Generation and Characterization of Mice Carrying a Conditional Allele of the \textit{Wwox} Tumor Suppressor Gene

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Abstract

\textit{WWOX}, the gene that spans the second most common human chromosomal fragile site, FRA16D, is inactivated in multiple human cancers and behaves as a suppressor of tumor growth. Since we are interested in understanding \textit{WWOX} function in both normal and cancer tissues we generated mice harboring a conditional \textit{Wwox} allele by flanking Exon 1 of the \textit{Wwox} gene with LoxP sites. \textit{Wwox} knockout (KO) mice were developed by breeding with transgenic mice carrying the Cre-recombinase gene under the control of the adenosivirus \textit{E1A} promoter. We found that \textit{Wwox} KO mice suffered from severe metabolic defect(s) resulting in growth retardation and all mice died by 3 wk of age. All \textit{Wwox} KO mice displayed significant hypocapnia suggesting a state of metabolic acidosis. This finding and the known high expression of \textit{Wwox} in kidney tubules suggest a role for \textit{Wwox} in acid/base balance. Importantly, \textit{Wwox} KO mice displayed histopathological and hematological signs of impaired hematopoiesis, leukopenia, and splenic atrophy. Impaired hematopoiesis can also be a contributing factor to metabolic acidosis and death. Hypoglycemia and hypocalcemia was also observed affecting the KO mice. In addition, bone metabolic defects were evident in \textit{Wwox} KO mice. Bones were thinner and smaller having reduced bone volume as a consequence of a defect in mineralization. No evidence of spontaneous neoplasia was observed in \textit{Wwox} KO mice. We have generated a new mouse model to inactivate the \textit{Wwox} tumor suppressor gene conditionally. This will greatly facilitate the functional analysis of \textit{Wwox} in adult mice and will allow investigating neoplastic transformation in specific target tissues.

Introduction

\textit{WW} domain-containing oxidoreductase (\textit{WWOX}) was cloned and identified as a potential tumor suppressor gene mapping to the chromosome region 16q23 [1]. The \textit{WWOX} gene spans >1.1 Mb and overlaps the common chromosomal fragile site, FRA16D, the second most common site for chromosomal breakage, instability and rearrangement of the whole genome [1–3]. Allelic losses and rearrangements affecting the \textit{WWOX} locus have been described in various human cancers [1–5]. Additionally, various studies have reported significant loss of \textit{WWOX} expression in multiple human neoplasias including breast, prostate, ovarian, lung and liver cancer [6,7].

\textit{Wwox} is a 46-kD protein, highly conserved through evolution, that contains two N-terminal \textit{WW}-domains and a short chain dehydrogenase/reductase domain (SDR domain) [1]. Our understanding of the NAD(P)(H)-dependent enzymatic functions of \textit{Wwox} is very limited. \textit{Wwox} structure is the archetypical representative of one of four separate clusters of classical SDRs conserved through evolution [8,9]. Based on the high expression of \textit{Wwox} in hormonally regulated tissues (testis, prostate and ovary) and its amino acid sequence homology to specific oxidoreductases, we postulated that \textit{Wwox} maybe an enzyme involved in sex-steroid metabolism [1]. The \textit{WW}-domains are required for protein-protein interactions through binding the conserved proline-rich sequence PPSXY. Several proteins have been identified as \textit{WWOX} protein partners such as SIMPLE, SCOTIN [10] and EZRIN [11] as well as several transcription factors such as the tumor suppressor p73[12], ERBB4-CTF [13], AP2[14], MEF- CTF [15] and the osteoblast differentiation master regulator, RUNX2 [16], to name a few. One role for \textit{WWOX} has emerged as a regulator of transcription by limiting transcription factor access to the nucleus through cytoplasmic sequestration [6].

The role of \textit{WWOX} as a tumor suppressor is supported by \textit{in vitro} studies with human cancer cells and \textit{in vivo} studies using mouse models. Several studies reported that ectopic \textit{WWOX} expression suppressed the \textit{in vivo} tumorigenicity of various human tumor types including, breast [5], lung [17], prostate [18] and ovarian [19] cancer cells when xenografted in nude mice. Mouse models with targeted disruption of \textit{Wwox} had increased spontaneous tumor incidence. Using a conventional mouse knockout approach Aqeilan et al. [20] reported development of osteosarcomas in some \textit{Wwox}$^{+/-}$ mice by the age of 2.5 weeks and as early as postnatal day 3. All \textit{Wwox} null mice died by 5–4 wk of age precluding any further studies of adult animals. However, development of spontaneous lung papillary carcinoma was observed in adult \textit{Wwox}$^{+/+}$ mice. Aqeilan et al [20] also reported that \textit{Wwox}$^{+/+}$ mice treated with chemical carcinogens displayed an increased incidence of lymphomas and lung tumors when compared
with wild type littermates. Using a gene trap approach we developed a Wwox hypomorphic mouse model [21]. Female Wwox hypomorphs had an increased incidence of spontaneously arising B-cell lymphomas. In addition, several female developed multiple neoplasias. Together, the in vitro and in vivo studies have provided significant evidence for Wwox as a tumor suppressor.

We are interested in understanding in more detail the normal and tumor suppressive role Wwox plays in the multiple tissues it is expressed. As mentioned, Wwox KO mice generated using conventional techniques die by four weeks of age thus impeding any studies in adult tissues. Therefore, we report here the generation of mice with a conditional allele for Wwox ablation for better understanding the roles of Wwox in different tissues and in pathological conditions.

Results

Generation of Mice with a Floxed-Wwox Allele

We devised a strategy for conditional ablation of Wwox gene expression using the Cre-Lox site-specific recombination system. Our approach, illustrated in Figure 1, utilized positive-negative selection gene targeting [22] to modify Wwox genomic sequences by flanking Wwox Exon 1 with two LoxP-recombination sites to generate a “floxed”-Wwox allele. We successfully targeted mouse ES cells and generated chimeric founders having germline transmission and subsequently bred a Wwox<sup>flu</sup> mouse.

Since it was possible that the inserted pgk-neo plasmid could impair Wwox gene transcription we determined whether the floxed-Wwox allele had any effect on viability and fertility. Breeding of heterozygous (Wwox<sup>+/flo</sup>) mice from two different founders resulted in pups having genotypes with the expected Mendelian ratios demonstrating that the floxed allele had no effect on embryonic development or viability. Mating of Wwox<sup>flu/flo</sup> males with Wwox<sup>flu/flo</sup> females resulted in normal litter size, genotype frequencies and pups had normal postnatal development. These results showed that the “floxed”-Wwox allele containing the insertion of the ~2 Kb neomycin resistance gene in the 5’ transcriptional control region of the Wwox gene had no detrimental affect on Wwox functions required for normal mouse growth, survival or reproduction. Therefore, this mouse model will be a useful tool for analyzing Wwox function(s) in specific mouse tissues.

Cre-Recombinase Deletion of the Wwox Gene Completely Ablates Wwox Expression

We showed that our strategy resulted in the specific modification of the Wwox allele resulting in the insertion of the two LoxP-recombination sequences flanking Exon 1 however it is possible that small changes to the inserted sequence could have been introduced during the procedure. Therefore, to determine whether Cre-recombinase could utilize the inserted LoxP sites to delete Wwox, we generated mice combining the Wwox<sup>flu</sup> allele with the Cre-recombinase gene under the control of the adenovirus E1B promoter. The adenovirus E1B promoter directs transcription of Cre-recombinase at the zygote stage of embryonic development resulting in deletion of floxed sequences in all cells of the developing embryo including the germ cells [23]. To determine whether Cre-recombination was successful we analyzed genomic DNA using PCR with oligonucleotide primers specific for wild-type or Cre-deleted Wwox sequences (Figure 2). Using this strategy we were able to test the functionality of the LoxP sites, determine whether our strategy of deleting Exon 1 would eliminate Wwox expression and give rise to a phenotype.

We then mated Wwox<sup>flu/fl</sup> mice with homozygous E1B-Cre transgenic mice that resulted in the first familial generation (F<sub>1</sub>) of
pups having one Wwox allele with wild-type sequences and one Wwox allele with Exon 1 deleted (Wwox/DCre). Since the Wwox/DCre allele was present in the germ cells a F1 6 F1 cross should result in pups having all possible genotypes (Wwox+/D, Wwox+/DCre, Wwox/DCre/DCre) with Mendelian ratios of 1:2:1 (Table 1). Successful matings from all mating pairs (n = 10) were obtained demonstrating that Wwox/DCre mice were fertile having litter sizes ranging between 3 to 11 pups (average = 7.75). Three-day old pups were genotyped by PCR (Figure 2) and correct Mendelian ratios were obtained after genotyping 154 animals (p = 0.257 using the χ2-test). To demonstrate that our targeting strategy lead to complete ablation of Wwox expression we performed immunoblot analysis of total protein extracts from a variety of tissues (Figure 2). As expected Wwox protein was undetectable in Wwox/DCre mice and was reduced by approximately one-half in heterozygotes confirming that Cre-mediated deletion of Wwox Exon 1 lead to the complete absence of Wwox protein.

Ablation of Wwox expression lead to significant growth retardation that was noticeable at birth (Figure 3A). To further address pup growth we weighed pups periodically and found that Wwox KO mice had a severe growth defect averaging only 4.2 g at postnatal day 14 compared to heterozygous and wild-type mice (6.82 and 7.07 g, respectively). Although some Wwox KO mice can survive up to 3 weeks of age, the mortality rate was very high. As early as 72 h after birth 43% (15 of 35) of Wwox KO and 77% by 17 days after birth and no mice survived past weaning. These findings demonstrated that ablation of Wwox expression by deleting Exon 1 using our Cre-loxP strategy lead to growth retardation and postnatal death in agreement with the observations of Aqeilan et al [16,20] using conventional knockout technology.

**Table 1.** Genotypes of pups from Wwox/DCre crosses.

| Genotype       | WT | HET | KO | Total |
|----------------|----|-----|----|-------|
| Actual # of pups | 39 | 85  | 30 | 154   |
| Expected # of pups | 38.5 | 77  | 38.5 | 154   |

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**Figure 2.** Strategy for Cre-mediated ablation of Wwox expression. (A) To promote deletion of Wwox alleles we used transgenic mice carrying the Cre-recombinase gene under control of the adenoviral EIIA-promoter. EIIA-regulated Cre-recombinase is expressed in pre-implantation embryos leading to site-specific deletion of LoxP flanked (floxed) sequences in all tissues including germ cells. (B) PCR-based strategy to demonstrate Cre-recombinase deletion of the Wwox target sequences. (C) Wwox protein expression is abolished in Wwox KO mice. Total protein extracts from the indicated tissues were analyzed by immunoblotting using Wwox specific antibodies. Anti-actin was used as a loading control. WT-wild-type, HET-heterozygous, KO-knockout.

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**Figure 3.** Wwox KO mice have reduced postnatal growth. (A) Photograph of WT and KO newborn littermates. (B) F2 pups were weighed on postnatal days 3, 10, 14 and 17. 100% of Wwox KO mice died before weaning (21 days). WT, n = 18; HET, n = 43; KO, n = 8. Error bars represent ±SEM.

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Ablation of Wwox Expression by EIIA-Cre Mediated Deletion Resulted in Impaired Hematopoiesis and Splenic Atrophy

To try to understand the cause of death of Wwox KO mice we performed necropsies and analyzed blood chemistries of mice at postnatal day 18. Necropsy results found significantly reduced relative weights of multiple organs and increased brain weight in KO mice consistent with wasting (Table 2). Interestingly, spleens of KO mice were dramatically reduced in size and weight when compared with those of wild-type and heterozygous. The average
Wwox KO spleen weighed 9.5 mg on average, representing 0.21% of whole body weight, while spleens from wild-type and heterozygotes mice weighed on average 4 times as much representing up to 0.53–0.46% of whole body weight, i.e more than 2 fold that relative weight of KO mice (p = 0.0015) (Table 2). The histology of the Wwox KO mice spleens was very abnormal displaying signs of spleen atrophy. As can be observed in Figure 4, the red pulp displayed dramatically reduced cellularity, although hematopoietic cells of all series appear to be present. Lymphoid aggregates of the white pulp of the KO spleens were also reduced in size compared to the WT spleens.

We also compared the histology of thymus that also displayed reduced relative weight (p = 0.05). We observed that in spite of preserving relatively normal organ architecture, the cortex of the thymus of KO mice is significantly thinner than that of wild type counterparts (Figure 4).

Given these histopathological findings we proceeded to perform some hematological analyses. White blood counts (WBC) were estimated manually on blood smears obtained from mice of the different genotypes. WBC for wild-type mice was estimated at an average of 9.45×10⁶ cells per μl (±2.25, n = 2) while Wwox KO mice showed signs of leukopenia with lower WBC at an average of 3.9×10⁶ cells per μl (±1.20, n = 2). No major differences were observed in the relative representation of the various white blood cell subtypes (not shown). However, one of the two KO mice analyzed displayed 3% of nucleated red blood cells (NRBC)/100 WBCs, a clear sign of severe anemia, while none of the WT mice showed any NRBCs. In summary, the histopathological and hematological findings suggest significantly impaired hematopoiesis in Wwox KO mice affecting white and red blood cell lineages.

**Table 2. Organ Weights.**

| Organ   | WT (n = 3) | HET (n = 3) | KO (n = 3) | p-value*** |
|---------|------------|------------|------------|------------|
| Spleen (mg) | 39.4*      | 33.3       | 9.30       | 0.0015     |
| Liver (g)   | 0.320      | 0.279      | 0.144      | 0.0367     |
| Kidney (g)  | 0.120      | 0.110      | 0.059      | NS         |
| Adrenal (mg)| 2.27       | 1.67       | 2.97       | NS         |
| Thymus (mg)| 60.0       | 55.0       | 25.1       | 0.0495     |
| Heart      | 0.063      | 0.056      | 0.034      | NS         |
| Brain      | 0.390      | 0.371      | 0.356      | 0.0003     |
| Brain      | 5.0(±0.31)%| 5.2(±0.28) | 8.5(±0.65)%| NS         |

Organ weights are given as average *absolute weight or **absolute weight/organ weight as a % of body weight (±SEM)*.

***p-value using student’s t-test comparing WT+HET vs. KO; NS-p > 0.05.

**Table 3. Blood Chemistry Analysis.**

| Test                     | WT (n = 3)   | HET (n = 3) | KO (n = 4) | p-value** |
|--------------------------|--------------|-------------|------------|-----------|
| BUN (mg/dL)              | 17.67±2.60   | 18.33±1.20  | 37.25±5.73 | 0.01086   |
| Glucose (mg/dL)          | 250.6±5.04   | 227.0±11.7  | 143.50±8.81| 0.000131  |
| Total Protein (g/dL)     | 4.20±0.058   | 4.10±0.000  | 4.10±0.252 | NS        |
| Albumin (g/dL)           | 2.70±0.000   | 2.63±0.067  | 2.60±0.200 | NS        |
| Globulin (g/dL)          | 1.50±0.580   | 1.47±0.067  | 1.50±0.058 | NS        |
| Calcium (mg/dL)          | 11.13±0.120  | 10.87±0.120 | 10.18±0.295| 0.0000385 |
| Phosphorus (mg/dL)       | 12.60±0.306  | 12.37±0.318 | 12.73±0.544| NS        |
| Sodium (Eq/L)            | 143.00±0.577 | 143.67±0.67 | 142.00±6.00| NS        |
| Potassium (mEq/L)        | 8.97±0.219   | 8.90±0.252  | 9.90±1.19  | NS        |
| Chloride (mEq/L)         | 108.67±0.667 | 108.67±1.20 | 112.50±1.50| 0.02769   |
| Total CO₂ (mEq/L)        | 21.67±0.333  | 21.33±0.333 | 14.50±3.5  | 0.006227  |
| Osmolarity (mOsm/kg)     | 306.23±0.517 | 306.50±1.55 | 304.75±14.2 | NS       |

*Values are presented as the average ± SEM.

**p-value using student’s t-test comparing WT+HET vs. KO; NS-p > 0.05.

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neoplastic growth by any of the methodologies. Importantly, X ray
histopathology. We did not observed any lesions compatible with
complete necropsies, X ray analyses and multiple organ
analyses did not reveal any bone lesions compatible with
osteosarcoma.

Discussion

The WWOX tumor suppressor gene is inactivated in many types of
human cancers by genomic rearrangements, aberrant mRNA
splicing, homozygous deletions and epigenetic silencing [1–5,24].
Because Wwox KO mice do not survive to adulthood previous
studies of Wwox tumor suppression required chemical carcinogen treatment of Wwox<sup>−/−</sup> mice [20]. Therefore, we have generated mice carrying a conditional allele of the Wwox tumor suppressor gene.

In this report we describe the generation and initial characterization of a novel mouse model with a conditional Wwox allele. We utilized adenovirus EIIA-regulated Cre-recombinase transgenic mice to delete Wwox Exon 1 in all mouse tissues and ultimately generated Wwox KO mice. We found that Wwox KO mice suffered from severe metabolic defect(s) resulting in significant growth retardation, hypoglycemia, impaired hematopoiesis, and signs of metabolic acidosis that ultimately lead to premature death of all mice by 3 weeks of age. Bone formation of Wwox KO was also significantly affected. Bones from KO mice were analyzed and found to be smaller and thinner having reduced bone volume as a consequence of a defect in mineralization.

The results of the blood chemistry analysis demonstrated that Wwox KO mice had severe metabolic defects. Hypoglycemia can result from multiple causes such as disorders in carbohydrate, fat or amino acid metabolism as well as endocrine system disorders. Since Wwox is expressed in a variety of endocrine, neuroendocrine and non-endocrine tissues it is not possible from these studies to identify the cause of hypoglycemia. The combination of increased BUN with reduced bicarbonate levels (hypocapnia) suggests impaired kidney function and a disturbance in the blood acid-base balance that would result in acidosis. Protracted acidosis disturbs many bodily functions and can lead to growth retardation, bone disease, possibly total kidney failure and ultimately death. Renal tubular acidosis (RTA) is a pathology that occurs when the kidneys fail to excrete acids into the urine, which causes the blood to remain too acidic due to defects of cells in the proximal and distal convoluted tubules. The convoluted tubules of healthy kidneys help maintain acid-base balance by excreting acids into the urine and returning bicarbonate to the blood. Interestingly, we have previously demonstrated that Wwox is highly expressed in distal convoluted tubules and to a lesser extent in the proximal tubules of the kidney in mice (Figure 6) and humans [25]. Given the pathology observed and the location of Wwox expression in the kidney we hypothesize that WWOX may play a role in the regulation of blood pH and maintenance of electrolyte balance. Thus, we speculate that the lack of Wwox expression in the kidney tubules in KO mice is likely responsible for the development of severe metabolic acidosis of renal tubule origin (RTA) that ultimately leads to death. In addition, as significant contributing factors to morbidity and death, we also observed severe histopathological abnormalities affecting hematopoietic organs such as spleen and thymus in addition to white blood cell counts compatible with a significant suppression on hematopoiesis affecting the Wwox KO mice. Specifically, the spleens of Wwox KO mice displayed signs of atrophy with dramatic reduction in red pulp cellularity. All studies indicate that Wwox KO mice suffered from anemia and leukopenia. Anemia can be also intimately linked to kidney function and to metabolic acidosis. Additional studies will be required to determine the exact causes of the impaired hematopoiesis observed as a result of Wwox ablation.

Wwox KO mice have been described to suffer alterations in bone metabolism [16]. We developed this conditional mouse model to facilitate the exploration of the cell autonomous function(s) of Wwox that contribute to the variety of abnormalities we and others have observed. Therefore, we wanted to determine whether our mouse model of EIIA-Cre mediated ablation of Wwox expression recapitulated the observed defects in normal mouse bone development. We used micro-CT and histomorphometry to measure several static bone parameters. We observed a decrease of bone formation in Wwox KO mice associated with a reduction of mineralization and concomitant increase in osteoid compared to WT and HET mice. Proper bone formation results from a fine balance between bone formation and bone resorption. This balance is mostly maintained by the activity of two bone cell types, osteoblasts and osteoclasts, that make bone and degrade bone respectively. We measured osteoblast activity and found no significant differences in the number of active and non-active osteoblasts in femurs between genotypes. Measurements of osteoclast activity did not show any differences in femurs from mice of any genotype either. This is in sharp contrast to the observations of Aqeilan et al [16] who observed consistently higher numbers of osteoclast activity in bone sections of KO mice however they did not perform quantitative analyses in those studies. We conclude that Wwox KO mice have reduced bone formation due to a bone mineralization defect. This is consistent with previous results that demonstrated a significant defect in differentiation of Wwox KO calvarial osteoblasts ex vivo compared to WT osteoblasts [16]. Notably, heterozygous mice did not show any abnormalities in postnatal growth and survival as well as blood or bone parameters highlighting that only one Wwox allele is sufficient for full function.

Importantly, Aqeilan et al. also reported that 4 of 13 (31%) mice Wwox KO mice developed focal lesions along the femoral diaphysis compatible with periostal osteosarcoma, lesions observed in one mice as early as 3d of age [20]. It is worth noting that in contrast with those previous observations, we did not detect any lesions compatible with spontaneous osteosarcomas in any of the Wwox KO mice analyzed macroscopically, histopathologically and radiographically. Furthermore, none of the long bones that were subjected to MicroCT analyses revealed any malignant lesions either. The reason(s) for the discrepancies between studies remain to be determined.

Very recently a spontaneous mutation in the Wwox gene was identified in rats that showed severe growth retardation, experienced epileptic seizures and died without reaching maturity [26]. The mutation, named lde for lethal dwarfism with epilepsy, was identified as a 13-bp deletion in Exon 9 of the Wwox gene resulting in a frame shift mutation causing aberrant amino acid sequences (371-424aa) at the C-terminal of the Wwox protein. Remarkably, lde/lde rats displayed phenotypic characteristics similar to Wwox KO mice including severe dwarfism and early lethality. The cause of dwarfism is not clear, however, lde/lde rats had slightly lower bone density and no osteosarcomas developed during their short life span [27]. Interestingly, lde/lde rats displayed...
significantly higher levels of BUN compared to normal rats [27] consistent with the blood chemistry of our Wwox KO mice.

In conclusion, we have generated a novel mouse model with a conditional allele at the Wwox locus. We demonstrated that homozygous Cre-recombinase mediated deletion of Wwox Exon 1 lead to complete ablation of Wwox expression. Loss of Wwox expression by this approach resulted in a phenotype similar to the previously reported Wwox KO generated by conventional techniques making this Wwox<sup>floxed</sup> strain an important reagent for studying Wwox function in normal mouse physiology and tissue specific tumorigenesis.

Materials and Methods

Animal Husbandry

Mice used in this were kept in a clean, modified-barrier animal facility, fed regular commercial mouse diet (Harlan Lab., Indianapolis, IN) under controlled light (12L:12D) and temperature (68–74°F). All animal research was conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) at the University of Texas, M.D. Anderson Cancer Center, Science Park Research Division, following international guidelines and all research was approved by the corresponding Institutional Bioethics Committee (IACUC).

Gene Targeting and Generation of a Wwox-Floxed Allele

The Wwox targeting construct was generated using ~6 Kb of Wwox gene obtained from BAC clone 9N99 containing a 5′ portion of the Wwox gene from 129/svJ mouse genomic DNA. Primers used for generating the targeting construct were: Right arm forward, 5′-CAAACCAGGAAACTGATTACGC-3′; Right arm reverse, 5′-CCTAGACTGTGGTCGTCCCAA-3′; Left arm forward, 5′-AGGGAGGAAGAGGAAGATGAGG-3′; Left arm reverse, 5′-CAGCTACTGGCGG-GAAAGGG-3′. First, a ~4 Kb genomic sequence containing Wwox Exon 1 and a portion of Intron 1 was PCR amplified and cloned into plasmid pK-11 [28]. pK-11 contains the neomycin phosphotransferase gene under control of the phosphoglycerol kinase promoter (pgk-neo) used as the positive-selection element and a single LoxP Cre-recombinase recognition site. Another LoxP Cre-recombinase recognition site was inserted into Wwox Exon 1 in the same orientation as the pK-11 LoxP site in order to “flox” Wwox Exon 1 (Wwox<sup>floxed</sup>) resulting in the plasmid pK-11-Wwox-flox. The left arm of the targeting construct was generated by cloning ~2 Kb of the Wwox 5′ upstream sequence into the plasmid pBJ101-DT [29]. Plasmid pBJ101-DT contains the diptheria toxin A-chain gene (DT-A) used as the negative-selection element. The final targeting construct was generated by transferring the DT-A gene and Wwox left arm sequences to pK-11-Wwox-flox plasmid (Figure 1A).

The Wwox target construct was then used by the UT MD Anderson Genetically Engineered Mouse Facility (GEMF) for generation of targeted ES cells and mouse chimeras. ES cell clones were electroporated with the targeting vector that had been linearized by digest with Not I restriction endonuclease. Transfected ES cells were selected for G418-resistance and individual clones screened by Southern hybridization. Genomic DNA was digested with Bgl II and hybridized to a probe corresponding to a region of Wwox Exon 1 outside of the targeting construct sequences (Figure 1A). Correctly targeted ES cells were identified by hybridization to two Bgl II restriction fragments of ~9 Kb and ~4 Kb representing the Wwox<sup>−</sup> and Wwox<sup>floxed</sup> alleles, respectively (Figure 1A,B). The hybridization probe was generated by PCR amplification with the following primers: HYB probe reverse, 5′-TCCTTCTGCGAAATCCCGTATGC-3′; HYB probe forward, 5′-CAAAACTCAAGTGAAGAAACAGG-3′ using 129/sv mouse genomic DNA as a template. Two correctly targeted ES cell lines were identified from 240 ES cell clones screened.

Injections of targeted ES cells into C57BL/6 blastocysts were performed to generate male chimeras that were mated to C57BL/6 females to test for germline transmission of the Wwox<sup>−</sup> allele. Tail DNA samples from agouti offspring were genotyped by Southern hybridization. Wwox<sup>−</sup>flox offspring were then used as the F<sub>1</sub> generation for subsequent use. After establishing Mendelian transmission of the Wwox<sup>−</sup> allele genotyping was performed using a multiplex PCR method (Figure 1C,D). The following primers were used for PCR genotyping: Primer A: 5′-ATGGGAGAAGACTGGAGCTCAGAA-3′; Primer B: 5′-TCAGCAACTCACCTCTGGCTTCAAC-3′; Primer C: 5′-GCATAGATTA-TAGAAGGTATTTGAG-3′.

Generation of Wwox Knockout Mice Using EIIA-Cre Mediated Deletion

Female Wwox<sup>floxed/flox</sup> mice were bred with male mice homozygous for the Cre-recombinase gene under the control of the adenovirus EIIA-promoter to generate the Wwox<sup>−/−</sup>KO F<sub>1</sub> generation. EIIA-Cre transgenic mice [23] were obtained from The Jackson Laboratory (Bar Harbor, ME; stock number: 003724). Genotypes were determined by PCR using the oligonucleotide primers: Primer X: 5′-GCCCTCAAGAAGAGGCTGCAAATTT-3′; Primer Y: 5′-GAGCCAGATCCCCCTAAATACGG-3′; Primer Z: 5′-GCCCTCAAGAAGAGGCTGCAAATTT-3′.

Western Blot Analysis

Mouse tissues were dissected and flash frozen in liquid nitrogen then crushed into a fine powder with a mortar and pestle. Total protein lysates were made by suspension of crushed tissue with lysis buffer (1% Igepal CA-630, 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol) containing protease inhibitors and incubation at 4°C for 30 min. with constant agitation. Insoluble material was then removed by centrifugation at 10,000 x g for 10 min. at 4°C. Protein concentration was determined using the BCA method (Pierce, Rockford, IL). Western blot analysis was performed as previously described [21] using 30 µg of total protein. WWOX protein was detected using a rabbit polyclonal antibody raised against the WWOX WW-domains [24]. The amount of β-actin in each sample was used as an internal control. β-actin was detected using a mouse monoclonal antibody (Sigma Aldrich, St. Louis, MO).

Mouse Necropsy and Histological Analyses

Mice were euthanized by CO₂ asphyxiation, organs harvested and immediately weighed using a Mettler AE50 analytical balance. Organs were then fixed in formalin for 24 hours, then 70% ethanol and embedded in paraffin. Tissue sections were stained in H&E or processed for immunostaining. Blood was collected immediately following euthanasia by intracardiac puncture on the left ventricle and allowed to clot at room temperature for 30 minutes. Serum was collected following centrifugation for 10 minutes at 14,000 rpm at 4°C. Blood chemistries were measured using serum from each individual mouse using the Olympus AU400 automated chemistry analyzer. Manual differential and WBC were obtained from feathered blood smears using fresh whole blood from each individual animal.
Bone Analyses
Limbs were dissected and soft tissue was carefully removed then fixed in formalin for 48 hours followed by 70% ethanol. Microcomputed tomography (μCT) was performed at the Baylor College of Medicine MicroCT Core facility. Bone histomorphometric analyses were performed at the UT MD Anderson Bone Histomorphometry Core Laboratory (Director, Dr Nora Navone) following the guidelines established by the American Society for Bone and Mineral Research (ASMBR) histomorphometry nomenclature committee.

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Author Contributions
Conceived and designed the experiments: JHLM MB CMA. Performed the experiments: JHLM HK JPT DK. Analyzed the data: JHLM DK MB CMA. Wrote the paper: JHLM CMA.