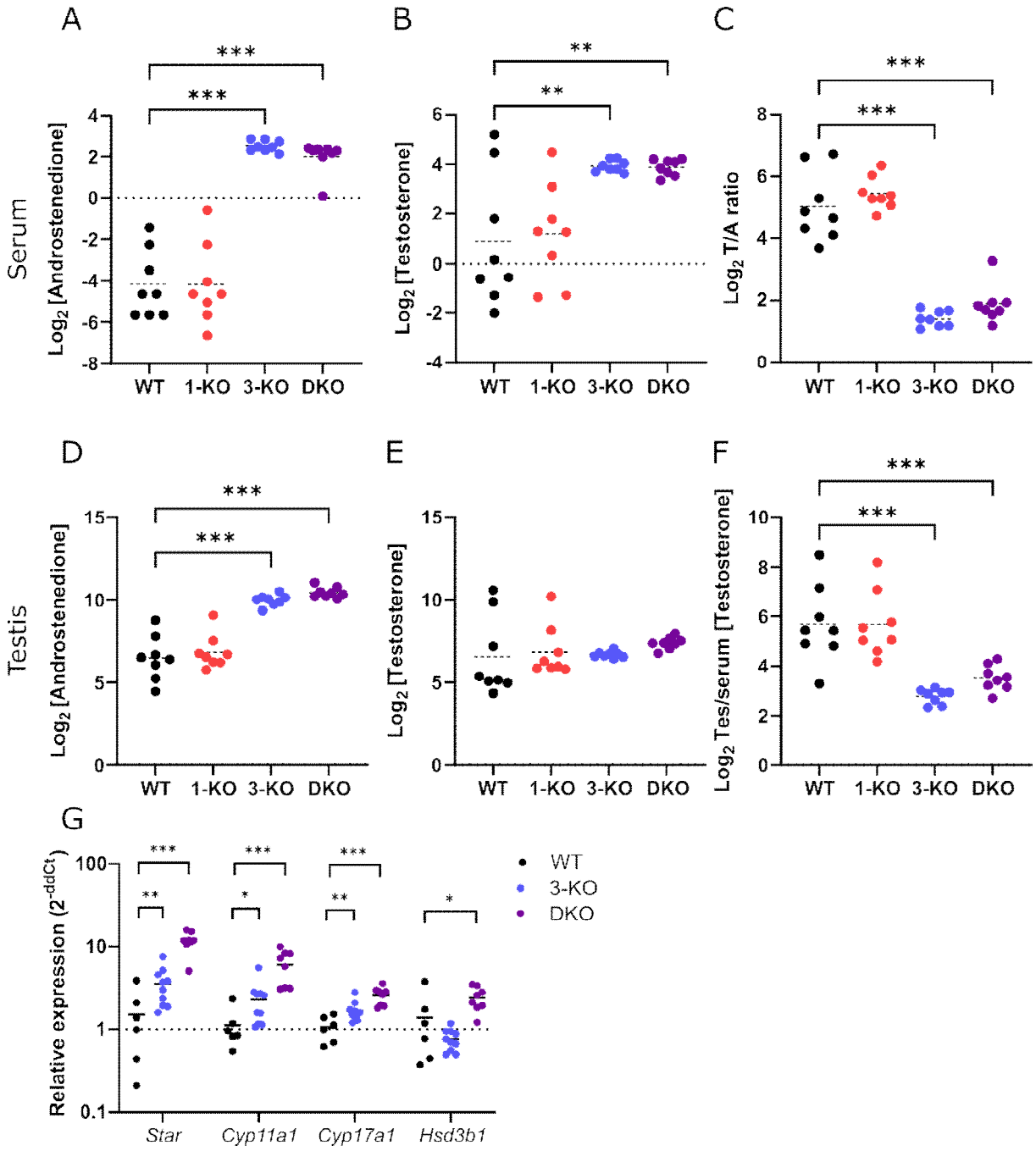
Figure 3


A
WT
K
K
SV
SV
T
T
E
E
DKO
B
Anogenital distance (mm)
C
Testis weight (mg)
Epididymis weight (mg)
D
Seminal vesicle weight (mg)
Kidney weight (mg)
Levator ani weight (mg)
WT
1-KO
3-KO
DKO

597 Figure 4



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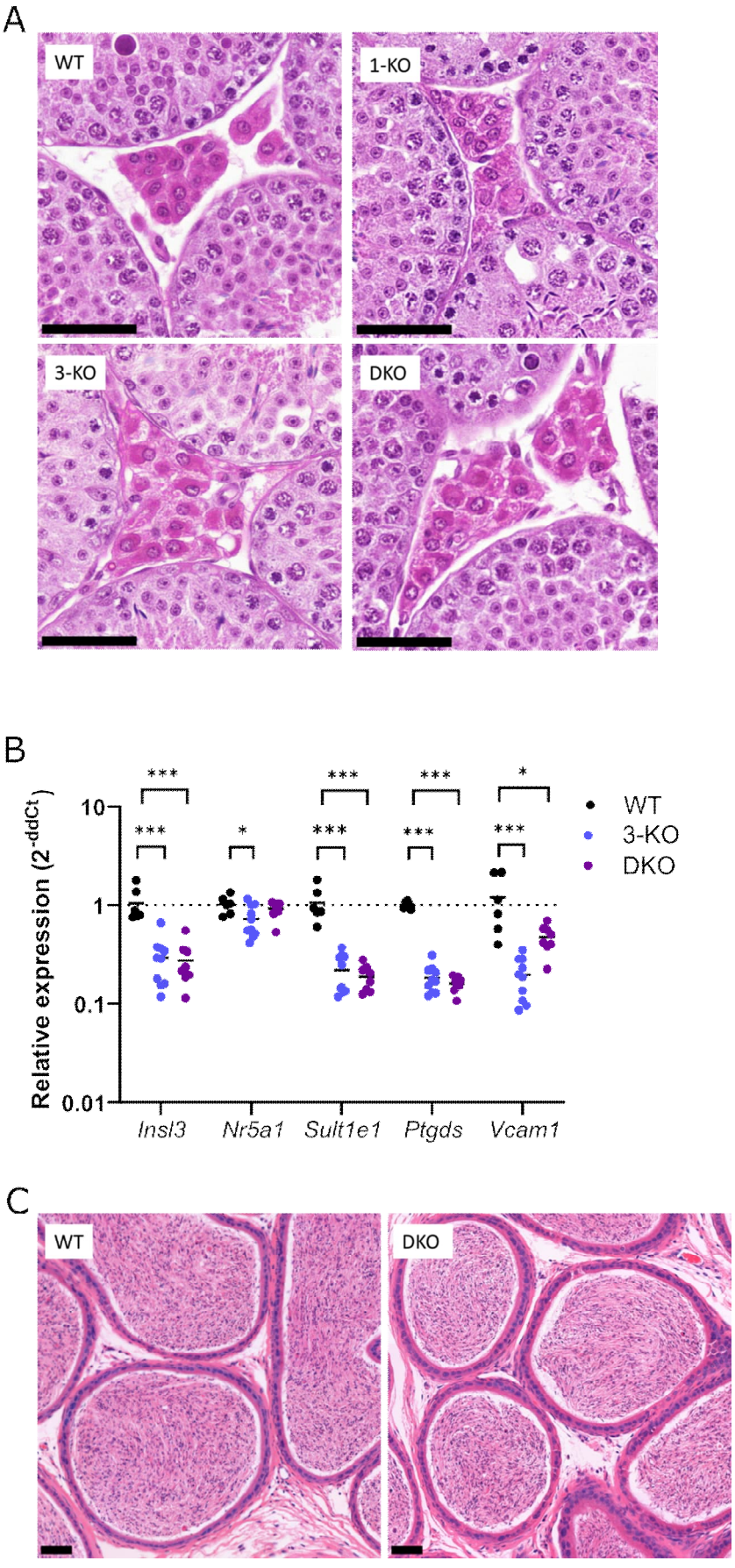
600 Figure 5



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