Research Article

Alterations of sex determination pathways in the genital ridges of males with limited Y chromosome genes[†]

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Abstract

We previously demonstrated that in the mouse only two Y chromosome genes are required for a male to produce an offspring with the help of assisted reproduction technologies (ART): testis determinant *Sry* and spermatogonial proliferation factor *Eif2s3y*. Subsequently, we have shown that the function of these genes can be replaced by transgenic overexpression of their homologs, autosomally encoded *Sox9* and X-chromosome encoded *Eif2s3x*. Males with Y chromosome contribution limited to two ($X^{Eif2s3y}OSry$), one ($X^{Eif2s3y}OSox9$ and $XOSry,Eif2s3x$), and no genes ($XOSox9,Eif2s3x$) produced haploid germ cells and sired offspring after ART. However, despite successful assisted reproductive outcome, they had smaller testes and displayed abnormal development of the seminiferous epithelium and testicular interstitium. Here we explored whether these testicular defects originated from altered pro-testis and pro-ovary factor signaling in genital ridges at the time of sex determination. Timed pregnancies were generated to obtain transgenic $X^{Eif2s3y}OSry$, $X^{Eif2s3y}OSox9$, $XOSry,Eif2s3x$, $XOSox9,Eif2s3x$, and wild-type XX and XY fetuses at 12.5 days post coitum. Dissected genital ridges were assessed for their morphology and anatomy, and expression of pro-testis and pro-ovary transcripts. All transgenic males displayed incomplete masculinization of gonadal shape, impaired development of testicular cords and gonadal vasculature, and decreased expression of factors promoting male pathway. Fetal gonad masculinization was more effective when sex determination was driven by the *Sry* transgene, in the presence of Y chromosome genes, and to a lesser extent a double dosage of X genes. The study adds to the understanding of the role of Y chromosome genes and their homologs during sex determination.

Summary Sentence

Males with limited Y chromosome gene contribution undergo incomplete gonadal masculinization during fetal development.

Key words: testis, sex determination, sex differentiation, X chromosome, Y chromosome.

Introduction

Testis and ovary are sexually dimorphic and highly specialized organs that develop from a common embryonic precursor—the genital ridges. The Y chromosome encoded *Sry* initiates molecular sig-

naling that leads genital ridge development towards testicular fate [1, 2]. In mice, *Sry* is expressed transiently, starting at 10.5 days post coitum (dpc) and ending at 12.5 dpc [3–5]. *Sry* expression has also been observed in adult testes; while these transcripts are

thought to not be translatable [6] they might function as epigenetic regulator(s) [7]. The primary molecular target of SRY in the developing XY gonads is autosomally encoded *Sox9* [8], which directs subsequent male-specific differentiation of presupporting somatic cell lineage into Sertoli cells (reviewed in [9–11]). Once activated, SOX9 upregulates expression of other testis-specific markers such as *Fgf9*, *Amb*, *Cyp26b1* [12–14] and through direct or indirect inhibition keeps ovarian markers such as *Foxl2*, *Wnt4*, canonical β -catenin, and *Rspo1* signaling at low levels [15–17]. In male gonads, sufficient levels of SOX9 are needed for both specification and active maintenance of Sertoli cell lineage, testis cord formation, differentiation of the primordial germ cells into male germ line progenitor cells, and formation of the coelomic blood vessel (reviewed in [10, 18–20]).

For normal gonadal development to occur, sex determining molecular signals must sufficiently antagonize gene expression that promotes the opposite sex. Failure to fully inhibit pro-ovary signaling in the future testis, or pro-testis signaling in the future ovary, often translates into abnormal gonadal development that is characteristic of disorders of sex development. Genetically derived perturbations can lead to a deviation from expected gene expression levels at the time of sex determination and abnormal testicular function in the adulthood (reviewed in [11, 18]). Interference with testicular differentiation pathways leads to abnormal supporting lineage development, failure of proper testis cords formation, and ovary-like tissue organization in the gonadal mesenchyme [21, 22].

In our previous studies, we showed that in the mouse only two Y chromosome genes are sufficient for successful assisted reproduction: *Sry* and the spermatogonial proliferation factor *Eif2s3y* [23]. Subsequently, we demonstrated that the function of these two “minimal” Y chromosome genes can be replaced by overexpression of either an autosomal target (*Sry*-to-*Sox9* replacement) [24, 25] or an X chromosome-encoded homolog (*Eif2s3y*-to-*Eif2s3x* replacement) [25]. Males with Y chromosome contribution limited to two genes ($X^{Eif2s3y}OSry$), one gene ($X^{Eif2s3y}OSox9$ and $XOSry,Eif2s3x$), and no genes ($XOSox9,Eif2s3x$) were capable of producing haploid germ cells and father offspring after assisted reproduction [25]. However, they had smaller testes and displayed abnormal development of seminiferous epithelium and testicular interstitium [25, 26]. The most severely affected were some of the $XOSox9,Eif2s3x$ males; these males also had an increased expression of pro-ovary factors (*Foxl2*, *Wnt4*, and *Rspo1*) in the testes [25].

These observations prompted us to hypothesize that testicular abnormalities observed in adult males with limited Y chromosome genes originate from altered pro-testis and pro-ovary factor signaling in genital ridges at the time of sex determination. Here, we investigated the effects of limited presence of Y chromosome genes on early gonadal development and sex determination-specific molecular signaling. We chose to focus our investigations on the time (12.5 dpc) when male genital ridges are morphologically distinguishable based on organ shape, presence or absence of testis cords and vascular development, and when sexually dimorphic gene expression patterns are clearly established. Our unique genetic system allowed us to evaluate functional interplay between four key genes (*Sry*, *Sox9*, *Eif2s3y*, and *Eif2s3x*) in three different sex chromosome contexts (XO, XX, and XY^{Tdym1}). We demonstrated that males with limited Y gene contribution displayed impairment in gonadal development and alterations in sex-specific molecular signaling, with transgene and sex chromosome context-dependent differences.

Materials and methods

Animals

The mice used in the study were bred “in house” as described below. The mice were fed ad libitum with a standard diet and maintained in a temperature- and light-controlled room in accordance with the guidelines of the Laboratory Animal Services at the University of Hawaii and guidelines presented in National Research Council’s Guide for Care and Use of Laboratory Animals published in 1996 by Institute for Laboratory Animal Research (ILAR) of the National Academy of Science (Bethesda, MD).

The primary genotypes of interests used in this study were male mice with a single X chromosome (XO) and a limited Y chromosome gene complement:

1. Male mice with two Y chromosome genes: $X^E OSry$, $X^{Eif2s3y} OSry$ (abbreviated as $X^E OSry$) carry an autosomally encoded *Sry* [Tg(*Sry*)2Ei] [27] and X chromosome encoded *Eif2s3y* [28] (designated as X^E) transgene in the context of a single X chromosome.
2. Male mice with one Y chromosome gene: $XOSry,Eif2s3x$. $XOSry,Eif2s3x$ have male sex determination initiated by the autosomally encoded *Sry* transgene [27] and spermatogenesis initiated by overexpression of the autosomally encoded *Eif2s3x* transgene [25].
3. Male mice with one Y chromosome gene: $X^E OSox9$. $X^{Eif2s3y} OSox9$ (abbreviated as $X^E OSox9$) have male sex determination initiated by overexpression of the autosomally encoded *Sox9* transgene driven by the *Wt1* (Wilms tumor 1) promoter [29] and carry the X chromosome-encoded *Eif2s3y* transgene [28].
4. Male mice with no Y chromosome genes: $XOSox9,Eif2s3x$. $XOSox9,Eif2s3x$ have male sex determination resulting from overexpression of the autosomally encoded *Sox9* transgene driven by the *Wt1* promoter [29] and spermatogenesis driven by the autosomally encoded *Eif2s3x* transgene [28].

In addition to the four primary genotypes of interest (“XO males”), we also examined analogous male genotypes with two X chromosomes (“XX males”) carrying the same transgenes needed for male sex determination and spermatogenesis initiation: $X^E XSry$ (two Y chromosome genes), $XXSry,Eif2s3x$ and $X^E XSox9$ (one Y chromosome gene), and $XXSox9,Eif2s3x$ (no Y chromosome genes). We also examined gonadal development in males carrying an X chromosome and a Y chromosome with a 11-kb deletion removing endogenous testis determinant *Sry* (Y^{Tdym1}) (*dl1Rlb*) [1, 30] and *Sry,Eif2s3x* or *Sox9,Eif2s3x* transgene sets ($XY^{Tdym1}Sry,Eif2s3x$ and $XY^{Tdym1}Sox9,Eif2s3x$, respectively) for comparison with analogous XX and XO males. Finally, mice carrying XY and XX sex chromosomes were used as wild-type controls.

The males of interest were obtained by breeding XO females with males carrying the Y^{Tdym1} chromosome and one of the two transgenes, *Sry* [27] or *Sox9* [29], needed for male sex determination, and one of the two transgenes, *Eif2s3y* [28] or *Eif2s3x* [25], needed for spermatogenesis initiation (Figure 1). The males of interest were on a mixed genetic background (outbred MF1 female $\times$ inbred C57BL/6 male). This is because the only stocks that allowed us to generate XO mothers “in house” were on the MF1 background while the transgenic fathers were only available on the C57BL/6 background. Outbred CD1 mice were used as wild-type controls (XX and XY) due to lack of wild-type genotypes on the MF1 background; both MF1 and CD1 are outbred Swiss stocks.

(A) Cross used to obtain males with two Y chromosome genes: *Sry* and *Eif2s3y* (B) Cross used to obtain males with one Y chromosome gene: *Eif2s3y* (C) Cross used to obtain males with one Y chromosome genes: *Sry*

(D) Cross used to obtain males with no Y chromosome genes

Figure 1. Breeding schemes. The breeding schemes that were used to produce male progeny with limited Y chromosome genes. The primary genotypes of interest are shown in yellow boxes: X^EOSry (A), X^EOSox9 (B), XOSry,Eif2s3x (C), XOSox9,Eif2s3x (D). Female genotypes and male genotypes are shown in pink and blue font, respectively. X and Y represent wild-type sex chromosomes. X^E is an X chromosome carrying the *Eif2s3y* transgene. Y^{Tdym1} is a Y chromosome carrying a deletion of endogenous *Sry*. *Eif2s3x*, *Sry*, and *Sox9* are autosomally encoded transgenes. Embryos lacking an X chromosome do not survive (crossed). Progeny genotype distribution is shown as a number and an average percentage (mean ± SEM; with n = number of litters: 18, 8, 15, and 14 for A, B, C, and D, respectively) of pups of each genotype. One-way ANOVA analysis revealed significant differences in progeny genotype distribution: $P = 0.003$ (A); $P = 0.0052$ (B); $P = 0.0032$ (C); and $P < 0.0001$ (D). For other analyses, see Supplementary Figures S2–S4.

Figure 2. Morphometric evaluation of gonadal shape in the context of XO. Four types of males with limited Y chromosome gene complement (tested: X^EOSry, X^EOSox9, X^EOSry, Eif2s3x, and X^EOSox9, Eif2s3x) were compared to wild-type XY and XX controls. (A) Diagrammatic presentation of measures for morphometric analysis; (B) gonadal width; (C) gonadal length: width ratio. (D) Exemplary images of genital ridges. Graphs are mean $\pm$ SDev ($n \geq 4$, except X^EOSox9 $n = 2$). Statistical significance (t -test, $P < 0.05$): graph bars marked with different letters are significantly different from each other while those marked with the same letters do not differ. tg = transgene. Bar = 100 μ m.

Chemicals

Chemicals were obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise stated.

Isolation of genital ridges

Embryos were collected from timed matings, with noon of the day on which the mating plug was observed designated as 0.5 dpc. The embryos were collected at 12.5 dpc into ice-cold DEPC-PBS (diethyl pyrocarbonate-phosphate buffer solution). Tail somites were counted from the first somite at the mid-region of hind limb to the tip of the tail. Genital ridges with mesonephros attached were dissected using surgical instruments (fine tip forceps and 25G $\times$ 5/8" syringe needles) under a stereomicroscope, photographed, snap-frozen in liquid nitrogen, and kept at -80°C until further processing.

Genotyping

Embryos of interest were identified by a combination of PCR and quantitative PCR (qPCR) as described before [25]. First, all embryos were screened using conventional PCR amplifying selected Y chromosome genes (*Ssty*, *Smcy*, *Zfy*) to detect genotypes carrying the Y^{Tdym1} chromosome, and then amplifying desired transgenes (*Eif2s3x*, *Eif2s3y*, *Sry*, *Sox9*). Transgenic XX and XO embryos were then screened for X chromosome copy number using qPCR to amplify X-linked *Prdx4* and *Amelx*, and autosomally linked *Atr* sequences. All qPCR samples were tested in quadruplicate per assay using single X chromosome genotype samples as a reference control. Copy number estimation for each gene was calculated with the $\Delta\Delta\text{Ct}$ method. Briefly, ΔCt values were calculated as a difference between the tested gene and *Atr*. $\Delta\Delta\text{Ct}$ values were calculated by subtracting ΔCt of tested genes from the reference samples. The copy numbers

were calculated by raising 2 to the power of $\Delta\Delta\text{Ct}$ ($2^{\Delta\Delta\text{Ct}}$). The genotypes were inferred from the copies of each target gene: XO, 1 *Prdx4* + 1 *Amelx*; XX, 2 *Prdx4* + 2 *Amelx*. Primer sequences are shown in Supplementary Table S1.

Evaluation of genital ridge morphology

Dissected 12.5 dpc genital ridges (with mesonephroi attached) were placed in the dissection medium (DEPC-PBS) in a culture dish and imaged using stereomicroscope under $\times 8$ magnification. Images were processed for contrast and saturation adjustment using *ImageJ* software [31]. Data collected from the photographs included measurements of gonadal length, gonadal width, coelomic gonadal surface (distal from mesonephros), length of the coelomic artery, testis cord count, and testis cord width.

Genital ridge width measurements were obtained at the midpoint of the gonadal longitudinal axis by drawing a line from the gonadal coelomic margin to the intersection with mesonephros, which could easily be determined due to change of tissue density. Genital ridge length measurements were acquired using segmented line tool that connected gonadal mid-width points starting with the anterior margin to the center of the gonad, and then to the distal margin (Figure 2A). Overall gonadal shape was defined as genital ridge length: width ratio.

Testis cord formation was inferred from the evidence of parallel vertical thickenings formed in the interstitial compartment, which gave the examined genital ridges a characteristic male pattern. The cords were identified as elongated regions of higher transparency running vertically across the transverse plane of the genital ridges. The number of individual and well-defined arc-like structures that were visible along the coelomic margin of the genital ridges (Figure 3A) was used for testis cord counts. Cords that displayed

Figure 3. Testis cord and gonadal vasculature development evaluation in the context of XO. Four types of males with limited Y chromosome gene complement (tested: X^EOSry, X^EOSox9, XO Sry, Eif2s3x, and XO Sox9, Eif2s3x) were compared to wild-type XY controls in terms of testis cord and gonadal vasculature development. (A) Exemplary images of XY (top) and XO Sox9, Eif2s3x (bottom) genital ridges displaying parameters used in morphometric analyses of testis cord and gonad vasculature development. Black dashed lines are outlines of the testis cords; blue arrows show testis cord width; red arrowheads point to coelomic artery; red arrows show interstitial arterial branches; bar = 100 μm; (B) testis cord number; (C) testis cord width; (D) coelomic artery development expressed as percentage of genital ridge coelomic surface coverage; (E) overall vascular score expressed using 6 point system, with maximum two points derived from each category: coelomic artery visibility, coelomic artery surface coverage, and number of vascular clusters in the interstitial domain (see Methods section for more details). Graphs are mean ± SDev (B, D, and E, n > 3, except X^EOSox9 n = 2) and mean ± SEM (C, n > 4). Statistical significance (t-test, P < 0.05): graph bars marked with different letters are significantly different from each other while those marked with the same letters do not differ. tg = transgene.

branching patterns and were arising close to the middle or mesonephric surface were not included in the counts. Testis cord width was measured at the coelomic end of genital ridges as the distance between parallel thickenings representing mesenchymal interstitium. The most rostral and most caudal cords that were within the rounded edges of the genital ridges were omitted from the testis cord width counts.

Development of the coelomic artery was evaluated by calculating a percentage of genital ridge coelomic surface coverage. Overall vascular score was determined using a 6-point scoring system, where 2 points were derived from each of three categories: (a) coelomic vessel visibility with 2 points = clearly visible, 1 point = barely visible, but with clear evidence of linear trace; 0 points = absent; (b) coelomic artery surface coverage: 2 = 90%–60%, 1 point = 59%–30%, 0 points = 29% or less; (c) evidence of vascular clustering in the interstitial compartment: 2 points = 8 or more branches observed; 1 point = 7–4 branches observed; 0 points = 3 or less branches observed. For the interstitial vasculature evaluation category, only those vascular structures were considered that had a vertical pattern, were perpendicular to the mesonephroi, and laid within the interstitial domain. The 90th percentile was considered top limit for the coelomic artery coverage of the coelomic surface because this number never exceeded in XY controls.

RNA isolation and real-time RT-PCR

Genital ridge RNA was isolated from individual pairs of gonads using Micro RNA kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Reverse transcription of polyadenylated RNA was performed with Superscript Reverse Transcriptase III, according to the manufacturer's guidelines (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using SYBR Green PCR Master mix on an ABI QuantStudio 12K Flex machine (Applied Biosystems, Carlsbad, CA, USA). At least three biological samples were used

for each genotype, except for X^EOSox9 (n = 2; only two males of this genotype were identified among 65 fetuses). All reactions were carried out in quadruplicate per assay and normalized to three ubiquitously expressed genes, *Sdha*, *Rps29*, and *Tbp*, which are considered suitable and stable normalizing genes for quantitative real-time PCR analysis of gene expression in fetal mouse gonads [32]. The ΔCt value for each individual sample was calculated by subtracting the Ct value of a tested gene from the geometric mean of the three loading controls. The ΔΔCt value was calculated by subtracting the ΔCt of each tested male from the average ΔCt of reference samples (XY males). The data were expressed as a fold value of expression level. Primers sequences are shown in Supplementary Table S1.

Statistics

Student t-test was used for most analyses, with P < 0.05 considered as statistically significant difference. If data were expressed as percentages, the percentages were converted to angles for statistical analyses. One-way ANOVA was used to assess offspring genotype differences.

Results

XO males with two, one, and no Y chromosome genes appear rarely among expected progeny genotypes

The breeding themes required to produce males with two, one, and no Y genes yield a variety of offspring genotypes, not all of which are viable (Figure 1). Crosses allowing to obtain X^EOSry, XO Sry, Eif2s3x, X^EOSox9, and XO Sox9, Eif2s3x males resulted in 114, 74, 65, and 115 offspring, respectively. All transgenic crosses were less efficient than breeding of XX × XY controls, evidenced as an approximately twofold litter size reduction (Supplementary Figure S1). All expected progeny genotypes were obtained from

crosses expected to yield $X^E OSry$, $X OSry, Eif2s3x$, $X^E OSox9$ males (Figure 2A–C). However, the cross allowing generation of $X OSox9, Eif2s3x$ males did not yield 4 out of 12 expected viable genotypes (Figure 1D; missing genotypes: $X OEif2s3x$, $XY^{Tdim1} Sox9$, $XX Sox9$, and $X OSox9$).

When progeny frequency data were analyzed, the following observations were made. First, XO genotypes, and XO males in particular, were obtained less frequently than other genotypes (Supplementary Figure S2). This was especially true in the context of the *Eif2s3y* transgene. $X^E Y^{Tdim1} Sry$ and XO cross yielded only three $X^E OSry$ fetuses (2.6% of all progeny). Similarly, after approximately 9 months of mating of $X^E Y^{Tdim1} Sox9$ males and XO females only two $X^E OSox9$ fetuses (3.6% of all offspring) were obtained (Figure 1A and B; Supplementary Figure S2A and B). The analogous transgene combinations in the context of XY^{Tdim1} or XX chromosomes appeared more frequently; this was especially prominent in the context of transgenic *Eif2s3y*, where $X^E XSry$ and $X^E X Sox9$ males appeared five to eight times more frequently than $X^E OSry$ and $X^E OSox9$ (Figure 1A and B; Supplementary Figure S2A and B). In the context of transgenic *Eif2s3x*, $XX Sox9, Eif2s3x$ progeny were approximately two times more frequent than $X OSox9, Eif2s3x$ but there was no difference in the yield of $XX Sry, Eif2s3x$ and $X OSry, Eif2s3x$ males (Figure 1C and D; Supplementary Figure S2C and D).

Because the *Eif2s3y* transgene is encoded on the paternal X chromosome, it cannot be transmitted to offspring who inherit the paternal Y^{Tdim1} chromosome (Figure 1A and B). The distribution of progeny positive and negative for the *Eif2s3y* transgene is thus reflective of combined XX/XO vs XY^{Tdim1} distribution. No differences were observed in the frequency of *Eif2s3y* positive and negative offspring when sex determination was driven by the *Sry* transgene (Figure 1A; Supplementary Figure S3A). However, when sex determination was driven by the *Sox9* transgene, progeny transgenic for *Eif2s3y* were more abundant (Figure 1B; Supplementary Figure S3B). The incidence of offspring carrying or lacking the *Eif2s3x* transgene in the context of transgenic *Sry* was similar (Figure 1C; Supplementary Figure S3C). However, when the *Eif2s3x* transgene was paired with the paternal *Sox9* transgene, an unusual offspring genotype distribution was observed. Three expected viable male genotypes carrying transgenic *Sox9* but lacking the *Eif2s3x* transgene were not obtained. This resulted in an abundance of male progeny carrying *Sox9* and *Eif2s3x* transgene pair. Among the females this trend was reversed, with a predominance of genotypes lacking the *Eif2s3x* transgene (Figure 1D; Supplementary Figure S3D).

In regard to sex distribution, crosses yielding $X^E OSry$ and $X^E OSox9$ males had fewer male offspring, a cross yielding $X OSry, Eif2s3x$ had fewer female offspring, and a cross yielding $X OSox9, Eif2s3x$ had a similar frequency of male and female offspring (Supplementary Figure S4).

XO males with two, one, and no Y chromosome genes display incomplete masculinization of gonadal shape and impairment in development of testis cord and gonadal vasculature

For evaluation of genital ridge morphology, we chose three parameters that display sexual dimorphism at 12.5 dpc: gonadal shape, testis cords development, and gonadal vascularization. All XO male genotypes, regardless of the transgene complement, displayed some degree of impairment in terms of gonadal shape (Figure 2). The characteristic increase of genital ridge width, which gives XY gonads a distinct male-specific appearance, was reduced in all

transgenic males, by 28.4% on average (Figure 2B–D). Gonadal length: width ratio examination revealed a similar trend of incomplete masculinization with genital ridges from $X^E OSry$, $X^E OSox9$, and $X OSry, Eif2s3x$ males displaying XX-like gonadal shape, while $X OSox9, Eif2s3x$ males having an intermediate gonadal length: width ratio different from both XX and XY controls (Figure 2C and D). *Sry*-to-*Sox9* and *Eif2s3y*-to-*Eif2s3x* replacement did not appear to affect gonadal shape development as there were no significant differences between $X^E OSry$ vs $X^E OSox9$ and $X OSry, Eif2s3x$ vs $X OSox9, Eif2s3x$, and between $X^E OSry$ vs $X OSry, Eif2s3x$ and $X^E OSox9$ vs $X OSox9, Eif2s3x$ (Figure 2B–D).

The fetal gonads from all transgenic XO males had fewer testis cords than XY controls (13 ± 1.25 cords per gonad) (Figure 3B). In the males transgenic for *Sry*, testis cord number was reduced to approximately 9.5 cords per gonad while in males transgenic for *Sox9* transgenics a more severe testis cord number reduction was observed (approximately 7.5 cords per gonad). The analyses of testis cord width revealed a similar trend: all XO males exhibited impairment when compared to XY (Figure 3C). The *Sry*-to-*Sox9* substitution resulted in further reduction while the *Eif2s3y*-to-*Eif2s3x* substitution had no effect (Figure 3B and C).

Vasculature development was examined with a focus on the coelomic artery, which is the most prominent vascular attribute, and using an overall vasculature score, which is inclusive of additional vascular features (Figure 3D and E). In XY males, coelomic artery covered on average 76% of the coelomic genital ridge surface. This was two- to threefold reduced in $X^E OSry$, $X^E OSox9$, and $X OSox9, Eif2s3x$ transgenic males. In the $X OSry, Eif2s3x$ males, gonadal vasculature development was impaired in comparison to XY, but was more advanced in comparison to the remaining transgenic XO males (Figure 3D). The overall vascular score yielded similar results and further emphasized better vascular development in males transgenic for *Eif2s3x*, and $X OSry, Eif2s3x$ in particular.

Together, these results support that XO males display incomplete masculinization of gonadal shape and impairment in the development of testis cord and gonadal vasculature, and some aspects of this phenotype are exacerbated when sex determination is driven by the *Sox9* transgene.

XO males with two, one, and no Y chromosome genes display altered sex-specific molecular signaling in fetal gonads

Transcript levels of a master regulator *Sox9* and male-specific (*Amb*, *Fgf9*, and *Cyp26b1*) and female-specific (*Foxl2*, *Rspo1*, and *Wnt4*) factors were assessed by real-time PCR.

Sox9 expression in transgenic males was decreased when compared to XY, except for $X OSox9, Eif2s3x$; the lack of difference in XY vs $X OSox9, Eif2s3x$ comparison is likely due to high variability between $X OSox9, Eif2s3x$ males (Figure 4A). The expression of *Amb*, *Fgf9*, and *Cyp26b1* was also altered. The *Amb* signaling was the most severely impaired, especially in males carrying the *Sox9* transgene. The levels of *Amb* and *Fgf9* in $X^E OSox9$ males were similar to those of XX control while the remaining three XO male genotypes ($X^E OSry$, $X OSry, Eif2s3x$, and $X OSox9, Eif2s3x$) had an approximately two- to threefold reduction of *Amb* and *Fgf9* expression when compared to XY (Figure 4B). The *Sry*-to-*Sox9* substitution in the context of the *Eif2s3y* transgene, but not in the context of the *Eif2s3x* transgene, resulted in a decrease of *Amb* and *Fgf9* transcript levels (Figure 4B). The *Cyp26b1* expression in males with sex determination driven by the *Sry* transgene was comparable to

Figure 4. Transcript expression in the genital ridges of XO males. Four types of males with limited Y chromosome gene complement (tested: $X^E O Sry$, $X^E O Sox9$, $X O Sry, Eif2s3x$, and $X O Sox9, Eif2s3x$) were compared to wild-type XY and XX controls. Transcript expression was defined for a master regulator *Sox9* (A), male-specific targets of SOX9: *Amh*, *Fgf9*, *Cyp26b1* (B), and ovarian markers known to be downregulated by SOX9 signaling: *Foxl2*, *Rspo1*, *Wnt4* (C). The loading controls were with three ubiquitously expressed genes (*Sdha*, *Rps29*, and *Tbp*), and normalization was achieved by geometric averaging of these genes. The reference controls were XY (A and B) and XX (C). Values are mean $\pm$ SDev with $n > 3$ (except $X^E O Sox9$, $n = 2$). Statistical significance (t -test, $P > 0.05$): graph bars marked with different letters are significantly different from each other while those marked with the same letters do not differ.

that of XY males, while in the males carrying the *Sox9* transgene it was reduced approximately twofold (Figure 4B). This reduction did not lead to increase in expression of meiosis marker *Stra8* (Supplementary Figure S5). Males with sex determination driven by the *Sox9* transgene, but not males transgenic for *Sry*, had elevated expression of ovarian markers *Foxl2* and *Rspo1*. The expression of *Wnt4* was not altered except in $X^E O Sox9$ males, in which it was slightly elevated (Figure 4C).

Together, the data support that XO males display an altered expression of male- and female-specific factors, and that males with sex determination driven by the transgenic *Sox9* are more strongly affected.

Sex chromosome context and sex determination driver influence male gonad development

The breeding crosses utilized to produce XO males with limited Y chromosome gene contribution (Figure 1) gave us an opportunity to directly investigate the effects of sex chromosome context and sex determination and spermatogenesis drivers.

To test for the effects of X chromosome dosage, we compared four sets of analogous genotypes that differed only by the X chromosome copy number, XX males vs XO males (Figure 5). Presence of a

second X chromosome improved masculinization of gonadal shape in XX males transgenic for *Eif2s3y*, but not XX males transgenic for *Eif2s3x*, when compared to their XO counterparts (Figure 5B and C). There were no differences between XX and XO males in regard to the development of testis cord, with the exception of $X^E X Sry$ that scored higher than $X^E O Sry$ (Figure 5D and E). The vasculature development was better in XX in the context of *Eif2s3y*, with $X^E X Sry$ and $X^E X Sox9$ scoring higher than their XO counterparts (Figure 5F and G). *Sox9* expression was similar in analogous XX and XO male genotypes, except for XX males carrying the *Sox9* and the *Eif2s3y* transgenes scoring approximately twofold higher than the analogous XO males (Figure 6A). The XX males transgenic for *Eif2s3y* had higher levels of *Amh* and *Fgf9* compared with their XO counterparts; *Amh* expression was also higher $XX Sry, Eif2s3x$ males compared to $X O Sry, Eif2s3x$ (Figure 6B). There were no differences between XX and XO males in the expression of *Cyp26b1*, *Foxl2*, *Rspo1*, and *Wnt4*, regardless the transgene combination (Figure 6B and C). Overall, we observed a beneficial effect of a second X chromosome on some aspects of gonadal masculinization and sex-specific signaling but almost exclusively in the context of the *Eif2s3y* transgene.

To test directly for the effects of the sex determination driver, we compared males carrying the *Eif2s3x* transgene and either the *Sry* (*Sry, Eif2s3x*) or the *Sox9* (*Sox9, Eif2s3x*) transgene; this analysis was

performed in three different sex chromosome contexts: XY^{Tdim1} , XX, and XO. A similar examination could not be performed for males transgenic for *Elf2s3y* because this transgene is inherited from the paternal X chromosome and is therefore present only in XX and XO offspring (Figure 1A and B). Sex determination driven by transgenic *Sry* resulted in more masculinized gonadal shape in XY^{Tdim1} and XX, but not XO sex chromosome contexts, when compared to sex determination driven by the *Sox9* transgene (Supplementary Figure S6A and B). Testis cord and vasculature development were also enhanced in presence of the *Sry* transgene (Supplementary Figure S6C–F). Although similar levels of *Sox9* transcripts were observed in gonads from *Sry* and *Sox9* transgenics (Supplementary Figure S7A), the former had higher levels of *Amb* and *Cyp26b1* while the latter displayed elevated expression of *Foxl2* and *Rspo1* (Supplementary Figure S7B and C). The effect of *Sry*-to-*Sox9* substitution on reduction of *Cyp26b1* and elevation of *Foxl2* and *Rspo1* levels could also be seen in XX vs XO comparison (Supplementary Figure S7B and C).

When the effect of sex chromosome context (XY^{Tdim1} vs XX vs XO) was considered, a trend was observed with masculinization of fetal gonads moving progressively from XY-like to XX-like

with the sex chromosome pairs changing from XY^{Tdim1} to XX and XO; this was most distinctly observed in gonadal shape of the *Sry* transgenics (Supplementary Figure S6A and B), and testis cord number (Supplementary Figure S6C) and development of coelomic artery (Supplementary Figure S6E) of both the *Sry* and the *Sox9* transgenics; the differences in molecular signaling between XY^{Tdim1} , XX and XO sex chromosome contexts were less pronounced (Supplementary Figure S7).

Together, these findings support that the *Sry* transgene is more effective in driving the development of male gonad during sex determination than the *Sox9* transgene and allows for a better fetal gonad masculinization and more XY-like molecular signaling. The data also support that presence of Y chromosome genes, and to a lesser extent the double dosage of X genes, enhances fetal gonad masculinization positively influencing genital ridge morphogenesis and vascularization.

DISCUSSION

This study is a continuation of our previous work on mice with limited Y chromosome gene contribution [23–26]. We investigated the

Figure 6. The effects of X chromosome number on transcript expression in the genital ridges. Four sets of male genotypes carrying an analogous transgene complement in the context of either XO or XX were compared (tested pairs: $X^E O Sry$ vs $X^E X Sry$, $X^E O Sox9$ vs $X^E X Sox9$, $XO Sry, Eif2s3x$ vs $XX Sry, Eif2s3x$, and $XO Sox9, Eif2s3x$ vs $XX Sox9, Eif2s3x$). Transcript expression was defined for a master regulator *Sox9* (A), male-specific targets of *SOX9*: *Amh*, *Fgf9*, *Cyp26b1* (B), and ovarian markers known to be downregulated by *SOX9* signaling: *Foxl2*, *Rspo1*, *Wnt4* (C). The loading controls were with three ubiquitously expressed genes (*Sdha*, *Rps29*, and *Tbp*), and normalization was achieved by geometric averaging of these genes. The reference controls were XY (A and B) and XX (C). Values are mean $\pm$ SDev with $n > 3$ (except $X^E O Sox9$, $n = 2$). Statistical significance (t-test, $P < 0.05$): ^a different from XX; ^b different from XY; the differences between XX vs XO are marked by the horizontal lanes with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

development of fetal gonads with the overall aim to clarify the origin of the testicular phenotypes that we observed earlier in adult males. We demonstrated that males with two, one, or no Y chromosome genes have impaired fetal gonadal development and alterations in sex-specific molecular signaling, with transgene- and sex chromosome context-dependent differences.

Breeding and offspring genotype distribution

To examine testicular development in mice with limited Y gene contribution, we performed timed matings of transgenic fathers with XO females, followed by dissection of fetuses at 12.5 dpc. The breeding efficiency, measured as a number of fetuses per litter, was reduced when compared to breeding of wild-type controls. We have previously shown that XY^{Tdm1} males transgenic for *Sry/Sox9* have normal fecundity [24]; therefore, the poor breeding we noted here was likely due to reduced reproductive abilities of XO females, in agreement with previous reports [33–35]. Poor breeding efficiency was paired with a low incidence of XO fetuses among offspring. This phenomenon was observed before in breeding trials with XO females [36] and is thought to result from nonrandom segregation of the single X chromosome during oogenesis, with the X chromo-

some being transmitted to the oocyte about two-thirds of the time (as opposed to the predicted one-half) and yielding XO embryos only half as frequently as XX and XY embryos [37]. It has also been shown that some XO embryos that implant near the cervix develop extremely poorly, which could further reduce the number of XO progeny that are born [38]. The analysis of progeny genotype frequencies revealed an interesting anomaly among offspring from $XY^{Tdm1} Eif2s3x$, *Sox9* fathers. Four offspring genotypes, expected to be viable, were not detected; three of them were male genotypes lacking the *Eif2s3x* transgene. This suggests that overexpression of *Eif2s3x*, warranted by the presence of the *Eif2s3x* transgene, positively influences viability of males lacking all Y chromosome genes. The elevated levels of *Eif2s3x* were not beneficial for development of female offspring since nontransgenic females were more abundant than females carrying the *Eif2s3x* transgene. We suspect that *Eif2s3x* (and likely *Eif2s3y*) plays distinct roles in males and females during sex determination. *Eif2s3x* is one of the few mouse genes that escape X chromosome inactivation and is expressed from both X chromosomes in females. In analyses carried out as part of a different project, we noted that at 12.5 dpc the expression of *Eif2s3x* in XX females was similar to that of XY males, suggesting that preferentially enhanced expression of *Eif2s3x* in male genital ridges compensates for

the effect of having only one rather than two expressing X chromosomes. Future studies involving analyses of *Eif2s3x/y* expression at various time points during sex determination, coupled with fetal gonad characterization, are needed to clarify the roles of *Eif2s3* X-Y gene pair during this critical period of development.

Gonadal morphology

We have previously shown that adult XO males with limited Y chromosome gene contribution had decreased testis size and displayed various testicular abnormalities [25, 26]. Here, genital ridge morphology assessment of the same genotypes revealed variable degrees of faulty sex-specific organ remodeling. By 12.5 dpc male genital ridges are expected to display a clear size increase and coelomic epithelium thickening, which doubles in size between 11.5 and 12.5 dpc [39]. In contrast, female genital ridges undergo minimal shape remodeling and retain undifferentiated-like morphology longer. The changes of genital ridge shape are attributed to *Sry*-dependent endothelial cell migration from the mesonephric region [40], clustering of interstitial cells around forming testis cords [39], and Sertoli cell proliferation [41]. It has been shown that the inhibition of Sertoli cell proliferation soon after initiation of *Sry* expression (between 10.8 and 11.2 dpc) results in decreased expression of *Sox9* and *Amb*, reduced numbers of testes cords, and ultimately development of male gonads with reduced size [41]. It is, therefore, possible that the mechanism behind testicular abnormalities in adult XO males [25] along with the feminized genital ridge size and shape and reduced *Sox9* expression observed in this study result from a failure of sufficient Sertoli cell proliferation prior to 12.5 dpc. Future experiments involving quantification of Sertoli cells in the fetal gonads from males with limited Y chromosome genes are needed to validate this proposition. Nevertheless, if there indeed was a decrease in fetal Sertoli cell number, this insufficiency is overcome prior to adulthood as we have shown that Sertoli cell counts in mature gonads from males with limited Y gene contribution, was similar or higher than in wild-type testis [25, 26].

Testis cords and vasculature development

Reorganization of gonadal primordium into seminiferous epithelium and testicular interstitium is another significant milestone in testicular development and is first initiated around 11.5 dpc [42]. The formation of rudimentary testis cords involves aggregation of somatic cells around germ cell clusters; the resulting pre-Sertoli/germ cell units continue to acquire increasingly more tubular and wider morphology [39, 43]. The testis cord evaluation performed here revealed that formation of clear tubular cord structures was impaired in all transgenic XO males, but especially when the sex determination was driven by the *Sox9* transgene.

Male-specific gonadal vascularization takes place at the same time as testis cord assembly. A prominent artery is established along the coelomic border of the genital ridge. Endothelial cell migration from the mesonephroi towards the genital ridges tends to follow a controlled pattern and consequently subdivides the coelomic epithelium into a set number of alternating vascular and avascular regions [44]. Establishment of male-specific gonadal angiogenic program appears to serve not only the primary purpose of providing blood supply to the gonads but is also required for testis cord formation [21, 45]. All transgenic XO males displayed some impairment in gonadal vascularization. The XO and XX males carrying the *Sox9* transgene had a more severe phenotype compared to XY males and to analogous *Sry* transgenics, especially in the coelomic

artery development. This impairment might result from an additive effect of lacking Y chromosome genes and functional impairment in one or more sex determination pathways. Studies done by others have shown that formation of the coelomic vessel in XY gonad relies on several redundant, signaling pathways, and that multiple known (like members of TGF- β family) and unknown factors act to induce endothelial cell migration towards the gonad [46, 47]. AMH was shown to act as Sertoli-cell-derived chemoattractant that can induce mesonephric cell migration and male-like vascular development in XX gonads in culture [46]. Here, all *Sox9* transgenic males had reduced *Amb* expression, which in XO and XX males correlated with impaired vasculature development. Since a loss of *Amb* alone does not lead to abnormal vasculature development in XY *Amb*^{-/-} males [46], it is likely that multiple pathways with overlapping functions in male-specific gonadal vascularization are affected.

Alterations in molecular signaling

The more severe impairment in fetal gonad morphology (shape, testis cord formation, and vasculature development) observed with the *Sox9* transgenics is reflective of changes in molecular signaling. The quantification of expression of key sex differentiation pathway factors revealed that XO transgenic males had comparable levels of global *Sox9* mRNA. However, SOX9-dependent male-specific signaling was better established in the *Sry* transgenics supporting that the *Sox9* transgene-driven sex determination was less favorable. It has been shown before that even if the *Wt1: Sox9* transgene expression is high, the protein may fail to accumulate and does not participate in its own expression maintenance [48]. This can negatively affect functional ability of the *Wt1: Sox9* transgene and lead to insufficient induction of *Amb*, *Fgf9*, and *Cyp26b1* upregulation. *Fgf9* and *Cyp26b1* play a special role in guarding timely differentiation in males by preventing germline from entering meiosis prematurely [49–51]. Both in males and in females meiosis is stimulated by retinoic acid, which is produced by the mesonephroi of both sexes starting 10.5 dpc [50]. In response to retinoic acid accumulation in the genital ridge, female germ cells initiate expression of *Stra8* and enter meiosis by 13.5 dpc [50, 51]. In male gonads meiosis is retarded by elevated levels of CYP26b1, which degrades retinoic acid [52], and upregulation of *Fgf9*, which induces prolonged expression of pluripotency genes in the germ cells and keeps them unresponsive to retinoic acid signaling [49]. In addition to its function in suppressing male meiosis initiation, FGF9 also plays a key role in promoting germ cell survival at 12.5 dpc [53] and in testis cord formation [54, 55]. Germline integrity influences supporting cell proliferation and testis cord development; at 13.5 dpc germ-cell-devoid W^c/W^c mutant mice had shorter, narrower, and more disorganized testis cords and fewer Sertoli cells than wild-type mice [56]. The reduction of *Fgf9* and *Cyp26b1* expression that we observed in males carrying the *Sox9* transgene hints at the possibility of impaired germline development in these mice. If true, the reduction of testis cord width would be a reflection of a loss of prospermatogonia due to *Fgf9* signaling insufficiency.

Effects of the *Sry* and the *Sox9* transgenes

All assays performed in this study showed that males with sex determination driven by the *Sox9* transgene displayed more atypical testis development than males carrying the *Sry* transgene. The mismatch between gonadal morphology and molecular signaling patterns between the analogous genotypes carrying the *Sry* versus the *Sox9* transgenes may indicate the differences in regulatory features that

are inherent to these two transgenic systems. The *Sry* transgene is directed by the endogenous *Sry* promoter and is expected to drive the expression of *Sry* (and its direct target *Sox9*) in the transgenic males similarly as the endogenous *Sry* does in XY males, with initiation at the center of the gonad and subsequent center-to-pole decrease in expression pattern. In contrast, the *Wt1* promoter drives the *Sox9* transgene expression throughout the genital ridge, not following the center-to-pole fashion [48]. Moreover, the *Wt1*-driven *Sox9* expression is initiated in male gonad at 10.5 dpc, 1 day earlier than the onset of endogenous *Sox9* expression [48]. A common notion is that the primary function of SRY is to upregulate *Sox9* signaling after which SOX9 takes over as the master facilitator of testis differentiation. However, a recent study employing chromatin immunoprecipitation and whole-genome promoter tiling microarray (ChIP-Chip) on fetal gonads at the time of sex determination has shown that SRY regulates a wide spectrum of targets involved in early events of testis differentiation, some of which could precede the normal *Sox9* activation [57]. These events include Sertoli cell fate determination, proliferation and recruitment, arrest of meiosis in germ cells and definition of their male lineage, inhibition of ovarian development, and eventual formation of the testis cords. However, it has also been shown that SOX9 is capable of assuming certain regulatory functions of SRY, and that both SRY and SOX9 regulate a significant number of common targets playing important functions in early events in testis differentiation. Together, this combined evidence supports that the weakened gonadal masculinization in males transgenic for *Sox9* observed in our study may be explained by the differences in spatial, temporal, and quantitative expression of the *Sry* and the *Sox9* transgenes.

Effects of sex chromosome contexts

An interesting aspect of this study was the comparison of males with the analogous transgene sets in different sex chromosome contexts. The fact that males carrying the *Y^{Tdym1}* chromosome, encoding all Y chromosome genes except *Sry*, scored better than XX and XO males in fetal gonadal masculinization is not surprising and verifies the importance of Y genes in male reproduction. However, the beneficial effect of a second X chromosome on gonadal masculinization was unexpected. Presence of two X chromosomes has been previously shown to be incompatible with spermatogenesis [58, 59]. Adult XX*Sry* males have testes essentially devoid of germ cells due to germ cell loss taking place during the first few days after birth [58, 60, 61]. The current belief is that this demise results from altered X chromosome dosage. Altered X chromosome dosage was also shown to impair germ cell development during early stages of testis differentiation; XXY males displayed significantly reduced germ cell numbers when compared to XY males from 15.0 dpc onwards [58]. Since the somatic constitution of XXY fetal testis was normal and the germ cells from the XXY gonads proliferated normally in vitro, it has been suggested that germ cell death reflects a defective somatic-germ cell communication [58]. The aforementioned studies compared XX and XY males. Here, we were able to expand these analyses by including XO males. When we quantified germ cell (prospermatogonia and spermatogonia) numbers in testes from neonatal XY^{Tdym1}*Sry*, XO*Sry*, and XX*Sry* males, the XX*Sry* males had significantly fewer cells at 3 dpp (days post partum) and by 11 dpp almost all cells were gone; XO*Sry* males were similar to XY at 3 dpp and their germ cells persisted in testes but did not proliferate due to the lack of *Eif2s3y* (our unpublished data). Contrary to what we observed in neonatal and adult males, at 12.5 dpc the presence of a second X

chromosome was beneficial to gonadal masculinization, especially for gonadal shape and vasculature development. We are not sure why a double dosage of X genes positively influences masculinization of fetal gonad. Perhaps during this early period of development, a double dosage of X genes belonging to X-Y gene pairs [62] can serve as a compensation for the lack of a Y chromosome.

Effects of the *Eif2s3y* and the *Eif2s3x* transgenes

The analyses of fetal gonads from males with limited Y gene content pointed to the specific effects of the *Eif2s3y* and the *Eif2s3x* transgenes. In adult males with limited Y chromosome contribution, spermatogenesis progression was correlated with the global levels of *Eif2s3x/y* [25]. While spermatogonial proliferation and differentiation took place in a broad window of *Eif2s3x/y* expression, progression through meiosis was more sensitive and required higher transcript levels. The differences in *Eif2s3x/y* expression between various genotypes with limited Y chromosome contribution originated from the differences in transgene copy number (*Eif2s3y*: 10 copies [63]; *Eif2s3x*: 4 copies [25]) and the differences in expression of endogenous genes. In wild-type mature testes, endogenous *Eif2s3x* and *Eif2s3y* are expressed in Sertoli cells and in spermatogenic cells with *Eif2s3y* transcripts significantly more abundant, 4- to 8-fold higher in Sertoli, premeiotic, and meiotic cells, and 33-fold higher in postmeiotic cells [64]. Thus, high expression of *Eif2s3y* is required to drive spermatogenesis. Contrary to what is seen in adult testes, in wild-type fetal male gonads (11.5–13.5 dpc) endogenous *Eif2s3y* and *Eif2s3x* are expressed at similar levels [65]. Thus, poorer gonadal development in males carrying the *Eif2s3y* transgene, as compared to the *Eif2s3x* transgenics, may be due to a negative effect of *Eif2s3y* overexpression. Future work is clearly needed to understand the roles of *Eif2s3x/y* gene pair in gonadal development. This could involve examination of various developmental time points in conjunction with genetic manipulation of *Eif2s3x/y* expression. Such analyses are not feasible with the transgenic models that we described here due to an extreme inefficiency in obtaining necessary samples. With the advent of CRISPR/Cas9 technologies, it should now be possible to generate mice with cell- and time-specific knockout of the *Eif2s3y* and *Eif2s3x* genes.

Conclusions

This study is the first to analyze fetal gonad development in males with Y chromosome contribution limited to two, one, and no Y chromosome genes. We demonstrated abnormal gonadal development, evidenced as reduced levels of expression of male-specific factors and only partial masculinization of genital ridge anatomy. These effects were especially prominent in the *Sox9* transgenic males, which in most cases displayed female-like or an intermediate gonadal phenotype. Our findings clarify the origin of testicular abnormalities and spermatogenic defects in adult males with limited Y chromosome genes. This study supports that Y chromosome is an essential genetic component required for healthy testicular development and provides new evidence regarding the function of *Eif2s3y/x* gene pair and X chromosome dosage on gonadal development.

Supplementary data

Supplementary data are available at [BIOLRE](https://doi.org/10.1016/j.biolre.2019.05.010) online.

Supplementary Table S1. Primer sequences.

Supplementary Figure S1. Breeding efficiency. Breeding efficiency of $X^{EY}T_{dym1}Sry$, $X^{EY}T_{dym1}Sox9$, $XY^{T_{dym1}}Sry$, $Eif2s3x$, $XY^{T_{dym1}}Sox9$, $Eif2s3x$ males bred to XO females, with XY males bred to XX females serving as control, presented as average number of pups per litter. $X^{EY}T_{dym1}Sox9 \times XO$ cross had the smallest overall progeny count and produced only two pups of interest ($X^E O Sox9$) in spite of the longest breeding time (over 9 months). In many cases, vaginal plugs were observed but resulted in no viable pregnancies. Bars are mean $\pm$ SEM. Statistical significance (t -test): ^a different from all other groups; * $P < 0.05$; ** $P < 0.01$.

Supplementary Figure S2. Progeny distribution—sex chromosome effect. The offspring were obtained after mating of $X^{EY}T_{dym1}Sry$ (A), $XY^{T_{dym1}}Sry$, $Eif2s3x$ (B), $X^{EY}T_{dym1}Sox9$ (C), and $XY^{T_{dym1}}Sox9$, $Eif2s3x$ males (D) with XO females. The graphs show the distribution of offspring carrying specific sex chromosome pairs ($XY^{T_{dym1}}$, XX and XO), with distinction between male and female offspring as well as with sexes pooled. Bars are mean $\pm$ SEM. Statistical significance (t -test; $P < 0.05$): graph bars marked with different letters are significantly different from each other, while those marked with the same letters do not differ; borderline significances are shown with horizontal lanes.

Supplementary Figure S3. Progeny distribution— $Eif2s3x/y$ transgene effect. The offspring were obtained after mating of $X^{EY}T_{dym1}Sry$ (A), $XY^{T_{dym1}}Sry$, $Eif2s3x$ (B), $X^{EY}T_{dym1}Sox9$ (C), and $XY^{T_{dym1}}Sox9$, $Eif2s3x$ males (D) with XO females. The graphs show the distribution of offspring positive (+) or negative (−) for the $Eif2s3y$ (A&B) or $Eif2s3x$ (C and D) transgene, with distinction between male and female offspring as well as with sexes pooled. Bars are mean $\pm$ SEM. Statistical significance (t -test): ** $P < 0.01$; *** $P < 0.001$.

Supplementary Figure S4. Progeny distribution—sex effect. The offspring were obtained after mating of $X^{EY}T_{dym1}Sry$ (A), $XY^{T_{dym1}}Sry$, $Eif2s3x$ (B), $X^{EY}T_{dym1}Sox9$ (C), and $XY^{T_{dym1}}Sox9$, $Eif2s3x$ males (D) with XO females. The graphs show the distribution of male and female offspring. Bars are mean $\pm$ SEM. Statistical significance (t -test): * $P < 0.05$; ** $P < 0.01$.

Supplementary Figure S5. *Stra8* transcript expression in the genital ridges of XO males. Four types of males with limited Y chromosome gene complement (tested: $X^E O Sry$, $X^E O Sox9$, $X O Sry$, $Eif2s3x$, and $X O Sox9$, $Eif2s3x$) were compared to wild-type XY and XX controls. Transcript expression was defined for a meiosis marker *Stra8*. The loading controls were with three ubiquitously expressed genes (*Sdha*, *Rps29*, and *Tbp*), and normalization was achieved by geometric averaging of these genes. The reference control was XX. Values are mean $\pm$ SDev with $n > 3$ (except $X^E O Sox9$, $n = 2$). Statistical significance (t -test, $P > 0.05$): graph bars marked with different letters are significantly different from each other while those marked with the same letters do not differ.

Supplementary Figure S6. The effects of sex determination driver on male gonad development. Males transgenic for *Eif2s3x* with sex determination driven either by *Sry* (*Sry*, *Eif2s3y*) or *Sox9* (*Eif2s3x*, *Sox9*) in the context of $XY^{T_{dym1}}$, XO or XX were compared. (A) Gonadal width; (B) gonadal length/width ratio; (C) testis cord number; (D) testis cord width; (E) coelomic artery development expressed as percentage of genital ridge coelomic surface coverage; (F) overall vascular score expressed using 6 point system, with maximum two points derived from each category: coelomic artery visibility, coelomic artery surface coverage, and number of vascular clusters in the interstitial domain (see Methods section for more detail). Graphs are mean $\pm$ SDev (A–C and E–F, $n > 3$, except $X^E O Sox9$ $n = 2$) and mean $\pm$ SEM (D, $n > 3$). Statistical significance (t -test, $P < 0.05$):

^a different from XX; ^b different from XY; the differences between *Sry*, *Eif2s3y* and *Eif2s3x*, *Sox9* are marked by the horizontal lanes with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Supplementary Figure S7. The effects of sex determination driver on transcript expression in the genital ridges. Males transgenic for *Eif2s3x* with sex determination driven either by *Sry* (*Sry*, *Eif2s3y*) or *Sox9* (*Eif2s3x*, *Sox9*) in the context of $XY^{T_{dym1}}$, XO or XX were compared. Transcript expression was defined for a master regulator *Sox9* (A), male-specific targets of SOX9: *Amb*, *Fgf9*, *Cyp26b1* (B) and ovarian markers known to be downregulated by SOX9 signaling: *FoxL2*, *Rspo1*, *Wnt4* (C). The loading controls were with three ubiquitously expressed genes (*Sdha*, *Rps29*, and *Tbp*), and normalization was achieved by geometric averaging of these genes. The reference controls were XY (A and B) and XX (C). Values are mean $\pm$ SDev with $n > 3$ (except $X^E O Sox9$, $n = 2$). Statistical significance (t -test, $P < 0.05$): ^a different from XX; ^b different from XY; the differences between *Sry*, *Eif2s3y* and *Eif2s3x*, *Sox9* are marked by the horizontal lanes with * $P < 0.05$, ** $P < 0.01$.

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