Endocrinology. First published ahead of print October 30, 2008 as doi:10.1210/en.2008-1087

Targeted ablation of the Wwox tumor suppressor leads to impaired steroidogenesis

Rami I. Aqeilan[*,**§], John P. Hagan[*], Alain de Bruin[*,†], Maysoon Rawahneh[*], Zaidoun Salah[**], Eugenio Gaudio[*], Hasan Siddiqui[*], Stefano Volinia[*], Hansjuerg Alder[*], Jane B. Lian[‡], Gary S. Stein[‡], and Carlo M. Croce[*]

[*]Department of Molecular Virology, Immunology and Medical Genetics, Comprehensive Cancer Center, Ohio State University, Columbus, Ohio 43210, [**]Lautenberg Center for General and Tumor Immunology, Hebrew University of Jerusalem, Jerusalem Israel 91120, [‡]Department of Cell Biology and Cancer Center, University of Massachusetts Medical School, Worcester, Massachusetts 01655,

[§] Corresponding author:
Rami I. Aqeilan, Human Cancer Genetics Program, Ohio State University-Biomedical Research Tower, Room 1088, 460 West 12th Avenue, Columbus, OH-43210. Phone: 614-292-5906; Fax: 614-292-4097; Email: rami.aqeilan@osumc.edu.

[†] Current Address: Department of Pathobiology, Faculty of Veterinary Medicine, University Utrechet, Utrechet 3584CL, Netherlands

DISCLOSURE STATEMENT: The authors have nothing to disclose

Key words: WW domain, Knockout, Leydig cells

Abstract

The WW domain-containing oxidoreductase (*WWOX*) gene encodes a 46kDa tumor suppressor. The Wwox protein contains two N-terminal WW domains that interact with several transcriptional activators containing proline-tyrosine motifs and a central short-chain dehydrogenase/reductase (SDR) domain that has been suggested to play a role in steroid metabolism. Recently, we have shown that targeted deletion of the *Wwox* gene in mice leads to postnatal lethality and defects in bone growth. Here, we report that *Wwox* deficient mice display impaired steroidogenesis. Mutant homozygous mice are born with gonadal abnormalities including failure of Leydig cells development in testis and reduced theca cells proliferation in ovary. Furthermore, *Wwox*$^{-/-}$ mice displayed impaired gene expression of key steroidogenesis enzymes. Affymetrix microarray gene analysis revealed differentially expressed related genes in steroidogenesis in knockout mice testis and ovary as compared to control mice. These results demonstrate the essential requirement for the Wwox tumor suppressor in proper steroidogenesis.

Introduction

WW domain-containing oxidoreductase (Wwox) is a 46kDa protein that contains two N-terminal WW domains, that mediate protein-protein interaction, and a central short-chain dehydrogenase/reductase (SDR) domain that has been suggested to play a role in steroid metabolism (1, 2). Recently, we reported the generation of a mouse carrying a targeted deletion of the *Wwox* gene (3). We demonstrated that loss of both alleles of *Wwox* resulted in the formation of some juvenile osteosarcomas while loss of one allele increased the incidence of spontaneous and chemically-induced tumors (3, 4) indicating that *Wwox* is a bona fide tumor suppressor. Indeed, *WWOX* expression is lost or reduced in several types of cancer including breast, prostate, lung, stomach and pancreatic carcinomas (5, 6). Restoration of *WWOX* expression in different types of cancer cells lacking expression of endogenous Wwox results in significant growth inhibition and prevents the development of tumors in athymic nude mice (7, 8). More recently, Aldaz group reported the generation of Wwox hypomorphic mice that have a low level of Wwox expression and display more frequent B cell lymphoma compared to wild type mice (9). Collectively, these results indicate that Wwox has a tumor suppressor function.

The generation of the *Wwox* deficient mice had also shed light on Wwox *in vivo* requirement. Our recent analysis has shown that *Wwox* deficient mice die by three weeks of age due to a severe metabloic defect. Analysis of serum chemistry of *Wwox* knockout mice at age of 2 weeks revealed that mice suffer of hypoglycemia, hypolipidemia and hypoproteinemia when compared to wild type littermates. Furthermore, *Wwox* deficient mice are smaller in size and exhibit an osteopenic phenotype. We found that WWOX physically associates with RUNX2, the principal transcriptional regulator of osteoblast differentiation and functionally suppresses *RUNX2* transactivation ability in osteoblasts.

Analysis of the Wwox expression pattern in mouse tissues revealed that Wwox is ubiquitously expressed with prominent expression in hormonally regulated tissues, such as testis, ovary, prostate and mammary epithelial cells (3, 10). Distribution of Wwox expression in normal tissues provided some insight on the potential physiological role of this protein. Further investigation of gonadal tissues in *Wwox* knockout mice indicated an aberrant steroidogenesis both in testis and ovary.

Material and Methods

Mice.

C57Bl/6J × 129/SvJ- F_1, F_2, F_3, F_4, and F_5 mice (B6-129 F_1–F_5) were produced in Ohio State University animal facility. The *Wwox* offspring were differentiated by genotyping of tail DNA using PCR-based method (3). Animals were sacrificed; tissues of all organs were removed,

fixed in 10% buffered formalin, and examined for histologically abnormalities by two pathologists after H&E staining.

Histology and Immunohistochemistry. Tissue from different organs were processed, embedded, and sectioned (4μ) according to standard methods. Antibodies used for immunohistochemical staining were polyclonal anti-Wwox (kindly provided by Dr. Kay Huebner; dilution 1:8000) and staining was performed as described (11); rat anti-mouse Ki67 antibody (Dako, dilution 1:500) was used as a marker for proliferation and rat anti-mouse P450 scc (Chemicon International, dilution 1:50) as a marker for Leydig cells; anti Fsh and anti-Lh (Abcam, Cambridge, MA). The detection system used was Vectastain Elite (Vector). Detailed protocol is available upon request. Pictures were taken using 10x or 40x objective lens; no magnification was used.

Real-Time PCR. Total RNA (1 μg) was isolated from the different tissues followed by homogenizing in Trizol Reagent (Invitrogen, Carlsbad CA) according to the manufacturer's protocol. cDNA was synthesized with oligo-dT primers using the SuperScript first Strand Synthesis kit (Invitrogen) according to the manufacturer's protocol. Gene expression was assessed by semi-quantitative and quantitative real-time PCR (Cyber green and TaqMan) using primers listed in supplementary data (SI-1). The expression of cyclophilin A or Gapdh was used as a control.

Affymatrix analysis. Total RNA from each testis and ovary were hybridized with Affymetrix mouse gene-chip 430 2.0 arrays. Normalization and analysis were performed as described in (12).

Results

Testicular abnormalities and impaired steroidogenesis in male *Wwox*$^{-/-}$ mice

Macroscopic examination of the *Wwox*-deficient mice testis revealed that the testis size is reduced (12) indicating a possible defect in steroid biosynthesis. To define the cause of the testicular hypoplasia, we examined testis histologically. Whereas abundant Leydig cells were readily apparent in the interstitium between the seminiferous tubules of control wild type mice, testis of *Wwox*$^{-/-}$ mice contained very few Leydig cells of smaller size (Fig 1A). Furthermore, staining of testis sections with anti-Wwox antibody revealed intense staining of Leydig cells and Sertoli cells in wild type mice while in mutant mice Wwox staining was not detected (Fig 1A, *a*&*c* vs. *b*&*d*). To verify further the hypoplasia of Leydig cells in mutant testis, we immunostained testis sections with

anti-cytochrome P450 side chain cleavage (P-450 ssc) antibody, a marker of Leydig cells, and we observed markedly reduced staining of P450 ssc in mutant testis compared to wild type testis (Fig 1A, *f*&*h* vs. *e*&*g*). Since it is known that Leydig cells produce testosterone, we measured serum testosterone levels and found that whereas in WT mice testosterone levels were ranging 50-65 pg/ml (13), in KO mice testosterone levels were undetectable.

To determine which of the Leydig cell genes encoding testosterone biosynthesis pathway enzymes are altered in the *Wwox* knockout mice, the expression of various markers was analyzed using real-time PCR. We found that Leydig cell markers, including *Cyp11a1*, *Cyp17a1*, *and Hsd3b6* were downregulated in KO testis compared to WT and HET ones (Fig 1B, 1C). We also observed that the expression of thrombospondin 2 (*Thbs2*) and *Star*, two fetal Leydig cell markers, are reduced in KO testis indicating a decreased number of fetal Leydig cells in the juvenile deficient testis. The seminiferous tubules of the KO mice were markedly reduced in size and contained immature germ cells. Based on the fact that KO mice died at juvenile age, we were not able to study effects of *Wwox* ablation during spermatogenesis.

Ovarian abnormalities in *Wwox*$^{-/-}$ female mice

Macroscopic and microscopic evaluation of female reproductive organs of juvenile KO mice revealed that ovaries were reduced in size and that uterus horns were thinner compared to WT littermates (data not shown). Ovaries derived from KO pups contained many primary follicles of normal appearance that were significantly smaller in size compared to WT littermate ovaries (Fig 2A). Immunohistochemical staining with anti-Wwox antibody showed a prominent expression of Wwox in WT ovary whereas no staining was observed in KO (Fig 2A). Staining with the proliferation marker Ki67 showed reduced expression of Ki67 in theca cells of the KO animals, indicating a decrease in proliferation (Fig 2A). Since the KO pups die at juvenile age, we did not study the maturation of the primary follicles towards secondary follicles, grafian follicles and corpora lutea.

Expression of many genes encoding steroid biosynthesis enzymes, including *Cyp11a1* and *Hsd3b6* were reduced in KO compared to WT and HET ovary (Fig 2B, 2C). Additionally, expression of the transforming growth factor-beta (TGFβ) superfamily members, including inhibin and activin were also reduced in the deficient ovary (Fig 2B, 2C).

Reduced expression of *Fsh* and *Lh* in pituitary gland of *Wwox*-deficient mice

Wwox protein level in pituitary gland, as assessed by immunohistochmeistry, is significantly high (3). To addresses whether Wwox may affect upstream targets that control steriodogenic enzymatic pathways, we analyzed the expression of follicle-stimulating hormone

and luteinizing hormone gene expression. We found that *Fshb* and, to a lesser extent, *Lhb* expression is downregulated in pituitary glands of *Wwox* KO mice as assessed by real-time PCR (Fig 3A). To further confirm downregulation of Fsh and Lh expression in pituitary gland of *Wwox* KO mice, we performed immunohistochemical staining using anti-Fsh and anti-Lh antibodies. As expected, we observed significant downregulation of Fsh and, to a lesser extent, Lh protein expression in *Wwox* KO pituitary compared to wild type (Fig 3B). Both intensity and number of positive cells stained for Fsh and Lh were less in KO compared to WT. These results may suggest that Wwox may affect upstream targets and thus affecting the normal function of gonads.

Gene expression profiling in gonads of *Wwox*-deficient mice

To investigate thoroughly the *in vivo* function of *Wwox* in gonads, we studied the differential expression of mRNAs in the *Wwox*-deficient mice gonads compared to their WT and HET littermates. mRNAs extracted from ovary and testis were analyzed using mouse Affymetrix microarray gene-chip 430 2.0 arrays. As shown in Table 1, our analysis identified the alteration of fifteen key genes involved in steroidogensis in both tissues including enzymes of the cytochrome P450 family. These data indicate that Wwox plays a central role in steroid metabolism.

Overexpression of Wwox in MLTC-1 Leydig cells modulates levels of steroidogenic enzymes

Since absence of Wwox expression leads to deregulation of many genes encoding steroid biosynthesis enzymes *in vivo*, we next addressed whether Wwox expression affect the levels of these enzymes *in vitro*. Transient expression of Wwox in MLTC-1 mouse leydig cells led to upregulation of *Hsd3b6* and, to a lesser extent, *Hsd3b1* and *Hsd17b1* expression as assessed by real-time PCR (Fig 4). Interestingly, expression of WwoxY33R mutant (containing a WW1 domain mutations that abrogates Wwox interaction with PPxY motifs (5, 14)) causes downregulation of these enzymes (Fig 4). No changes were observed in mRNA expression of *Hsd17b3*, *Cyp11a1*, and *Cyp17a1* following overexpression of Wwox or WwoxY61R (a WW2 domain mutation) *in vitro* (Fig 4). These results suggest that the first WW domain of Wwox is indispensable for Wwox function in regulation of steroid enzymes.

Discussion

Mice lacking *Wwox* die approximately three weeks after birth and display defects in growth rate bone and steroid metabolism. *Wwox* deficient mice display growth retardation of key organs that weighed less in KO pups compared with wild-type littermates (12). Interestingly, we observed that other organs such as adrenal and the pituitary gland of KO pups were heavier compared with WT littermates. Macroscopic and

histological examination of these glands showed no significant microscopic lesions (3, 12). The complexity of the mutant phenotype, together with early postnatal lethality, supports multiple roles for Wwox *in vivo* and suggest that Wwox functions may not be compensated for by redundant functions of related proteins of the WW domain-containing proteins or proteins containing SDR domain.

This steroidogenesis defect we observed in *Wwox*-deficient mice is contributed by several factors. First, the high expression of Wwox in Leydig cells and Sertoli cells, as well as in the ovarian follicles suggest that Wwox is required for proper gonadal function. Second, failure of Leydig cell formation and very low levels of serum testosterone in testis of KO mice and the reduced proliferation of theca cells and smaller primary follicles in ovary of KO mice suggests that Wwox is directly involved in steroidogenesis pathway. Third, our Affymetrix analysis identified fifteen differentially expressed genes in the *Wwox* KO gonads (Table 1) suggesting that absence of Wwox alter the expression of steroidogenesis-related genes. Our data also suggest that the follicle-stimulating hormone (*Fshb*) and, to a lesser extent, the luteinizing hormone (*Lhb*) expression is downregulated in pituitary glands of *Wwox* KO mice (Fig 3). These data suggest that Wwox may affect upstream targets that control steriodogenic enzymatic pathways. No changes were observed in mRNA expression of estrogen, progesterone and androgen receptors in KO mice (data not shown).

Interestingly, *Wwox* KO mice show a number of similarities to the LH-b-deficient mice (15). Ma et. al, have demonstrated that targeted disruption of LH-b-subunit leads to prominent Leydig cell hypoplasia, defects in expression of genes encoding steroid biosynthesis pathway enzymes and reduced testosterone levels (15). *Wwox* KO mice may also share some phenotypic changes similar to mice lacking FSH-β subunit or the FSH receptor (reviewed in (16, 17)). Since our mice die before sexual maturity, we did not study spermatogenesis and maturations of primary follicles to compare our KO mice to these various animal models. Recently, Wwox hypomorphic mice were generated and found to be viable (9) in contrast to the postnatal lethality we observed in the *Wwox*-null mice (12). Intriguingly, testis from Wwox hypomorphic males had high numbers of atrophic seminiferous tubules and reduced fertility when compared with wild-type counterparts (9). These results suggest that total loss of Wwox leads to failure of Leydig cell development and reduced testosterone levels while reduced expression of Wwox leads to testicular atrophy. Altogether, these data indicate the indispensable role of Wwox in steroidogenesis.

Wwox protein contains two WW domains predicted to mediate protein-protein interaction. Indeed, the first WW domain of Wwox belongs to class I of WW domains (18,

19, 20). We have previously shown that Wwox binds PP*x*Y motifs of several transcription factors, sequesters them in the cytoplasm and reduces their transactivation abilities (19, 21-23). Here, we show that the first WW domain of Wwox is also important for proper steroid related gene expression. It is possible that Wwox, via its first WW domain, associates with key transcription factor(s) that regulates steroid enzymes. Work by the Chang laboratory (24), demonstrated that 17beta-estradiol (E(2)) and androgen activate Wwox, via phosphorylation on tyrosine (Y) 33, in different cell lines and that this activation positively correlates with cancerous progression of prostate and breast to a premetastatic state. Nevertheless, we cannot exclude that other domains of Wwox also contribute to Wwox function in steroid metabolism. For example, Wwox also possesses a typical short-chain dehydrogenase/reductase domain that has been predicted to play a role in steroid metabolism (1). The exact contribution of each of Wwox domains toward these phenotypes is still to be determined.

Absent or reduced Wwox expression has been suggested to be associated with prostate carcinoma (25, 26). The normal prostate and early-stage prostate cancers depend on androgens for growth and survival, and androgen ablation therapy causes them to regress (27). Our results suggest that loss of Wwox expression results in reduced levels of testosterone, a condition that may affect prostate normal function and may also contradict with early role of Wwox during prostate carcinoma. Histological examination of prostate gland in juvenile *Wwox* deficient mice did not show any abnormal morphology (3). This may suggest that alternative androgen sources, other than Leydig cells, are adequate for prostate normal morphology. Of note, expression of cytochrome P450 side-chain cleavage (Cyp11a1) was not affected in adrenal gland (data not shown) which expresses high levels of Wwox (3). This may suggest that Wwox regulation of testosterone synthesis could be specifically restricted to Leydig cells in the testis. On the other hand, it is possible to speculate that Wwox tumor suppressor function may also be important during tumor progression in prostate cancer, a condition when tumor cells become androgen independent.

In conclusion, our phenotypic analysis of the *Wwox* knockout mice demonstrates an important role of Wwox in fundamental cellular processes including survival, growth, and steroidogenesis. The defect in steroid and bone metabolism in *Wwox* KO mice may suggest that both phenotypes are related. Further analysis of mechanisms underlying these phenotypes and contribution of Wwox different domains toward these phenotypes is to be determined.

Acknowledgment.

We are grateful to Dean Marshall and Darshna Bhatt for technical assistance. This work was supported by Ohio Cancer Research Associates

and Kimmel Scholar Award (R.I.A.), NCI grant (CMC).

References.

1. **Bednarek AK, Laflin KJ, Daniel RL, Liao Q, Hawkins KA, Aldaz CM** 2000 WWOX, a novel WW domain-containing protein mapping to human chromosome 16q23.3-24.1, a region frequently affected in breast cancer. Cancer Res 60:2140-2145
2. **Ried K, Finnis M, Hobson L, Mangelsdorf M, Dayan S, Nancarrow JK, Woollatt E, Kremmidiotis G, Gardner A, Venter D, Baker E, Richards RI** 2000 Common chromosomal fragile site FRA16D sequence: identification of the FOR gene spanning FRA16D and homozygous deletions and translocation breakpoints in cancer cells. Hum Mol Genet 9:1651-1663
3. **Aqeilan RI, Trapasso F, Hussain S, Costinean S, Marshall D, Pekarsky Y, Hagan JP, Zanesi N, Kaou M, Stein GS, Lian JB, Croce CM** 2007 Targeted deletion of Wwox reveals a tumor suppressor function. Proc Natl Acad Sci U S A 104:3949-3954
4. **Aqeilan RI, Hagan JP, Aqeilan HA, Pichiorri F, Fong LY, Croce CM** 2007 Inactivation of the Wwox Gene Accelerates Forestomach Tumor Progression In vivo. Cancer Res 67:5606-5610
5. **Aqeilan RI, Croce CM** 2007 WWOX in biological control and tumorigenesis. J Cell Physiol 212:307-310
6. **Paige AJ, Taylor KJ, Taylor C, Hillier SG, Farrington S, Scott D, Porteous DJ, Smyth JF, Gabra H, Watson JE** 2001 WWOX: a candidate tumor suppressor gene involved in multiple tumor types. Proc Natl Acad Sci U S A 98:11417-11422
7. **Bednarek AK, Keck-Waggoner CL, Daniel RL, Laflin KJ, Bergsagel PL, Kiguchi K, Brenner AJ, Aldaz CM** 2001 WWOX, the FRA16D gene, behaves as a suppressor of tumor growth. Cancer Res 61:8068-8073
8. **Fabbri M, Iliopoulos D, Trapasso F, Aqeilan RI, Cimmino A, Zanesi N, Yendamuri S, Han SY, Amadori D, Huebner K, Croce CM** 2005 WWOX gene restoration prevents lung cancer growth in vitro and in vivo. Proc Natl Acad Sci U S A 102:15611-15616
9. **Ludes-Meyers JH, Kil H, Nunez MI, Conti CJ, Parker-Thornburg J, Bedford MT, Aldaz CM** 2007 WWOX hypomorphic mice display a higher incidence of B-cell lymphomas and develop testicular atrophy. Genes Chromosomes Cancer 46:1129-1136
10. **Nunez MI, Ludes-Meyers J, Aldaz CM** 2006 WWOX protein expression in normal human tissues. J Mol Histol 37:115-125
11. **Aqeilan RI, Kuroki T, Pekarsky Y, Albagha O, Trapasso F, Baffa R, Huebner K, Edmonds P, Croce CM** 2004 Loss of WWOX expression in gastric carcinoma. Clin Cancer Res 10:3053-3058
12. **Aqeilan RI, Hassan MQ, de Bruin A, Hagan JP, Volinia S, Palumbo T, Hussain S, Lee SH, Gaur T, Stein GS, Lian JB, Croce CM** 2008 The WWOX tumor suppressor is essential for post-natal survival and normal bone metabolism. J Biol Chem 283:21629-21639
13. **Jeyaraj DA, Grossman G, Petrusz P** 2005 Altered bioavailability of testosterone in androgen-binding protein-transgenic mice. Steroids 70:704-714

14. **Chang NS, Hsu LJ, Lin YS, Lai FJ, Sheu HM** 2007 WW domain-containing oxidoreductase: a candidate tumor suppressor. Trends Mol Med 13:12-22
15. **Ma X, Dong Y, Matzuk MM, Kumar TR** 2004 Targeted disruption of luteinizing hormone beta-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. Proc Natl Acad Sci U S A 101:17294-17299
16. **Sairam MR, Krishnamurthy H** 2001 The role of follicle-stimulating hormone in spermatogenesis: lessons from knockout animal models. Arch Med Res 32:601-608.
17. **Kumar TR** 2005. Gonadotropin gene targeting and biological implications. Endocrine 26:227-233.
18. **Macias MJ, Wiesner S, Sudol M** 2002 WW and SH3 domains, two different scaffolds to recognize proline-rich ligands. FEBS Lett 513:30-37.
19. **Aqeilan RI, Pekarsky Y, Herrero JJ, Palamarchuk A, Letofsky J, Druck T, Trapasso F, Han SY, Melino G, Huebner K, Croce CM** 2004 Functional association between Wwox tumor suppressor protein and p73, a p53 homolog. Proc Natl Acad Sci U S A 101:4401-4406
20. **Ludes-Meyers JH, Kil H, Bednarek AK, Drake J, Bedford MT, Aldaz CM** 2004 WWOX binds the specific proline-rich ligand PPXY: identification of candidate interacting proteins. Oncogene 23:5049-5055
21. **Aqeilan RI, Palamarchuk A, Weigel RJ, Herrero JJ, Pekarsky Y, Croce CM** 2004 Physical and functional interactions between the Wwox tumor suppressor protein and the AP-2gamma transcription factor. Cancer Res 64:8256-8261
22. **Aqeilan RI, Donati V, Palamarchuk A, Trapasso F, Kaou M, Pekarsky Y, Sudol M, Croce CM** 2005 WW domain-containing proteins, WWOX and YAP, compete for interaction with ErbB-4 and modulate its transcriptional function. Cancer Res 65:6764-6772
23. **Gaudio E, Palamarchuk A, Palumbo T, Trapasso F, Pekarsky Y, Croce CM, Aqeilan RI** 2006 Physical association with WWOX suppresses c-Jun transcriptional activity. Cancer Res 66:11585-11589
24. **Chang NS, Schultz L, Hsu LJ, Lewis J, Su M, Sze CI** 2005 17beta-Estradiol upregulates and activates WOX1/WWOXv1 and WOX2/WWOXv2 in vitro: potential role in cancerous progression of breast and prostate to a premetastatic state in vivo. Oncogene 24:714-723
25. **Watson JE, Doggett NA, Albertson DG, Andaya A, Chinnaiyan A, van Dekken H, Ginzinger D, Haqq C, James K, Kamkar S, Kowbel D, Pinkel D, Schmitt L, Simko JP, Volik S, Weinberg VK, Paris PL, Collins C** 2004 Integration of high-resolution array comparative genomic hybridization analysis of chromosome 16q with expression array data refines common regions of loss at 16q23-qter and identifies underlying candidate tumor suppressor genes in prostate cancer. Oncogene 23:3487-3494
26. **Qin HR, Iliopoulos D, Semba S, Fabbri M, Druck T, Volinia S, Croce CM, Morrison CD, Klein RD, Huebner K** 2006 A role for the WWOX gene in prostate cancer. Cancer Res 66:6477-6481
27. **Feldman BJ, Feldman D** 2001 The development of androgen-independent prostate cancer. Nat Rev Cancer 1:34-45

Fig legends.

Fig 1. Gonadal phenotype in *Wwox* knockout male mice. A. Leydig cell defects. Histology section of testis showing normal Leydig cells in WT testis (*a*&*c*) stained with anti-WWOX antibody, whereas the KO testis (*b*&*d*) shows sparse interstitium and few Leydig cells and negative staining of Wwox. A positive marker for Leydig cells, anti-P450 scc (*e*, *g, f & h*), was used to confirm the marked reduction of Leydig cells in KO testis section. Arrows point at Leydig cells. **B.** Real-Time PCR analysis of testis cDNA identifies impaired steridogenesis in KO testis. Expression of key genes in the steridogenesis pathway was analyzed. **C.** Semi-quantitative RT-PCR analysis of testis cDNA identifies impaired steridogenesis in KO testis. Expression of key genes in the steridogenesis pathway was analyzed. The expression of cyclophilin A was used as a control.

Fig 2. Gonadal phenotype in *Wwox* knockout female mice. A. Histology of the ovary shows multiple follicles at different stages in WT whereas in KO mice follicles tended to be smaller in size. Staining with anti-WWOX antibody in *a*&*b*. Panels *c*&*d* demonstrate reduced proliferation in theca cell in KO (*d*) compared with WT (*c*) after staining with anti-Ki67 antibody. **B.** Real-Time PCR analysis of ovaries cDNA identifies impaired steridogenesis in KO ovary. The expression of cyclophilin A was used as a control in both C&E. **C.** Semi-quantitative RT-PCR analysis of ovary cDNA identifies impaired steridogenesis in KO testis. Expression of key genes in the steridogenesis pathway was analyzed. The expression of cyclophilin A was used as a control.

Fig 3. Reduced expression of *Fshb* and *Lhb* in Pituitary gland of *Wwox*-null mice. A Real-time PCR (TaqMan, Applied Biosystem) showing downregulation of *Fshb* and *Lhb* in different *Wwox* KO mice compared to wild-type mice. **B.** Immunohistochemical staining of Fsh and Lh in pituitary gland showing reduced expression in *Wwox* KO mice.

Fig 4. Wwox expression modulates steroidogenic gene expression in MLTC-1 Leydig cells *in vitro*. MLTC-1 mouse Leydig cells transiently expressing Wwox as indicated were harvested and total RNA was extracted. Results show real-time PCR analysis of steroidogenic related gene expression (as indicated) following the different transient expression relative to GFP expression. Results represent fold difference relative to expression of Gapdh.

Gene symbol	Description	Fold Difference	
		Testis	Ovary
Downregulated in WWOX Knockout			
Wwox	WW domain-containing oxidoreductase	0.16	0.36
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	0.21	0.16
BB144871	Expressed sequence BB144871	0.28	0.23
Car3	Carbonic anhydrase 3	0.32	0.23
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	0.36	0.33
Ren1	Renin 1 structural	0.37	0.43
Hao3	Hydroxyacid oxidase (glycolate oxidase) 3	0.37	0.29
Scd1	Stearoyl-Coenzyme A desaturase 1	0.39	0.35
Cbr2	Carbonyl reductase 2	0.43	0.42
Hp	Haptoglobin	0.44	0.29
Slc38a5	Solute carrier family 38, member 5	0.49	0.24
Upregulated in WWOX Knockout			
Galntl2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2	2.12	2.67
Cyp2e1	Cytochrome P450, family 2, subfamily e, polypeptide 1	2.34	3.75
Dlk1	Delta-like 1 homolog (Drosophila)	2.38	2.35
Inmt	Indolethylamine N-methyltransferase	3.13	2.40

Table 1. *Wwox* regulated genes in mouse gonads. Transcriptional profiling using Affymetrix Mouse Genome 430 2.0 array identified fifteen differentially expressed genes in the mouse *Wwox* knockout that displayed at least two fold expression differences both in testis and in the ovary. Mouse ovary RNA was isolated from three HET and three KO mice and hybridized without pooling. Likewise, testis RNA from four WT, two HET, and six KO mice were also analyzed. For calculating fold differences, WT and HET mice were grouped together and compared to KO. Data have 90% level of confidence that the false discovery rate was less than 10%.

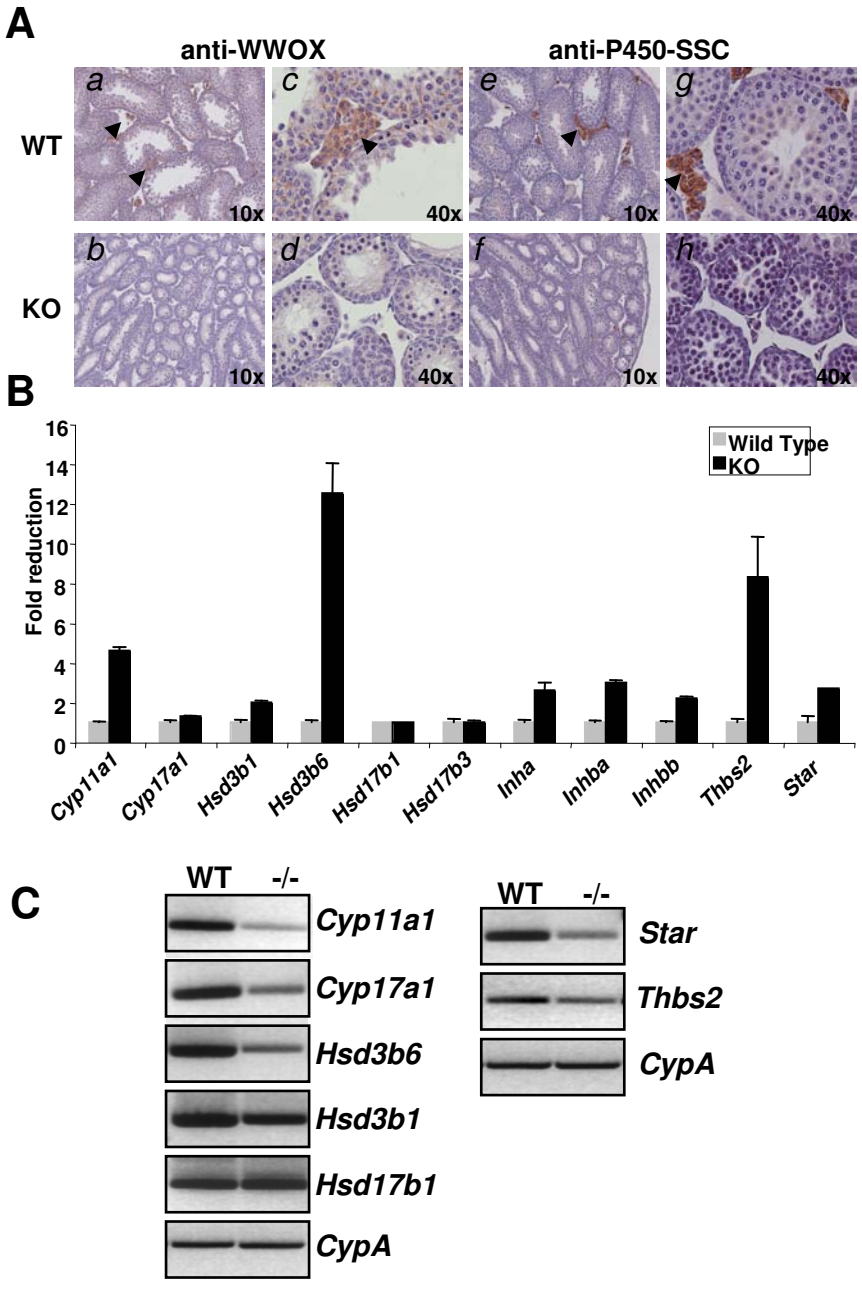

A
anti-WWOX
anti-P450-SSC
WT
KO
a
b
c
d
e
f
g
h
10x
40x
B
Fold reduction
Wild Type
KO
Cyp11a1
Cyp17a1
Hsd3b1
Hsd3b6
Hsd17b1
Hsd17b3
Inha
Inhba
Inhbb
Thbs2
Star
C
WT
-/-
Cyp11a1
Cyp17a1
Hsd3b6
Hsd3b1
Hsd17b1
CypA
Star
Thbs2

Figure 1

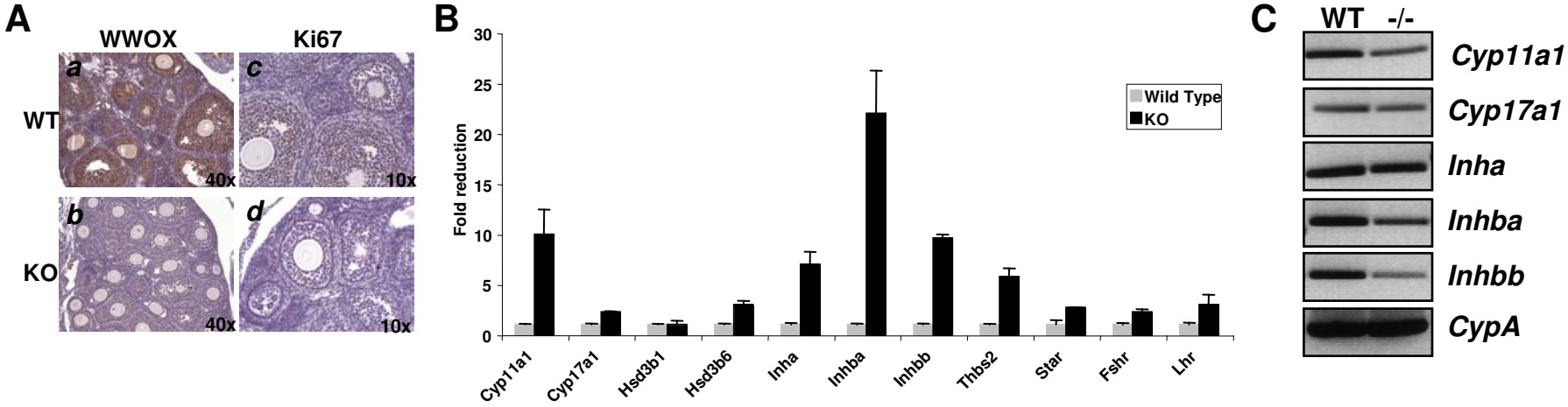
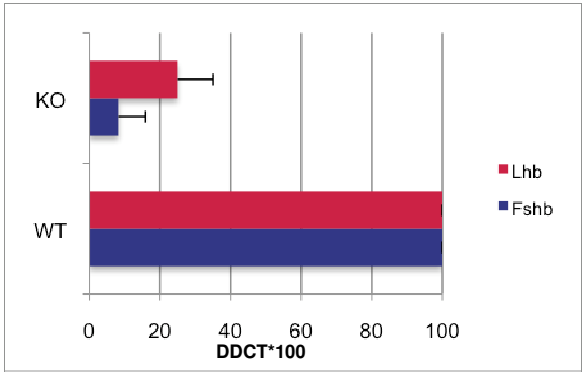

A
WWOX
Ki67
a
c
WT
40x
10x
b
d
KO
40x
10x
B
Fold reduction
30
25
20
15
10
5
0
Wild Type
KO
Cyp11a1
Cyp17a1
Hsd3b1
Hsd3b6
Inha
Inhba
Inhbb
Thbs2
Star
Fshr
Lhr
C
WT
-/-
Cyp11a1
Cyp17a1
Inha
Inhba
Inhbb
CypA

Figure 2

A



B

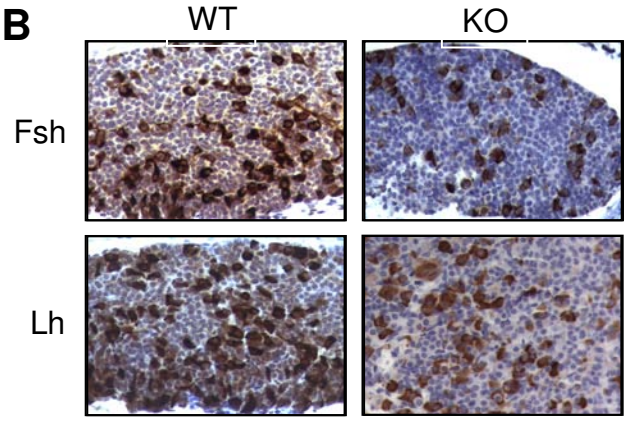


Figure 3

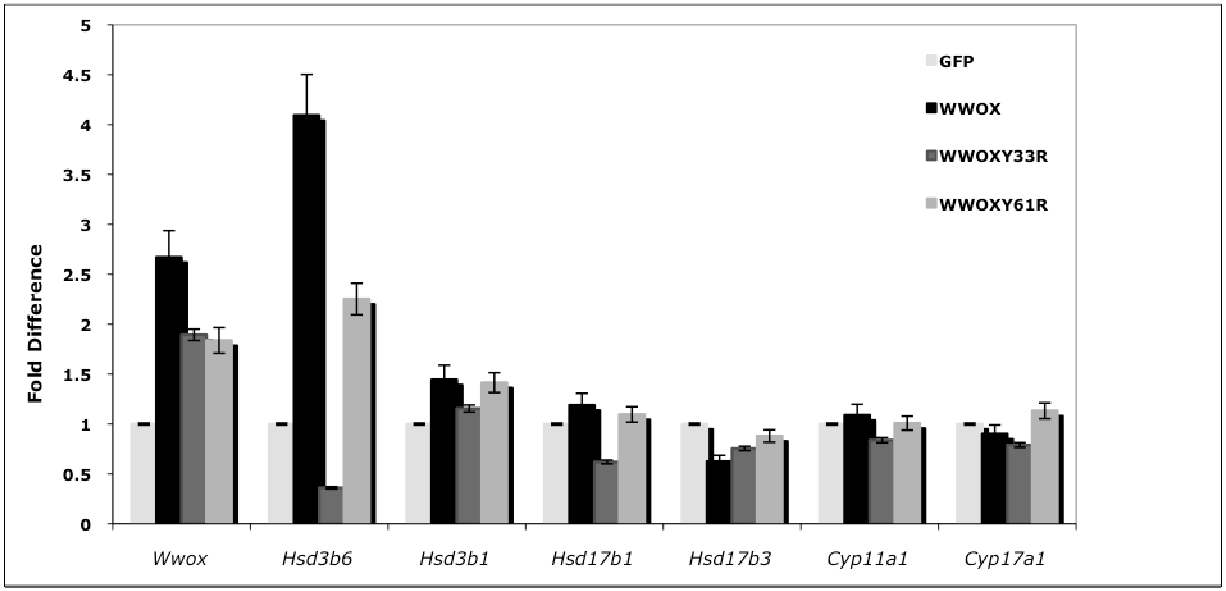

Fold Difference
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
GFP
WWOX
WWOXY33R
WWOXY61R
Wwox
Hsd3b6
Hsd3b1
Hsd17b1
Hsd17b3
Cyp11a1
Cyp17a1

Figure 4